Novel T cell function and specificity at the human Maternal-Fetal Interface

By Richard Morgan Powell

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Abstract

The human placenta is the key interface at which the maternal immune system interacts with the semi allogeneic fetus. It is known that fetal cells can cross into the placenta into the maternal circulation, and that during pregnancy the maternal immune system is able to induce a potential cytotoxic response to fetal antigens. However, the complex equilibrium of immune tolerance at the maternal-fetal interface is maintained by a range of modifications to the local and systemic maternal immune system, which leads to protection of the fetus from the maternal immune response.

Lymphoid infiltration is seen at the implantation site at the maternal-fetal interface, and decidual Natural Killer (NK) cells have been demonstrated to facilitate extravillous trophoblast (EVT) invasion into maternal decidua during the first trimester, facilitating haemochorial placentation. However, although there is also considerable T cell infiltration of the maternal decidua during pregnancy, the functional properties of this T cell response remain poorly defined. Indeed, a widely reported pathology of pregnancy, Villitis of Unknown Etiology (V.U.E), is associated with massive inflammatory invasion of cytotoxic T cells, causing detrimental fibrosis of the placental villi. This can lead to intrauterine growth restriction, fetal death and perinatal morbidities (Redline et al 2007).

To further investigate the role of T cells that have infiltrated the maternal-fetal interface, and how they respond to the semi allogeneic fetus, we investigated the

phenotype, functionality, specificity and regulation of CD4+ and CD8+ T cells obtained from healthy human third trimester placenta, and compared these with matched maternal peripheral blood lymphocytes and cord blood. We demonstrated that there are large populations of highly differentiated effector CD4+ and CD8+ T cells at the decidua in comparison to peripheral blood. These cells also displayed a unique functional profile with simultaneous production of interferon-γ (IFN-γ) and interleukin (Il-4). Decidual T cells had a significantly increased proliferative response to fetal tissue, and HY-specific T cells were detectable in the decidua of women with male pregnancies. Fetal-specific T cells expressed high levels of Programmed Death Protein 1 (PD-1) and depletion of T regulatory cells led to an increase in fetal-specific proliferation, suggesting a dual mechanism of T cell regulation. Finally, transcriptional analysis of CD4+ and CD8+ decidual T cells revealed a unique gene profile characterized by elevated expression of proteins associated with the response to interferon signalling, as well as regulatory response pathways.

Together, these findings have considerable importance for the study of healthy placentation and, furthermore, provide insights into the etiology of allo-reactive immune responses within disorders of pregnancy such as V.U.E.

Dedication

Til mine skatterbasser Ditte og Morgan

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List of Abbreviations

ANG2: Angiopoietin-2

APC: Antigen Presenting Cell

CTLA-4: Cytotoxic T-lymphocyte antigen-4,

DC: Dendritic Cell

EMRA: Revertant effector memory

EVT: Extravillous Trophoblast

FOXP3: Forkhead Box P3

G-CSF: Granulocyte Colonoy Stimulating Factor

GM-CSF: Granulocyte Macrophage Colony Stimulationg Factor

HLA: Human Leukocyte Antigen

HSC: Haematopoietic Stem Cell

IDO: Indoleamine 2,3- dioxygenase

IL: Interleukin

IRF: Interferon regulatory factor

IUGR: Intrauterine Growth Restriction

KIRs: killer-cell immunoglobulin-like receptors

Lag-3: Lymphocyte activation gene-3

mHAgs: Minor Histocompatibilty Antigens

MHC: Major Histocompatibility Complex

NK: Natural Killer Cell

P1FG: Placental growth factor

PD-1: Programmed Death Protein

RORγt: Receptor-related orphan receptor gamma

SGA: Small for Gestational Age

STAT: Signal transducer and activator of transcription

TCR: T Cell Receptor

TGFβ: Transforming Growth Factor Beta

Th: T helper

TIGIT: T cell Immunoreceptor with Ig and ITIM domains

Tim-3: T cell immunoglobulin domain and mucin domain 3

TNF: Tumor Necrosis Factor

TRAIL: TNF-related apoptosis-inducing ligand

V.U.E: Villitis of Unknown Etiology

VEGF: Vascular Endothelial Growth Factor

Chapter 1. Introduction

1.1. The Adaptive and Innate immune response

The human immune system is principally comprised of the Innate immune system and the Adaptive immune system. Innate immunity is stimulated almost instantly following recognition of allo-antigens, often in the form of invading pathogens, or in a clinical setting during allo-graft transplantation. Leukocyte components of the Innate immune response include phagocytic dendritic cells (DCs), macrophages, neutrophils, basophils and eosinophils, and cytotoxic natural killer (NK) cells (Janeway & Medzhitov 2002). Upon receptor binding, these cells are stimulated to phagocytose or release cytotoxic molecules to lyse target cells. Phagocytic leukocytes, such as dendritic cells, will process antigens following phagocytosis, and present these to the components of the adaptive immune response. NK cells detect aberrant MHC molecules, such as non-self MHC, and release cytotoxic cytokines. Together, the innate immune system sets an inflammatory response in motion.

The adaptive immune system works in synergy with the innate immune system to provide an almost immediate primary response (and secondary responses) to subsequent infections and allo-recognition. The adaptive immune system is primarily composed of T cells and B cells. These two cell types must be primed and tailored to specific aberrant or foreign antigens before being able to induce a response and undergo expansion and proliferation. Once activated these cells are able to directly affect or induce the killing of target cells. A proportion of the adaptive immune cells will form a “memory” subset, already primed to specific antigens, and are able to generate a rapid inflammatory response to secondary exposure to the allo-antigen.

Antigen processing, presentation and recognition is key to developing a fully functional immune response. The Major Histocompatibility Complex (MHC) which displays processed antigens, plays a pivotal role in antigen presentation. MHC molecules are separated into two classes: MHC class I and MHC class II. Class I are expressed on almost all nucleated cells, and present peptides derived from intracellular antigens. In humans, MHC class I molecules include Human Leukocyte Antigen (HLA) -A, -B, -C and also specifically on fetal trophoblast, HLA-G (Gregori 2016) . CD8+ T cells that have been primed to a specific antigen are able to bind via their T cell receptor to MHC class I presented peptides. Once bound with additional co-stimulatory markers, CD8+ T cells are able to elicit a cytotoxic response. MHC class I molecules can also present exogenous antigen via cross-presentation performed by antigen presenting cells (APC). MHC class II molecules are presented on specialised APCs, including dendritic cells, macrophages and B Cells. MHC class II molecules include HLA-DR, -DQ and -DP. MHC class II molecules often present peptide derived from endocytosing extracellular antigens. Once an antigen is captured and processed, APCs can migrate to lymph nodes where they present the antigen via MHC class II to CD4+ T cells (Roche & Furuta 2015). Figure 1.1 summarises the differences between MHC class I and class II antigen presentation pathways.

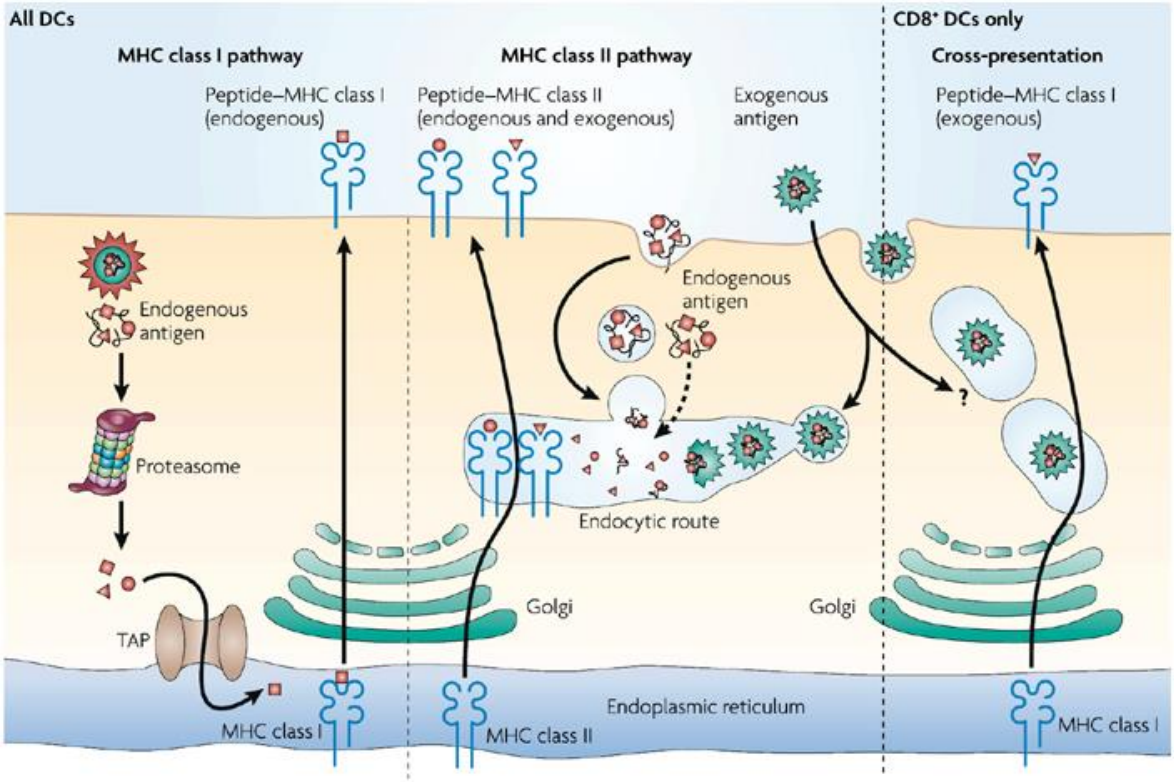


Figure 1. 1 MHC-peptide complex presentation pathways

Briefly, MHC class I molecules present peptides that are derived from proteins degraded predominately in the cytosol, which comprise almost exclusively endogenous proteins. MHC class II molecules expressed by antigen presenting cells present endogenous and exogenous derived antigens. MHC class II molecules acquire peptide cargo that is generated by proteolytic degradation in endosomal compartments. The precursor proteins of these peptides include exogenous material that is endocytosed from the extracellular environment, and also endogenous components, such as plasma membrane proteins. Antigen presenting cells can also present exogenous antigen to CD8 + T cells through MHC class I via cross presentation however the mechanism involved are still poorly understoond. (Villadangos & Schnorrer 2007).

1.2. T cell development

T cells are derived from haematopoietic stem cells (HSC), which are produced in bone marrow. Haematopoietic precursors that migrate to the thymus undergo T cell maturation and develop into naïve T cells. Lymphoid committed HSC that enter the thymus lack TCR, CD4 and CD8 expression. As they progress through the outer cortex they rearrange α and β TCR chains to a complete αβTCR and become CD4 and CD8 double positive. They next interact with thymic cortical epithelial cells, which present self-antigens via both MHC class I and II. Depending on which MHC molecule they bind, and provided correct TCR/co-receptor signalling is successful, T cells mature into single positive CD4+ or CD8+. T cells that are unable to recognise the MHC peptide complex undergo apoptosis. This stage is known as positive selection. Single positive CD4+ and CD8+ T cells then traffic to the medulla where they encounter medullary epithelial cells or DCs. Here, T cells are again subject to self-antigen presented via MHC molecules. T cells that bind to these complexes with high avidity undergo apoptosis. This stage is known as negative selection. Once complete, the T cells that have passed both rounds of selection, are then able to leave the thymus and circulate as naïve T cells. Mature naïve T cells can be primed in lymph nodes by antigen presenting cells, such as DCs. Upon antigen presentation and co-stimulatory signals, CD4+ and CD8+ T cells will begin differentiation and upregulation of activation markers and cytokines (Zúñiga-Pflücker 2004).

When primed T cells are able to form interactions with their cognate antigen on the target cell and they initiate an activation response. An immunosynapse forms at the site of activation, which involves TCR, CD3, CD4/CD8 and additional co- receptor interactions with

the target cell. CD4 and CD8 will interact with the corresponding MHC allele to stabilise antigen stimulation. Co-stimulatory signals are critical for a fully functional T cell response. TCR engagement in the absence of co-stimulatory signals can lead to anergy of the T cell resulting in inefficient cytokine expression and activation (Huppa & Davis 2003).

1.3. T cell subtypes

Effector CD4+ T cells are primarily comprised of Type 1 helper cells (Th1), Th2 and Th17 subsets, each defined by a set of transcription factors, which allows them to reach a particular differentiated state. CD4+ differentiation state is often determined by the cytokine environment in which they are situated once activated. They may be also defined by their cytokine expression, related to their individual effector function. In addition each subset expresses chemokine receptors that directs their recruitment to inflammatory sites, where specific tissues will express different chemokine ligands (Zhu & Paul 2008). Figure 1.2 illustrates the different chemokine, cytokine and transcription factors that are associated with different T cell subtypes.

Th1 cells express T-bet and signal transducer and activator of transcription (STAT) 4 as their signature transcription factors, and secrete interferon- γ (IFN-γ) as their dominant cytokine, as well as tumor necrosis factor (TNF)-α, interleukin (IL-2) and lymphotoxin-α. They also express CXCR3 as their main chemokine receptor. Their key role is to eradicate virus infected cells and intracellular pathogens. Expression of IFN-γ promotes macrophage activation and also endothelial cell expression of the chemokines CXCL9, CXCl10 and

CXCL11, which are all ligands for the chemokine receptor CXCR3. They are therefore able to elicit their own recruitment, as well as recruitment of cytotoxic T cells to sites of inflammation (Nakanishi et al. 2009). Due to Th1 roles in surgical allo-graft rejection, it is thought that they may play a significant role in driving rejection of the fetus in some pathologies of pregnancy.

Th2 cells express GATA3 and STAT6 as their signature transcription factors, along with cytokines IL-4, IL-5 and IL-10 and chemokine receptor CCR4. Their primary function is regulation of antibody switching in areas of allergic inflammation and destruction of helminths. Th2 are seen to be highly relevant in maintenance of pregnancy due to their less cytotoxic cytokine profile and their ability to inhibit Th1 differentiation. This gives rise to the often cited paradigm of Th2:Th1 skew during pregnancy, to minimize cytotoxic damage by Th1 cells (Saito et al 2010).

Th17 cells express receptor-related orphan receptor-γ (RORγt), STAT3 and interferon regulatory factor (IRF) 4 as their signature transcription factors, along with cytokine IL-17 and chemokine receptor CCR6. Their main function is to enhance an inflammatory response to extracellular bacteria and fungi. IL-17 induce epithelial cells to express CXCL1, which attracts neutrophils to sites of inflammation, and neutrophil survival factor granulocyte colony stimulating factor (G-CSF).

CD8+ T cells, otherwise known as cytotoxic T cells share their characteristic properties with Th1 cells. They express transcription factors T-Bet and STAT4 as well as cytokines IFN-γ and TNFα and chemokine receptors CXCR3 and CCR5. Their main function is to eradicate intracellular pathogens by expressing the cytolytic molecules perforin and

granzyme. These cytolytic molecules are released upon binding of TCR to MHC-class I peptide complexes. Trophoblast down regulate MHC class I molecules on their surface, thereby limiting CD8+ T cells potential for allo-reactivity (Madeja et al. 2011). It has been suggested that DC and macrophage derived IL-12 and IL-18 can induce IFN-γ secretion by CD8+ T cells in a TCR independent pathway (Freeman et al. 2012) and their potential for detrimental cytotoxic activity in the decidua should not be discounted.

1.4. Effector T cell differentiation

Once an antigen-presenting cell presents pathogenic or aberrant antigen to naive T cells, T cells become activated, proliferate, and differentiate into effector cells which migrate to the site of infection or inflammation. The effector cells are short-lived, whilst memory cells are formed with the potential for long term survival (Golubovskaya & Wu 2016). Central Memory cells can be located in the secondary lymphoid organs, whilst Effector Memory cells reside in peripheral tissues. During re-exposure to their cognate antigen these memory T cells undergo rapid expansion and assist in the secondary immune response. CD8+ and CD4+ T cells can be divided into four subsets based on the expression of CD45 and the chemokine receptor CCR7. These are categorised as: naïve (CD45+ CCR7+), Central Memory (CD45RA-CCR7+), effector memory (CD45RA- CCR7-) and CD45RA revertant effector memory, otherwise known as EMRA (CD45RA+ CCR7-) (Sallusto et al. 1999). CCR7

is a chemotaxic receptor and its expression on central memory and naïve CD8 subsets is essential for migration to lymphoid tissue. Effector memory and EMRA subsets lose CCR7, which allows for their migration to peripheral tissue. CD45 has two isoforms, CD45RA expressed on naïve cells, which is then spliced into the second isoform CD45RO, following activation. EMRA CD8 subsets re-express CD45RA following activation and extensive cell division. This highly differentiated subset is, however, still cytotoxic. Expression of CD57 is a marker of cell differentiation and a proxy for telomere length, where naïve subsets are characterised by low CD57 expression, whilst EMRA subsets have a high CD57 expression (Brenchley et al. 2003). Effector memory subsets can be further sub-divided based upon the expression of co-stimulatory markers CD28 and CD27, where CD27- and CD28- are classed as having a high potential for cytolytic activity with expression of perforin, granzyme and IFN-γ (Romero et al. 2007).

1.5. Regulatory T cells

Despite negative and positive selection in the development of T cells, not all self and aberrant antigens will be presented by thymic and medullary epithelial cells and DCs in the thymus. There are therefore additional peripheral mechanisms of tolerance. One aspect of this peripheral tolerance is the immunomodulatory CD4+ T regulatory cells. These specialised T cells negatively regulate effector CD4+ and CD8+ T cells during inflammatory

responses to self and foreign antigens. The variety of suppressive mechanisms by CD4+ T regulatory cells include: i). expression of inhibitory cytokines IL-10 , transforming growth factor β (TGFβ) (Joetham et al. 2007) and vascular endothelial growth factor (VEGF) (Facciabene et al. 2011); ii). absorption of IL-2, which induces effector T cell apoptosis (Pandiyan et al. 2007) and iii). interaction with APCs, leading to a down-regulation of the their co-stimulatory markers (Qureshi et al. 2011), or with DCs, increasing expression of indoleamine 2,3- dioxygenase (IDO) (Fallarino et al. 2003).

Two subsets of T regulatory cells are believed to exist: the thymus derived T regulatory cells, which have high avidity to self-MHC complexes, are thought to maintain tolerance to self-antigens in the periphery; and the peripheral or induced T regulatory cells, which are generated from peripheral naïve T cells and are thought to have more restricted TCR to modulate the immune response to specific foreign antigens (Sakaguchi et al. 2010).

T regulatory cells are often characterised by their duel expression of CD25+ and transcription factor forkhead Box P3 (FOXP3). However, as these markers can also be expressed by non-regulatory activated CD4+ T cells, additional markers are often used to identify these regulatory cells, such as CD127 (IL-7 receptor) (Seddiki et al. 2006).

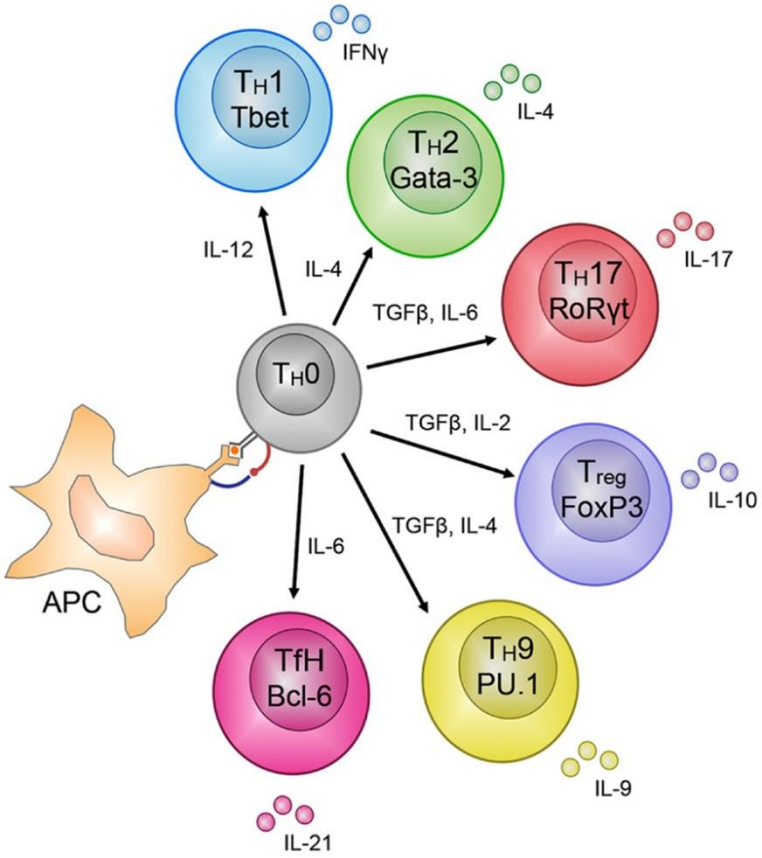


Figure 1. 2 CD4+ T cell differentiation

Summary of different cytokines and factors that influence CD4+ T cell differentiation upon interaction with APCs. Th1 are defined by the transcription factor Tbet and expression of IFNγ. Th2 are defined by transcription factor Gata-3 and expression of IL-4. Th17 are defined by RoRγt and the expression of IL-17. CD4+ T regulatory cells are defined as being FOXP3+ along with expression of IL-10.(Russ et al. 2013)

1.6. Establishment of pregnancy

Mature oocytes reside in the ampulla region of the oviduct. A fertilised oocyte travels from this region to the uterus, whilst undergoing compaction and cleavage. A morula is formed comprising of internal and external mass cells. The internal mass forms the embryo, whilst the external mass differentiates into the trophectoderm, which, in turn, forms the trophoblast cells of the placenta. During a process called cavitation the trophoblast cells form the blastocoel. The blastocoel grows and forms the blastocyst, which is implanted into the maternal endometrium (Cha et al. 2012). Once implanted, the trophoblast cells grow and differentiate into different cells types in parallel to the growing embryo and fetus. At the point of implantation, the maternal endometrium differentiates into the decidua, where fibroblasts of the uterine stroma transform and accumulate lipids and glycogen. At the point of direct contact with the trophoblast cells the decidua is known as decidua basalis, whilst the decidua parietalis forms from the remaining uterine wall.

Following implantation, the developing placenta is fixed into position by anchoring chorionic villi, whilst floating villi form the large surface area trophoblast labyrinth for gaseous and nutrient exchange. Villi are comprised principally of two cell types: mononucleated cytotrophoblast which form an “endothelial” layer, and multinucleated syncytiotrophoblast which an “epithelial” layer (Lunghi et al. 2007). Following implantation

of the blastocyst, some cytotrophoblast penetrate through the syncytiotrophoblast layer and attach to the uterine wall forming cytotrophoblast columns and extravillous trophoblast cells (Huppertz & Peeters 2005). These invasive extravillous trophoblast cells migrate through the decidua and reach the maternal spiral arteries of the maternal endometrium, and replace the endothelial cells of the arterial vessel walls. The maternal spiral arteries now provide a continuous blood flow via the transformed vessels and cytotrophoblast columns (Craven et al. 2000). Figures 1.3 and 1.4 illustrate the early and later stages of placental and decidual development in more descriptive detail.

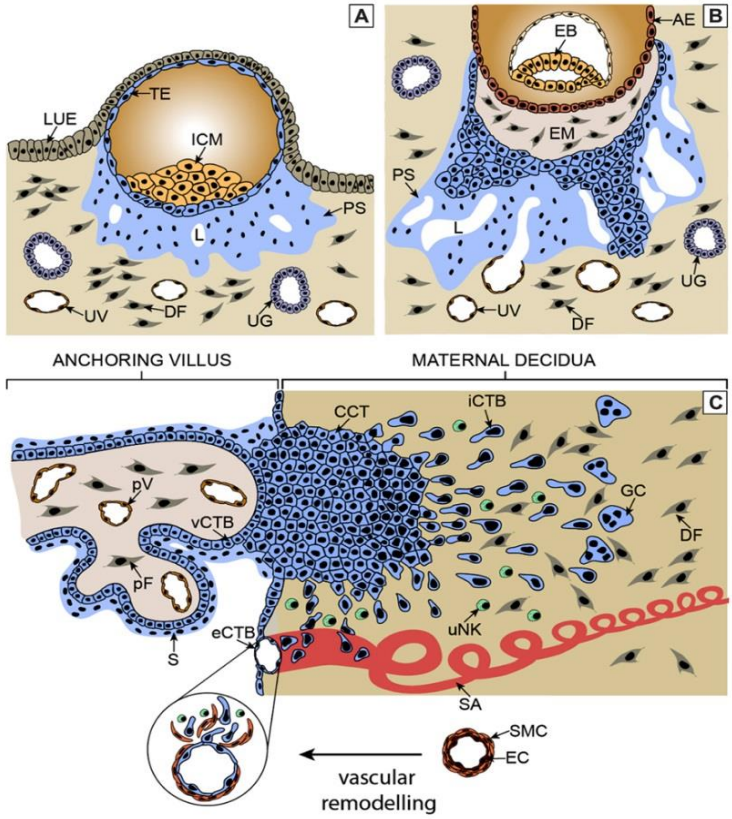


Figure 1. 3 Early stages of human placentation.

A*) After implantation, progenitors of the trophectoderm give rise to the primitive syncytium by cell fusion. In this region lacunae, the ancestor of the intervillous space, are formed.* ***B****) Later, proliferative cytotrophoblasts (CTBs) derived from the trophectoderm, break through the primitive syncytium and contact the basal plate forming primary villi.* ***C****) Tertiary villi are built upon migration of extraembryonic mesodermal cells into the primary structures and vascularization. At distal sites, proliferative cell columns are formed which give rise to different invasive extravillous trophoblast subtypes. iCTBs migrate into decidual stroma where they approach vessels from outside and eventually form giant cells as the end stage of the invasive differentiation pathway. Endovascular trophoblasts migrate into spiral arteries and contribute to uNK cell-initiated tissue re-modelling within the decidua and the upper part of the myometrium.*

AE, amniotic epithelium; CCT, cell column trophoblast; DF, decidual fibroblast; EB, embryoblast; EM, extraembryonic mesoderm; eCTB, endovascular cytotrophoblast; GC, giant cell; ICM, inner cell mass, iCTB, interstitial cytotrophoblast; LUE, luminal uterine epithelium; L, lacunae, pF, placental fibroblast; PS, primitive syncytium; pV, placental vessel; SA, spiral artery; S, syncytium; TE, trophectoderm; UG, uterine gland; uNK, uterine NK cell; UV, uterine vessel; vCTB, villous cytotrophoblast.

(Knöfler & Pollheimer 2013)

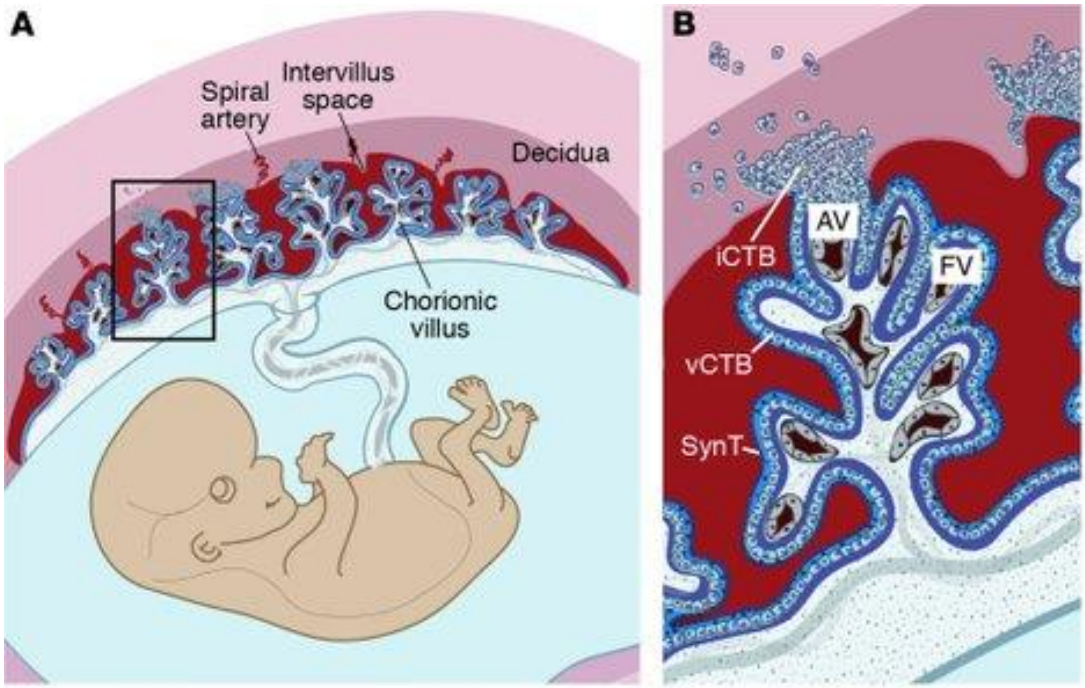


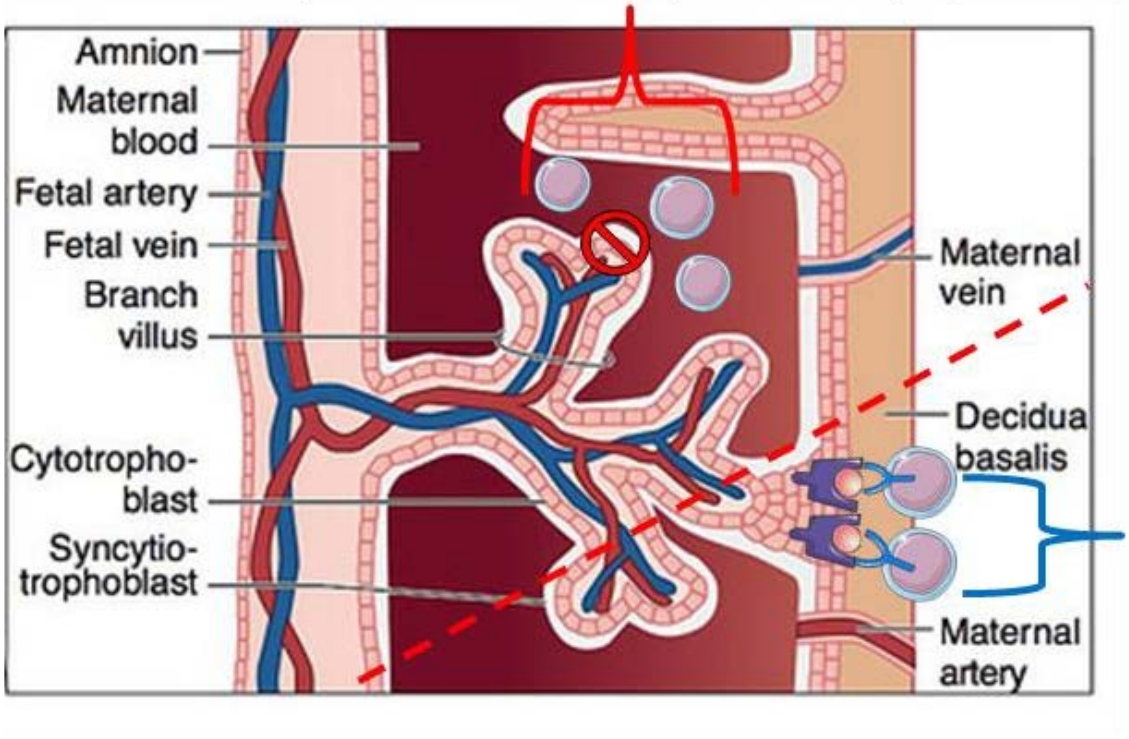
Figure 1. 4 Later stages of placentation and decidualisation.

***A**) Decidual spiral arterioles perfuse the chorionic villi that line the intervillous space. **B**) In floating villi (FV), a continuous layer of multinucleated syncytiotrophoblast (SynT) interfaces with maternal blood. Beneath lies a progenitor population of mononuclear villous cytotophoblast (vCTB). At the uterine wall, invading cytotrophoblast (iCTBs) differentiate along the invasive pathway to form anchoring villi (AV) along with formation of the decidual layer*

(Zeldovich & Bakardjiev *2012*)

It is important to address the sites at which the maternal immune system interacts directly with the placenta. The first site, which is concentrated on throughout this thesis, is within the decidua. It is at this site that immune cells including NK cells and T cells interact with invading extravillous trophoblast. This interaction allows for comprehensive engraftment of the placenta to uterine tissue and facilitates alterations to maternal spiral arteries to provide maternal blood to the intervillous space. Extravillous trophoblast express a restricted repertoire of HLA class I molecules, HLAC-C, G and E. Allo-reactive responses directed against extravillous trophoblast by T cells within the decidua will therefore be governed by interactions with these MHC molecules. The second site where maternal leukocytes can interact with the placenta is within the inter villous space. Maternal leukocytes are transported to the intervillous space through the supply of maternal blood via maternal spiral arteries, flooding the intervillous space. T cells within the intervillous space should not be able to interact or elicit an allo-reactive response to floating placental villi, as syncytiotrophoblast lack any MHC molecules. Figure 1.5 illustrates the differences in T cell interactions between these two sites. The interaction of maternal T cells with placental trophoblast is distinct at these two sites, and therefore any developmental or pathological outcome of these interactions will be discussed in context of this throughout the rest of this thesis.

Syncytiotrophoblast do not express HLA molecules. T cells that enter the intervillous space via maternal blood flow are incapable of alloreactive response to the syncytium.



Decidual T cells can interact with **HLA– C , G** or **E** expressed by Extra villous Trophoblast. Potential alloreactivity however restricted to specific fetal HLA molecules.

Figure 1. 5 T cell interaction with fetal tissue at the decidua and intervillous space.

Extravillous trophoblast express specific repertoire of HLA Class I molecules, HLA-C, G or E. Decidual T cells will only be able to elicit a response to antigens restricted to HLA molecules. Syncytiotrophoblast do not express HLA Class I therefore T cells in the intervillous space cannot be induced to a fetal specific response.

1.7. Immune modulation and immune tolerance during gestation

The mechanisms that control maternal immune tolerance have not been fully elucidated, however, the consensus is that a combination of the maternal immune system, hormones, pregnancy cytokines and chemokines as well as cellular interactions of the fetal trophoblast play an important role. These mechanisms have a direct impact on pregnancy success and the quality of outcome.

From a historical perspective, Peter Medawar attempted to put forward his theories and hypothesis of why the semi-allograft fetus was not rejected by the mother (Medawar 1953; Ribatti 2015) . The three key aspects postulated for this immune modulation were: the inertness of the maternal immune system; the immaturity of fetal antigens; and the physical separation of the mother and fetus. Today, these early hypotheses have been largely disproven. The fetal trophoblast of the placenta is in direct contact with maternal blood, and there is constant transfer of reactive antigens to T cells of the maternal immune system (Zenclussen et al. 2007). Pregnancy is an active adaptive mechanism towards tolerance of the semi-allogeneic fetus, both locally at the intrauterine interface and in the peripheral immune system (Khosrotehrani et al. 2005).

During pregnancy the maternal immune system undergoes several phenotypic changes that coincide with the growth of the semi-allogeneic fetus. Whether these

changes are necessary for the tolerance of semi-allogenic tissue is not fully understood. The syncytium of floating villi is devoid of MHC Class I, therefore it is theoretically impossible for a fetal specific immune response to occur within the intervillous space (see Figure 1.5). T cells could only mount an allo-reactive response if there is a breakdown of the syncytium during growth and shedding of the trophoblast layers, therefore providing access to fetal structures within the villi (potentially in the case of V.U.E). Indeed T cells found in the intervillous space may provide a protective role against bacterial infections of the placenta, rather than being a source for an anti-fetal response. Solders et al. (2017) demonstrated that human placental intervillous blood has an enriched population of MAIT T cells, a subset of T cell that recognizes microbial derived vitamin B metabolites, providing potential antimicrobial responses.

Despite the lack of MHC class I on syncytium, it is well documented that immunological changes occurring at the maternal-fetal interface is initiated with an inflammatory environment which is thought to allow for implantation (Dimitriadis et al. 2005). This process is followed by progression towards an anti-inflammatory profile followed by a switch towards an inflammatory environment again in order to facilitate parturition (Christiaens et al. 2008).

The earliest stage at which the maternal immune system is potentially primed for fetal tolerance is by the exposure to paternal semen. In the female reproductive tract, seminal plasma ellicit an inflammatory response and secretion of regulatory cytokine from cervical cells (Sharkey et al. 2007). The first critical step in successful pregnancy is implantation which requires constant communication between the embryo and the endometrium. During implantation there is an excess of cytokines, growth factors and hormones released from both fetal cells and maternal cells to initiate comprehensive implantation and decidualisation (Norwitz et al. 2001). Extravillous trophoblast facilitate the development of the decidua, and do express MHC Class I HLA-C, G and E. If an adaptive immune response was to develop at the maternal-fetal interface it would be based on this interaction with the extravillous trophoblast (Figure 1.5). Indeed the relative number of immune cell subsets in the decidua changes over time of gestation to allow for implantation, decidualisation and growth of the placenta. At the beginning of pregnancy the decidua is predominantly populated by uterine NK cells which interact with HLA-C and is critical for comprehensive implantation and decidualisation. As gestation progresses the NK cells populations decrease and at term the decidua has subsets of macrophages, T cells and granulocytes (Gomez-Lopez et al. 2010). The original dogma for fetal tolerance during pregnancy was the general hypothesis of Th1 and Th2 balance. During pregnancy it was

thought that there was a shift to a more modulatory Th2 environment from a Th1 response (Wegmann et al. 1993). However, this does not fully explain the complexity of maternal fetal immune interaction. Indeed, studies in mice have shown IL-4 null mutations have normal pregnancies (Svensson et al. 2001). In addition, Th1 associated cytokines are important in implantation, therefore the phenomenon of fetal tolerance during pregnancy is more complex than a shift from one cytokine environment to another (Trowsdale & Betz 2006). As with intervillous T cells, the importance of the decidual T cells is not fully understood, and a shift towards Th2 cytokine anti-inflammatory profile might be a result of tissue repair environment that adapts to the growing placenta rather than to specifically limit a Th1 type anti-fetal tissue response.

1.8. Peripheral immune changes

Maternal peripheral changes of the immune system during pregnancy deviate towards a more tolerogenic state. Whether the peripheral change is a result of localised change at the decidua or whether peripheral immune tolerance in itself is an adaptive response is not yet fully understood.

Murine studies have demonstrated that female pregnant mice accept engraftment of paternal allogeneic tumor tissue during pregnancy more readily than non-pregnant mice

(Adams et al. 2007). Immune communication via cytokines and chemokines play an important role in fetal tolerance, both systemically and locally (Robertson et al. 2011). It is also known that elevated levels of hormones, including progesterone, play an important role in maintenance of pregnancy. Progesterone induces progesterone-induced blocking factor (PIBF) which is expressed by T cells and has been shown to inhibit NK and Th2 activation in mice (Blois et al. 2004).

The main hypothesis of CD4+ T cells and their role in maintenance in pregnancy is that there is an establishment of a Th2 bias and a down-regulated Th1 response (Wegmann et al. 1993). It has been shown in murine models that TNF-α and IFN-γ can induce miscarriage; however, this can be ameliorated with the administering of Th2 secreted cytokine IL-10, which has an anti-inflammatory effect (Chaouat et al. 1995).

T regulatory cells are of great importance for normal pregnancy and fetal tolerance. Studies in mice have shown that deletion of T regulatory cells cause low implantation rates and high fetal reabsorptions (Shima et al. 2010).

In humans it is well documented during pregnancy autoimmune conditions, such as rheumatoid arthritis where Th1 has a role in the pathophysiology of the condition, improve during pregnancy (Østensen & Villiger 2002; de Man et al. 2008), whilst conditions such as systemic lupus erythematosus, where a Th2 immune response is involved, increase in

severity. Furthermore, a Th2:Th1 shift causes reduced cell-mediated responses, which is potentially responsible for increased susceptibility to influenza and leprosy where intracellular pathogenesis is primarily involved.

Leukocyte count is elevated during pregnancy and studies have shown there is innate activation (Sacks et al. 2003). Lissauer et al (2012) have also shown potential for adaptive immune responses towards male histocompatibility antigen, i.e.HY fetal antigens, during pregnancy. In pre-eclampsia the innate immune response is altered when compared to healthy pregnancy, which is related the systemic pathogenesis of pre-eclampsia (van Nieuwenhoven et al. 2008).

Several studies conducted on pregnant women verses non-pregnant controls have demonstrated an increase in IL-4 and IL-10 with a concurrent decrease in IFN-γ and IL-2 by ELISA and flow-cytometric experiments. This observation has been supported by analysis of mRNA levels where IL-4 mRNA has been shown to peak at month 7 with a decrease in levels of IFN-γ (Tranchot and Diallo et al. 1997). Th17 cells have a role in several autoimmune diseases and immune based disorders, and also in complications during pregnancy. It has been shown that there are higher levels of Th17 cells in preeclampsia and recurrent miscarriages compared to healthy pregnancies, and that this also coincides with a decrease in CD4+ T regulatory cells (Santner-Nanan et al. 2009; Lissauer et al. 2014).

In humans it has been shown that the peripheral levels of T regulatory cells are higher in pregnancy and that low numbers during gestation are associated with miscarriage and preeclampsia (Sasaki et al 2007).

1.9. Decidual immune changes

1.9.1. Decidual effector T cells

Both murine and human studies have shown that T cells are critical for fetal tolerance, and reduced levels at the decidua can lead to pregnancy complications (Guerin et al. 2009). In humans, it has been shown that there are higher numbers of Th17 cells in the decidua of spontaneous abortion, however, the function of these subsets in tolerance at the maternal interface has not yet been elucidated (Wang et al 2010).

At the human maternal interface, expression of IL-4, IL-10 and m-GSF by T cells at the decidua is largely associated with successful pregnancy (Piccinni 2006). The localised tissue environment contributes to Th2 cytokine production, with IL-4, IL-6 IL-10 and IL-13 being expressed by trophoblast tissue (Dealtry et al. 1998). Also, co-culture of T-cells with trophoblast cells results in an increase of Th2 regulatory transcription factors GATA-3 and STAT-6, along with a reduction of Th1 STAT-4 regulatory transcription factor (Liu et al. 2011). The anti-inflammatory effects of IL-4 and IL-10 results in an inhibition of Th1 cells

and macrophages, as well as inhibition of inflammatory cytokines such as TNF-α. This allows for cellular tolerance of the fetal-allograft. Despite this anti-inflammatory environment, Tilburgs et al (2010) demonstrated that in human decidual tissue, there are significant populations of differentiated effector memory CD8+ T cells, however their specificity was not identified.

During labour, there is a shift from the immune tolerance equilibrium to a Th1 inflammatory response at the maternal-fetal interface, which allows parturition. Progesterone is an important hormone for maintenance of pregnancy and is at a high level throughout pregnancy. However, at parturition, progesterone and IL-10 levels being to decline steeply, which, in turn, allows for an inflammatory response to occur (Zakar & Hertelendy 2007). At the decidua, amnion and chorion site there is an increase in IL-8, MIP-1α MIP-1β and G-CSF and TNFα during parturition, all of which would be detrimental during the maintenance of pregnancy. These cytokines are associated with macrophages and neutrophils, which are involved in tissue degradation and phagocytic cell death. Interestingly, these cytokines are also detectable in cases of preterm labour, indicating a labour-induced inflammatory response (Leong et al. 2008).

1.9.2. Decidual T regulatory cells

CD4+ T regulatory cells are deemed to play a critical role in immune modulation at the maternal-fetal interface (Aluvihare et al. 2004a). Murine studies have demonstrated that recruitment of T regulatory cells depends on the time of intercourse in respect to the menstrual cycle as well as hormonal levels (Robertson et al. 2013).

Induced CD4+ T regulatory cells are generated upon stimulation of high affinity cognate TCRs by TGFβ and retinoic acid (Chen et al. 2003). These are distinct from thymic T regulatory cells, where it has been observed that specific induction of enhancer of Foxp3, CNS1 and retinoic acid receptor binding sites facilitate Foxp3 and differentiation in peripheral T regulatory cells (Zheng et al. 2010). It has been demonstrated that blocking CNS1 in a mouse model abrogates peripheral T regulatory cells but not thymic T regulatory cells, and induces abortion of allogeneic pregnancies (Robert M. Samstein et al. 2012). Despite the importance of peripheral T regulatory cells in the maintenance of pregnancy during gestation, it has been demonstrated that thymic T regulatory cells mediate the implantation of mice embryos (Chen et al. 2013). Therefore, it is suggested that both thymic and peripheral T regulatory cells play a dual role in establishment and maintenance of pregnancy.

Regulatory T cell specificity in pregnancy has only been demonstrated in mouse models thus far, principally by Rowe et al. (2012). They were able to demonstrate that transgenic mice expressing a surrogate fetal antigen, were able to generate peptide specific T regulatory cells and, furthermore, accumulate these throughout gestation and post-partum. These T regulatory cells were furthermore able to proliferate at an

accelerated rate upon subsequent pregnancies with the same partner (Rowe et al. 2012). However, it is not known whether these peptide specific T regulatory cells were generated within the decidua, or in the periphery via circulating shed trophoblast debris or exosomes.

Human studies have shown that peripheral circulation and decidual levels of T regulatory cells peak midway through gestation (Somerset et al. 2004). Studies performed by Saito et al. (2005; 2007) demonstrated the presence of FOXP3 mRNA in endometrial biopsies during the menstrual cycle, and that these levels are decreased in patients with infertility complications. Additionally, it was found that T regulatory cells peak at the late follicular phase and that women with recurrent spontaneous abortions had reduced numbers of T regulatory cells. Furthermore, changes in T regulatory cell populations were not significant during the menstrual cycle. These lower levels of T regulatory cells were found to be similar to those of post-menopausal women, and their suppressive capacity was both reduced and deemed functionally inept at immune regulation.

In the context of cytokine levels, reduction in T regulatory cell numbers is not correlated with reduced levels of IL-6, IL-4, IL-10, TNFα IFN-γ or GM-CSF and, therefore, it is suggested that IL-27 plays a major role in regulation of T regulatory cells during gestation (Wang et al. 2013). In addition to hormonal changes and cytokines, placental trophoblast produce high levels of TGF-β, which is able to recruit and induce peripheral CD4+ T regulatory cells (Ramhorst et al. 2012). Tilburgs et al. (2009) also suggest that due to reduction of peripheral T regulatory cells in matched maternal PBMCs compared to an increase in term decidua, there is specific recruitment to this site.

Further evidence of the importance of T regulatory cells in maintenance of pregnancy is their significance in pre-eclampsia. Several studies have indicated that imbalance and reduction of T regulatory cell levels correlate with pre-eclampsia (Toldi et al. 2008; Hsu et al. 2012).

1.9.3. Uterine NK cells

Uterine NK (uNK) cells constitute 70% of lymphocytes at the maternal–fetal interface during early pregnancy and are deemed to be the most important cell for successful pregnancy. Their interaction with other decidual leukocytes points to key functional cross-talk during gestation. They are phenotypically different from peripheral blood NK cells and have an important functional role in the maintenance of fetal tolerance and decidualisation. uNK cells are characterised as being CD56 high CD16 negative with a loss of cytotoxic ability (Moffett-King 2002). Despite being rich in granzyme and perforin, they are cytotoxically weak and do not kill trophoblast *in vivo* (Veljkovic Vujaklija et al. 2013). They produce VEGF, placental growth factor (P1FG) and angiopoietin-2 (ANG2), all of which are important for trophoblast invasion of uterine tissue and subsequent vascularisation (Li et al. 2001). Interestingly, IFN-γ, a cytokine associated with potential detrimental cytotoxic activity, is also produced by uNKs and is critical for spiral artery remodelling (Ashkar et al. 2000). IFN-γ along with IL-10 are thought to be required for maintaining immune

equilibrium as well as remodelling of decidual arteries (Vigano 2001). Indeed, studies have shown that mice deficient for NK cells have abnormal spiral artery modelling (Hofmann et al. 2014) and that in humans, pregnancy complications such as pre-eclampsia and fetal growth restriction are often associated with poor spiral artery formation and shallow trophoblast invasion (Pijnenborg et al. 1998). Important receptor interaction for uNK cells is the interaction of killer-cell immunoglobulin-like receptors (KIR) and fetal HLA-C ligand. Expression of HLA-C stops missing self activation of NK cells and it has been shown that non-compatible KIR and fetal HLA-C have an adverse effect on pregnancy (Rodrigues et al. 2013). This crucial allo-recognition of trophoblast HLA-C and G elicits decidual angiogenic cross talk.

1.9.4. Uterine Dendritic cells

Along with uNKs, uterine dendritic cells are integral during early pregnancy and angiogenesis (Pollard 2008). In mice, when DCs are exposed to seminal and fetal antigens they elicit migration of other immune cells to the mucosa (Blois et al. 2014). uDCs are potent antigen presenting cells, that interact with trophoblast cells and recognise allo-antigens during implantation and decidualisation (Tagliani & Erlebacher 2011). Despite the potential of initiating an allogeneic response, in murine models it is suggested that the

decidua itself “entraps” uDCs, making them unable to traffic to lymphoid organs and thereby limiting their antigen presenting capability (Collins et al. 2009a). Dendritic cells are skewed towards a tolerogenic function following secretion of granulocyte colony stimulating factor (GCSF) and granulocyte macrophage colony stimulating factor (GMCSF) by immune cells, and can recruit, generate and differentiate T regulatory cells via cytokine and IDO (Chen 2011). Depletion of dendritic cells and the maternal fetal interface in mice has shown to impair implantation (Plaks et al. 2008). It should be noted however than in human decidua, uterine dendritic cells are a very rare population, making up 1.7% of total CD45+ cells (Gardner & Moffett 2003)

1.9.5. Decidual Macrophages

Macrophages derive from myeloid progenitors found in bone marrow, which differentiate into promonocytes and then circulating monocytes. These cells next migrate transendothelially and differentiate into macrophages at tissue and organ sites which is where they play a crucial role in presentation of aberrant antigen peptide complexes to T cells. Upon fertilisation, macrophages traffic to the early decidua and are found to interact with trophoblast cells, which are partly responsible for macrophage chemotaxic signalling (Ivanisevic et al. 2010). Macrophages make up 10% of total leukocytes at the decidua, and

are seen to increase towards the end of gestation (Gomez-Lopez et al. 2010). Decidual macrophages in healthy pregnancy appear to exhibit an immunomodulatory and tissue remodelling role. Immunosuppressive molecules expressed by these "M2" type macrophages include IL-10, and IDO, an enzyme of tryptophan, which is crucial for T cell metabolism and function (Nagamatsu & Schust 2010). Indeed studies have shown that a reduced population of M2 macrophages at the decidua can influence pathologies of pregnancies, such as pre-eclampsia (Schonkeren et al. 2012).

Figure 1.6 demonstrates how the majority of cell types discussed accumulate throughout gestation.

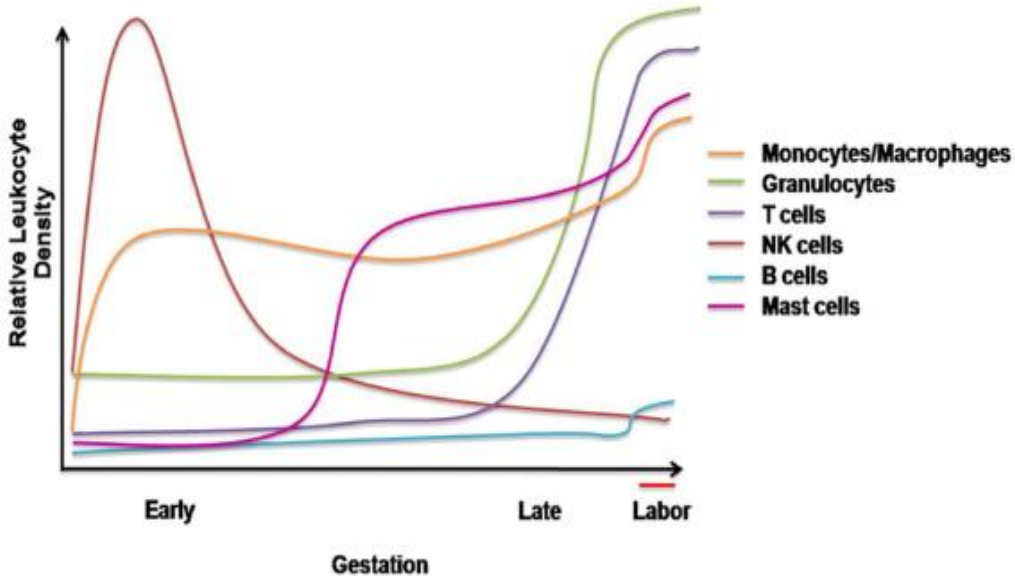


Figure 1. 6 Timing of the presence of leukocytes at the fetal-maternal interface during gestation

(Gomez-Lopez 2010).

1.10. Fetal tissue adaptations

In addition to maternal adaptions, fetal cells and tissue also have a direct role in immune tolerance during pregnancy. One of the most important aspects of fetal derived immune tolerance is the absence of expression of MHC class I molecules on villous cytotrophoblast and syncytiotrophoblast cells. Due to their absence, there is direct prevention of a class MHC class I presentation to T cells which, in turn, prevents the recognition of allo-antigens (Munoz-Suano et al. 2011). However, HLA-C is expressed on distinct extravillous trophoblast which are responsible for invasion of the uterine wall, forming the decidua, and are essential to avoid non-self cytotoxic activation of NK cells and CD8 cytotoxic cells. HLA-G is also a MHC class I molecule expressed on extravillous trophoblast tissue, however, it is non-classical. HLA- G possess low polymorphism and can modulate allo-reactivity by down-regulating CD8 and CD4 expression, thereby assisting in generation of T regulatory cells (Gregori et al. 2009). T regulatory cells are able to secrete IL-10 and TGFβ that can supress cytolytic activity in a contact independent manner. Increased levels of soluble HLA-G has also been associated with increase in IL-10 and graft survival (Naji et al. 2007). This suggests that HLA-G is able to promote tolerance locally at the placenta and also in the maternal peripheral immune system. HLA-G's inhibitory receptors are found on NK cells, APCs, and T cells and consist of inhibitory receptor Ig-like 2 (ILT-2), ILT-4 and

KIR2Dl4. Interaction with these receptors grants protection from allogeneic responses from NK and cytotoxic T cells (Hunt et al. 2005).

Trophoblast cells also express Fas ligands, which may bind to Fas receptors on T cells and induce T cell apoptosis. This mechanism has been shown to be important in modulating responses of CD8+ HY specific T cells in mouse models of pregnancy (Vacchio & Hodes 2005). IDO is also expressed by trophoblast cells. This reduces interaction of tryptophan with T cells which in turn inhibits T cell proliferation (Mellor & Munn 2004). In addition to apoptosis inducing Fas ligand, trophoblast cells also express tumor necrosis factor related apoptosis inducing ligand (TRAIL), which consequently induces apoptosis upon binding (Phillips et al. 2001).

Figure 1.6 illustrates mechanisms and contributing factors from the decidual cells and trophoblast cells that ensure the immunomodulation of the maternal-fetal interface.

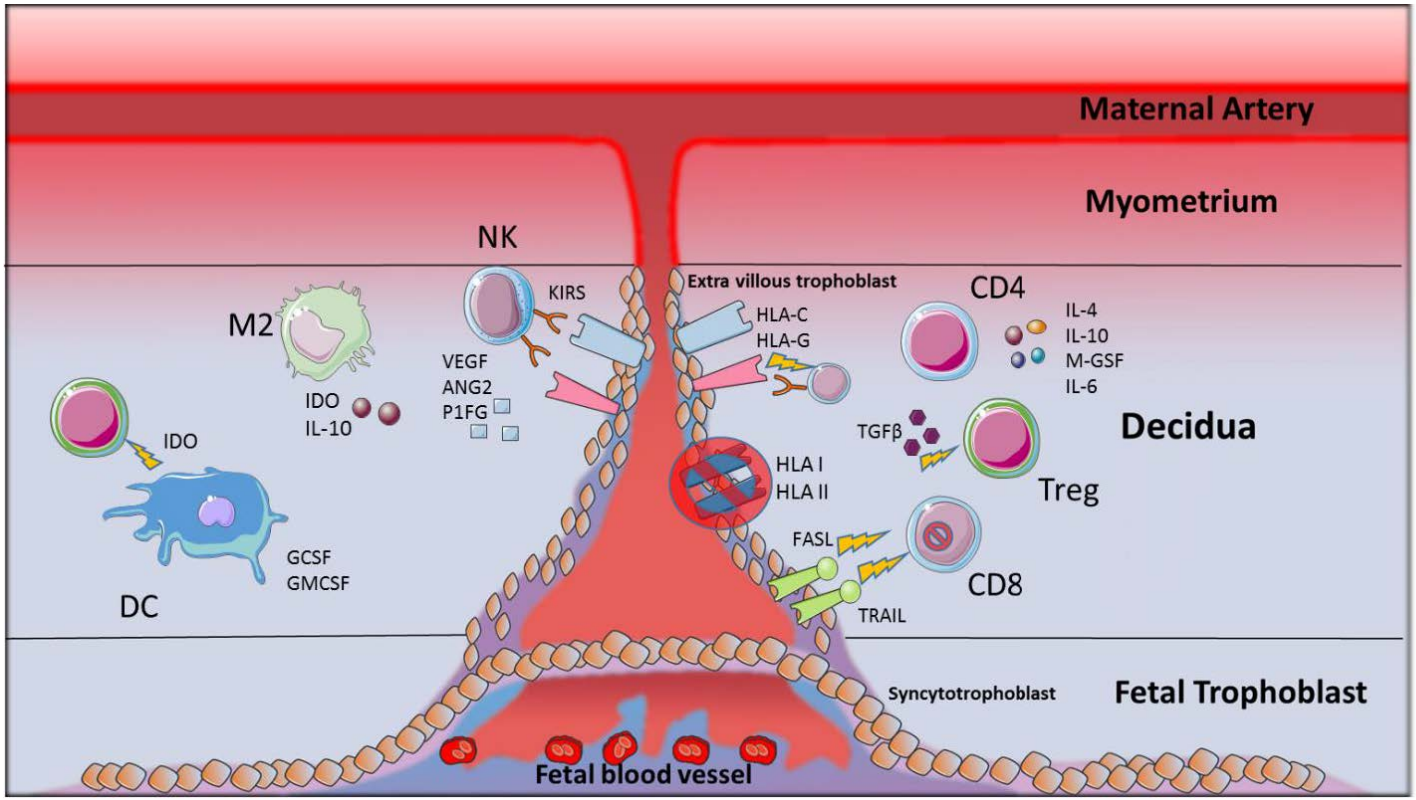


Figure 1. 7 Immune modulation at the maternal-fetal interface

At the decidua, there are multiple mechanisms to ensure that the placenta is tolerated by the maternal immune system. DCs express GCSF and GMCSF and also IDO, which induces CD4+ T regulatory cell formation,however low numbers of DCs in human placenta. M2 macrophages express immune suppressive IDO and IL-10. NK cells express VEGF, ANG2 and P1FG and interact with HLA-C present on extravillous trophoblast through KIR receptors which avoids non-self activation. HLA-G present on extravillous trophoblast interacts with inhibitory receptors on T cells, and reduction in other HLA class I and II molecules inhibits adaptive immune responses. Apoptosis inducing ligands FASL and TRAIL are also expressed on trophoblast cells. Placental derived TGFβ induces T regulatory cells and T cell derived IL-4, IL-10, M-GSF and IL-6 ensures an immunomodulatory

1.11. T cells and responses to fetal antigen

Allo-reactive responses against fetal tissue can be conducted towards mismatched non-self MHC or minor histocompatibility antigens (mHAgs) (Tafuri et al. 1995; Goulmy 2006). Whereas MHC are involved in the introduction of peptides to immune cells, mHAgs are proteins that often present with different alleles between individuals due to deletions, single nucleotide polymorphisms (SNP) and/or the antigen being present on the Y chromosome, and can initialise an immune response (den Haan et al. 1998).

mHAgs are presented to CD4+ and CD8+ T cells via MHC class I and class II molecules as previously discussed in section 1.2. It is important to point out at this stage that due to cross-presentation by DCs, CD8+, as well as CD4+ cells, can be activated toward exogenous derived antigens provided by fetal matter despite the lack of MHC class I expression on trophoblast cells (Moldenhauer et al. 2009). Indeed, it has been shown that placental debris in the maternal immune system can be processed by DCs, presented to CD8+ T cells, which subsequently instigates an immune response in the maternal peripheral blood (Erlebacher et al. 2007). However, only HLA-C restricted antigen specific T cells would be able to carry out an allo-reactive response to placental tissue if trafficked to the decidua. Additionally, as syncytium of floating villi do not express MHC Class I, fetal specific T cells trafficked to the intervillous space would be unable to elicit an immune response (See figure 1.5)

The role of mHAgs mismatch and the concurrent immune response was first demonstrated in transplantation studies. The cells were initially discovered for their role in

modulating graft versus host disease in bone marrow transplantation therapy. Goulmy et al. (1976) showed how a HLA matched transplant was rejected in a male donor to female recipient transplantation. It was shown that the female T cells could lyse HLA matched male donor cells, thus indicating a mHAg on the Y chromosome (Goulmy et al. 1977). Additionally, mHAgs were shown to elicit graft versus host disease, where donor female cells were able to lyse male recipient cells (Goulmy et al. 1983). These findings influenced the hypothesis that mHAgs may play a role in the outcome of pregnancy. In further support of the hypothesis one observation includes the instance where females with previous male pregnancies elicit graft versus host disease in transplant recipients, which was potentially due to maternal memory immune response to HY mHAgs, (Flowers et al. 1990). Holland et al (2012) have shown several fetal mHAgs are expressed in human cord blood and trophoblast cells, including HY proteins RPS4Y1, DDX3Y and KDM5D (Holland et al. 2012). In addition, murine studies (James et al. 2003) have shown that maternal CD4+ and CD8+ T cells are able to elicit responses to endogenous HY antigen. Given the proximity of maternal blood to syncytiotrophoblast cells throughout pregnancy, this provides a substantial source of potential activation. However, previously discussed mechanisms such as T regulatory cells, T cell deactivation, MHC class I and II restriction on trophoblast, and limitation of maternal dendritic cells to migrate from the pregnant uterus to conduct antigen presentation (Collins et al. 2009a), suggest why fetal-specific responses are not generated to cause widespread fetal rejection (Kahn & Baltimore 2010; Nancy et al. 2012).

Despite this, fetal microchimerism provides an alternative source for allo-reactivity towards fetal antigens. Fetal microchimerism describes fetal cells that are trafficked to the

maternal peripheral blood supply, which in turn, provide a source of fetal mHAgs to the peripheral immune system (Evans et al. 1999). Syncytiotrophoblast is constantly shed during pregnancy where there is renewal of cytotrophoblast forming the placental villi. Studies have shown that up to 1g per day of this tissue is shed from a term placenta, and therefore provides an additional supply of fetal mHAgs (Burton & Jones 2009). Studies in humans have shown that following pregnancy there is a generation of responses to mHAgs which persist for long periods of time post-pregnancy, (Verdijk et al. 2004). More recently, Lissauer et al (2012), demonstrated that CD8+ T cells specific to paternal HY antigens were present during pregnancy in maternal peripheral blood in pregnancies with a male fetus, and that these cells were capable of producing IFN-y (Lissauer et al. 2012). The epitope that was detected in this case was derived from the HY protein KDM5D. The fetal antigens are assumed to be cross-presented by maternal, or even potentially by fetal microchimeric antigen presenting cells, such as monocytes and macrophages (Seavey & Mosmann 2006; Moldenhauer et al. 2010; Moldenhauer et al. 2009; Erlebacher et al. 2007). Additionally, scavenging DCs can phagocytose fetal antigen in the periphery, and traffic to lymph nodes where the antigen is presented (Taglauer et al. 2010). It is therefore possible that an allo-reactive fetal specific response is generated away from the immunodulatory environment of the decidua.

The role of fetal mHAg and its clinical impact on immune dysregulation during pregnancy is deemed to be highly correlated. One example is that placentas in pre-eclamptic pregnancies show upregulation of minor histocompatibility antigen HA-1. It has been suggested that this introduces an additional load of antigenic immune response and contributes to an overall immune imbalance involving increased proinflammatory effects

which are characteristic of pre-eclampsia (Kronborg et al. 2011). mHAgs have also been suggested to play a role in recurrent miscarriage. Epidemiologically it has been shown that the likelihood of secondary miscarriage is increased if the first pregnancy was male (Ooi et al. 2011). Additionally, women that have had secondary recurrent miscarriage possess upregulated MHC class II molecules capable of presenting HY mHAgs (Christiansen et al. 2010). This suggests that recurrent miscarriage can be linked to the generation of Y-chromosome linked T cell memory during the first pregnancy, which later affects the outcome of subsequent pregnancies resulting in a male fetus (Christiansen et al. 2011).

Murine models of pregnancy have provided a greater understanding of the trafficking of fetal cells, immune function at the placenta and antigen specific allo-reactivity. However, due to specific anatomical and immunological differences, such as murine trophoblast expressing MHC class I later in gestation (Jiang & Vacchio 1998), there is a limit to how much murine pregnancy immunology is translatable. For instance, murine studies have suggested that epigenetic silencing of key chemokines in the decidual stroma may limit T cell access to the decidua and thus their activation to allo-antigen is restricted (Nancy et al. 2012). However Villitis of Unknown Etiology, a condition characterised by a high CD8+ inflammatory T cell response, is relatively common, even in healthy pregnancy. Tilburgs et al (2010) have also demonstrated differentiated CD8+ T cells to be present in the human decidua. Importantly, despite examples of fetal specific T cells being detected in the human periphery, no studies, to date, have demonstrated fetal specific T cells *in situ* at the maternal-fetal interface. This project aim to address this, and suggest that despite evidence in murine models that fetal specific T cells are limited in their trafficking to the decidua, this is not necessarily the case in a human setting.

1.12. Co-stimulatory factors and immune checkpoint proteins in pregnancy

For effective T cell activation to occur, two signals are required via APCs. Firstly, there is the antigen specific interaction of the TCR and the MHC-peptide complex. Secondly, an antigen independent co-stimulatory signal is required, which involves specific ligands expressed by APC, interacting with their cognate receptors on T cells. In the absence of co-stimulatory interaction, T cells can enter a state of anergy or apoptosis (Mueller et al. 1989). Co-stimulatory signalling pathways can be both positive in that they promote T cell activation or negative, where T cell responses are mediated and tempered. Studies have shown that certain negative co-stimulatory interactions play a role in the maintenance of pregnancy. Figure 1.7 summarises the negative co-stimulatory molecules and their cognate ligand discussed in the following sections. Negative co-stimulatory molecules can also be referred to as immune checkpoint proteins or exhaustion markers. For simplicity, these molecules will be collectively referred to as immune checkpoint proteins throughout this thesis.

1.12.1. PD-1

Programmed death-1 receptor (PD-1) and its ligands PD-L1 and PD-L2 are one such co-stimulatory pathway, which has been demonstrated to deliver inhibitory signals and balance T cell activation (Sharpe et al. 2007). Binding of PD-1 to its ligand enacts a state of

"exhaustion" of T cells in tumour microenvironments (Driessens et al. 2009) and it is seen to elicit a similar suppressive environment at the maternal-fetal interface.

Despite being also expressed during thymic development, PD-1 is expressed classically upon cell activation and has been found to be present on DCs, monocytes, and B cells, as well as T cells (Keir et al. 2008). Additionally, cytokines IL-2, IL-17 and IL-15 have been found to induce PD-1 expression (Kinter et al. 2008). PD-L1 is expressed on a broad range of haematopoietic cells as well as non-haematopoietic cells and tissues, including epithelial cells and the placenta. PD-L1 expression can be upregulated in the presence of inflammatory cytokines such as IFN-γ, TNFα and can therefore establish an activation-mediation feedback loop.

Standard T cell activation involves TCR signalling and CD3/CD28 co-stimulation, which in turn activates phophoinosotide 3-kinase (PI3K) followed by protein kinase B (AKT). Whilst this pathway elicits cytokine secretion and T cell proliferation, PD-1 inhibits PI3K and the downstream AKT activation. Also PD-1 reduces zeta-chain associated protein kinase (ZAP)-70 expression and inhibits protein kinase θ phosphorylation and extracellular signal regulated kinase (Erk) activation. This results in reduced cytokine production, proliferation and survival signalling (Zhang et al. 2004; Parry et al. 2005; Gianchecchi et al. 2013). In addition to modulating effector T cell activation, PD-1 signalling is critical for the development of T regulatory cells. Studies involving blocking of PDL-1 on APCs results in minimal development of T regulatory cells, and instead can cause an increase in Th17 cells in pregnant mice, which affects maternal-fetal tolerance (Francisco et al. 2009; D'Addio et al. 2011). As both APCs and T regulatory cells can present PD-L1 in the decidua, the

combination of these inhibitory signals is crucial for reducing the magnitude of T cell allo-reactive responses. Decidual stromal cells constitutively express PD-L1 and their contact with decidual T cells is suggested to be a crucial in modulating T cell activation at this site. Indeed, blocking PD-L1 on decidual stromal cells resulted in an increase of CD4+ cytokine production *in vitro*. In addition to stromal decidual cells, PD-L1 is expressed on cytotrophoblast, and even more so on syncytiotrophobalst from the second trimester (Holets et al. 2006). However, their interaction with T cells may be inhibited due to MHC molecule down-regulation on trophoblast. Extra-villous trophoblast, and more specifically invading cytotrophoblast cells in the decidua, express HLA-C and HLA-G and a potential suppressive signalling pathway with decidual T cells is therefore possible. Macrophages make up 20 to 30% of total leukocytes in decidual tissue and only decrease at term. Despite specific immunomodulatory mechanism not being characterised between decidual T cells and macrophages, studies have shown that they can supress IFNγ secretion via PD-L1 and PD-1 interaction (Sayama et al. 2013).

As previously discussed in section 1.10, the Th2:Th1 skew at the maternal-fetal interface is seen as a crucial mechanism of fetal tolerance, in which PD-1 pathways may be involved. Several studies have demonstrated that blockade of PD-L1 in mice can lead to abortion, fetal reabsorption, and increase in Th17 proportion (Guleria et al. 2005; D'Addio et al. 2011). T regulatory cells constitutively express PD-L1, indicating that this co-stimulatory pathway is central to mediating Th1, Th2 and Th17 balance at the maternal-fetal interface. In humans, PD-1 expression on T cells is elevated in healthy pregnancy (Grozdics et al. 2014) and an increase in Th17, along with a decrease in T regulatory cells

(Darmochwal-Kolarz et al. 2013), suggests that PD-1 co-stimulatory pathways are crucial for the avoidance of pregnancy related pathologies.

1.12.2. Tim-3

T cell immunoglobulin domain and mucin domain 3 (Tim-3) is another co-stimulatory marker that has a suppressive or inhibitory effect on T cell activation. Its ligand is Galectin-9 (Gal-9), which engages downstream apoptotic signalling (Zhu et al. 2005). Tim-3 was originally identified on Th1 cells and was later found to be important in Th17 and CD8+ T cell modulation, where blockade of Tim-3 in mouse models resulted in an increased autoimmune responses (Sánchez-Fueyo et al. 2003). As with PD-1, Tim-3 has more recently been regarded as a crucial marker for tumour associated immune suppression (Sakuishi et al. 2011) and modulation of pregnancy (Li et al. 2014).

Gal-9 is expressed by T cells, B cells and macrophages as well as endometrial tissue during the decidual phase of gestation (Shimizu et al. 2008) and is regulated by the expression of IFN-γ. A feedback loop is initiated in an inflammatory environment, where Gal-9 expression is induced in T cells, B cells and macrophages, which leads to increased interaction with Tim-3 on Th1 cells, the major IFNγ producers, and subsequent apoptosis is induced. Furthermore, CD8+ T cells that express Tim-3 have shown reduced ability to clear virus and tumour cells (Fourcade et al. 2010). However, decidual CD8+ T cells that are Tim-3+ do not show a classical exhausted phenotype. Instead, they exhibit increased proliferative capacity along with secretion of anti-inflammatory cytokines (Meggyes et al. 2014). Subsequent studies in mice demonstrated that blockade of Tim-3 resulted in

decreased proliferation of decidual CD8+ T cells and a shift to IFN-γ secretion, which resulted in increased fetal loss (Wang et al. 2015). In addition, CD4+ T regulatory that express Gal-9, have been shown to have increased suppressive function (Wu et al. 2014). Although Tim-3+ T regulatory cells at the maternal-fetal interface have not been reported in the literature, one would suggest that Tim-3 Gal-9 signalling pathways play an important part in the this system, given the importance of T regulatory cells in the modulation of successful pregnancy.

1.12.3. CTLA-4

Another significant negative costimulatory molecule involved in mediating T cell responses is cytotoxic T-lymphocyte antigen-4 (CTLA-4). T cell activation often involves the binding of CD28 to CD80/86 molecules presented on antigen presenting cells. However, if CTLA-4 bind CD80/86 in this co-stimulatory signal pathway an inhibitory response is enacted (Boussiotis et al. 1996). Blocking of CD80/86 has led to long term allograft acceptance and also successful embryo implantation in abortion prone murine models (Jin et al. 2005). CTLA-4 was found to be significantly reduced in human decidual T cells in cases of miscarriage compared to normal pregnancy (Jin et al. 2009). In addition, the expression of CTLA-4 was also negatively correlated with IL-2 and IFNγ cytokine production. Human studies have also demonstrated that CTLA-4 is expressed on T regulatory cells stimulated by trophoblast (Svensson-Arvelund, Ratnesh B Mehta, et al. 2015). Despite the lack of direct studies into CTLA-4 and its function in modulation of pregnancy, it has been

suggested that that it is another important costimulatory marker in regulating inflammatory response at the maternal-fetal interface.

1.12.4. Lag-3

Lymphocyte activation gene-3 (Lag-3) is upregulated on activated CD4+ and CD8+ T cells and some NK cells (Triebel et al. 1990). Lag-3 is structurally similar to the CD4 co-stimulator receptor, and competes with this molecule for MHC class II binding. As CD8+ T cells do not interact with MHC class II it is thought that Lag-3 binds to alternative ligands on these cells, such as liver sinusoidal endothelial cell lectin (LSECtin) expressed on tumour cells (Xu et al. 2014). Lag-3's significance in modulating immune responses has been demonstrated in murine models. Here data show that Lag-3 deficient mice are unable to control T cell expansion following allo-antigen activation (Huang et al. 2004). In addition to effector T cells, T regulatory cells also express Lag-3, and it has been demonstrated that blockade of Lag-3 on these cells abrogates their suppressive function (Huang et al. 2004). As with CTLA-4 there is a lack of literature that directly address Lag-3 expression in maintenance of pregnancy, however, its role in tumour suppressive environments, expression on T cells to control unmitigated expansion and T regulatory cell function suggest that it may play an important part in maternal-fetal allo-reactive responses.

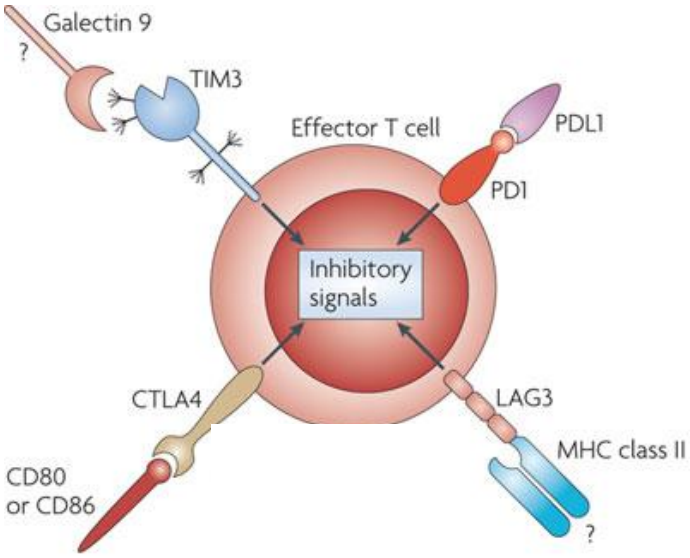


Figure 1. 8 Summary of inhibitory receptors and their cognate ligands

Effector T cells upregulate immune checkpoint proteins as PD-1, Tim-3, Lag-3 and CTLA-4 on their surface. Ligation of these receptors with PD-L1, Gal9, MHC class II molecules and CD80 or CD86, respectively, delivers inhibitory signals to the effector T cells and controls their inflammatory activity. (Rouse & Sehrawat 2010).

1.13. T cells and Pathologies of Pregnancy

There are many pathologies that affect the outcome of pregnancy involving various bacterial and viral infections and autoimmune diseases. However, for brevity I shall address pathologies that are most commonly associated with an immunological phenotype associated with the breakdown of immune modulation towards the placenta.

1.13.1. Pre-eclampsia and spontaneous abortion.

Pre-eclampsia is a syndrome which is often associated with first time pregnancies, but it can also occur in subsequent pregnancies. The condition is associated with high blood pressure, protein in the urine (proteinuria) and edema (Zhou et al. 2002). However, what clearly defines the onset of pre-eclampsia is the ineffective vascularisation of the maternal-fetal interface, which is why pre-eclampsia is also often associated with intrauterine growth restriction (Redman & Sargent 2005). The trophoblast cells identified in patients suffering pre-eclampisa, are characterised by their inability to express adhesion receptors that are needed for invasion of the uterine wall (Damsky & Fisher 1998). Invasion of the uterine wall is therefore shallow and spiral artery remodelling is incomplete. This results, ultimately, in less maternal blood bathing the placental villi and reduced gaseous and nutrient exchange.

One cell type that may be a crucial factor in preventing pre-eclampsia is T regulatory cells. Several studies have suggested that a reduced number of peripheral blood and decidual T regulatory cells are associated with pre-eclampsia and spontaneous abortion (Toldi et al. 2012; Sasaki et al. 2007; Wang et al. 2010). As a result of T regulatory

cell immunosuppressive capacity, involving expression of IL-10 and TGF-β these cells have been seen to limit effector T cell activity over the course of pregnancy, where more and more allo-antigen ultimately will be processed and presented. Without this limiting effect, Th1 and Th17 cells would induce detrimental cytotoxic responses effecting spiral artery remodelling. Indeed studies observed a correlation between an increased in Th17 cell frequencies with pathogenesis of spontaneous abortion (Wang et al 2010).

1.13.2. Villitis of Unknown Etiology

Villitis of Unknown Etiology (V.U.E.) is an inflammatory lesion of the placental villi, with an absence of any bacterial or viral source of inflammation (Kim et al. 2008). It is the most common of placental lesions, occurring in up to 15% of term pregnancies and is usually visualised during routine diagnostic histology with a higher incidence during 3rd trimester of gestation (Redline 2007). V.U.E is defined histologically by large T cell infiltrate, predominately CD8+, along with fetal derived Hofbauer macrophages (Kim et al 2008). V.U.E. was first recognised in the 1960's; however, it was only identified as not having an infective etiology in 1975 (Altshuler et al. 1975), and to this day, the exact inflammatory factor has not yet been discovered.

V.U.E is only diagnosed following histopathological analysis and exists in many variants depending on the pattern of inflamed trophoblast. Classically V.U.E. is characterised by inflammatory cell infiltrate of the placental villi, often incurring disruption

and destruction of the vascular structure and widespread deposition of fibrin at areas of necrosis. It differs from bacterial or viral villitis as it has a non-uniform inflammation of villi, with areas completely unaffected and maintaining normal histological architecture (Redline 2007). In addition, V.U.E commonly affects only the distal placental villi; however, less commonly it can also affect the chorionic plate and stem villi. As a result of infiltrating lymphocytes, macrophages, and granular cells there is often potential for obliterative fetal vascularopathy which has significant complications in pregnancy (Boog 2008). It is often described as occurring in up to 15% of term placenta, however, the reported range varies between 2% and 33.8% (Labarrere et al. 2005).

Cellular composition of V.U.E is described as predominantly maternal derived T cell lymphocytes (42%) with a higher proportion of CD8+, macrophages (54%) of both maternal and fetal origin, along with small numbers of B-cells (2%) (Kapur et al. 2004). Interestingly, despite a strong association of T regulatory cells in the maintenance and immunotolerance of the fetus, it has been shown that T regulatory cells are increased in V.U.E compared to normal placentas (Katzman et al. 2011).

Even though V.U.E. is relatively common occurrence in normal placentas the syndrome is associated with adverse pregnancy outcomes, including intrauterine growth restriction (IUGR) and small for gestational age (SGA) (Becroft et al. 2005), neurological

impairment (Redline et al. 2000), and still birth and perinatal mortality (Redline & Abramowsky 1985). As well as diagnosis of V.U.E it's severity, i.e. high grade and diffuse is also associated with adverse pregnancy outcome. More severe V.U.E is likely to reoccur during subsequent pregnancies with increased adverse effects and risk of intrauterine death (Redline et al 2000).

Despite peripheral, decidual and fetal mechanisms of maternal-fetal tolerance during pregnancy, it is widely considered that V.U.E is a form of allograft rejection, where the maternal–fetal tolerance has degraded to a level where there is significant cytotoxic lymphocyte recruitment and infiltrate (Taglauer et al. 2010). Indeed, it has been shown that women with autoimmune diseases, where there is already a breakdown of systemic and localised immunotolerance, there is an increased incidence of V.U.E. (Labarrere et al. 1986). Even though there is still no clear etiology of V.U.E., we can compile evidence from numerous studies of the immunology of the maternal-fetal interface to construct potential pathways for V.U.E to develop out of a number of immune abnormalities.

Whilst the antigenic etiology of V.U.E has not yet been defined, it is widely assumed that it is of fetal origin. Indeed, it has been shown that in peripheral blood, a maternal CD8+ T cell immune response to HY fetal antigens can be detected. These fetal specific T cells increase as pregnancy progress and have been shown to have functional ability and

cytotoxicity (Lissauer et al. 2012). A separate study has also shown that these fetal specific T cells persist following pregnancy for many years and increase following subsequent pregnancies (Piper et al. 2007). Due to V.U.E being characterised as a lesion comprising of predominately a CD8+ T cell infiltrate, it can be hypothesised that the maternal immune system is primed substantially to fetal antigens and that these traffic to maternal-fetal interface.

As previously mentioned, tolerance from the maternal immune system is required for a successful pregnancy, and of particular importance are T regulatory cells. This has been shown in both mouse models, where the absence of the T regulatory cells results in loss of pregnancy and in human tissue studies, where the cells have a predominant role in maternal allo-reactive response to fetal antigen modulation (Tilburgs et al. 2008a). Having said this, it has been shown that the numbers of T regulatory cells in V.U.E. tissue was comparatively higher than that of controls (Katzman et al. 2011). Whether these have a direct role in progression of V.U.E. or that they accumulate in response to a high inflammatory response in V.U.E. has not been studied.

During pregnancy the cytokine environment is skewed towards a Th2 profile of IL-4, IL-5 and IL-10. This in turn limits pro inflammatory Th1 associated cytokines IFNγ and TNFα and the cytotoxic potential of CD8+ T cells and macrophages (Walker & Abbas 2002).

However, studies have shown that both Th1 or Th2 dominant profiles have a detrimental effect on the outcome of pregnancy (Saito et al. 2010). This implies that the assumption that Th1 increases risk of V.U.E. whilst Th2 decreases cytotoxicty is unreliable, and that a more complex cytokine environment may play a part, involving Th17 cells, T regulatory cells and fetal and maternal derived macrophages. T regulatory cells can be generated by tolerogenic antigen presentation cells in presence of a diverse range of factors including IL-10, TGFβ GM-CSF and IL-4. In addition, Th17 cells and IL-17 are involved in autoimmunity and inflammation, and may therefore also have a role in V.U.E. Disruption of these cytokine environments may induce multiple pathways leading to loss of tolerance and an increased likelihood of cytotoxic activation, proliferation and infiltration (Guerin et al. 2009).

In addition to the role of inflammatory and tolerogenic cytokines involved in V.U.E., chemokines and cell trafficking are also of particular interest. Immunohistochemistry has demonstrated that the lymphocytes present in V.U.E. affected villi are of both maternal and fetal origin (Myerson et al. 2006) . Subsequently, unless there is significant homeostatic proliferation of activated T cells, there must be important roles for chemokine associated trafficking to inflammatory sites. Kim et al. (2009) performed microarray analysis of human derived V.U.E. tissue and normal placental tissue and were able to

demonstrate Th1 chemokines CXCL9 and CXCL10 were upregulated in V.U.E, as well as their receptor CXCR3 (Kim et al. 2009). Conversely, a separate study demonstrated that in normal decidual tissue there is epigenetic silencing in decidual cells of CXCL9, CXCL10, and it is hypothesised that T cell trafficking to the decidua is limited (Nancy et al. 2012). This might be purported as another mechanism of immunotolerance at the maternal-fetal interface. However, this analysis was carried out on a small sample size of mice and may therefore not reflect accurately chemotaxis in human placenta.

Infiltration through the trophoblast layer to the villous stroma is decreased by reduced expression of intercellular adhesion molecule 1 (ICAM-1) (Petroff 2005). However, it has been shown that in V.U.E., ICAM-1 expression is increased and TNFα and IFNγ have been shown to upregulate ICAM-1 on trophoblast. Therefore, it is suggested that a pro-inflammatory environment upregulates expression of ICAM-1 and intravillous invasion of lymphocytes in V.U.E. (Labarrere et al. 2005).

It has been suggested that V.U.E. is a form of semi-allograft rejection, where maternal T lymphocytes are primed against fetal antigens and elicit an inflammatory response.

1.14. Flow cytometric analysis of T cells

Flow cytometry is a widely used technique in the study of leukocytes, as well as other cells. It allows the quantification and assessment of size, granularity and cell surface or intracellular peptide expression. Through the use of fluorochrome conjugated monoclonal antibodies binding to specific peptides, cells pass through a high-speed fluidic system, and different lasers (often 405nm, 488nm and 635nm) excite the fluorochomes conjugated to cells. Light emission is then detected by photomultiplier tubes and "events" corresponding to individual cells are recorded and presented using various software programs. Size and granularity of cells are determined by diffracted (forward scatter) and refracted (side scatter) light respectively, whilst markers such as CD3 or CD4 are detected using antibody-conjugated fluorochromes. Some of the first analyses of decidual T cell using flow cytometric techniques were performed by Starkey et al. (1988) and Watanabe (1987), who were able to describe CD2+ and CD3+ populations.

For detection of antigen specific T cells, MHC-peptide complexes are used to detect specific T cell receptors. Due to TCRs having low affinity to MHC-peptide complexes, it can be difficult to detect or distinguish between similar specificities by using monomer based complexes. This is why multimers are now often used for antigen specific TCR detection. By having multiple MHC-peptide complexes able to bind the TCR at multiple sites on one cell, the technique allows for increased sensitivity and stability. Recently, dextramers, which possess a dextran polymer backbone, have been developed. These are able to carry more MHC-peptide complexes and fluorochrome molecules, allowing for enhanced staining intensity and the detection of potential rare TCR specificities. Due to their high avidity,

background noise is reduced, which allows for a more confident assessment of populations. Figure 1.8 illustrates the general structure of a dextramer antibody.

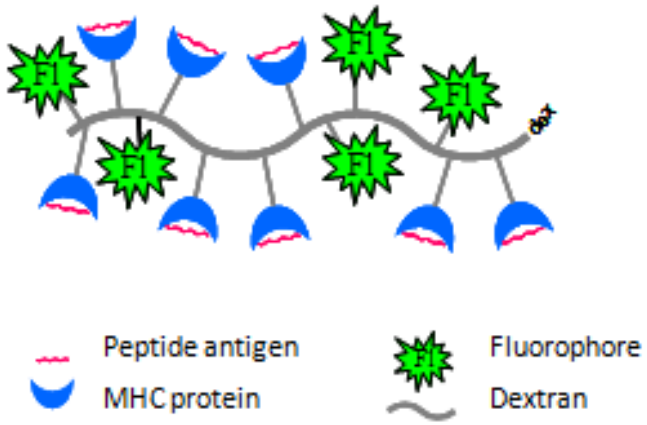


Figure 1. 9 Schematic of dextramer antibody for flow cytometry.

Immunodex

1.15. Analysis of the cellular transcriptome

Transcriptomics is the measurement of all the cell(s) gene transcripts, which includes non-coding RNAs, mRNAs and small RNAs. This is, therefore, a highly sensitive measurement of the molecular mechanisms of cells at a transcriptional level in response to applied conditions, treatments and pathologies. To date, DNA microarrays and RNA sequencing are the two techniques commonly used for global transcriptome analysis (Fang et al. 2012).

DNA microarrays consist of highly densely packed oligonucleotide sequences coupled to precise locations on a chip. mRNAs are then allowed to hybridise to these probes and measurement of transcripts relative to a control transcriptome or differing conditions can be performed making it therefore possible to elucidate whether a variety of

cellular pathways are affected by measuring the relative increase or decrease of transcripts from a particular gene. cDNA microarrays consist of cloned probe molecules that correspond to well-characterised expressed sequences, whilst oligonucleotide microarrays utilize synthesised probes based on prior knowledge of specific transcripts (Murphy 2002; Maughan et al. 2001). A typical microarray experiment is demonstrated in Figure 1.9. First, total RNA is isolated from the cell type in question and quantified. These mRNAs are then converted to cDNA in a reverse-transcriptase enzyme reaction. The library of cDNA afterwards is then labelled with fluorescent dye and then hybridised with target probes on the microarray chip. Following washing procedures, chips are scanned and fluorescence measured. Using specialist software, the transcriptome of each sample is outputted as a raw format, ready for normalisation procedures, bioinformatics and statistical analysis.

Koopman et al. (2003), was one of the first groups to utilise microarray analysis on decidual leukocytes, in this case NK cells. More recently, studies by Tilburgs et al. (2010) and Apps et al. (2011) have used microarray technology to study trophoblast tissue to provide a more in-depth profile of how this tissue may interact with the maternal immune system.

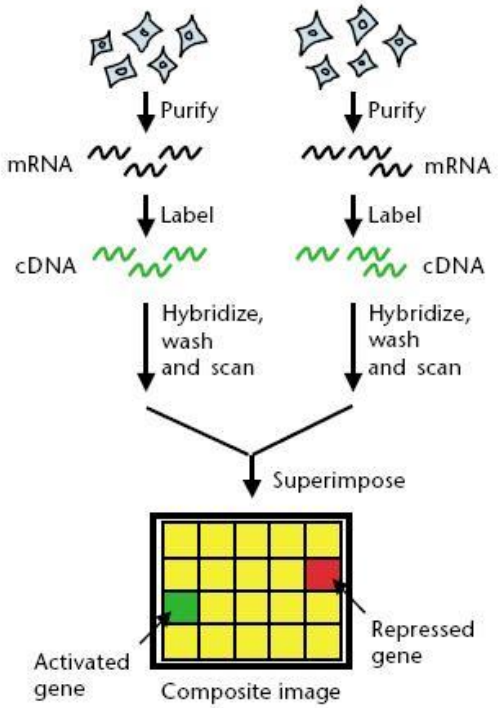


Figure 1. 10 Summary of procedures for single colour microarray experiment.

One-color expression analysis uses a single fluorescent label (green) and two microarray chips to generate transcriptome expression profiles for two or more cell samples. Upregulated and downregulated genes (green and red squares, respectively) are obtained by superimposing images obtained from different microarray chips.(Stears et al. 2003)

1.16. Aims of project

The aims of this project are:

1) To fully characterise effector T cells at the maternal-fetal interface in healthy pregnancy:
    a. The phenotype and functionality of T cells at the maternal–fetal interface, i.e. the decidua basalis and decidua parietalis, trophoblast tissue, will be assessed and compared to matched maternal peripheral blood and cord blood.
2) To elucidate mechanisms by which these T cells may be regulated during pregnancy:
    a. CD4+ T regulatory cell populations will be investigated, as well as inhibitory receptor expression on effector T cells.
3) To assess the allo-reactivity of T cells at the maternal-fetal interface towards fetal antigen:
    a. Populations of HY specific decidual CD8+ T cells will be determined in pregnancies of male fetuses, which might suggest specific allogeneic response.

b. Mixed lymphocyte reactions of decidual T cell and peripheral blood against matched cord blood leukocytes will determine intensity of allo-reactive response to fetal antigen, and how regulatory factors may inhibit this.

4) To assess how potential transcriptional differences between decidual effector T cells and maternal peripheral blood T cells may play a role in maternal-fetal immune response.

   a. A microarray of decidual CD4+ and CD8+ effector memory T cells against matched maternal peripheral blood will be carried out and the analysis of these will assist in defining important immune pathways that may be responsible for maternal-fetal immune responses.

Chapter 2.
Materials and Methods.

2.1. Media and Solutions

Wash media - RPMI 1640 (Sigma, UK)

100U/ml Penicillin (Gibco, UK)

100 µg/ml Streptomycin (Gibco, UK)

General media- RPMI 1640 (Sigma, UK)

100U/ml Penicillin (Gibco, UK)

100 µg/ml Streptomycin (Gibco, UK)

2mM Glutamine (Gibco, UK)

10% Fetal Calf Serum (SBS Biologicals, UK)

Freezing media- Fetal Calf Serum (SBS Biologicals UK)

10% DMSO (Sigma, UK)

MACS buffer- 1 x Phosphate Buffered Saline (PBS) (University of Birmingham)

0.5% Bovine serum albumin (Sigma, UK)

2mM EDTA (Sigma,UK)

Sterile filtered

TBE Buffer- 89nM Tris HCL

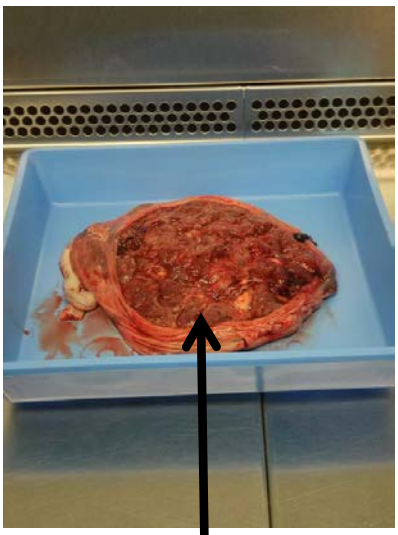
89nM Boric Acid

3mM EDTA

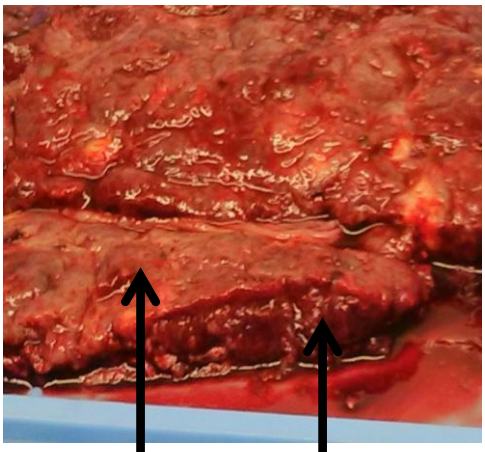
2.2. Mononuclear cell isolation from placental tissue and peripheral blood

Placental tissue was obtained from healthy mothers undergoing 3rd trimester elective caesarean section. Peripheral blood and cord blood samples were also obtained at this time. Decidua basalis, decidua parietalis and trophoblast tissue was dissected. Sterile scalpel and tweezers were used on the main body of the placenta to cut away the top layer of decidua basalis from underlying trophoblast tissue. Figure 2.1 demonstrates the macroscopic areas of dissection. Additionally, the maternal side of the decidua parietalis was gently scraped with a scalpel and tissue was collected. All tissues were dissected and collected from multiple sites of the placenta. Each tissue sample was washed three times sterile PBS to remove contaminating blood. Decidua basalis, decidua parietalis and trophoblast were then dissociated via Miltenyi GentleMACs using program "Human Spleen" in 7.5mls of wash media using C-Tubes (Miltenyi, US). Samples were then enzymatically digested by adding 1mg/ml collagenase (Sigma, UK) and 200U/ml (Sigma, UK) and then placed on a rotator for 45 minutes at 37^{O}C. Digested tissue was then filtered into new 50 ml tubes using Smart Strainers 50µm (Miltenyi, US) along with 30mls of sterile wash wash media. Samples were centrifuged at 300g for 10 minutes. Pellets were re-suspended in wash media and were pipetted on to a density gradient medium Lymphoprep (Alere, NO) and centrifuged for 30 minutes at 758g without brake to isolate mononuclear cells. Maternal and cord blood mononuclear cells were also separated using the density gradient method. The layer suspended above the lymphoprep was pipetted into a new 50 ml tube, and re-suspended in up to 50mls of wash media. Samples were centrifuged at

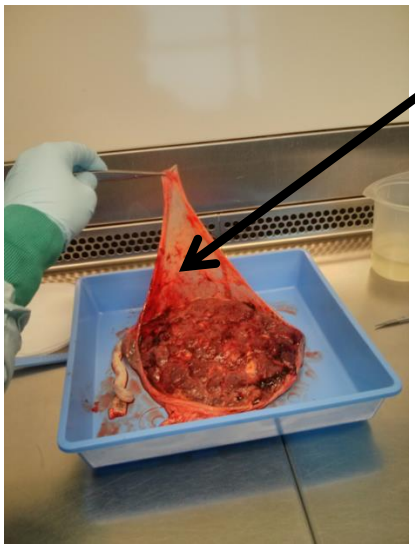
300g for 10 minutes. Supernatant was removed, and cells were re-suspended again in up to 50 mls of wash media and centrifuged at 300g for 10 minutes. Supernatent again was removed and then re-suspended in 20 mls of general media. An aliquot of each sample was pipetted on to a haemocytometer, and leukocytes per ml and total cells were counted. Separately, a full thickness section of the placenta was cut and fixed in 4% formalin and was subsequently paraffin embedded by the Birmingham Women's Hospital Pathology Department. Sections of each placenta were analysed for presence of Villitis of Unknown Etiology.



Maternal- Fetal interface: Decidua



Decidua basalis Trophoblast



Decidua Parietalis

Decidua basalis and trophoblast tissue washed in PBS

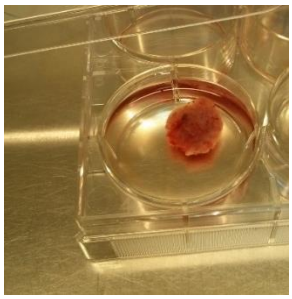


Figure 2. 1 Placental Tissue Dissection

2.3. Flow cytometry using LSRII

A general gating strategy using flow cytometry was used to identify CD4+ and CD8+ T cells throughout the study. Single cells were identified on a forward scatter area vs forward scatter height plot, and lymphocytes gated on the basis of standard forward and side scatter properties. A "dump" channel was used to exclude dead cells (using PI), monocytes (CD14) and B cells (CD19). T lymphocytes were then selected by CD3 expression. CD3+ T cells were then categorised again based on CD4+ and CD8+ expression (Figure 2.2) "Fluorescence minus one" (FMO) controls were used to determine gating thresholds.

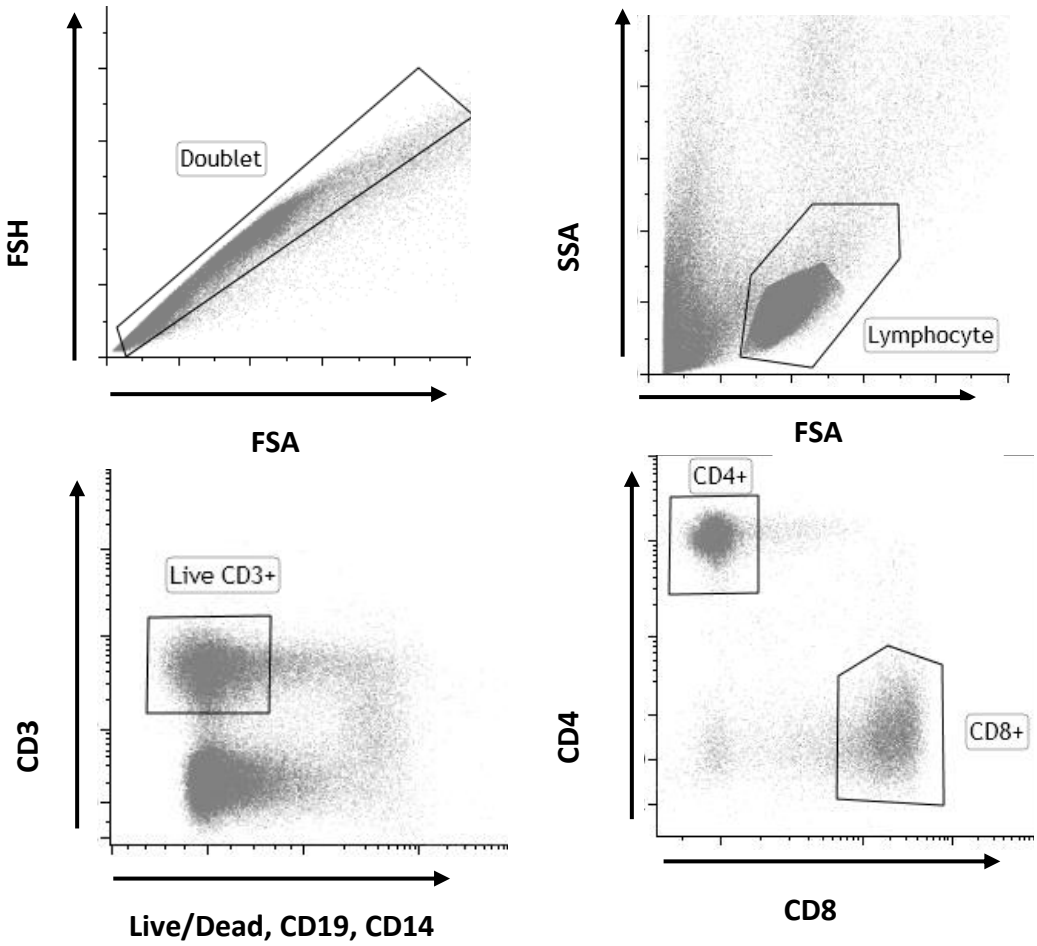


Figure 2. 2 Flow cytometric gating strategy for CD4+ and CD8+ T cells

2.4. Characterisation of T cells phenotype and functionality using flow cytometry

To phenotype T cell memory status, 10^6 fresh maternal, cord blood, decidual or trophoblast mononuclear cells were centrifuged at 300g for 5 minutes in 5 mls of PBS, supernatant removed and re-suspended in 100ul of MACs buffer. Table 2.1 lists the antibodies used:

Antibody	Fluorophore	Clone	Company
CD3	Amcyan	SK7	Becton Dickinson UK
CD4	PE	RPA-T4	Cambridge Bioscience UK
CD8	Efluor450	OKT8	eBiosciences UK
CD45RA	AF700	HI100	Biolegend UK
CCR7	FITC	150503	Becton Dickinson UK
CD27	APCeFluor 780	0323	eBiosciences UK
CD28	PerCPCy5.5	L293	Becton Dickinson UK
CD57	APC	HCD57	Biolegend UK
CD14	ECD	RM052	Beckman Coulter UK
CD19	ECD	J3-119	Beckman Coulter UK
Propidium Iodide	ECD	NA	Becton Dickinson UK

Table 2. 1 T Cell Memory Phenotype Antibodies

Samples were then analysed using LSRII (Becton Dickinson, UK).

To phenotype intracellular cytokines and T regulatory cell status, prior to antibody staining, monocular cells were incubated in RPMI supplemented with L-glutamin (Sigma, UK), bovine foetal calf serum (Sigma, UK) with 50ng/ml of PMA (Sigma) and 1ug/ml of Ionomycin (Sigma, UK) for 4 hours. After 30 minutes, 1.25ug/ml of monencin was added. Unstimulated samples were set up in parallel. Samples were then prepared for surface antibody staining as previously described. Following surface stain incubation, samples were centrifuged at 300g for 5 minutes in 5 mls of PBS, supernatant removed and re-suspended in 1ml/ 10^6 cells of Fix/Perm solution (eBioscience, UK) for 30 minutes. Samples were centrifuged at 300g for 5 minutes in Fix/Perm buffer (eBioscience, UK), supernatant removed and then re-suspended in 2ul per 10^6 cells of Rat Blocking Serum (eBioscience, UK) to remove background of FOXP3 staining. Table 2.2 lists surface and intracellular antibodies used.

Surface			
Antibody	Fluorophore	Clone	Company
CXCR3	PECy7	IC6	Becton Dickinson UK
CD25	FITC	IM0478U	Beckman Coulter UK
CD127	eFluor 780	ebioRDR5	eBiosciences UK
CD8	BV510	RPA-T	Biolegend UK
CD4	PerCP CY5.5	RPA-T4	Biolegend UK
CD3	BV605	SK7	Becton Dickinson UK
CD14	ECD	RM052	Beckman Coulter UK
CD19	ECD	J3-119	Beckman Coulter UK
Live/Dead Fixable Red DYE	ECD	N/A	Life Technologies UK
Intracellular			
IL-4	APC	8D4-8	Biolegend UK
IL-17	Pacific Blue	BL168	Biolegend UK
IFNγ	AF700	4S.B3	Biolegend UK
FOXP3	PE	PCH101	eBioscience UK

Table 2. 2 Antibodies for Intracellular Cytokine Staining

For immune checkpoint protein phenotyping, maternal and decidual samples were prepared for antibody staining as described previously and Table 2.3 lists the antibodies used.

Antibody	Fluorophore	Clone	Company
CD3	Amcyan	SK7	Becton Dickinson UK
CD8	APCCY7	SK1	Biolegend UK
CD4	BV510	RPA-T4	Biolegend UK
CCR7	FITC	150503	Becton Dickinson UK
CD45RA	AF700	HI100	Biolegend UK
PD-1	BV421	EH2.2H7	Biolegend UK
CTLA-4	PECY5	BNI3	Becton Dickinson UK
Tim-3	PeCy7	F382E2	Biolegend UK
Lag-3	APC	FAB2319P	R and D Systems UK
CD14	ECD	RM052	Beckman Coulter UK
CD19	ECD	J3-119	Beckman Coulter UK
Propidium Iodide	ECD	NA	Becton Dickinson UK

Table 2. 3 Antibodies for Immune Checkpoint Proteins

2.5. Mixed lymphocyte reaction assay

Fresh maternal and decidual mononuclear cells were prepared as previously described. To each sample 1ul per 10^6 cells of Easy Sep T-cell enrichment antibody mix (StemCell, US) was added and incubated at room temperature for 10 minutes. Then, 1ul of magnetic

beads per 10^6 of cells was added and incubated at room temperature for 5 minutes. Samples were re-suspended in 3mls of MACs buffer and inserted into an Easy Sep Purple Magnet (Stemcell, US) and incubated for 5 minutes. The T cell enriched suspensions were then transferred into a new FACS tubes, centrifuged at 300g for 5 minutes in MACs buffer, re-suspended and counted using haemocytometer. Cells then split between non T regulatory cell depleted and T regulatory depleted samples. Suspensions that were retained for T regulatory depletion were centrifuged at 300g for 5 minutes in MACs buffer and re-suspended into 20ul of CD25 Miltenyi Microbeads and 80ul of MACs buffer. The samples were incubated on ice for 15 minutes. 2mls of MACs buffer was added and samples were centrifuged at 300g for 5 minutes and re-suspended in 500ul of MACs buffer. Cell suspensions were then applied to a magnetic MACs micro-column and run through was collected. Samples were re-suspended to 10^6 cells per ml in PBS. Purity of T regulatory cell depleted fractions are demonstrated in Figure 2.3. 1ul of Violet cell trace dye (Invitrogen, UK) was added per 10^6 of cells and incubated at 37 degrees for 20 minutes. 5x the original amount of cell suspension of enriched media was added to each sample and incubated at 37 degrees for 5 minutes. Samples were then washed and counted.

Cord blood mononuclear cells were isolated using the method previously described. 5mls of red blood cell lysis buffer was added to each sample and incubated at room temperature for 5 minutes. Cell suspensions were then centrifuged and re-suspended in 20mls of enriched media. Cord blood lymphocytes were then irradiated for 3000 RADS. Cells and then counted using a haemocytometer.

Irradiated cord blood lymphocytes were added at a 2:1 ratio to each maternal and decidual sample in a 96 well round-bottom plate in enriched media. Each sample assay was carried out in duplicate, along with T regulatory cell depleted samples in parallel. Positive control assays were constructed using 5ul of CD3 dynabeads (Becton Dickinson, UK) in the place of cord blood. Negative control assays were constructed by using enriched media in place of effector cells/beads. To each sample, 10 units of IL-2 cytokine was added. Samples were incubated for 5 days. At day three, 100ul of media per well was replaced with fresh media along with 10units of IL-2.

Prior to harvesting on day 5, 1.25ug of Monensin was added to each sample and incubated for a further 4 hours. Each sample was harvested and centrifuged at 300g for 5 minutes in 5mls of PBS. Following re-suspension surface antibodes were added. After incubation on ice for 30 minutes, samples were centrifuged at 300g for 5 minutes in 5 mls of PBS and re-suspened. To each sample 100ul of 4%PFA was added and incubated on ice for 30 minutes. Each sample was again centrifuged at 300g for 5 minutes, resuspended and 10ul of 4% Saponin was added. After permeabilisation for 10 minutes on ice, intracellular antibodies were added. Samples were centrifuged and re-suspended in MACs buffer before flow cytometry was performed. Table 2.4 lists antibodies used.

Surface			
Antibody	Fluorophore	Clone	Company
CD3	BV605	SK7	Biolegend UK
CD8	BV510	RPAT-8	Biolegend UK
CD4	PerCP CY5.5	RPAT-4	Biolegend UK
Live/Dead Fixable Red Stain Dye	ECD	NA	Life Technologies UK
Intracellular			
IL-10	PE	JES3-9D7	eBiosciences UK
IL-4	APC	8D4-8	Biolegend UK
IL-17	APC-CY7	BL168	Biolegend UK
IFNγ	AF700	4S.B3	Biolegend UK
Granzyme B	FITC	GB11	Biolegend UK
Perforin	PECY7	dG9	eBiosciences UK

Table 2. 4 Antibodies for Mixed Lymphocyte Reaction

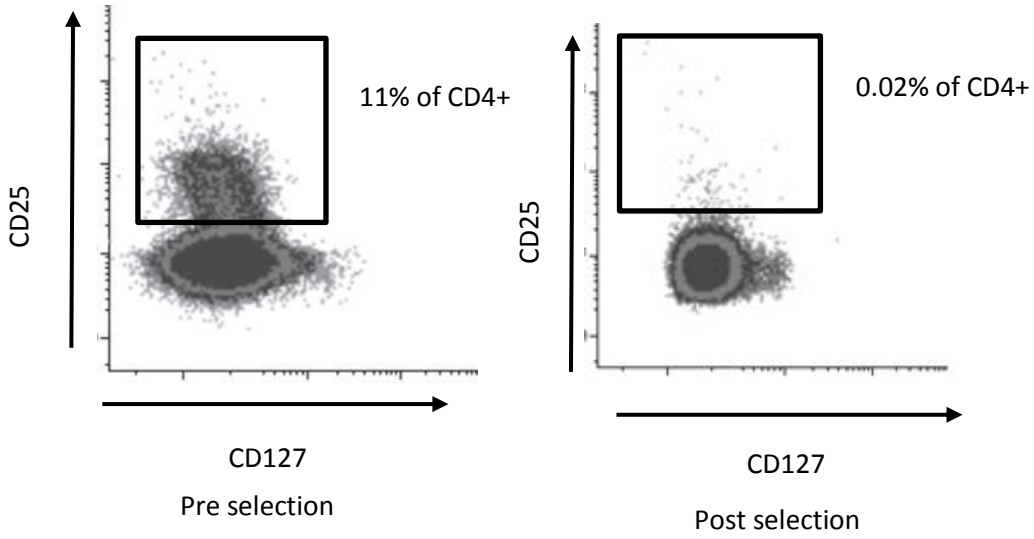


Figure 2. 3 T regulatory cell depletion

Decidual and maternal T cells were depleted for CD4+ T regulatory cells using CD25 Magnetic Bead separation. Flow plots illustrate population of CD25+ T cells pre and post magnetic bead separation.

2.6. HY specific CD8+ T cells in Decidual and Maternal peripheral blood

Mononuclear cells from the decidua basalis and maternal peripheral blood were prepared as previously described. DNA was prepared for maternal and cord blood using the DNeasy Blood and Tissue Kit (Qiagen, UK). Patient samples were selected, based on MHC A2 and B7 positivity, and whether the child was male. HLA typing was carried out. Briefly, a master mix of 70.87µl TDMH, 35µl nuclease-free water, 2uM of APC control primers (ATGATGTTGACCTTTCCAGGG) and (TTCTGTAACTTTTCATCAGTTGC) and 3.75 units of Taq polymerase (Bioline, UK) was prepared. 8.5µl of master mix was removed for a no DNA control tube. 5µl of the test DNA sample, adjusted to a concentration of 28ng/µl, was then added to the master mix, which was aliquoted into the PCR tubes including the HLA A2 and HLA B7 primer combinations, as per Table 2.5. A thermal cycler was used for the following heating programme; 60 seconds (95.^{o}C), 5 cycles of 25 seconds (94.^{o}C), 45 seconds (70.^{o}C), 45 seconds (72.^{o}C) followed by 21 cycles of 25 (96.^{o}C), 50 seconds (65.^{o}C), 45 seconds (72.^{o}C) followed by 4 cycles of 25 seconds (96.^{o}C), 60 seconds (55.^{o}C) and 120 seconds (72.^{o}C), then 72.^{o}C for 5 minutes before being held at 10 ^{o}C. PCR products were electrophoresed in 1% agarose gels containing 1X TBE buffer supplemented with 0.05% ethidium bromide. A 100bp molecular weight marker was added to each row so that any positive bands could be identified according to their size. Gels were run for approximately 40 minutes at 120V and then visualised using an ultraviolet transilluminator.

HLA	Sense primer sequence	Anti-sense primer sequence	Final concentration (μM)
A2	5-GTGGATAGAGCAGGAGGGT	5CCAAGAGCGCAGGTCCTCT	3.3
B7	5-GGAGTATTGGGACCGGAAC	5-TACCAGCGCGCTCCAGCT	3.3
B7	5-GAGCCTGCGGACCCTGCT	5-TACCAGCGCGCTCCAGCT	3.3
Positive Control	5-TGCCAAGTGGAGCACCCAA	5GCATCTTGCTCTGTGCAGAT	3.3
No DNA Control	5-TGCCAAGTGGAGCACCCAA	5-GCATCTTGCTCTGTGCAGAT	3.3

Table 2. 5 HLA typing primers

Once A2+ and B7+ samples were identified, aliquots were prepared for flow cytometry as previously described. Samples were incubated with PE dextramers derived from either A2 (FIDSYICQV) or B7 (SPSVDKARAEL) epitopes of HY protein KDM5D (Immundex, DK), for 20 minutes prior to full flow cytometer staining protocol. Table 2.6 lists the antibodies used. A negative control dextramer was used that contained an irrelevant peptide. This enabled levels of background staining to be determined. Background staining was calculated as a percentage of the total CD8 population and subtracted from the HY specific T cell frequency to determine the final frequency of HY specific T cells. Fluorescence minus one controls were used to determine gating thresholds. Phenotypic gating was initially carried out by reference to the negative dextramer population, where populations could be clearly

separated due to the larger cell populations, with the same gating template, then applied to the dextramer positive population.

Antibodies	Fluorophore	Clone	Company
CD3	Amcyan	SK7	Becton Dickinson
CD8	eFluor450	OKT8	eBiosciences
PD-1	PECY7	EH12.2H7	Biolegend UK
CD69	AlexaFluor 647	B176236	Biolegend UK
CD45RA	Af700	HI100	Biolegend UK
CCR7	FITC	150503	Becton Dickinson UK
CD14	ECD	RM052	Beckman Coulter UK
CD19	ECD	J3-119	Beckman Coulter UK
Live/Dead fixable red stain dye	ECD	NA	Life technologies UK

Table 2. 6 Antibodies for HY Dextramer staining

2.7 Microarray

24 samples selected for the microarray consisted of 6 pairs of maternal and decidual CD4+ and CD8+ effector memory T cells. Mononuclear cell suspensions were prepared for each pair of maternal and decidual samples as previously described. Antibodies used for flow cytometric cell sorting were CD3, CD4, CD8 , CD45RA, CCR7 and Viability Dye. See Table 2.1 for antibody details. Maternal and Decidual samples were sorted into separate CD4+ and CD8+ effector memory samples. Figure 2. 4 demonstrates populations that were isolated

following flow cytometric cell sorting. Sorted populations were deposited into separate eppendorfs containing PBS. RNA was immediately isolated and extracted using the Qiagen PLUS MICRO RNA easy kit. The RNeasy Plus Micro Kit purifies total RNA from up to 5 x 10^5 cells. Briefly, cells were first lysed and homogenized. The lysate was passed through a gDNA Eliminator spin column, ethanol was added to the flow-through, and the sample was applied to an RNeasy spin column. RNA bound to the membrane and contaminants were washed away. High-quality RNA was eluted in RNA free water 14 µl and quantified. RNA samples were immediately stored at -80 degrees. In total, 24 RNA samples were sent to Eurofins Genomics services. Affymetrix Pico labelling kit was used to amplify RNA, and the Affymetrix Human Gene 2.0 ST array was used to carry out the measurement of transcripts.

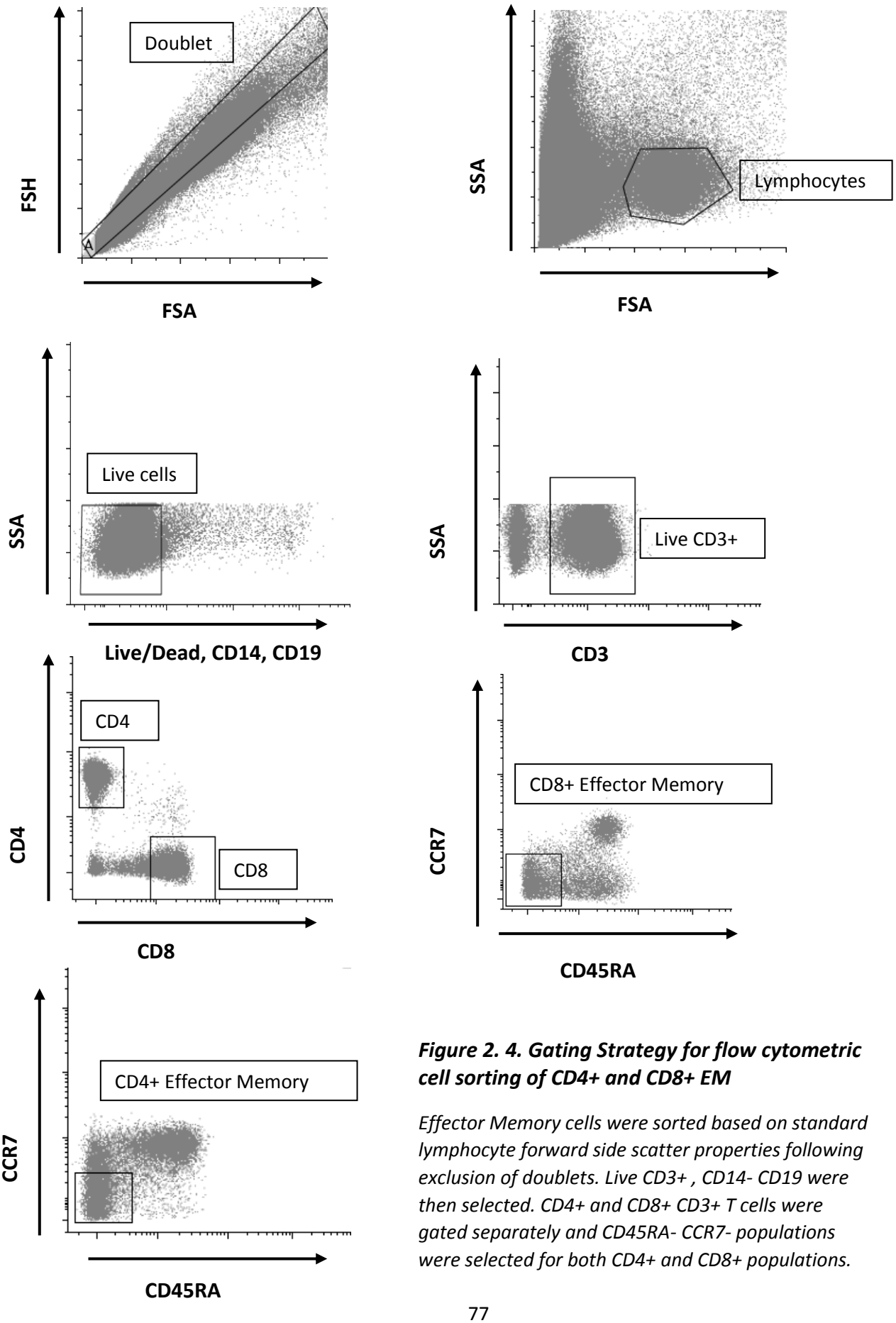


Figure 2. 4. Gating Strategy for flow cytometric cell sorting of CD4+ and CD8+ EM

Effector Memory cells were sorted based on standard lymphocyte forward side scatter properties following exclusion of doublets. Live CD3+ , CD14- CD19 were then selected. CD4+ and CD8+ CD3+ T cells were gated separately and CD45RA- CCR7- populations were selected for both CD4+ and CD8+ populations.

2.8 Statistics

Flow cytometry data was analysed using FACS Diva (Becton Dickinson, UK) and statistical analysis was performed using Prism (GraphPad, US). Non Gaussian distribution was applied to all samples: Wilcoxon matched pairs signed rank test was used to identify significance between two sets of data, whilst Freidmans test with Dunns multiple comparison was used for multiple sets.

Microarray data was analysed with the R Limma Package (Bioconductor). Normalisation was performed with the Loess (intra-array) and Aquantile (inter-array) methods. An adjusted p-value (Benjamini and Hochberg's method) of 0.05 and below was taken as significant for differences in gene expression. The empirical Bayesian shrinkage of moderated t-statistics function (eBayes) was used to generate Bayes factors for significantly upregulated or downregulated genes in decidua compared to maternal peripheral blood. Volcano plots were generated using R, and genes annotated based on significant Bayes value (>5). A fold change value of 1.65 (log2FC >0.7) was observed as significantly up/down regulated. Microarray data deposited to Array Express under accession number: E-MTAB-5517, website URL: www.ebi.ac.uk/arrayexpress

Gene Set Enrichment Analysis (Broad Institute) was performed as follows: Matrices of differently expressed genes in decidual CD4 and CD8 effector memory T cells compared to maternal blood were generated using R Limma Package. Expression data was then applied to GSEA analysis. Enriched gene sets were observed as significant if <FDR 0.25.

2.9 Study Approval and Patients Cohort

Written informed consent was obtained from all women recruited into the study. Women that were targeted for recruitment did not have any known pregnancy complications. Matched maternal blood, cord blood and placenta were collected with the approval of Health Research Authority- West Midlands, Edgbaston Research Ethics Committee (RG_14-194). Table 2.7 demonstrates basic information of the patient cohort. All placental samples used in this study did not contain Villitis of Unknown Etiology.

	N=90
Age	32 ±4.5
Parity	
primiparous	37 (41%)
multiparous	57 (58%)
Gestational age	279 (median)
Delivery	Caesarian
Birthweight	3791.8 ± 475.3

Table 2. 7 Cohort information

Chapter 3. Results
Memory and Functional Phenotype of CD8 and CD4 T cells at the 3rd trimester of pregnancy.

3.1. Introduction

In early pregnancy, the decidua sees the accumulation of large numbers of specialized uterine NK cells (CD56bright CD16-), and their interaction with extravillous trophoblast plays a key role in successful placentation (Lash et al. 2011; Moffett & Colucci 2014). However, as pregnancy progresses the number of NK cells declines and by the third trimester T lymphocytes become the predominant leukocyte population (Williams et al. 2009). Despite this, the role that decidual T cells play during healthy pregnancy is not fully understood. Historically, T cells at the maternal fetal interface have been described in both early and late pregnancy, and their phenotype and functionally only partially explained (Tilburgs et al. 2010; Slukvin et al. 1996; Saito et al. 1994). Pathologies predominately associated with cytotoxic T cell infiltrate, such as Villitis of Unknown Etiology, are well characterised (Redline 2007), however, research into pathways from what is seen in healthy pregnancy through to destructive pathologies that are caused by T cells is distinctly lacking. To expand on current data, we provide comprehensive phenotypic analysis of CD4+ and CD8+ decidual T cells isolated from the decidua basalis and parietalis as well as T cells isolated from fetal trophoblast tissue (see Figure 2.1) , and compare these with their matched maternal peripheral blood and cord blood counterparts. The memory status of these T cells was elucidated, along with their co-expression of CD28 and CD27 to distinguish their differentiation stage. Functional capacity was conducted by assessing their ability to produce cytokines, IFNy, IL4 and IL-17, all relevant in cytotoxic responses at sights of allograft responses and inflammation. Lastly, CXCR3 expression, a chemokine receptor

previously identified as an important marker for homing of an allograft response (Croudace et al. 2012) expression was assessed, which provides an important insight in the potential of decidual T cell trafficking. It is important to point out that T cells extracted from fetal trophoblast tissue might be of both maternal and fetal origin and therefore any results will be addressed in this context.

3.2. Decidual effector memory T cells dominate the T cell repertoire at the maternal fetal interface.

In order to investigate and fully characterise the T cell infiltrate of healthy 3rd trimester placenta (<37 weeks) we collected (n=45) matched maternal peripheral blood mononuclear cells (PBMCs), cord blood, decidua basalis, decidua parietalis and trophoblast tissue, and isolated mononuclear cells. Using flow cytometric analysis, we first analysed memory status of CD4+ and CD8+ T cells. Figures 3.1 A and 3.1 B shows the gating strategy used to identify the different memory phenotypes that we analysed. CD45RA and CCR7 was used to categorise the memory populations as follows: Naïve= CD45RA+ CCR7+; Central memory CD45RA- CCR7+; Effector memory CD45RA- CCR7-; EMRA= CD45RA+ CCR7-.

Figures 3.1 C, D, E and F demonstrate the distribution of CD8+ T cell memory phenotype in the tissues we examined. When comparing maternal CD8+ T cells to their decidual counterparts, there is a striking difference in the population composition. Naïve populations are relatively reduced in decidua parietalis (12.36%) and decidua basalis (14.80%) when compared to both maternal PBMCs (40.74%, p= 0.0001) and cord blood (87.12%, p=0.0001). Conversely, activated effector memory CD8+ T cells are significantly increased at the decidua parietalis (56.25%) and decidua basalis (51.25%) when compared to maternal PBMCs (25.87% p=0.0001), cord blood (2.84%, p= 0.0001) and trophoblast (30%, p= 0.0001 and 0.0068). There are no significant differences in EMRA populations between maternal PBMCs, decidua basalis, decidua parietalis and trophoblast, and all

approach 18% of CD8+ population. Despite the fact that a significant majority of cord blood CD8+ T cells possess naive phenotype, it is not completely devoid of immunological activity with central memory, effector memory and EMRA phenotypes comprising 6%, 2% and 5% respectively. Interestingly, trophoblast tissue displays significant differences towards decidual CD8+ T cells with increased naïve (43.9%) and decreased effector memory populations (30%). This might be due to contaminating cord blood cells within the isolated lymphocyte cells obtained from the trophoblast tissue, which may skew the population ratio towards a naïve phenotype. Figure 3.2 illustrates the data using charts of median proportions of memory phenotype for tissue and blood samples. Differences can clearly be seen between the dominant effector memory phenotype (red) seen in decidual tissues, and naïve populations (blue) seen in maternal, cord and trophoblast tissue T cells.

Figure 3.1.

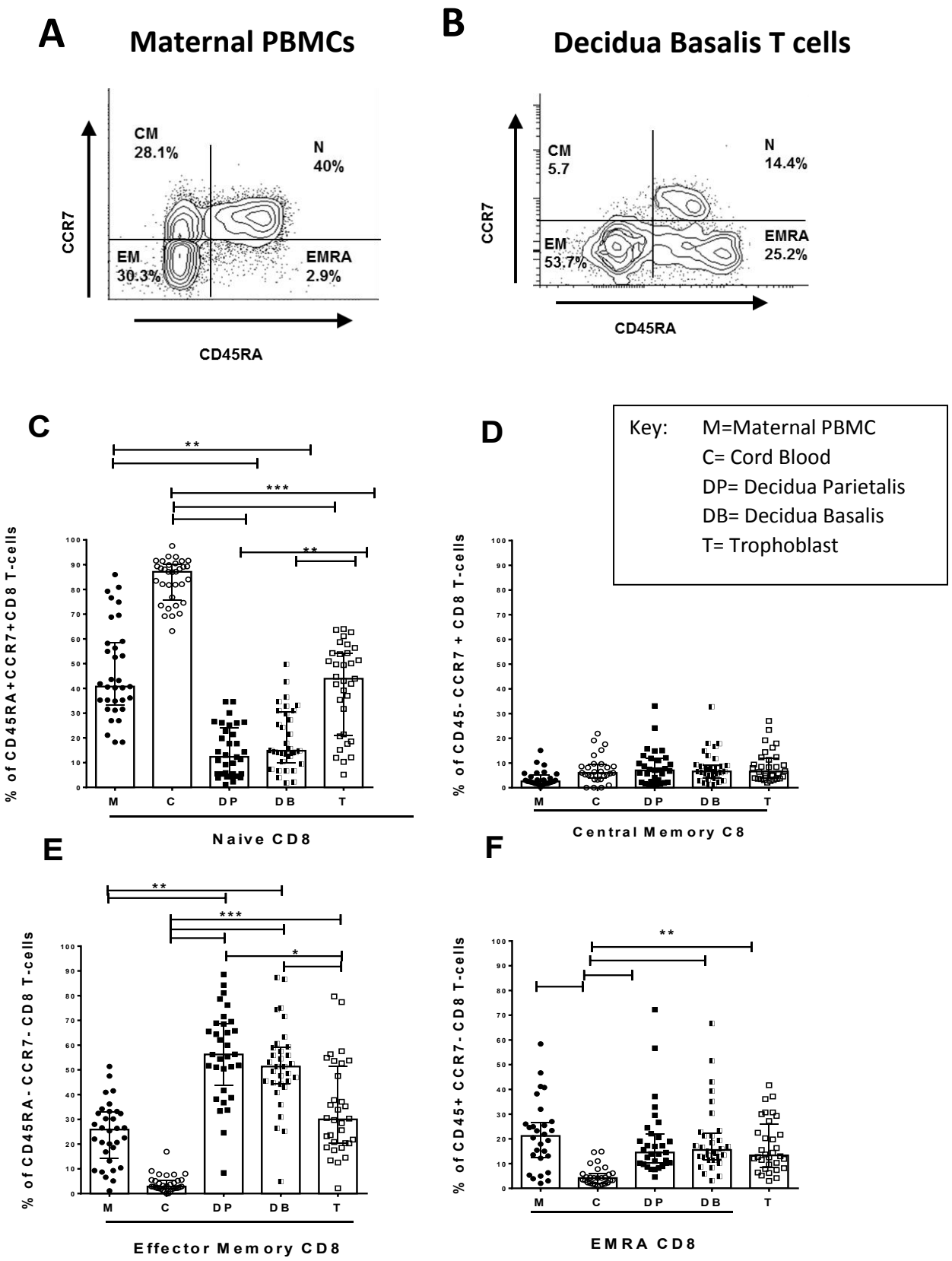

A
Maternal PBMCs
CM
28.1%
N
40%
EM
30.3%
EMRA
2.9%
CCR7
CD45RA
B
Decidua Basalis T cells
CM
5.7
N
14.4%
EM
53.7%
EMRA
25.2%
CCR7
CD45RA
C
% of CD45RA+CCR7+CD8 T-cells
M
C
DP
DB
T
Naive CD8
D
Key:
M=Maternal PBMC
C= Cord Blood
DP= Decidua Parietalis
DB= Decidua Basalis
T= Trophoblast
% of CD45- CCR7 + CD8 T-cells
Central Memory C8
E
% of CD45RA - CCR7- CD8 T-cells
Effector Memory CD8
F
% of CD45+ CCR7- CD8 T-cells
EMRA CD8

Figure 3. 1. Memory Status Phenotype of CD8+ T cells at 3rd trimester of pregnancy

*CD8+ T cell memory status was analysed in a cohort of n=45 maternal PBMCs, cord blood, decidua basalis, decidua parietalis and trophoblast tissue. (**A**) and (**B**) demonstrates gating strategy of maternal PBMCs and decidual mononuclear cells gated on live CD3+, CD8+ and examined for memory status using CD45RA and CCR7 expression. Memory status was classified into 4 categories: Naïve (N) CD45RA+ CCR7+; Central Memory (CM) CD45RA- CCR7+; Effector Memory (EM) CD45RA- CCR7-; Effector Memory RA (EMRA) CD45RA+ CCR7. (**C**) (**D**) (**E**) and (**F**) demonstrate Naïve, Central memory, Effector memory and EMRA populations respectively as percentage of total CD8 T-cells in all tissues. Lines represent median and interquartile range. Significance was calculated using Friedman's test and Dunn multiple comparison test where *P=<0.05, **P=<0.01, ***P=<0.001.*

Figure 3.2.

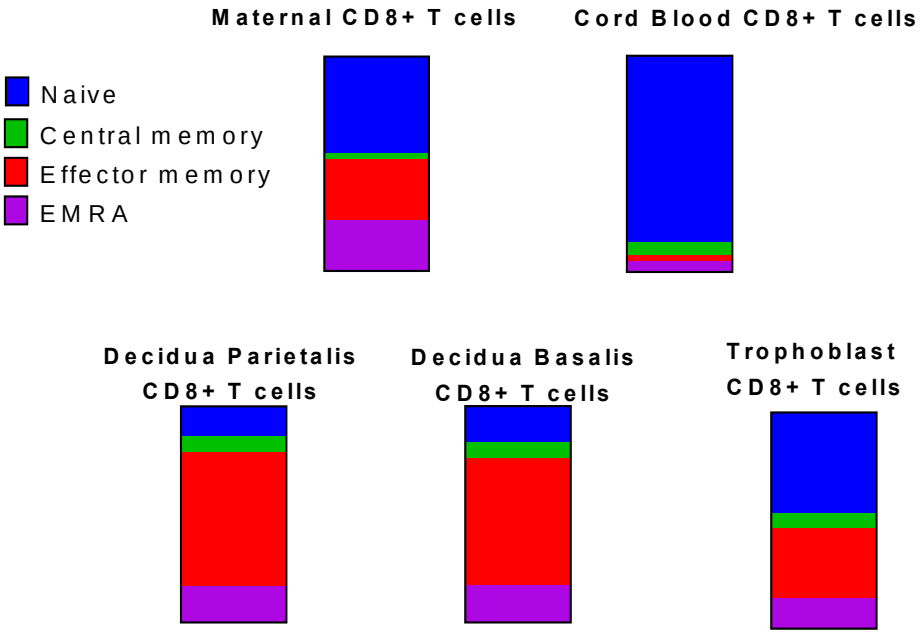


Figure 3. 2. Comparison of CD8+ T cell memory phenotypes between maternal and fetal tissues at 3rd trimester.

Proportions of CD8+ memory T cell populations are shown for maternal PBMCs, cord blood cells, dedidua basalis, decidua parietalis and trophoblast tissue. Pie charts were generated by calculating the median of each CD8+ T cell memory population: Naïve- Blue; Central memory- Green; Effector memory- Red; EMRA- Purple.

CD4+ T cell memory status was also elucidated in decidua basalis, decidua parietalis, trophoblast, cord blood and maternal PBMCs. Figure 3.3 A, B, C and D demonstrate the comparison of different memory populations between maternal PBMCs, cord blood T cells, decidua basalis decidua parietalis and trophoblast T cells. As with CD8+ T cells there is a distinct contrast between populations of naïve and effector memory T cells in the decidual tissues when compared to other tissues. Maternal PBMCs, cord bood T cells and trophoblast T cells all had significantly increased populations of naïve CD4+ T cells, 48.4%, 85.5%, 60.2%, compared to decidua basalis 28.78% (p= 0.0111; 0.0001; 0.0025), and decidua parietalis 13.55% (all p=0.0001). Maternal PBMCs had moderate levels of effector memory CD4+ T cells, however, decidua basalis and decidua pareitalis were still significantly increased, representing 53.2% (p= 0.0067) and 69.48% (p=0.0001) respectively. As with CD8+, CD4+ cord blood T cells, despite having a majority of naïve population, their effector memory, central memory and EMRA populations were still detected at, 3.8%, 6.2% and 3.9% respectively. CD4+ trophoblast T cells had significantly reduced levels of effector memory population (22.3%) when compared to other placental tissue, however, they did have significantly increased levels of EMRA CD4+ T cells in comparison to decidua parietalis, 5.1% to 2.3% respectively (p=0.01). This may suggest an increase in chronic stimulation or activation as T cells infiltrate into deep placental tissue. Figure 3.4 illustrates the data using charts of median proportions of memory phenotype for tissue and blood samples as in Figure 3.3. Differences in proportion of populations can clearly be seen between the dominant effector memory phenotype (red) seen in decidual tissues, and naïve populations (blue) seen in maternal, cord and trophoblast tissue CD4+ T cells.

Figure 3.3.

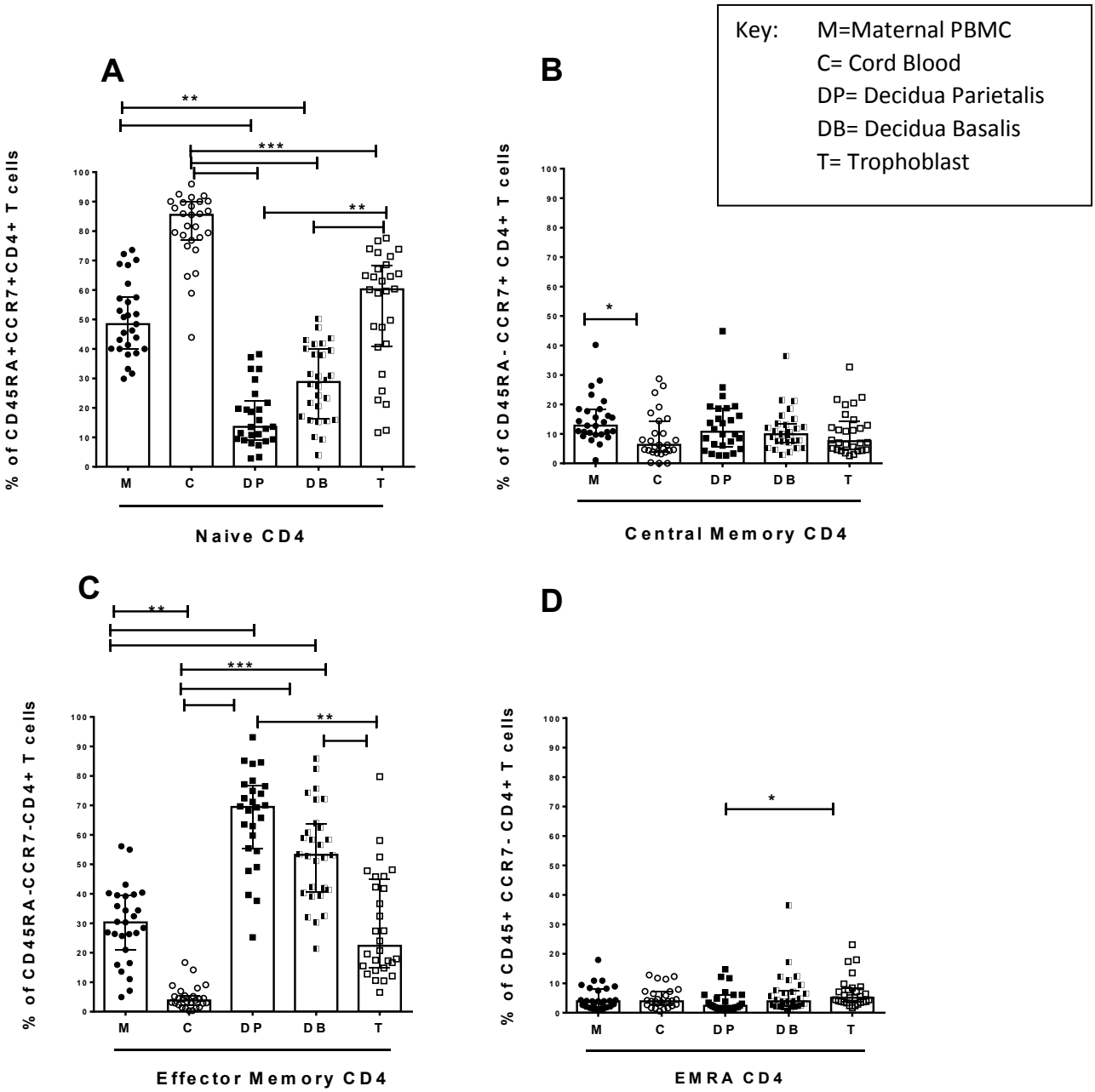

Key:
M=Maternal PBMC
C= Cord Blood
DP= Decidua Parietalis
DB= Decidua Basalis
T= Trophoblast
A
% of CD45RA+CCR7+CD4+ T cells
M
C
DP
DB
T
Naive CD4
B
% of CD45RA- CCR7+ CD4+ T cells
Central Memory CD4
C
% of CD45RA-CCR7-CD4+ T cells
Effector Memory CD4
D
% of CD45+ CCR7- CD4+ T cells
EMRA CD4

Figure 3. 3 Memory Status Phenotype of CD4+ T cells at 3rd trimester of pregnancy

*CD4+ T cell memory status was analysed in a cohort of n=45 maternal PBMCs, cord blood, decidua basalis, decidua parietalis and Trophoblast tissue. Memory status was classified into 4 categories: Naïve (N) CD45RA+ CCR7+; Central Memory (CM) CD45RA- CCR7+; Effector Memory (EM) CD45RA- CCR7-; Effector Memory RA (EMRA) CD45RA+ CCR7. (**A**) (**B**) (**C**) and (**D**) demonstrate Naïve, Central memory, Effector memory and EMRA populations respectively as percentage of total CD4+ T cells in all tissues. Lines represent median and interquartile range. Significance was calculated using Friedman's test and Dunn multiple comparison test where *P=<0.05, **P=<0.01, ***P=<0.001.*

Figure 3.4.

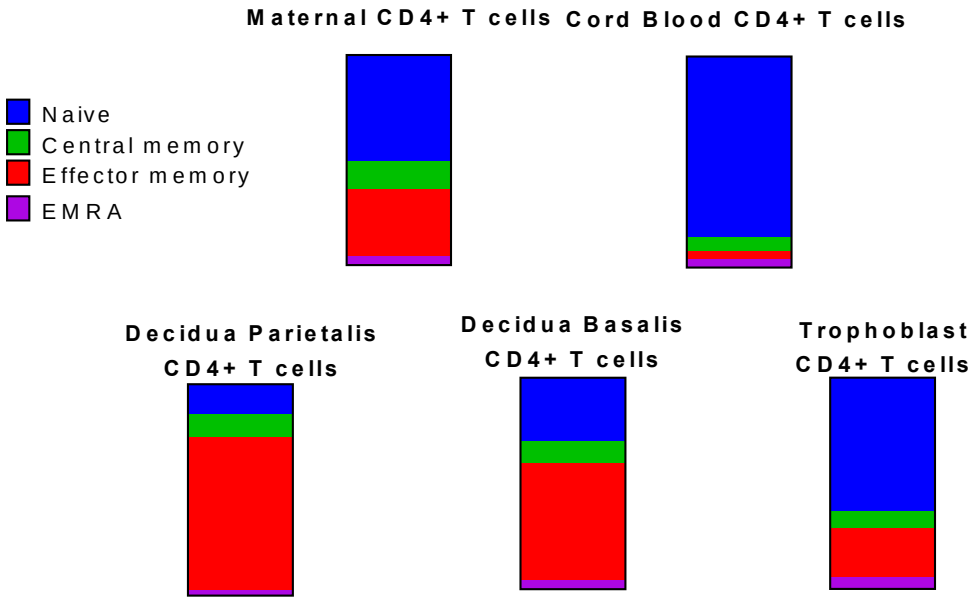


Figure 3. 4 Comparison of CD4+ T cell memory phenotypes between maternal and fetal tissues at 3rd trimester

Proportions of CD4+ memory T cell populations are shown for maternal PBMCs, cord blood cells, dedidua basalis, decidua parietalis and trophoblast tissue. Pie charts were generated by calculating the median of each CD4+ T cell memory population: Naïve- Blue; Central memory- Green; Effector memory- Red; EMRA- Purple.

3.2. Highly differentiated effector memory T cells are present in the decidua.

To categorise the level of differentiation in effector memory CD8+ T cells we measured expression of co-stimulatory markers CD27 and CD28 in all tissue types. Figure 3.5 A and B demonstrates the gating strategy used to define the differentiated subtypes: EM1= CD27+ CD28+; EM2= CD27+ CD28-; EM3= CD27- CD28-; EM4 = CD27- CD28+. EM1 phenotype of effector memory T cells are memory-like, expressing less effector mediators, and are less mature exhibiting both CD27 and CD28 co-stimulator markers (Romero et al, 2007). These are significantly higher in proportion in maternal and cord blood, 54.6% and 45.47% respectively, when compared to decidua parietalis, 14.7% (p=0.0001), decidua basalis, 18.63% (p=0.0001; 0.0004) and trophoblast T cells, 6.9% (p=0.0001) (Figure 3.5 C). In contrast, EM3 CD+ T cells that are more mature and characterised by increased cytolytic properties, are significantly increased at the decidua basalis and decidua parietalis, representing 40.62% and 41.30%, respectively, when compared to maternal PBMCs 13.54% (p= 0.0029; 0.0005) and cord blood 0.32% (p=0.0001) (Figure 3.5 E). Interestingly, despite trophoblast CD8+ cells, having significantly reduced levels of overall effector memory population in comparison to decidual T cells, they maintain a significantly increased pool of EM3 CD8+ T cells, representing 34.17% of the effector memory population compared to maternal PBMCs (p=0.0408). Additionally, EM4 T cells, similar to EM1, with central memory- like properties, are significantly increased in the trophoblast, with a value of 34.89%, in comparison to decidua parietalis at 15.88% (p=0.0002). This

suggests a potential difference in functionality of T cells in placental tissue, as they transit through different anatomical layers (Figure 3.5 E).

Figure 3.6 illustrates the data using median proportions of each subtype for all tissues. This demonstrates a significant trend of more mature effector memory subsets capable of cytotoxicity at the decidua, but less mature effector memory subsets in the maternal PBMCs and cord blood.

Figure 3.5.

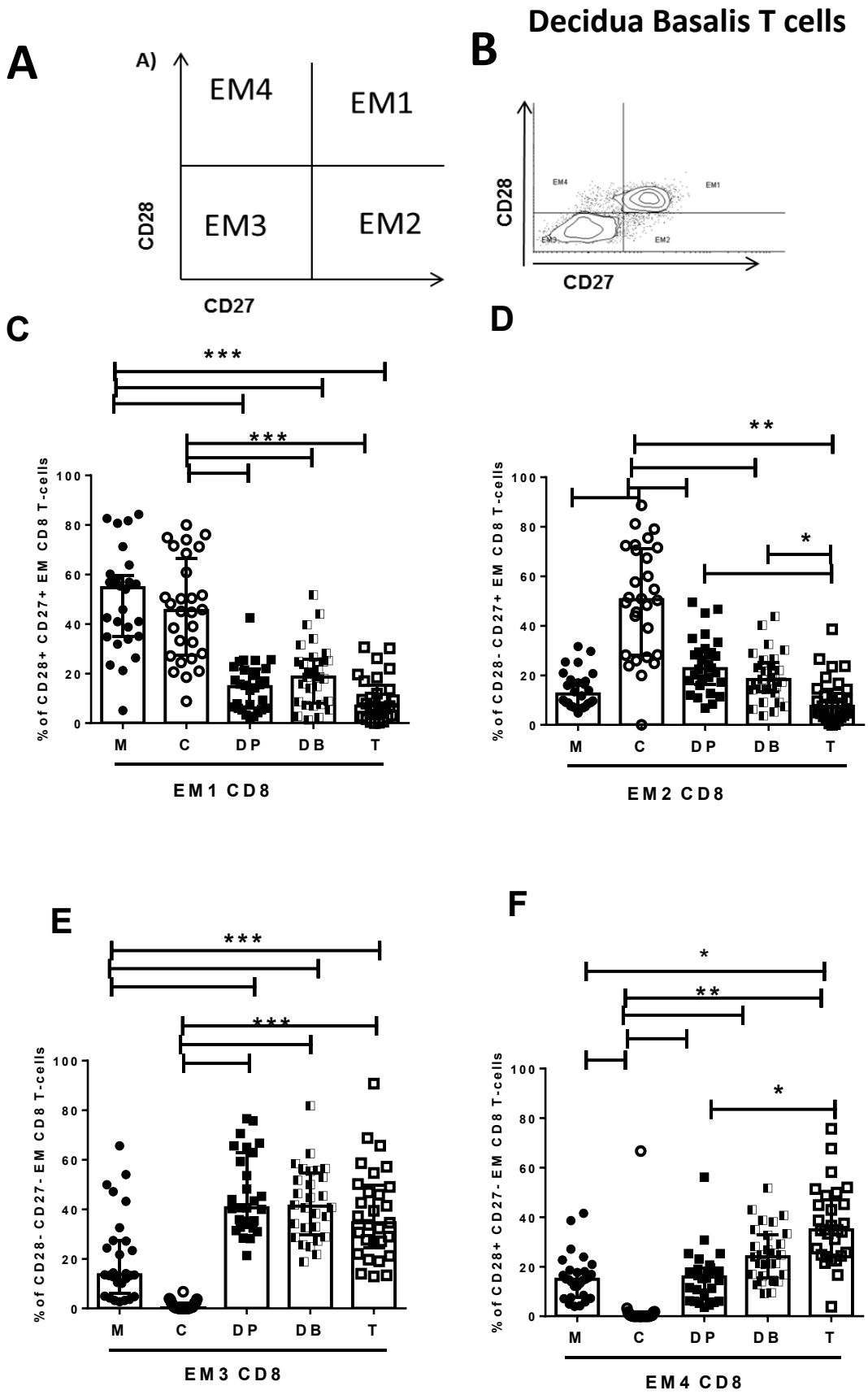

A
A)
EM4
EM1
EM3
EM2
CD28
CD27
B
Decidua Basalis T cells
CD28
CD27
C
% of CD28+ CD27+ EM CD8 T-cells
M
C
DP
DB
T
EM1 CD8
D
% of CD28- CD27+ EM CD8 T-cells
EM2 CD8
E
% of CD28- CD27- EM CD8 T-cells
EM3 CD8
F
% of CD28+ CD27- EM CD8 T-cells
EM4 CD8

Figure 3. 5. Effector memory phenotype of CD8+ T cells at 3rd trimester of pregnancy

*CD8+ T cell effector memory status was analysed in N=45 of maternal PBMCs, cord blood, decidua basalis, decidua parietalis and trophoblast tissue. CD8+ Effector Memory T cells were gated on live CD3+, CD8+ and CD45RA- CCR7- populations. (**A**) and (**B**) represent gating strategy of decidual effector memory (EM) phenotype based on CD27 and CD28 expression: EM1 CD28+ CD27+; EM2 CD28- CD27+; EM3 CD28- CD27-; EM4 CD28+ CD27-. (**C**), (**D**), (**E**) and (**F**) represent EM1, EM2 EM3 and EM4 populations respectively as percentage of total CD8+ effector memory CD8+ T cells in all tissues. Lines represent median and and interquartile range. Significance was calculated using Friedman's test and Dunn multiple comparison test where *P=<0.05, **P=<0.01, ***P=<0.001*

Figure 3.6.

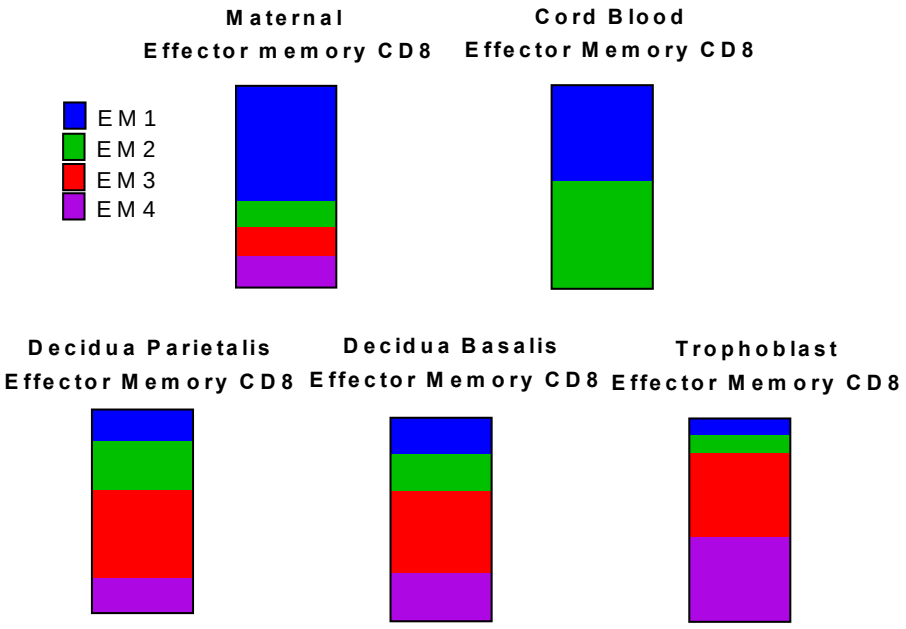


Figure 3. 6 Comparison of CD8+ effector memory phenotypes between tissues at 3rd trimester

Proportions of CD8+ T cell effector memory subsets are shown for maternal PBMCs, cord blood cells, dedidua basalis, decidua parietalis and trophoblast tissue. Pie charts were generated by calculating the mean of each effector memory subset population: Em1- Blue; Em2- Green; Em3- Red; Em4- Purple.

The level of differentiation of effector memory T cells was also measured in CD4+ T cells in all tissues. As with CD8+ effector memory T cells, there is a similar trend of reduced effector function, EM1 phenotype, in maternal PBMCs, 69.52%, in comparison to decidua basalis 27.06%, decidua parietalis 25.15% and trophoblast CD4+ T cells, 26.50% (all p= 0.0001) (Figure 3.7 A). Conversely, CD4+ EM3 effector memory T cells are significantly increased in all placental tissues, with decidua basalis, decidua parietalis and trophoblast representing 29.13%, 28% and 23.77% respectively in comparison to maternal PBMCs, 1.76% and cord blood T cells, 2.32% (all p=0.0001) (Figure 3.7 C). Mirroring CD8+ EM4 effector memory T cells, CD4 trophoblast T cells are significantly increased in comparison to decidua parietalis, 31.29% and 18.80% respectively (p=0.0144) (Figure 3.7 D).

Figure 3.8 illustrates the data using median proportions of each subtype for all tissues. Similar to CD8+ effector memory subsets, this demonstrates a significant trend of more mature effector memory subsets capable of cytotoxicity at the decidua and trophoblast, but less mature effector memory subsets in the maternal PBMCs and cord blood.

Figure 3.7.

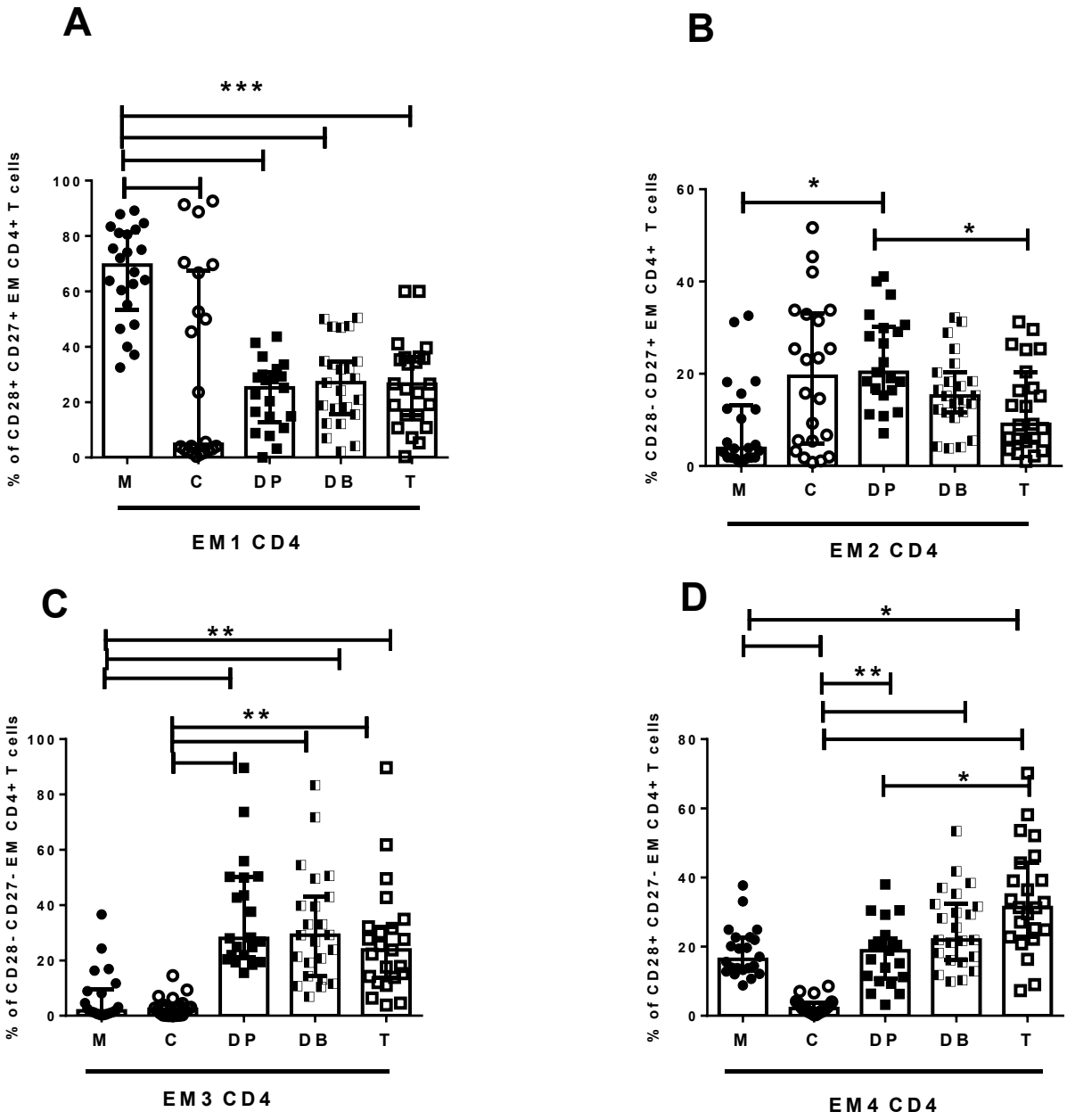

A
***
% of CD28+ CD27+ EM CD4+ T cells
100
80
60
40
20
0
M
C
DP
DB
T
EM1 CD4
B
*
*
% CD28- CD27+ EM CD4+ T cells
60
40
20
0
M
C
DP
DB
T
EM2 CD4
C
**
**
% of CD28- CD27- EM CD4+ T cells
100
80
60
40
20
0
M
C
DP
DB
T
EM3 CD4
D
*
**
*
% of CD28+ CD27- EM CD4+ T cells
80
60
40
20
0
M
C
DP
DB
T
EM4 CD4

Figure 3. 7. Effector memory phenotype of CD4+ T cells at 3rd trimester of pregnancy

*CD4+ T cell effector memory status was analysed in N=45 of maternal PBMCs, cord blood, decidua basalis, decidua parietalis and trophoblast tissue. CD4+ Effector Memory T cells were gated on live CD3+, CD4+ and CD45RA- CCR7- populations. (**A**) and (**B**) represent gating strategy of decidual effector memory (EM) phenotype based on CD27 and CD28 expression: EM1 CD28+ CD27+; EM2 CD28- CD27+; EM3 CD28- CD27-; EM4 CD28+ CD27-. (**C**), (**D**), (**E**) and (**F**) represent EM1, EM2 EM3 and EM4 populations respectively as percentage of total CD4+ effector memory T-cells in all tissues. Lines represent median and and interquartile range. Significance was calculated using Friedmans test and Dunn multiple comparison test where *P=<0.05, **P=<0.01, ***P=<0.001*

Figure 3.8.

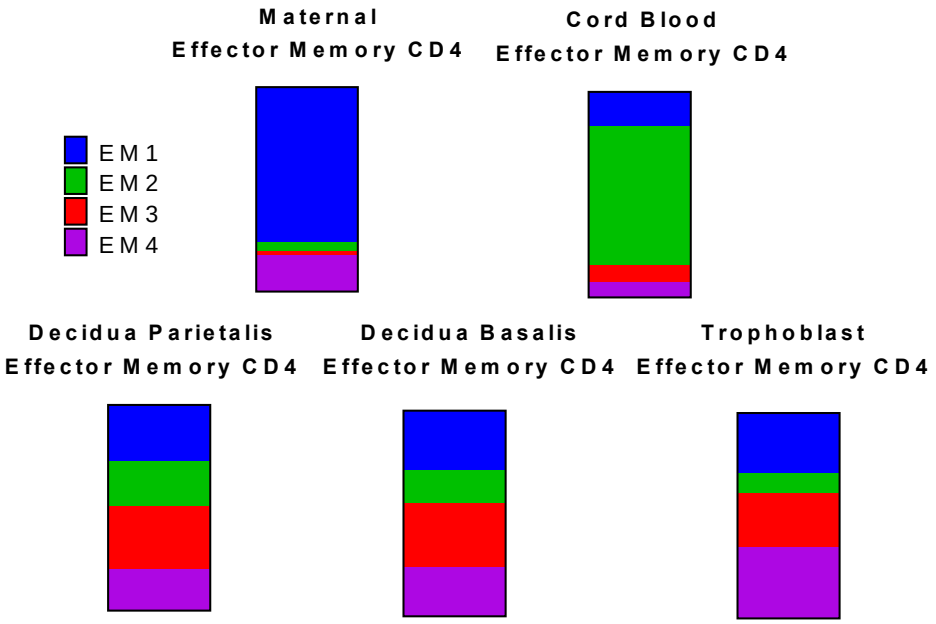


Figure 3. 8 Comparison of CD4+ effector memory phenotypes between tissues at 3rd trimester

Proportions of CD4+ T cell effector memory subsets are shown for maternal PBMCs, cord blood cells, dedidua basalis, decidua parietalis and trophoblast tissue. Pie charts were generated by calculating the mean of each effector memory subset population: Em1- Blue; Em2- Green; Em3- Red; Em4- Purple.

An additional measurement of differentiation was carried out on CD4+ and CD8+ effector memory T cells with the assessment of CD57. Higher levels of CD57 correlates with decreased telomere length and therefore is a proxy for cell division and differentiation. It was found that decidua basalis and decidua parietalis CD8+ effector memory T cells had significantly increased proportion of CD57 expressing cells, 45.2% and 43.9% respectively, in comparison to maternal peripheral blood CD8+ effector memory T cells, 31.95% (p=0026, 0.0039) (Figure 3.9 A). Decidua basalis and decidua parietalis CD4+ effector memory T cells had lower expression of CD57 when compared to CD8+ counterparts, 14.1% and 12.6 respectively, yet still had significantly increased proportions compared to CD4+ maternal peripheral blood T cells, 6.4% (p=0.0022, p=0.0006) (Figure 3.9 B).

Figure 3.9.

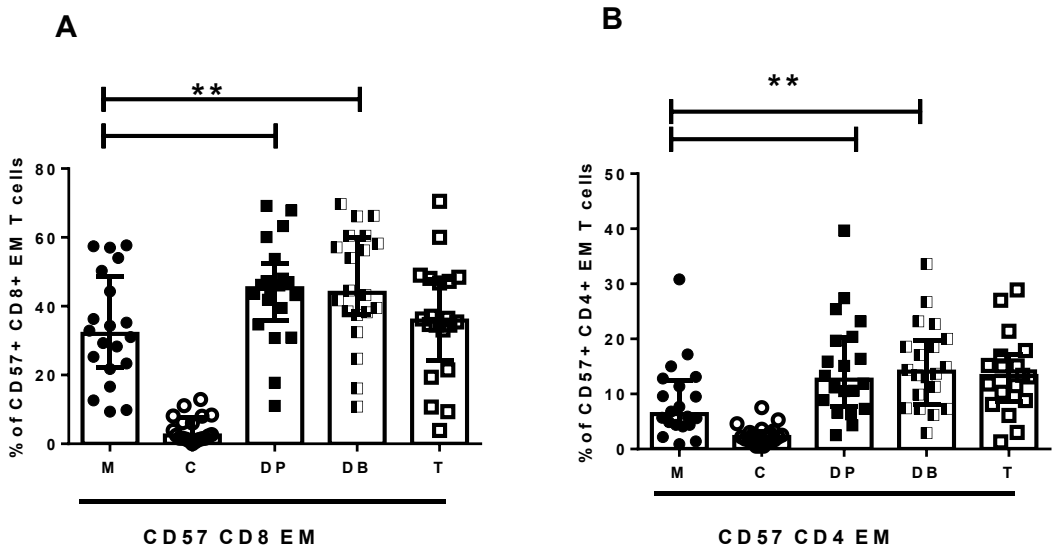


Figure 3. 9. CD57 expression on effector memory T cells in healthy 3rd trimester pregnancy

*The proportion of CD57+ (**A**) CD8+ and (B) CD4+ Effector memory T cells were measured in maternal PBMCs, cord blood, decidua basalis and decidua paritalis and trophoblast T cells (N=20). Lines represent median and and interquartile range. Significance was calculated using Friedmans test and Dunn multiple comparison test where *P=<0.05, **P=<0.01, ***P=<0.001*

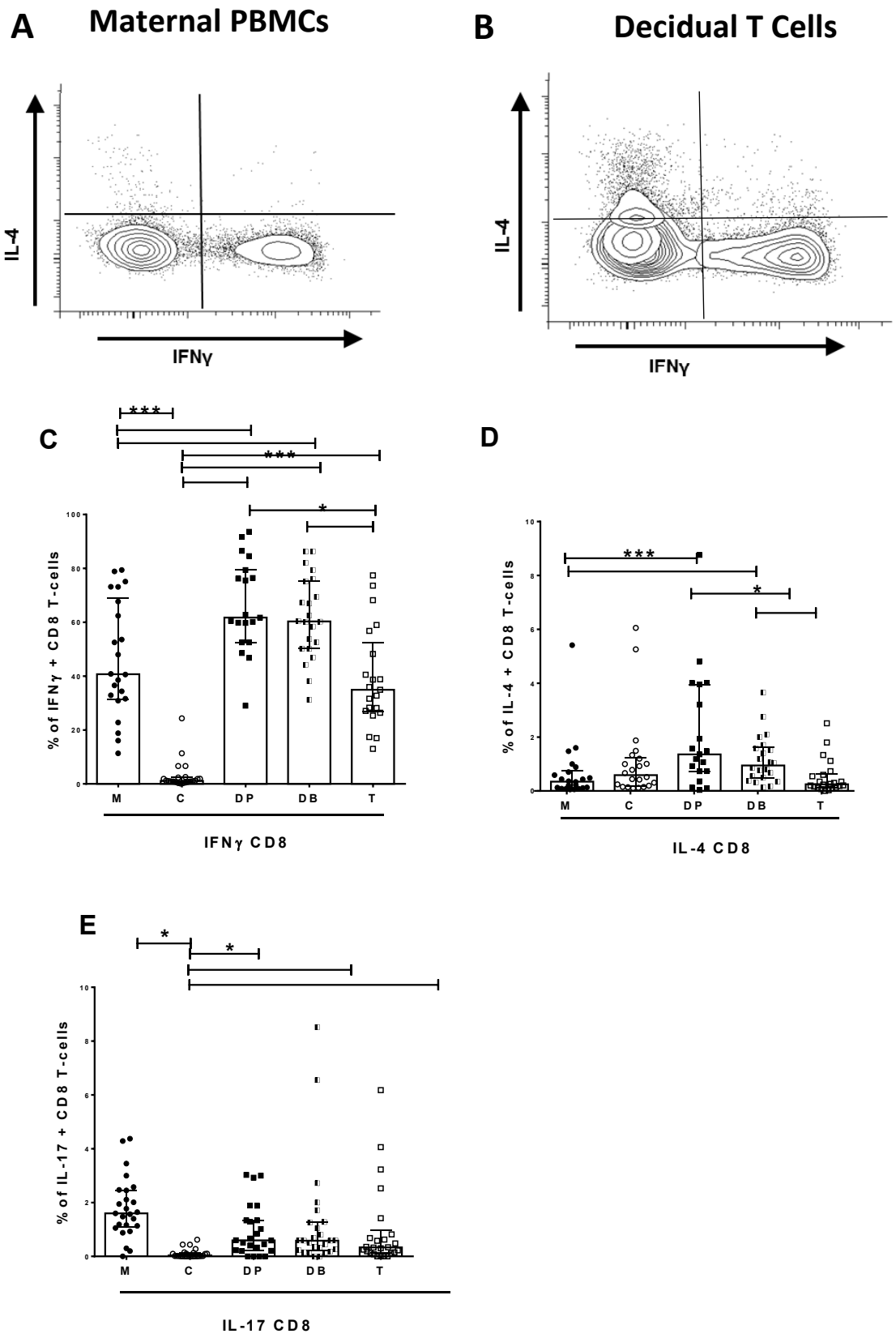
3.3. Cytokine and chemokine expression of Decidual CD8 and CD4 T cells

Following the phenotypic analysis of CD8+ and CD4+ T cells based on their memory status we next wanted to identify their functional capacity by measuring cytokine expression in decidual tissue, trophoblast, maternal PBMCs and cord blood. We measured cytokine levels following mitogen stimulation, to elucidate activation and cytotoxcity. Figure 3.10 A and B demonstrate the gating strategy to asses cytokine expression of T cells subjected to mitogen stimulation followed by intracellular cytokine flow cytometric staining. Figure 3.10 C demonstrates that CD8+ T cells at the decidua parietalis and decidua basalis express significantly increased IFNγ, 61.74% and 60.32% respectively, when compared to maternal PBMCs, 40.71% (p=0.0002; 0.0003), cord blood, 1.11% (both p=0.0001) and trophoblast, 34.95% (p=0.0122; 0.0446). Interestingly, despite relatively lower levels of IL-4 in comparison to IFNγ, IL-4 expression was also significantly increased in decidua basalis and decidua parietalis, 0.94% and 1.35% respectively, in comparison to maternal PMCs, 0.34% (p=0.0493; 0.0121) (Figure 3.10 D). Level of Il-17 expression in CD8+ T cells was very low throughout all samples, approaching a median of 0.5%, and did not have any significant increases between samples apart from cord blood T cells (Figure 3.10 E). Decidual CD4+ T cells demonstrated a similar pattern of increased dual expression of IFNγ and IL-4 expression to their CD8+ counterparts (Figure 3.11). Decidua basalis and decidua parietalis CD4+ T cells exhibited lower overall expression of IFNγ, 25.35% and 21.69% respectively, compared to CD8+, however they are significantly increased in comparison to maternal PBMCs, 15.84% (p=0.0006; 0.0052), cord blood, 0.30% (both p= 0.0001) and trophoblast

CD4+ T cells, 7.34% (both p=0.0039) (Figure 3.11 A). Decidua basalis and decidua parietalis IL-4 expression in CD4+ T cells was higher, 3.37% and 6.26% respectively, compared with CD8+, and significantly increased in decidua parietalis when compared to maternal PBMCs, 1.67% (p= 0.0046) cord blood, 1.62% (p=0.0162) (Figure 10 B). As with CD8+ T cells, IL-17 was not significantly increased in comparison between samples, apart from cord blood (Figure 3.11 C).

In addition to the characterisation of cytokine expression and memory status of CD4 at the maternal fetal interface, we also wanted to assess the expression of chemokine receptor CXCR3, which is reported to be important in trafficking T-cells in tissue and areas of allo-reactivity and inflammation. We were able to detect that CXCR3 has significantly increased expression at the decidua parietalis, 15.40% of total CD4+ T cells, when compared to maternal PBMCs, 5.61% (p=0.0021), cord blood, 2.73% (p=0.0001) and trophoblast, 7.32% (p=0.0431) (Figure 3.12).

Figure 3.10.


A
Maternal PBMCs
IL-4
IFNγ
B
Decidual T Cells
IL-4
IFNγ
C
***
***
*
% of IFNγ + CD8 T-cells
M
C
DP
DB
T
IFNγ CD8
D
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% of IL-17 + CD8 T-cells
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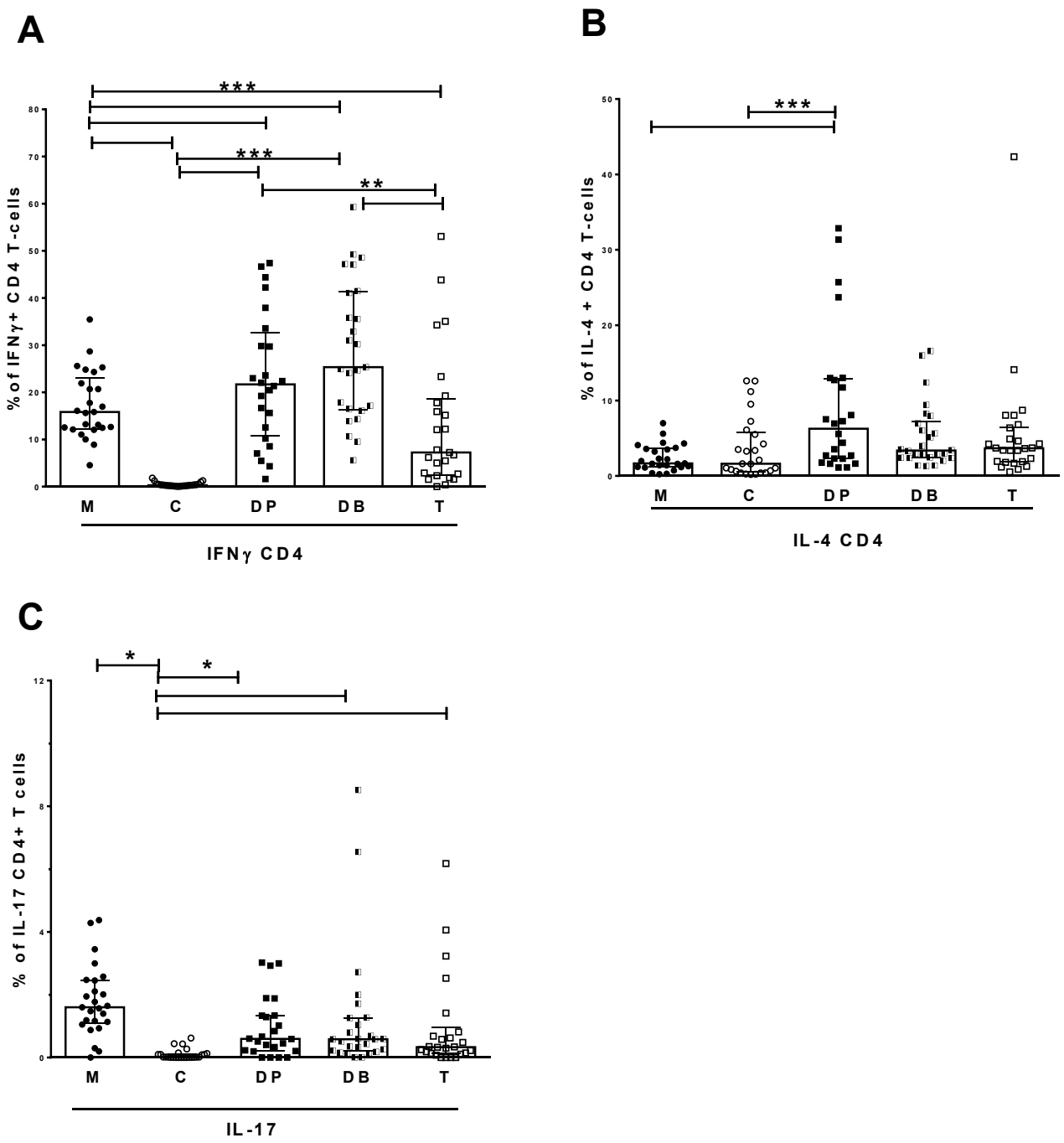
Figure 3. 10. CD8+ cytokine expression at 3rd trimester of healthy pregnancy

*Cytokine expression following mitogen stimulation of CD8+ T cells were analysed in n=30 maternal PBMCs, cord blood, decidua basalis, decidua parietalis and trophoblast tissue. (**A**) and (**B**) show gating strategy of Il-4 and IFNγ in maternal PBMCs and Decidua basalis respectively. (**C**), (**D**) and (**E**) show IFNγ, IL-4 and IL-17 expression respectively as a percentage of total CD8+ in maternal PBMCs, Cord blood, decidua basalis, decidua parietalis and trophoblast. Lines and bars represent median and interquartile range. Significance was calculated using Friedmans test and Dunn multiple comparison test, *P=<0.05, **P=<0.01, ***P=<0.001*

Figure 3.11.


A
***
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% of IFNγ+ CD4 T-cells
M
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IFNγ CD4
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% of IL-4 + CD4 T-cells
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IL-4 CD4
C
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*
% of IL-17 CD4+ T cells
M
C
DP
DB
T
IL-17

Figure 3. 11. CD4+ cytokine expression at 3rd trimester of healthy pregnancy

*Cytokine expression following mitogen stimulation of CD4+ T cells was analysed in n=30 maternal PBMCs, cord blood, decidua basalis, decidua parietalis and trophoblast tissue. (**A**), (**B**) and (**C**) show IFNγ, IL-4 and IL-17 expression respectively as a percentage of total CD4+ in maternal PBMCs, Cord blood, decidua basalis, decidua parietalis and trophoblast. Lines and bars represent median and interquartile range. Friedmans test and Dunn multiple comparison test, *P=<0.05, **P=<0.01, ***P=<0.001*

Figure 3.12.

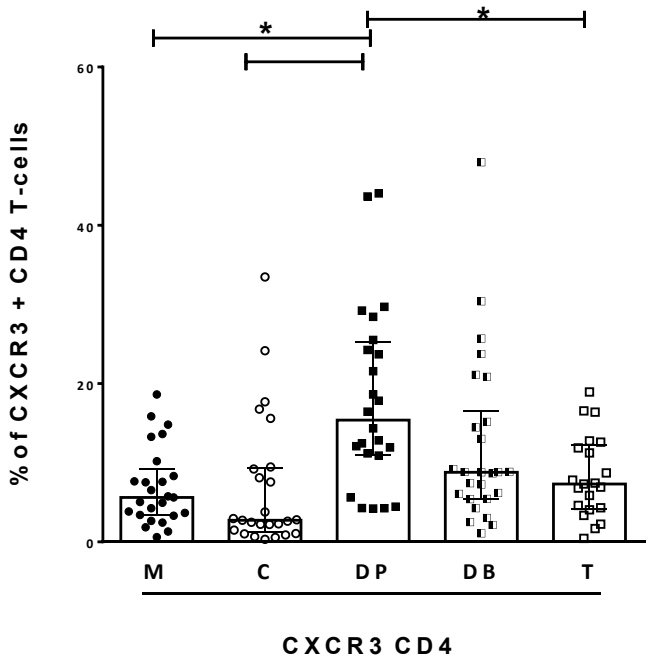


Figure 3. 12 CXCR3 expression in CD4+ T cells at 3rd trimester

*CXCR3 expression by CD4+ T cells was analysed in n=35 maternal PBMCs, cord blood, decidua parietalis, decidua basalis and trophoblast. Lines represent median and interquartile range. Significance was calculated using Friedman's test and Dunn multiple comparison test where *P=<0.05, **P=<0.01, ***P=<0.001*

Chapter 3.4. Discussion

So far we have established a comprehensive memory phenotype and cytokine expression profile of CD4+ and CD8+ T cells within placental tissue, encompassing the decidua basalis, decidua parietalis and trophoblast, together with donor matched maternal PBMCs and cord blood. The first striking feature of these data is the predominance of effector memory CD4+ and CD8+ T cells in decidual tissue and a reduced proportion of naive cells when compared to maternal, cord and trophoblast. Additionally, these decidual effector memory T cells are identified as having a significantly increased proportion of EM3 subtypes. These have been shown to have increased cytolytic properties and shortened telomere length (Romero et al. 2007) suggesting they have experienced allo-antigen and have undergone several rounds of cell division (Tilburgs et al. 2010). This is confirmed with significant increase of CD57 expression in decidual T cells in comparison to maternal PBMCs T cells. See Table 3.1 for summary of these results, comparing proportions of cell types between maternal PBMCs and decidua basalis T cells.

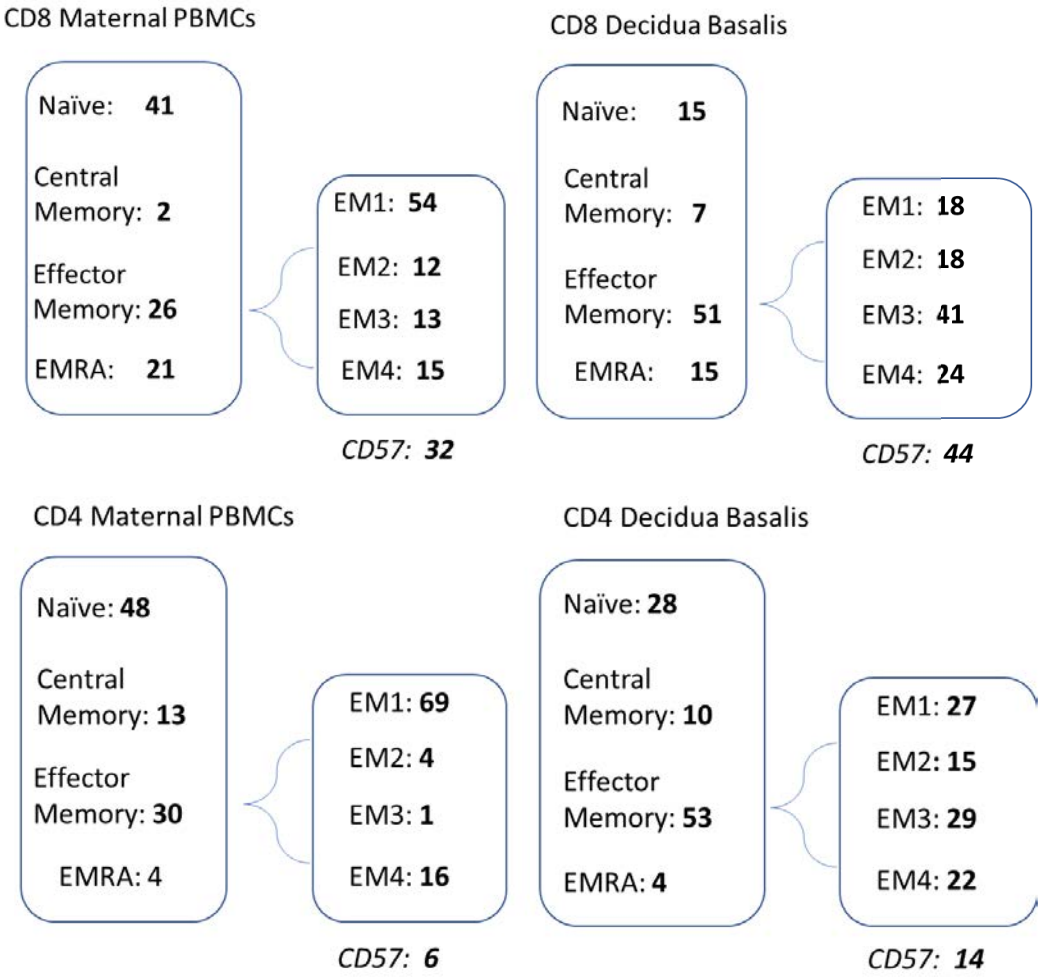


Table 3. 1 Summary of T cell Memory cell types in Maternal PBMCs and Decidua Basalis T cells

Breakdown of CD8 and CD4 memory populations in maternal PBMCs and decidual T cells compared. Numbers represent median percentage proportion of total CD4 or CD8 T cells. Additionally breakdown of effector memory subsets , numbers represent median percentage proportion of total effector memory CD4 or CD8 T cells. Median percentage of CD57 expression on total effector memory T cells is also listed .

With the measurement of increased EM3 effector memory subsets and CD57 expression on effector memory T cells within the decidua, we address the increase in ratio of effector memory to naïve T cells in comparison maternal peripheral blood is indeed a significant observation. Peripheral blood contains high numbers of naïve T cells and are less capable of entering tissues. Therefore, it could be suggested that our findings of increased proportions of effector memory T cells in decidual tissues does not represent specific

trafficking to the decidua and instead demonstrates that the natural occurring disparity in naïve and effector populations in blood and tissue. However, we have demonstrated that within effector memory populations between maternal PBMC and decidual samples, there is heightened activated cell types, suggesting that the presence of these cell populations at the maternal-fetal interface is significant. This activated phenotype of decidual T cells is also supported by the findings of elevated levels of IFNγ production upon mitogen stimulation, when compared to maternal, cord and trophoblast tissue. However, there is also additional increase of IL-4 producing an unusual profile, which may suggest the role of increased progesterone at this site (Lissauer et al. 2015). A Th1:Th2 shift during pregnancy is, despite an oversimplification, still an important concept in maintenance of a healthy pregnancy, and this balance is maintained not only by cellular processes distinct to maternal immune cells and fetal tissue, but that of maternal hormonal changes at the maternal-fetal interface. Progesterone in particular is known to induce increased levels of IL-4 in maternal CD4+ and CD8+ T cells and, furthermore, limit their cytotoxicity (Lissauer et al. 2015), (Raghupathy et al. 2005a).

Decidual T cells additionally expressed increased levels of the chemokine receptor CXCR3. Interestingly, Nancy et al (2012) suggested CXCR3 ligand gene silencing in decidua basalis of mice placenta led to reduced recruitment of T-cells to this site. This does not seem to be the case in human pregnancy, and highlights the importance further investigation of T immunology in healthy human pregnancy.

T cells isolated from trophoblast demonstrate increased proportions of naive T cells, however, unlike maternal peripheral blood cells, they exhibit increased proportions

of EM3 and EM4 effector memory subtypes. This observation may be due to contamination of cord blood cells in the trophoblast isolated T cells, therefore skewing the level of naivety of this tissue type. However, the identification of highly differentiated effector T cells, suggests these cells are indeed maternal in origin and have trafficked from the decidua, deeper into the fetal tissue. These trophoblast T cells express reduced levels of cytokine, in comparison to decidual T cells. Again, this may be due to fetal blood T cell contamination, or indeed may be a phenotype of chronically differentiated T cells, identified by their CD27 and CD28 status.

An interesting observation is the presence of potentially antigen experienced cord blood cells in the form of effector memory T cells. It is thought that as the fetus develops in a sterile environment, the only T cell subset to be present are T regulatory cells (Mold et al 2008). However Zhang et al (2014) have identified that these small populations of effector T cells in cord blood are capable of functional responses and are distinct from T regulatory cells. It is not known whether these are antigen specific or what endogenous functions would drive their differentiation; however, it is proposed that they represent a naturally derived pool of memory T cells able to mobilize quickly soon after antigenic stimulus. Other sources of potential antigenic stimulus include non-pathogenic commensal bacteria present in the placenta (Aagaard et al. 2014) and CD45R0+ T cells within the fetal intestine (Bunders et al. 2012).

In conclusion, we have demonstrated that within healthy term pregnancy, populations of CD4+ and CD8+ T cells at the decidua are enriched for highly differentiated and potentially antigen experienced effector memory phenotype. In addition, decidual

CD4+ and CD8+ T cells have increased expression of both IFNγ and IL-4, a cytokine profile indicative of both activation and modulation. Lastly, the significant increase of chemokine receptor, CXCR3 in CD4+ T cells suggest that these cells are trafficked to the maternal-fetal interface, which indicates an allo-reactive response. V.U.E is associated with an inflammatory phenotype of CD8+ T cells and our findings suggest that populations of these cells are also present in healthy pregnancy in the decidua. This therefore demonstrates a potential role of the breakdown of immune modulation at the maternal-fetal interface in generating V.U.E pathologies.

Chapter 4.Results
Regulation of T cells at the maternal-fetal interface.

4.1 Introduction

Our data has demonstrated that CD4+ and CD8+ T cells at the decidua parietalis and decidua basalis, have an increased proportion of activated and highly differentiated effector memory T cells, when compared to maternal PBMCs and cord blood T cells. The mechanisms by which decidual T cells are regulated in humans is unclear and indeed may depend on the potential of these cells to gain anatomical access to fetal tissue. Large numbers of T regulatory cells are generated during pregnancy (Rowe et al. 2012; Robert M. Samstein et al. 2012) and murine studies show that these are essential for fetal survival (Aluvihare et al. 2004a). Inhibitory checkpoint proteins such as PD-1 and T Cell Immunoglobulin And Mucin-Domain Containing 3 (Tim-3) play a critical role in murine models of transplantation and pregnancy (Guleria, et al. 2005) and antibody-mediated blockade results in allograft and fetal rejection (Chabtini et al. 2013; Tanaka et al. 2007). Expression of these inhibitory immune checkpoint proteins has been demonstrated on human decidual CD8+ T cells (Wang et al. 2016) whilst Programmed Death Ligand 1 (PD-L1) is also expressed in human decidua (Guleria, et al. 2005; Liang et al. 2003). For this study, we assessed regulatory components at the maternal-fetal interface by establishing the proportion of CD4+ T regulatory cell populations and the expression of checkpoint markers on effector memory T cells.

4.2 CD4+ T regulatory cells at the maternal-fetal interface

We analysed the percentage proportion of CD4+ CD25+ CD127- FoxP3 + T regulatory cells in maternal blood, cord blood, decidua basalis, decidua parietalis and trophoblast tissue. Figure 4.1 A represents the gating strategy used, focusing on CD25 + CD127 low FOXp3 + CD4+ T-cells. Figure 4.2, demonstrates that the decidua parietalis and decidua basalis contain increased populations of CD4+ T regulatory cells, 7.62% and 10.89% respectively, when compared to maternal PBMCs, 2.58% (both p=0.0001) and cord blood, 4.53% (p=0.011, 0.0004). This result is supported by previous data (Kahn & Baltimore 2010; Trowsdale & Betz 2006), suggesting increased populations of T regulatory cells in the decidua contribute to maintenance of immunological interaction between the mother and the fetus during pregnancy up to term.

Interestingly, there are increased proportions T regulatory cells at the decidua parietalis in comparison to the trophoblast, 4.5% (p=0.003), suggesting reduced infiltration of CD4+ T regulatory cells into the trophoblast layer of the placenta.

Figure 4.1.

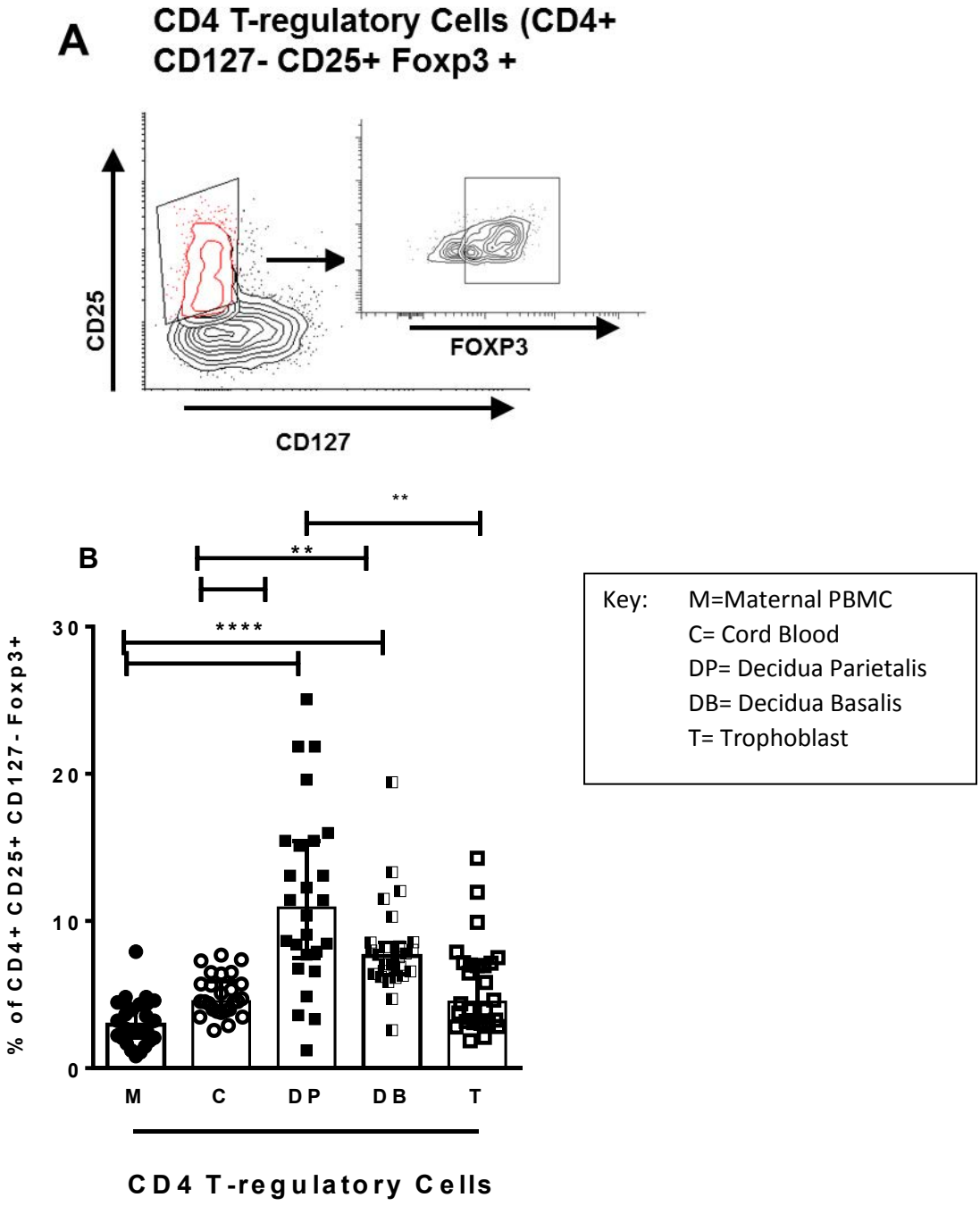


Figure 4. 1. T regulatory CD4 T-cells at 3rd Trimester.

*Populations of T regulatory CD4 T-cells were measured in n=30 maternal PBMCs cord blood, decidua parietalis and decidua basalis and trophoblast. (**A**) represents gating strategy of CD4 T-regulatory cells based on CD25+ CD127 low Foxp3+ expression. (**B**) shows proportion of T-regulatory cells as a percentage of total CD4 T-cells in all tissues. Significance was calculated using Friedman's test and Dunn multiple comparison test*

4.3. Checkpoint Markers are increased on decidual T cells

To further characterise an intrinsic process of regulation as opposed to extrinsic factors demonstrated by T regulatory cells, we analysed the level of expression of PD-1, Tim-3, CTLA-4 and Lag-3 on CD4+ and CD8+ effector memory T cells. These markers have been previously described as major immune checkpoint proteins indicating exhaustion in immune responses, from anti-tumour responses in solid tumours to allograft responses in bone marrow transplants. Using flow cytometric methods, we analysed these four immune checkpoint proteins in both matched maternal blood and decidua basalis effector memory CD4+ and CD8+ T cells (Figure 4.2 A and Figure 4.3 A respectively). All immune checkpoint proteins were significantly increased in decidua basalis effector memory T cells in comparison to maternal blood effector memory T cells. This was seen in both CD4+ and CD8+ subsets and was most marked in the case of PD-1, expressed on 45% and 56% of CD4+ and CD8+ T cells respectively (both p=0.0078). Tim-3 and CTLA-4 expression was very low on maternal effector memory CD4+ T cells, whilst decidua basalis expression significantly approached 10% (p=0.0078) along with LAG-3 increasing from 10% to 17% (p=00.78). In maternal blood CD8+ effector memory T cells, Tim3 and CTLA-4 were expressed at somewhat higher levels compared to CD4+. However, again, the percentage expression of Tim-3, CTLA-4 and LAG-3 significantly increased from 15%, 4%, 8% to 28%, 14%, 16% respectively on CD8+ decidua basalis effector memory T cells compared to maternal peripheral blood counterparts (p=0.0078;0.0078;0.0156). Using Boolean analysis we were able to examine the pattern of co-expression of these inhibitory proteins. Expression of two markers was the most frequent co-expression pattern, and was

dominated by PD-1. PD-1 was commonly found co-expressed with either Tim-3 (47%,37%), CTLA-4 (32%, 21%) or Lag-3 (35%, 41%) in CD4+ and CD8+ T cells respectively (Figure 4.2 B and 4.3 B).

Figure 4.2.

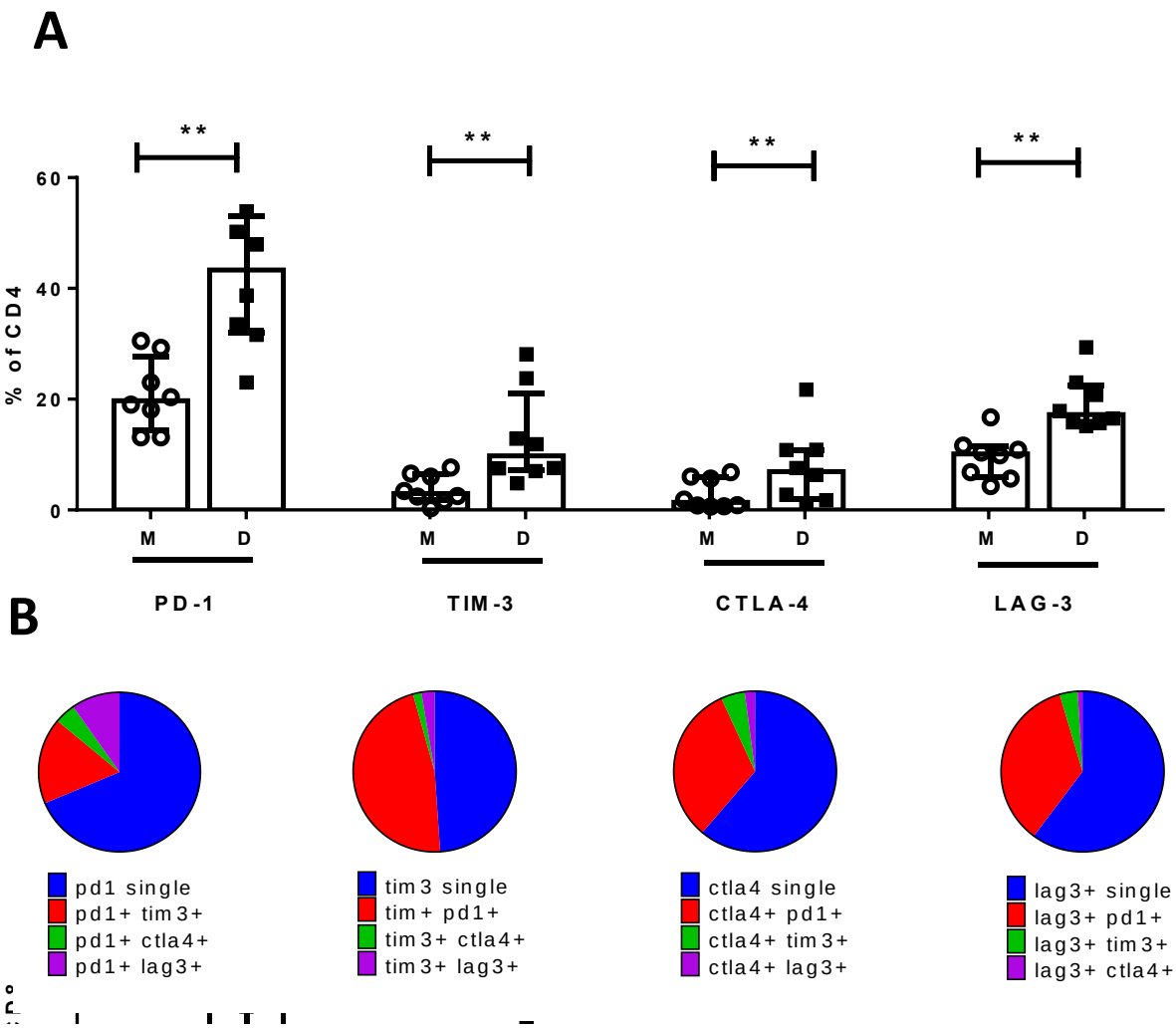


Figure 4. 2. CD4+ Effector Memory immune checkpoint protein expression at the maternal-fetal interface.

*Expression of PD-1, Tim-3, CTLA4 and Lag-3 were measured in n=10 maternal PBMCs, and decidua basalis tissue. (**A**) demonstrates expression of immune checkpoint proteins in maternal PBMCs and decidua basalis tissue as a percentage of total CD4 effector memory T-cells. Significance calculated using Wilcoxon paired sample test P=<0.05, **P=<0.01, ***P=<0.001. (**B**) Boolean gating analysis shows proportions of dual positivity of immune checkpoint proteins expressed by effector memory CD4 T-cells in decidual tissue.*

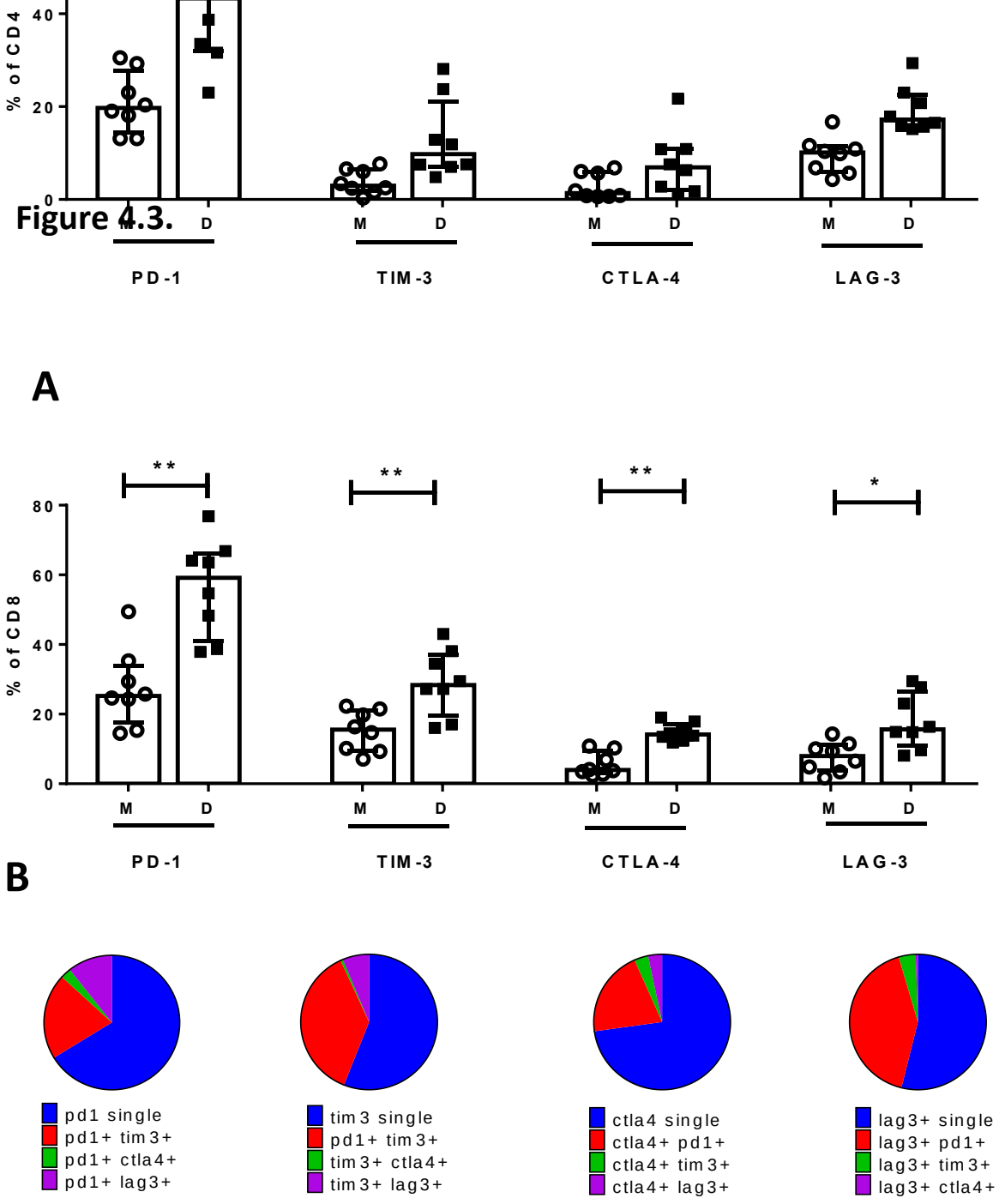


Figure 4.3.

Figure 4. 3. CD8+ Effector memory immune checkpoint protein expression at the maternal-fetal interface

*Expression of PD-1, Tim-3, CTLA4 and Lag-3 were measured in n=10 maternal PBMCs, and decidua basalis tissue. (**A**) demonstrates expression of immune checkpoint proteins in maternal PBMCs and decidua basalis tissue as a percentage of total CD8 effector memory T-cells. Significance calculated using Wilcoxon paired sample test P=<0.05, **P=<0.01, ***P=<0.001. (**B**) Boolean gating analysis shows proportions of dual positivity of immune checkpoint proteins expressed by effector memory CD4 T-cells in decidual tissue.*

Chapter 4.4 Discussion

In Chapter 3 we demonstrated a strong functional capacity of decidual T cells. We were, therefore, particularly interested in the mechanisms that might regulate decidual T cell activation and, consequently, our research focussed on mechanisms of extrinsic and intrinsic suppression. CD4+ T regulatory cells are deemed to play a critical role in immune modulation at the maternal-fetal interface (Aluvihare et al. 2004a) and several groups have reported an increase in T regulatory cells within decidua (Tilburgs et al. 2006). We were able to confirm this with a 2.5-fold increase in the proportion of T regulatory cells compared to maternal peripheral blood. Additionally we found that there were reduced populations of T regulatory cells in the trophoblast tissues. This may have an impact on inflammatory diseases of the placenta, if this regulatory cell type is unable to infiltrate into the deeper trophoblast tissue and therefore unable to modulate allo antigen immune responses.

We were further interested to study the expression of inhibitory checkpoint proteins on decidual T cells as these proteins mediate intracellular signalling pathways that limit the magnitude and outcome of T cell responses by negatively regulating T cell activation and function. Indeed, antibodies that block inhibitory receptors have a transformative impact on the treatment of many forms of cancer (Topalian et al. 2012). We observed that the expression of four of the major checkpoint proteins, PD-1, Tim-3, CTLA-4 and LAG-3, increased substantially on T cells within decidua when compared to matched maternal peripheral blood T cells. A similar profile has been observed on decidual T cells in the first trimester, and indicates that this profile is established early within

pregnancy and is maintained throughout gestation (Wang et al. 2015). PD-L1 expression has been demonstrated on syncytiotrophoblast, and intermediate trophoblastic cells located in the chorion laeve and implantation site, which provides further support for the importance of this inhibitory pathway in reproduction (Veras et al. 2016).

Combinatorial staining showed that checkpoint proteins were often expressed together on decidual T cell populations. PD-1 was the dominant partner in each case but 35% of both CD4+ and CD8+ PD-1+ T cells expressed an additional checkpoint protein. This indicates that checkpoint expression is not redundant and, furthermore that the physiological importance of these patterns of expression, and their relevance in relation to checkpoint blockade in oncology, will need further investigation. Due to the increased expression of a range of immune checkpoint proteins, dominated by PD-1, the data suggests that CD4+ and CD8+ T cells experience chronic activation, which may lead to restriction of their cytotoxic activity and allo-reactive potential.

The evidence of both extrinsic (CD4+ T regulatory cells) and intrinsic (immune checkpoint proteins) regulatory mechanisms at the maternal-fetal interface, and their relative elevated levels in comparison to peripheral blood, suggests that these are critical for healthy pregnancy, and in light of our findings in Chapter 3 are required to modulate the increased effector T cell populations in the decidua.

Chapter 5.Results
Fetal specific responses at the maternal-fetal interface.

5.1. Introduction

In Chapters 3 and 4, we established that decidual T cells are highly activated, functional, and differentiated; however, these cells express increased levels of immune checkpoint proteins. This indicates that decidual T cells cells are indeed antigen experienced and are potentially responding to allo-antigens present on fetal tissue. Villous cytotrophoblast and syncytiotrophoblast do not express MHC class I antigen presenting molecules however, previous reports have suggested that T cells can directly interact with extravillous trophoblast (EVT) and, therefore, become exposed to fetal antigen. EVT downregulates expression of HLA-A, HLA-B alleles, but does retain HLA-C expression (Blais et al. 2011). Indeed, significantly higher levels of activated T cells and T regulatory cells are induced within decidua of HLA-C mismatched pregnancies (Tilburgs et al. 2009). Murine models also suggest that fetal proteins are presented to the maternal immune system indirectly, through uptake and presentation by maternal antigen presenting cells (Erlebacher et al. 2007). Due to microchimerism of fetal tissue shed into the maternal circulatory system, allogeneic responses are detectable both during and post pregnancy (Lissauer et al. 2012; Piper et al. 2009; Verdijk et al. 2004). Research directly addressing antigeneic specificity within human decidual is however limited (Tilburgs et al. 2010). Previous research has shown that during pregnancy in which the fetus is male, the maternal immune system elicits T cell responses specifically to HY minor antigen. These CD8+ HY specific T-cells can be detected during and after pregnancy, and also have cytotoxic reactivity (Lissauer et al. 2012). The dextramers used to detect these HY specific CD8+ T cells were the A2 and B7 epitopes derived from HY protein KDM5D.

Using these same dextramers we were able to detect HY specific T cells at the decidua. Additionally proliferation to fetal antigen was measured using a mixed lymphocyte reaction assay utilising matched cord blood to stimulate maternal peripheral blood T cells and decidual T cells.

It is critical to state however that due to HLA-A2 and B7 not being expressed by extravillous trophoblast, A2/ B7 specific T cells in the decidua will not necessarily be able to carry out an allo-reactive response (See figure 1.5).

5.1. HY specific responses of CD8 decidual T cells

Thus far, we have established highly differentiated and activated CD4+ and CD8+ T cells are present in the decidua. To elucidate whether this activatory pattern is, in part due to an allo-reactive response to fetal antigen, we analysed T cell HY specificity in pregnancies of male fetuses. We carried out analysis of n=9 matched maternal and decidual T cells using HY specific antibodies in flow cytometric analysis. Dextramers specific to A2 and B7 epitopes derived from HY protein KDM5D were used. Maternal blood and cord blood was also HLA typed and samples with correct corresponding HLA type were chosen. Figure 5.1 A demonstrates the gating strategy used to identify CD8+ T cells that were specific to HY dextramer. We found that decidual basalis had a 10-fold increase in HY+ CD8+ T cells representing 0.6% of total CD8+ T cells when compared to maternal PBMCs, 0.06% ($p= 0.0313$) (Figure 5.1 B).

We further phenotyped these HY specific T cells by investigating their memory, activation and immune checkpoint status. Figure 5.2 A demonstrates an overview of HY specific T cells compared to overall T cells found at the decidua. When compared to maternal HY specific PBMCs, effector memory populations were significantly enhanced at the decidua, representing 20.53% and 56.49% of HY+ CD8+ T cells respectively, p=0.017 (Figure 5.2 D). The expression of immune checkpoint protein PD-1 was significantly increased in these effector memory decidual HY+ CD8+T cells, 66.06%, when compared to maternal PBMCs counterparts, 15.63% (p=0.0076) (Figure 5.3). We additionally measured the level of expression of the tissue resident activation marker CD69 and discovered that this was also significantly increased in the decidual effector memory HY+ CD8+ T cells in comparison to maternal PBMCs, 70% and 11.17 respectively (p= 0.0048) (Figure 5.3 B). These data suggest that T cells that are activated to fetal antigen, in this case male HY antigen, accumulate at the maternal-fetal interface and are of an effector memory phenotype.

Figure 5.1.

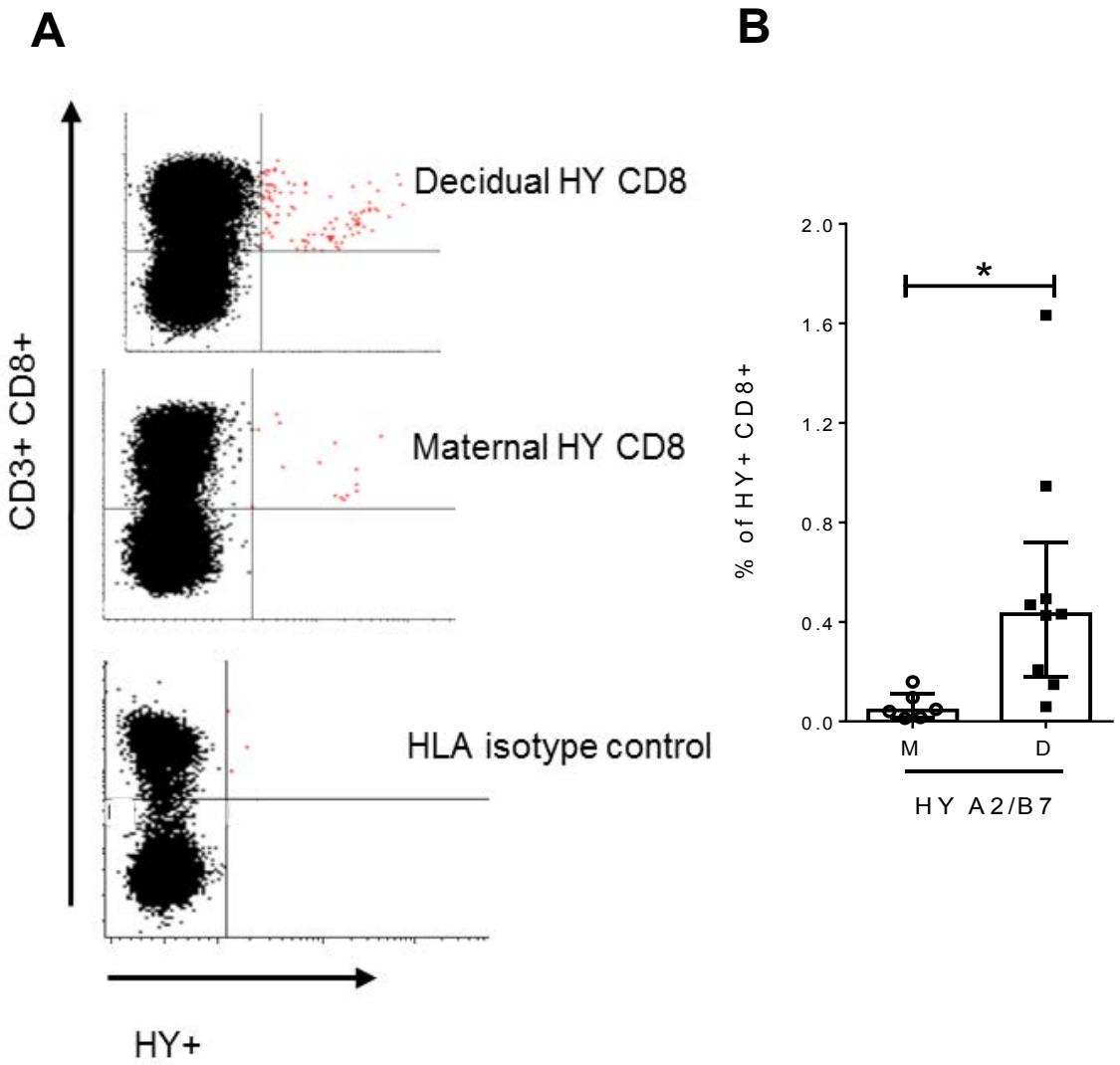


Figure 5. 1. Fetal minor histocompatability antigen specific CD8+ T cells present in the decidua

*(**A**) CD8 T cell responses to HY-minor histocompatability antigens were measured by gating on freshly isolated maternal and matched decidual live CD3+ CD8+ and HY dextramer+ positive cells, ex vivo ,n=9 (plots show HLA-A2 restricted dextramer results). An HLA specific isotype tetramer was used as –ve control. (**B**) HY specific CD8 T-cells are significantly increased at the decidua. Data shown combines results for A2 and B7 restricted dextramers, (Wilcoxon paired samples test, P*=<0.05). Horizontal lines represent median and interquartile range.*

Figure 5.2.

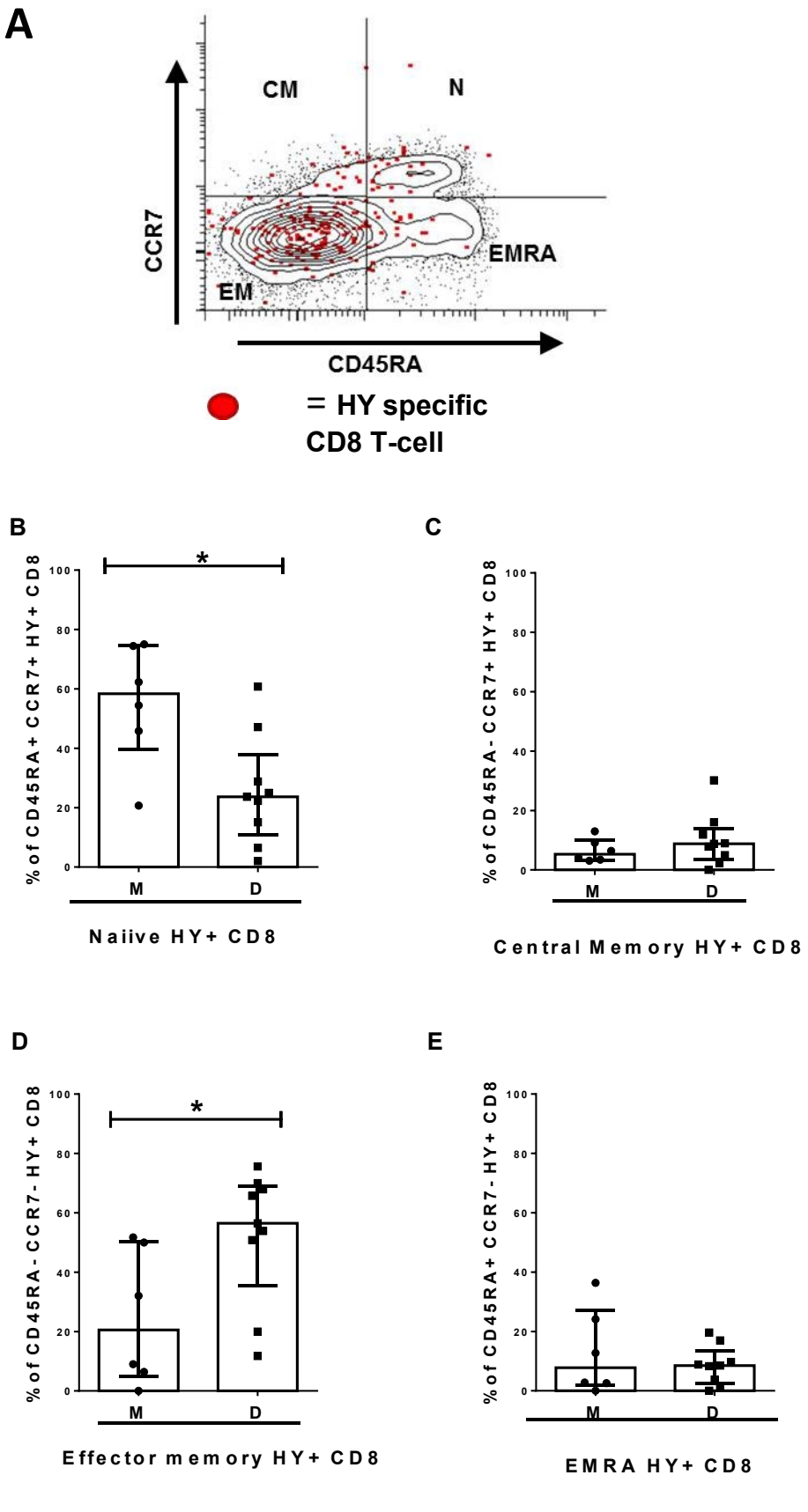

A
CM
N
EM
EMRA
CCR7
CD45RA
= HY specific CD8 T-cell
B
% of CD45RA+ CCR7+ HY+ CD8
M
D
Naiive HY+ CD8
C
% of CD45RA- CCR7+ HY+ CD8
Central Memory HY+ CD8
D
% of CD45RA- CCR7- HY+ CD8
Effector memory HY+ CD8
E
% of CD45RA+ CCR7- HY+ CD8
EMRA HY+ CD8

Figure 5. 2. Memory Phenotype of HY specific CD8 T-cells at the maternal- fetal interface

*(**A**) demonstrates memory phenotype of HY specific CD8 T-cells (in red) against a background of overall CD8 T cells of the decidua basalis. Memory phenotype categorised based on CD45RA and CCR7 expression : Naïve (N) CD45RA+ CCR7+; Central Memory (CM) CD45RA- CCR7+; Effector Memory (EM) CD45RA- CCR7-; Effector Memory RA (EMRA) CD45RA+ CCR7-. (**B**), (**C**), (**D**) and (**E**) represent Naïve, Central Memory, Effector memory and EMRA populations of HY specific CD8 T-cells respectively as percentage of total HY specific CD8 T-cells. Lines represent median and interquartile range. Significance was calculated using Wilcoxon paired sample test P=<0.05, **P=<0.01, ***P=<0.001*

Figure 5.3.

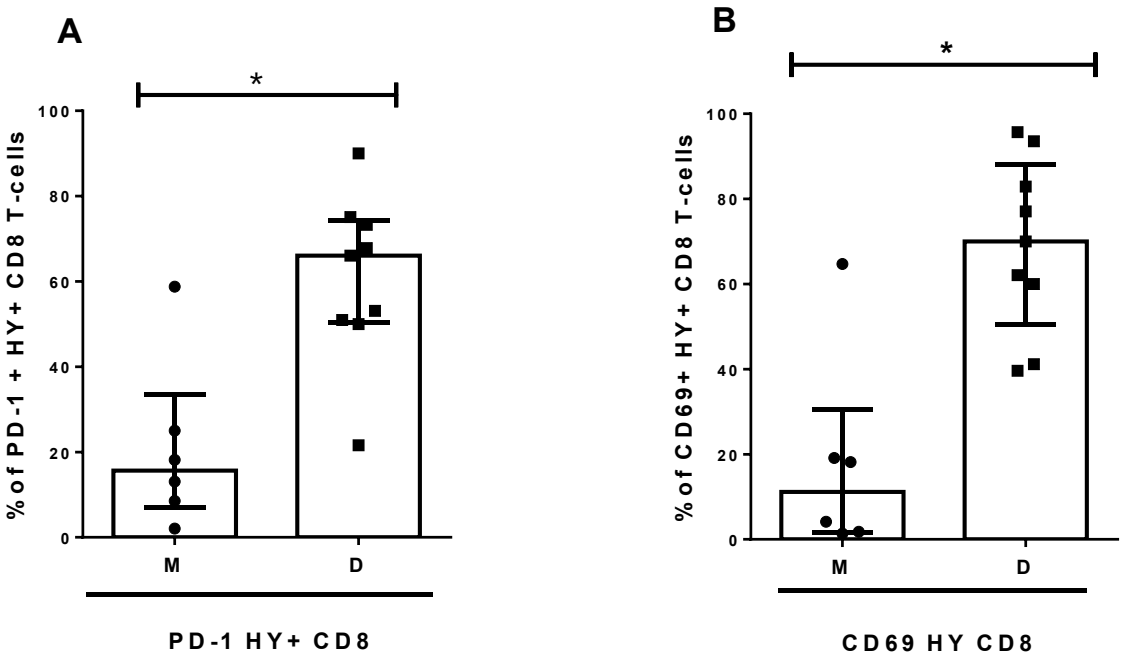


Figure 5. 3. PD-1 and CD69 in HY specific CD8 T-cells at the maternal fetal interface

*(**A**) demonstrates levels of PD-1 and CD69 expression of HY specific CD8 T-cells (red) against a background of total CD8 T-cells in decidua basalis. (**B**) and (**C**) show PD-1 and CD69 expression respectively of HY specific CD8 T-cells as percentage of total HY specific CD8 T-cells in maternal PBMCs and decidua basalis. Significance calculated using Wilcoxon paired sample test P=<0.05, **P=<0.01, ***P=<0.001*

5.3. Fetal antigen specific responses in CD4+ and CD8+ decidual T cells

By establishing that there are increased populations of CD8+ fetal specific T cells present in the decidua, we next wanted to elucidate the functionality of T cells following allo-antigen stimulation. Using a mixed lymphocyte reaction assay, we were able to measure the proliferation of matched maternal PBMC lymphocytes and decidual lymphocytes when subjected to fetal antigen in the form of matched cord blood. Additionally, by measuring granzyme B on proliferating cells, we were able to detect the level of cytotoxicity of allo responsive T cells. Figure 5.4 illustrates how the level of proliferation is measured in this mixed lymphocyte reaction, where dilution of CFSE dye is measured in maternal PBMC T cells, decidual T cells and decidual T cells that have been depleted for T regulatory cells. Figure 5.5 A and 5.6 A demonstrate that CD8+ and CD4+ decidual T cells, respectively, have an increased proliferative response towards fetal antigen in comparison to maternal PBMCs. In the case of CD8+ decidual T cells, the median proportion of proliferative cells 14.55% compared to 10.20% in maternal PBMCs, ($p=0.0039$). CD4+ decidual T cells had a similar level of proliferation, with a median 13.20% compared to 9.15% in maternal PBMCs ($p=0.0039$). Having already shown that T regulatory cells are increased at the decidua, we depleted this cell type in the mixed lymphocyte reaction to elucidate how this may affect the response to fetal antigen. Proliferation was significantly enhanced upon depletion of CD4+ T regulatory cells in both decidual CD4+ and CD8+ populations, representing an increase to 20.98% and 27.65% respectively ($p=0.0020$ and 0.0039). In addition to proliferation we measured expression of cytokine IL-4 and IL-10 and cytotoxic granzyme B on proliferating CD4+ and CD8+ maternal PBMCs and decidual T cells in the mixed

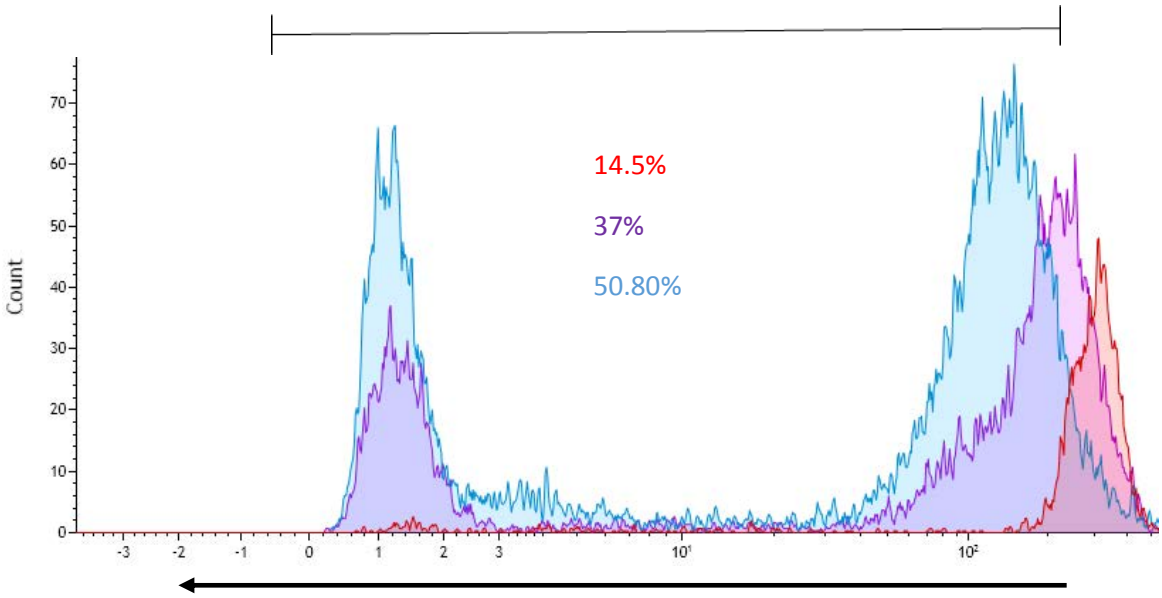
lymphocyte reaction. CD8+ decidual T cells expressed significantly increased levels of granzyme, 32% of proliferating T cells, compared to maternal PBMCs, 17.15% (p= 0.0192) (Figure 5.5 B). Upon depletion of T regulatory cells, expression of granzyme was significantly enhanced in decidual CD8+ T cells, with an increase to 37.88% (p=0.010). IL-4 was not significantly increased between decidual CD8+ T cells and maternal PBMCs, 3.24% and 1.52% respectively (Figure 5.5 C). Interestingly, however, expression of IL-10 was significantly increased in decidual CD8+ T cells, 2.26%, in comparison to maternal PBMCs, 0.16% (p=0.0273) (Figure 5.5D). There was no significant increase of IL-4 or IL-10 between decidual T cells and decidual T cells with depleted CD4+ T regulatory cells. Overall, expression of granzyme B was reduced in CD4+ T cells in comparison to CD8+ T cells, however, there was still significant increase between decidua and maternal PBMCs, 8.08% and 2.79% respectively (p=0.002%) (Figure 5.6 B). Upon depletion of CD4+ T regulatory cells granzyme B significantly increased to 10.88% (p=0.0068). T cells had increased expression of IL-4 in comparison to CD8+ T cells, and there was significant increase between decidua and maternal PBMCs, 11.32% and 3.42% respectively (p=0.0039) (Figure 5.6 C). As with granzyme B, this expression was significantly increased to 14.26% (p=0.0039) following depletion of CD4+ T regulatory cells. IL-10 expression was also increased in CD4+ T cells, again with a significant increase between decidua and maternal PBMCs, 3.77% and 1.27% respectively (p=0.0098)(Figure 5.6 D); however, Il-10 was not significantly increased following CD4+ T regulatory cell depletion.

Figure 5.4

Red: % of proliferating CD8 + Maternal PBMC T cells

Purple: % of proliferating CD8+ Decidual T cells

Blue: % of proliferating CD8+ Decidual T cells depleted T regulatory cells

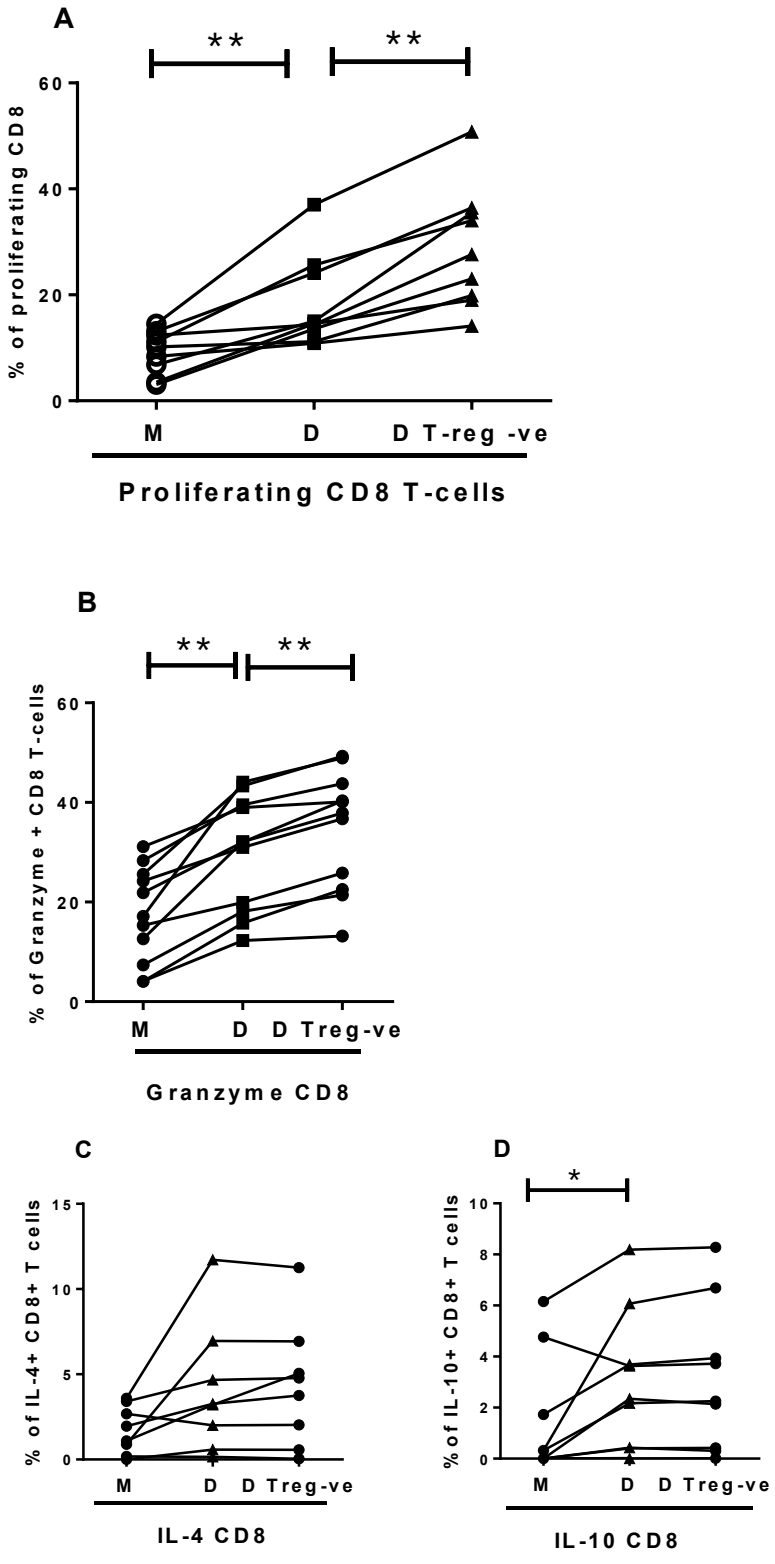


CFSE Dilution/Proliferation

Figure 5. 4. Decidual and Maternal PBMC T cells proliferating following exposure to fetal antigen.

Example of flow cytometric analysis of CFSE dye dilution, which corresponds to proliferation of T cells following an MLR assay. Red histogram plot corresponds to maternal PBMC T cells, purple to decidual T cells and blue to decidual T cells which have had T regulatory cell depletion. Values shown are a percentage of proliferating T cells as a proportion of total T cells.

Figure 5.5.


A
**
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% of proliferating CD8
60
40
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60
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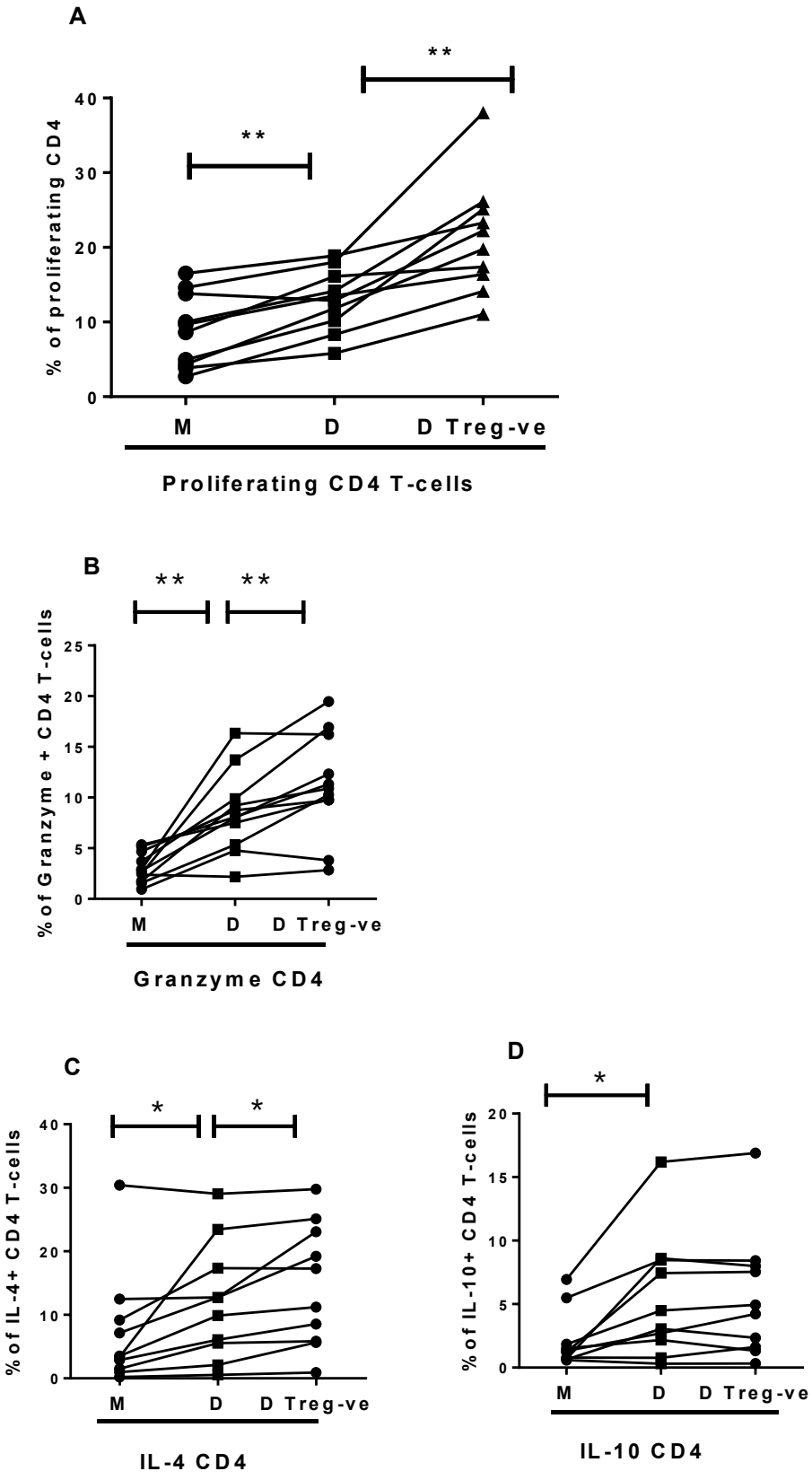
D Treg-ve
Granzyme CD8
C
% of IL-4+ CD8+ T cells
15
10
5
0
M
D
D Treg-ve
IL-4 CD8
D
*
% of IL-10+ CD8+ T cells
10
8
6
4
2
0
M
D
D Treg-ve
IL-10 CD8

Figure 5. 5. Proliferation and cytotoxicity of maternal and decidual CD8 T-cells to fetal antigen

*Mixed lymphocyte reactions were performed by matched cord blood as a stimulator and proliferation measured in n=10 maternal and decidua basalis T cells. (**A**) demonstrates percentage of proliferating CD8 T cells in maternal, decidua basalis and CD4 T regulatory cell depleted decidua basalis. (**B**), (**C**) and (**D**) demonstrates expression of granzyme, IL-4 and IL-10 as percentage total of proliferating CD8 T cells in maternal pbmcs and decidua basalis. Significance was calculated using Wilcoxon paired sample test P=<0.05, **P=<0.01, ***P=<0.001*

Figure 5.6.


A
**
**
% of proliferating CD4
40
30
20
10
0
M
D
D Treg-ve
Proliferating CD4 T-cells
B
**
**
% of Granzyme + CD4 T-cells
25
20
15
10
5
0
M
D
D Treg-ve
Granzyme CD4
C
*
*
% of IL-4+ CD4 T-cells
40
30
20
10
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D
D Treg-ve
IL-4 CD4
D
*
% of IL-10+ CD4 T-cells
20
15
10
5
0
M
D
D Treg-ve
IL-10 CD4

Figure 5. 6. Proliferation and cytotoxicity of maternal and decidual CD4 T-cells to fetal antigen

*(**A**) demonstrates percentage of proliferating CD4 T cells in maternal, decidua basalis and CD4 T-regulatory cell depleted decidua basalis. (**B**), (**C**) and (**D**) demonstrates expression of granzyme, IL-4 and IL-10 as percentage total of proliferating CD4 T cells in maternal pbmcs and decidua basalis. Significance was calculated using Wilcoxon paired sample test P=<0.05, **P=<0.01, ***P=<0.001*

Chapter 5.3. Discussion

This data suggests that at the maternal-fetal interface we see a functional and complex site of immunological interaction. We provide evidence that T cells are trafficked to the decidua, in the form of HY specific CD8+ T cells and are able to proliferate directly to fetal antigen. Previous murine work has suggested that the reason for the acceptance of the placenta to the uterine wall is due to immune silencing at maternal-fetal site. However, as demonstrated in humans, the immunological interplay is more complex. Our data shows that these immune responses aren't necessarily silenced, but limited due to intrinsic and extrinsic pathways.

Evidence of fetal antigen specific T cells in the maternal circulatory system during and after pregnancy (Lissauer et al. 2012; Piper et al. 2007) suggests that there is adequate fetal allo-antigen present to elicit an appropriate response. In this study, we have demonstrated that these fetal specific T cells are indeed present at the maternal fetal interface, which also suggests that these T cells are actively trafficked to the placenta. It is important in this case, however, that extra-villous trophoblast downregulates expression of HLA class I A2 and B7, which correspond to the dextramer restriction used in our studies. This suggests that other fetal matter is responsible for instigating an HY specific responses in this case. Additionally, these HY A2/B7 restricted T cells will not necessarily respond against placental trophoblast *in situ*. Nevertheless, the presence of these restricted T cells is still of importance as once they are able to access further fetal tissue beyond the maternal fetal interface, they may have the potential to cause cytotoxic damage.

Given the evidence of local activation of T cells within decidua it was important to assess if decidual lymphocytes could recognise proteins expressed on fetal derived cells. Interestingly, although both CD4+ and CD8+ decidual T cells proliferated in response to cord blood cells, this effect was more marked for CD8+ T cells. The profile of paternally-derived antigens presented on cord blood cells will represent only a subset of those expressed on all fetal tissues and, as such, we believe this assay would have underestimated the total magnitude of the fetal-specific immune response. Nonetheless, we were able to use it to demonstrate a role for T regulatory cells in the suppression of the decidual T cell response to fetal antigen. T regulatory cell depletion led to a 1.59 and 1.90 fold increase in decidual CD4+ and CD8+ T cell proliferation in response to cord blood and demonstrates the importance of this regulatory population in suppressing maternal response to fetal protein. Furthermore, the reduction in regulatory T cells enhanced cytotoxic expression of granzyme B in both CD8+ and CD4+ T cells. An interesting observation is the presence of increased expression IL-4 and IL-10 in decidual CD4+ T cells and increase in IL-10 in decidual CD8+ T cells. This profile suggests a protective cytokine response towards activation and potential evidence of a CD8+ T regulatory cell phenotype (Noble et al. 2006; Zhao et al. 2013). This may be an additional immunosuppressive response towards fetal antigen activation.

In order to investigate the potential importance of inhibitory protein expression on fetal-specific decidual T cells we were able to combine HLA-peptide tetramer staining of HY-specific T cells with assessment of PD-1 expression. This revealed a high level expression of PD-1 on HY-specific cells, further implicating this checkpoint protein in an important role in regulating the activity of fetal specific T-cells within the decidual

microenvironment. Unfortunately, the low frequency of such cells prevented us from assessing the functional impact of PD-1 inhibition.

The overall picture that emerges from these results is that maternal-fetal interface is home to functionally active and directly allo-reactive T cell populations, but both intrinsic and extrinsic regulation are utilized in control of fetal-specific T cell function within decidua.

Chapter 6.Results
Transcriptomic analysis of CD4 and CD8 effector decidual T cells.

6.1. Introduction

Our study has provided evidence for activated T cells located within the decidua that are able to elicit a response to fetal allo-antigen, express enhanced effector functions and increased cytotoxic potential. Additionally, we have demonstrated various potential mechanisms in which this cytotoxic response to the fetus may be inhibited by T cells themselves. However, pathologies of the placenta that are characterised by immune cell cytotoxic destruction, such as Villitis of Unknown Etiology (V.U.E.), highlights the potential damage that can be caused by activated T cells at the maternal fetal interface in healthy pregnancies. Studies such as Kim et al (2009) demonstrated that the transcriptomic profile of V.U.E is associated with up-regulation of CXC chemokines and MHC class II molecules, resulting in a genetic profile similar to that of graft versus host disease and allograft transplantation. Unfortunately, this research was performed on whole tissue isolated RNA, and therefore specific transcriptomic profiles of immune cell subsets was not analysed. Thus far, we have only demonstrated increase in fully transcribed proteins present on the surface of T cells through use of flow cytometry. To provide us with a greater understanding of the nature of any fetal allo-antigen response, analysis of the transcriptome of CD4+ and CD+ effector memory T cells reveals targets and pathways that might not have been fully elucidated with cellular based assays.

6.1. Transcriptional analysis of CD4+ and CD8+ effector memory T-cells reveals a strong response in interferon signalling within decidual T cells

In order to comprehensively describe the differences between the decidual and peripheral effector memory T cell population we went on to perform a transcriptional analysis using a microarray of CD4+ and CD8+ effector memory T cells purified from decidual tissue and matched maternal peripheral blood. In total of 6 sets of patient samples were analysed each comprised of paired maternal and decidual CD4+ effector memory T cells and CD+8 effector memory T cells. Statistical significance of upregulated of downregulated genes was calculated using a dual methodology of Bonferroni-Hochburg corrected p values followed by empirical Bayesian ranking. This provided us with a Bayes value, where >5 was regarded to be statistically significant. Additionally, fold changes greater than 1.65 (log2FC >0.7) were deemed significantly upregulated or downregulated. See Figure 6.1 for a breakdown of number of genes up or downregulated in CD4 and CD8 decidual T cells. 26 and 31 genes were significantly upregulated in the decidual CD4+ and CD8+ T cells respectively. In addition, 6 genes were shown to be downregulated within decidual CD4+ effector T cell. (Figure 6.2 and 6.3).

Eight genes, *RSAD2, IFI6, IFI27, IFI44L, IFIH1, EIF2AK2, DDX60* and *USP41*, were upregulated in both CD4+ and CD8+ from decidua. Interestingly, these are all associated with the cellular response to interferon signalling. RSAD2, also known as viperin, is an interferon-inducible antiviral protein induced by type I and type II interferon. It also facilitates T cell receptor (TCR)-mediated GATA3 activation and optimal T helper 2 (Th2) cytokine production (Lim et al. 2012; Hata et al. 2009). *IFI6, IFI27, IFI44L* and *IFIH1* are all

interferon-inducible genes that are associated with a type 1 interferon signature, and EIF2AK2 is a double-stranded RNA-dependent protein kinase R that is amongst the first proteins to become activated following viral infection or stress signalling (Wang et al. 2014). *DDX60,* also known as DEAD (Asp-Glu-Ala-Asp) box polypeptide 60 (DDX60), is a cytosolic DNA sensor and RNA helicase that is involved in RLR-mediated type I IFN production after viral infection and mediates viral RNA degradation via the RNA exosome (Oshiumi et al. 2016). The function of USP41 is unknown, but it has high sequence homology with USP18, (USP)-18, a Ubiquitin-specific peptidase which acts as a negative regulator of interferon α/β responses (Pinilla-Vera et al. 2016). Several differences were observed in the transcriptional profile between CD4+ and CD8+ T cells within decidua including the OAS1, OAS2 and OAS3 genes, which encode members of the 2-5A synthetase family involved in the innate immune response to viral infection and which were upregulated by approximately 2-5 fold in CD8+ decidual T cells. A specific finding of interest within CD4+ T cells was a marked increase in expression of the CLIC1 intracellular chloride channel, which has not previously been reported to be expressed in decidual T cells.

Of the 6 significantly downregulated genes in CD4+ effector memory decidual cells, 3 are described as non-coding RNAs. Despite an absence of documented functional relevance in T cell immunology, these non-coding RNAs may have a role in epigenetic regulation of gene expression, and the downregulation of these genes may be distinct in T cell activation in the decidua. Indeed, a recent study has suggested several upregulated and downregulated micro RNAS have a potential role in the decidua of patients of recurrent miscarriage (Dong et al. 2014).

The genes encoding Galectin-1 and Galectin-9 demonstrated increased expression in decidual CD4+ and CD8+ effector memory cells respectively. Galectin-1 expression has been previously observed on decidual NK cells and galectin-9 is also present on T regulatory cells in pregnancy (Koopman et al. 2003; Meggyes et al. 2014). As such, this particularly high expression by effector T cells within decidua is further evidence that these proteins are important immune-regulatory molecules during pregnancy (Deák et al. 2015). Further interrogation of the role of the differentially expressed genes using the Database for Annotation, Visualization and Integrated Discovery (DAVID) and Reactome identified several pathways that were significantly upregulated, most notably "interferon signalling", 'immune responses to virus' and 'regulation of apoptosis' (Table 6.1 and 6.2).

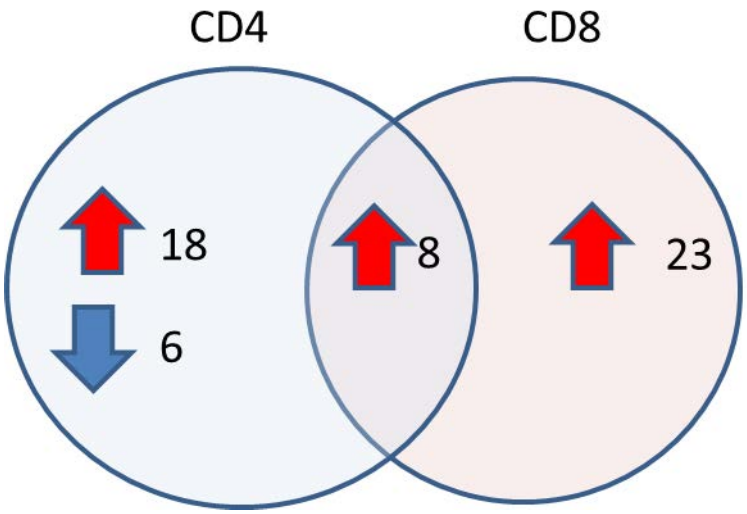

Figure 6. 1 Summary of Up/Downregulated genes in Decidual vs Maternal Effector Memory T cells.

In decidual CD4 effector memory T cells, there were 26 significantly upregulated and 6 downregulated genes when compared to maternal blood effector memory T cells. There were 31 significantly upregulated genes in decidual CD8 effector memory T cells. 8 significantly upregulated genes were shared between CD4 and CD8 decidual effector memory T cells.

We compared the genes that comprised the response to interferon and response to virus/RNA binding (see Table 6.1 and 6.2) gene list in both decidual CD4 and CD8 effector memory T cells, with gene sets in the Molecular Signature Database (Broad Institute). Specifically we computed overlaps of our gene lists with the C7 collection, a database of 4872 gene sets generated by manual curation of published studies in human and mouse immunology. There were no overlaps between our gene lists and gene sets derived from experiments on T cells in other tissues. This suggests that the specific gene signatures discovered in our microarray data regarding interferon signalling and RNA binding is unique to effector memory decidual T cells, and is not necessarily a component of activated T cells in other tissues.

Figure 6.2.

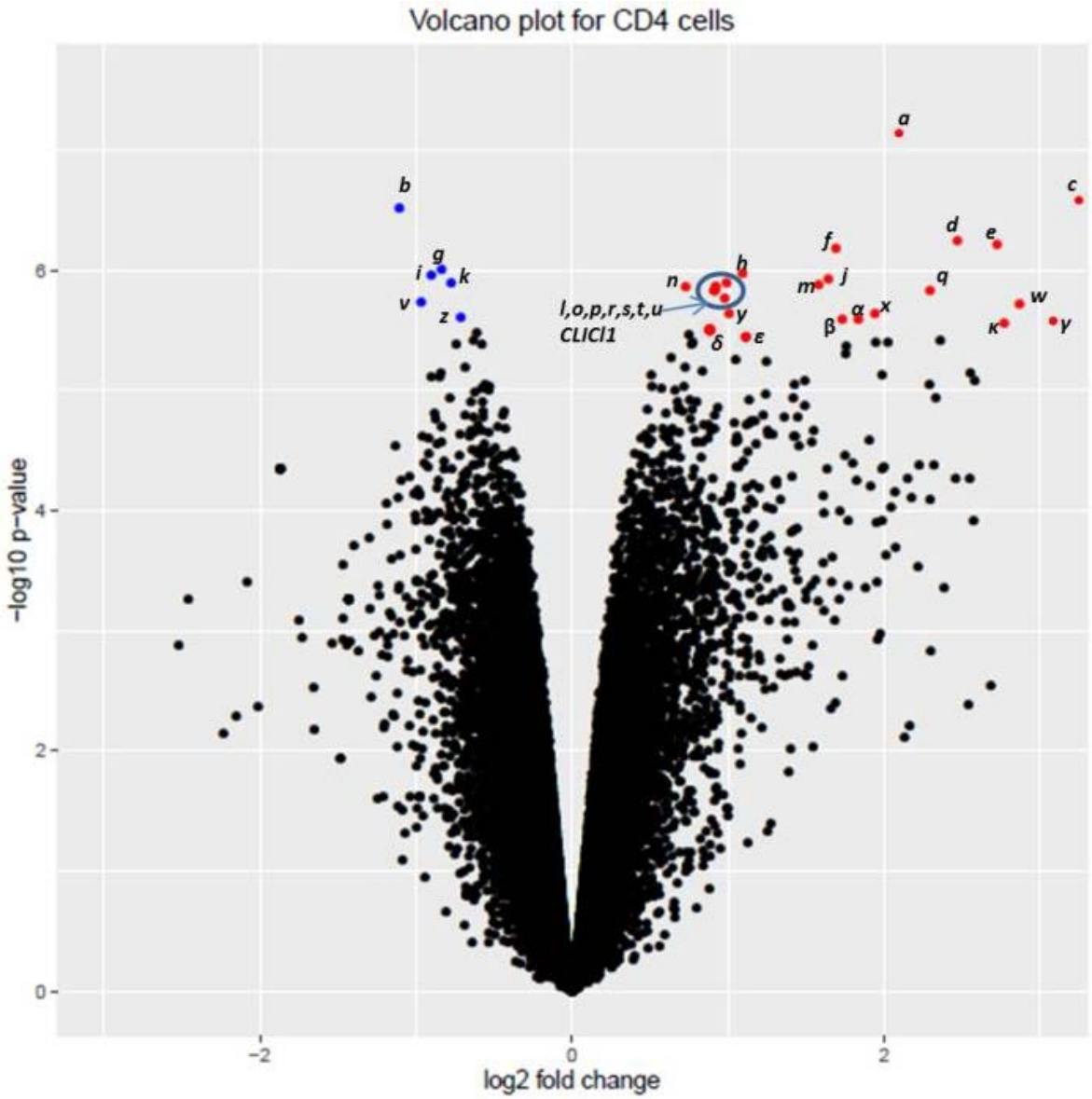
Volcano plot for CD4 cells
−log10 p−value
log2 fold change
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Figure 6. 2. Transctriptome of Effector memory CD4 T cells in Maternal PBMCs and Decidual Tissue

RNA was isolated from maternal and decidual CD4 effector memory T-cells and analysed using HTA 2.0 microarray (n=6). Volcano plots were generated, based on individual probes log fold change and P value associated with reproducibility of these changes between Maternal and Decidual T-cells. Bayesian analysis was used to calculate genes with significantly different expression profiles, where red represents upregulation and blue down regulation. Genes were appropriately annotated in order of Bayesian value (B=>5). Annotated dots refer to genes listed in Table 6.1

Table 6.1.

Pathway	Genes (log2 FC >0.7; Bayes >5)	Bayes Value	Fold Change (log2FC)
Interferon Signalling Reactome Bonferroni : $3.5x10^{-6}$	*c* -RSAD2	6.406	4.00
	d -USP18	6.299	2.471
	K -IFI6	5.070	2.770
	q -USP41	5.576	2.296
	X -EIF2AK2	5.229	1.942
	γ -IFI44L	5.121	3.030
	a -IFI44	9.144	2.020
	e -MX1	6.245	2.722
	w -IFI27	5.372	2.867
Response to virus/RNA binding D.A.V.I.D Bonferroni: $2.6x10^{-4}$	*c* -RSAD2	6.406	4.000
	K -IFI6	5.070	2.770
	X -EIF2AK2	5.229	1.942
	β -IFIH1	5.125	1.733
	γ -IFI44L	5.121	3.030
	j -DDX60	5.758	1.640
	h -DDX60l	5.838	1.095
	a -IFI44	9.144	2.020
	e -MX1	6.245	2.722
Regulation of Apoptosis D.A.V.I.D Bonferroni: $3.9x10^{-2}$	κ -IFI6	5.070	2.770
	m -LGALS1	5.656	1.581
	β -IFIH1	5.125	1.733
	ε -CD70	5.050	1.244
	δ -IFI16	5.060	0.749
	e -MX1	6.245	2.722
CLIC1 Chloride Channel Protein	*l* -CLIC1 (hap3)	5.686	0.993
	o -CLIC1(hap4)	5.625	0.923
	p CLIC1(hap6)	5.625	0.923
	r -CLIC1	5.570	0.916
	s -CLIC (hap5)	5.570	0.916
	t -CLIC1(hap7	5.570	0.916
	u -CLIC1(hap2)	4.460	0.980
Non coding RNAS	*f* -Noncode AX747531	6.190	1.1691
	g -Noncode linc_luo_1022	5.881	-0.835
	i -Noncode BC038574	5.798	-0.899
	v -NonCode AF520793	5.414	-0.966
	b -RNU6-878P(pseudogene)	6.733	-1.105
	k -TSPAN18	5.703	-.0775
	n -PHPT1	5.633	0.730
	y -CTNNAL1	5.226	1.009
	z -ATF7IP2	5.168	-0.712

Table 6. 1. Significantly upregulated genes in effector memory CD4+ decidual T cells

Genes that had significantly differential expression in decidua were entered into Database for Annotation, Visualisation and Integrated Discovery (DAVID) and significantly upregulated pathways were generated (Bonferroni corrected p=<0.05). The seven coding exons of CLIC1 are also categorised as well as non-coding RNAs. Red indicates upregulated genes, blue indicates downregulated genes. Bold and underlined indicates upregulated genes in both CD4+ and CD8+ effector memory T cells. Letter annotation for each gene represented in table matches annotated dots in Figure 6.2

Figure 6.3.

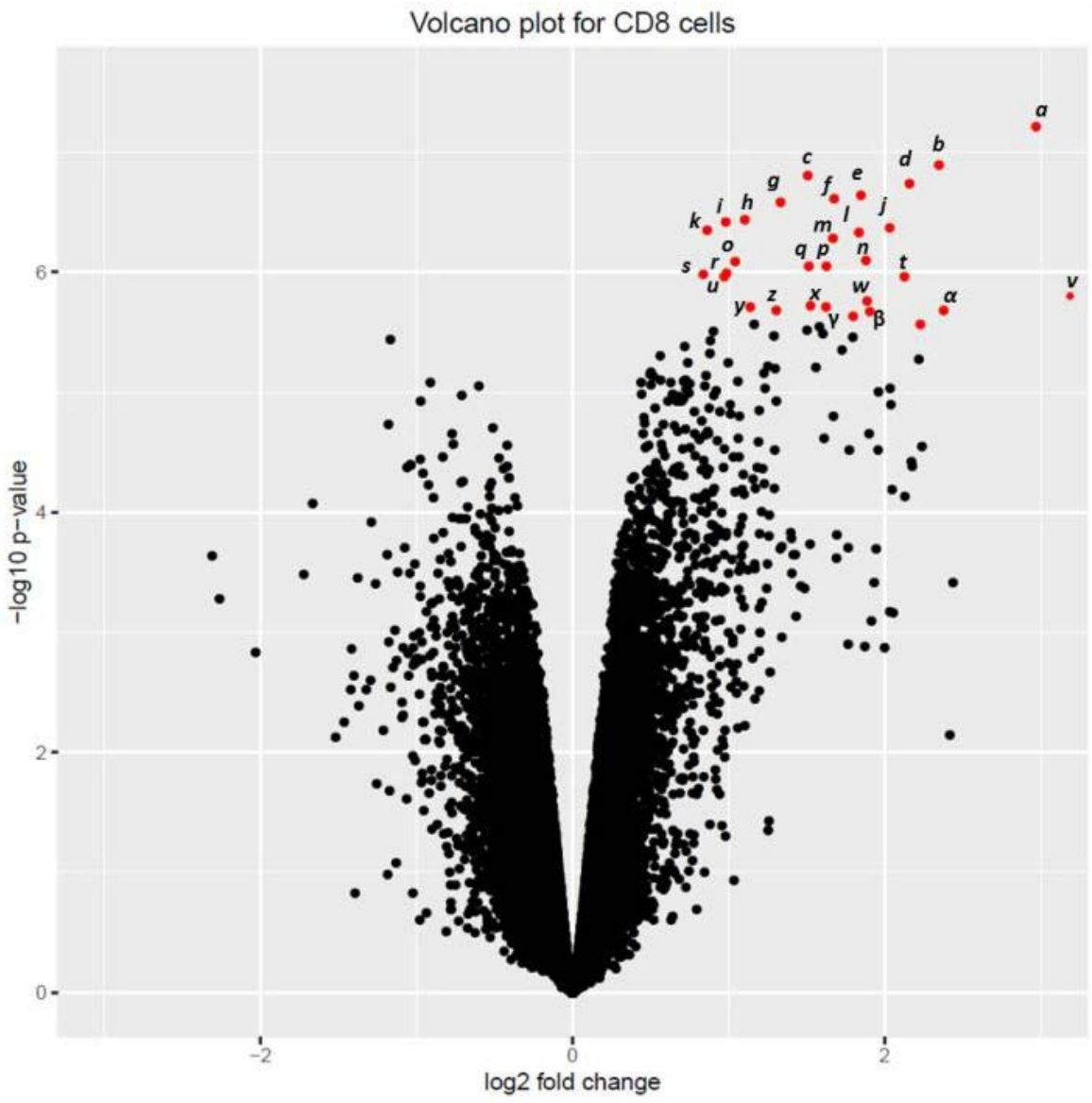
Volcano plot for CD8 cells
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Figure 6. 3. Transctriptome of Effector memory CD8 T cells in Maternal PBMCs and Decidual Tissue

RNA was isolated from maternal and decidual CD8 effector memory T-cells and analysed using HTA 2.0 microarray (n=6). Volcano plots were generated based on individual probes log fold change and P value associated with reproducibility of these changes between Maternal and Decidual T-cells. Bayesian analysis was used to calculate genes with significantly different expression profiles. Genes were appropriately annotated in order of Bayesian value (B=>5). Annotated dots refer to genes listed in Table 6.2

Table 6.2.

Pathway	Genes (log2 FC >0.7; Bayes >5)	Bayes Value	Fold Change (log2FC)
Interferon Signalling Reactome 4.03 x10 $^{-11}$	***a*** **-RSAD2**	8.610	3.582
	d **-Usp18**	6.932	2.157
	b **-IFI6**	7.164	2.346
	j **-Usp41**	6.370	2.032
	e **-EIF2AK2**	6.794	1.667
	v **-IFI44L**	5.449	3.352
	g -OAS2	6.709	1.332
	n -OAS1	5.930	1.880
	α **-**OAS3	5.214	2.376
	x -HERC6	5.257	1.622
	γ -IFI27	5.123	1.798
	z **-**BST2	5.216	1.306
Response to virus/RNA binding D.A.V.I.D Bonferroni: 2.9x10 $^{-5}$	***a*** **-RSAD2**	8.610	3.582
	b **-IFI6**	7.164	2.346
	e,m -**EIF2AK2**	6.794	1.667
	p -**IFIH1**	5.845	1.627
	v **-IFI44L**	5.449	3.352
	c -**DDx60**	7.030	1.505
	n -OAS1	5.930	1.880
	g -OAS2	6.709	1.332
	α **-**OAS3	5.214	2.376
	w -PLSCR1	5.340	1.887
	z **-**BST2	5.216	1.306
	β -CCR1	5.196	1.905
Regulation of Apoptosis D.A.V.I.D Bonferroni: 3.9x10 $^{-2}$	***b*** **-IFI6**	7.164	2.346
	q,t **-**LGALS9	5.844	2.126
	f,l -TNFSF10	6.784	1.676
	h **-**Tmem200a	6.484	1.103
	i,r **-** ANXA2P1	6.445	0.980
	k **-**N4BP1	6.344	0.862
	o **-**CHMP5	5.923	1.041
	s -DNAJA1	5.741	0.838
	u **-**ALOX5AP	5.695	0.971
	y **-**LY6E	5.255	1.138

Table 6. 2. Significantly upregulated genes in effector memory CD8+ decidual T cells

Genes that had significantly differential expression in decidua were entered into Database for Annotation, Visualisation and Integrated Discovery (DAVID) and significantly upregulated pathways were generated (Bonferroni corrected p=<0.05). Red indicates upregulated genes, blue indicates downregulated genes. Bold and underlined indicates upregulated genes in both CD4+ and CD8+ effector memory T cells. Letter annotation for each gene represented in table matches annotated dots in Figure 6.3

6.2 Targeted analysis of decidual effector memory T cell transcriptome

We also examined the combined pattern of gene expression within specific immune response and regulatory pathways. Gene Set Enrichment Analysis demonstrated that gene sets and pathways associated with apoptosis, allograft rejection, negative regulation of activation of T cells and IFNγ and IFNα responses were enriched in decidual CD4+ and CD8+ T cells.

Gene Set Enrichment Analysis allows for measurement of subtle increases and decreases in large groups of genes across entire microarray data sets. These trends of large numbers of differentially expressed genes are then measured against a data base of "gene sets". Any significantly enriched gene sets discovered during the analysis represent pathways that are potentially upregulated. Additionally, specific differentially expressed genes can contribute to the core enrichment of a gene set, allowing for discovery of upregulated candidate genes that might have been missed in conventional microarray analysis of significantly upregulated genes. Table 6.3 lists the significantly upregulated gene sets in both CD4 and CD8 decidual effector memory T cells along with the top 30 differentially expressed genes contributing to core enrichment of each gene set.

Consistent with our earlier findings, genes encoding checkpoint proteins Lag-3, Tim-3, CTLA-4 were differentially expressed in decidual effector CD8+ and CD4+ T cells, in comparison to peripheral blood, and contributed to the core enrichment of negative regulation of T cell activation gene sets and pathways within decidual T cells. In addition to these, another differentially expressed gene coding an inhibitory protein of particular

interest was T cell Immunoreceptor with Ig and ITIM domains (TIGIT). This is a recently discovered immune checkpoint protein (Yu et al. 2009), which is seen to be highly expressed in effector CD4 and CD8 T cells as well as NK cells and regulatory T cells (Lozano et al. 2012; Joller et al. 2014; Johnston et al. 2014). Its cognate ligand is CD155 and upon binding enacts similar inhibitory function that binding CTLA-4 causes. Indeed, TIGIT competes with CD226 for CD155 mirroring the CTLA-4/CD28 pathway. TIGIT and its role in tumor infiltrating lymphocytes has been thoroughly researched and various inhibitory pathways involving TIGIT have been discovered that contribute to immune senescence at the tumor microenvironment. Firstly, TIGIT on CD4 or CD8+ T cells has been shown to bind to dendritic cells, which in turn elicits downregulation of CD86, causing reduced costimulatory function, and increased expression of IL-10 (Yu et al. 2009). TIGIT present on CD4+ T regulatory cells can bind to CD8+ T cells and suppress effector function, as well as skew CD4+ T cells to a Th2 phenotype (Kourepini et al. 2016; Kurtulus et al. 2015).Lastly, TIGIT expressed on tumor cells has shown to directly inhibit CD8+ T cell cytotoxicity (Johnston et al. 2014). Currently, there have been no studies addressing TIGIT and its effect on T cell fetal antigen responses in the placenta; however, due to its highly suppressive mechanism in T cell inflammatory responses, it could be highly relevant in immunotolerance at the maternal-fetal interface.

Genes encoding Caspase 3, IL-2 receptor, CCR5, Granzyme B, CXCR3, IFN-γ, and CXCL13 were also differentially expressed in CD4+ and CD8+ decidual effector memory T cells and contributed to the enrichment of apoptosis and allograft response gene sets within decidual T cells respectively. Previous research by Kim et al. (2009) demonstrated that CCR5 and CXCL13 were both upregulated in placental tissue of V.U.E. and through our

own analysis of decidual CD4+ and CD8+ T cells, we find that these chemokine receptors and ligands both are present in term healthy pregnancy.

TNF-related apoptosis-inducing ligand (TRAIL) was also differentially expressed in decidual effector memory CD8 and CD4 and contributed to enrichment of apoptosis gene set pathway. Similar to another apoptosis signalling factor, FAS ligand (FasL) binding of TRAIL to its cognate receptors, induces extrinsic pathway of apoptotic signalling. During pregnancy it has been shown that both trophoblast and fetal derived exosomes exhibit TRAIL, resulting in immunomodulation against activated immune cells (Svensson-Arvelund, Ratnesh B. Mehta, et al. 2015; Stenqvist et al. 2013). Also, expression of TRAIL by activated T cells themselves, has shown to be an important factor against viral infected cells and tumor cell clearance as well as haematopoiesis (Falschlehner et al. 2009; Brincks et al. 2008). There has been no research addressing the specific role of TRAIL expression on T cells at the maternal-fetal interface; however, given that our data indicates that Caspase 3, an important downstream factor of apoptosis, is also upregulated, it might function in an immunomodulatory role.

Our data indicates that CD4+ and CD8+ T cells at the maternal-fetal interface demonstrate a unique transcriptional profile including a dominant response to interferon signalling and activation of inflammatory pathways, but also express a wide range of proteins associated with immune activation and regulation. Figure 6.4 highlights enriched pathways and genes derived from the GSEA analysis.

Figure 6.4.

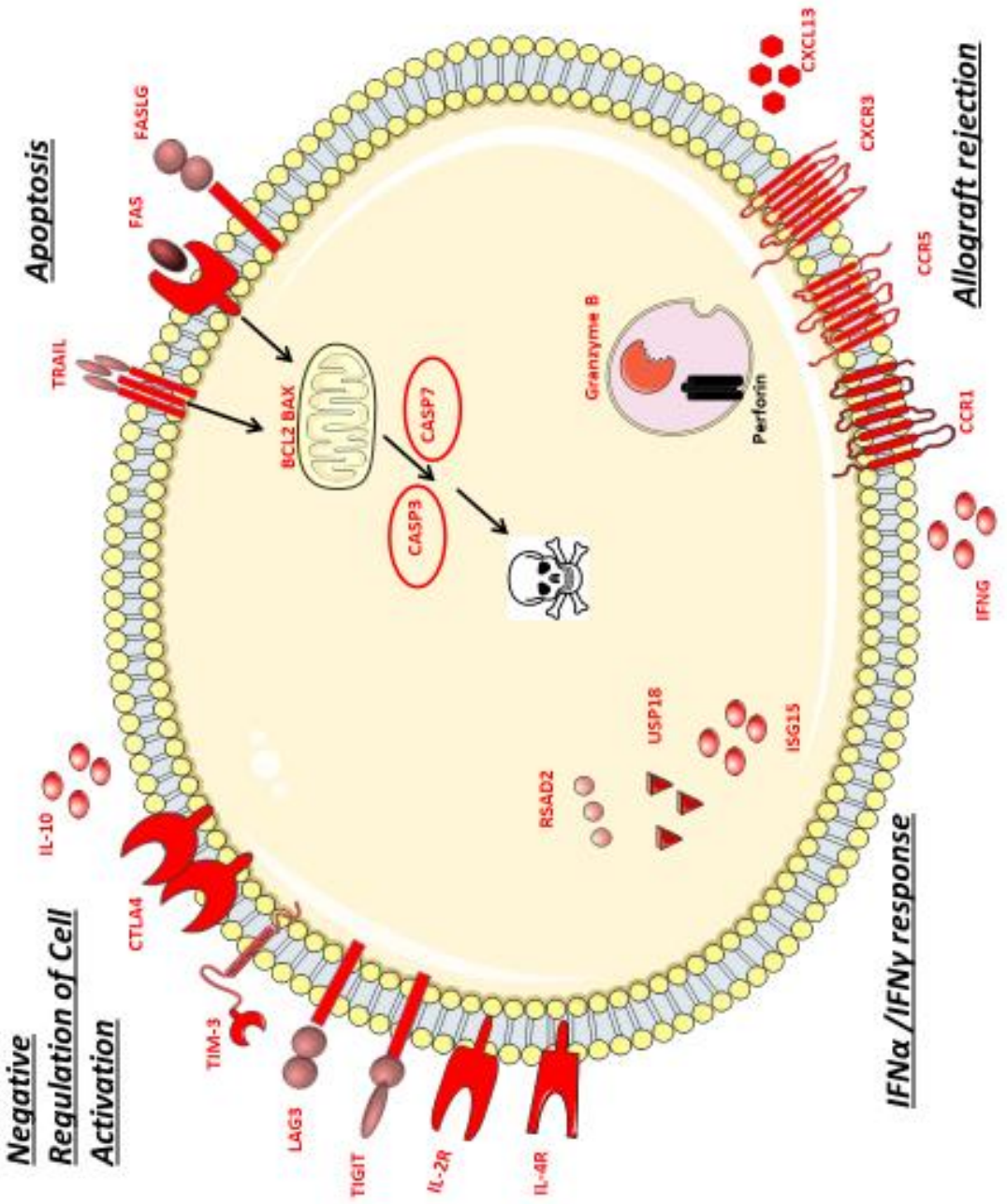

Figure 6. 4 GSEA significantly enriched gene sets and core enrichment genes

Allograft Rejection (NES: 1.34, FDRq: 0.164)	INFγ Response (NES:1.35, FDRq:0.176)	IFNα Response (NES:1.29, FDRq: 0.182)	Apoptosis (NES:1.65, FDRq:0.168)	Negative regulation of Cell Activation (NES:1.45, FDRq: 0.151)
IFNG	IFI44L	IFI44L	TNFSF10(TRAIL)	CTLA4
IL2RA	RSAD2	RSAD2	GZMB	IL2RA
CCR1	IFIT1	LAMP3	PMAIP1	XCL1
CXCL13	CMPK2	CMPK2	FASLG	LGALS9
TLR3	IFIT3	IFIT3	PSME2	HAVCR2 (TIM-3)
STAT1	OAS3	USP18	LMNB1	TNFSF4
IRF4	XCL1	OAS1	FAS	LAG3
WARS	USP18	IFI27	PSMA6	CD274
CSF1	CD38	PLSCR1	PSMD14	CEACAM1
GZMB	IFI27	IFI44	BCL2L11	SAMSN1
CD80	PLSCR1	PARP9	PSMA2	SLA2
FASLG	IFI44	IFIT2	PSMB5	FAS
CCR5	IFIT2	MX1	BCL2	TIGIT
IL1B	MX1	EIF2AK2	PSMA4	ANXA1
FAS	TNFSF10	DDX60	VIM	IL4R
GCNT1	EIF2AK2	IFIH1	CASP7	IL10
HIF1A	DDX60	EPSTI1	PSMB6	HLA-DRB5
CCL7	IFIH1	RTP4	LMNA	CASP3
NCF4	EPSTI1	SAMD9L	PSMB3	HLA-DRB1
CAPG	RTP4	HERC6	CASP3	JAK3
TAP2	SAMD9L	PNPT1	PSMA3	SERPINE2
ICAM1	HERC6	BST2	BMF	HLA-DRB4
IL4R	STAT1	IFI35	PSMC1	IFNA2
TAP1	IRF4	OASL	PSMA1	IRF1
CD2	PNPT1	PARP12	PSME1	HLA-DRB3
IL10	XAF1	LAP3	PSMB9	PDCD1LG2
CD7	BST2	ISG15	PSMD5	CD74
CXCR3	IFI35	WARS	BAX	BCL6
DARS	OASL	IFITM1	PSMD8	LGALS3

Table 6. 3 Gene Set Enrichment Analysis

Gene set enrichment analysis (Broad Institute) was performed to asses enriched gene sets present in microarray data of CD4 and CD8 effector memory decidual and maternal T cells. Enriched gene sets (<FDR 0.25) are listed along with top 30 genes from microarray data that contribute to core enrichment of each gene set.

6.3 Discussion

Microarray analysis revealed a wide range of genes whose transcriptional activity was differentially expressed in decidual effector memory CD4+ and CD8+ T cells compared to peripheral blood. Significantly, genes associated with interferon signalling receptivity were markedly increased in both CD4+ and CD8+ decidual T cells. Interestingly, high levels of interferon-γ have been reported within decidual tissue (Chard et al. 1986) and this appears to be localised primarily within cells of the monocyte linage (Khan et al. 1990). Interferon production is driven most strongly by viral infection and these observations raise the question as to whether or not localised viral infection is found within decidual tissue. Indeed, we would suggest that endogeneous retroviruses (ERV) are the likely trigger for interferon production within decidua. ERVs represent more than 8% of the human genome (Bannert & Kurth 2004; Tristem 2000) and became integrated into the mammalian genome between 40 and 0.1 million years ago via retroviral infection of germ cells (Turner et al. 2001). Notably, a protein encoded from the env gene of ERVW-1 called Syncytin-1 has an essential role placentogenesis (Blond et al. 1999; McCoy et al. 2000) through formation of the syncytiotrophoblast. Furthermore, Syncytin-1 has been shown to be released into the periphery via placental microvesicles that are able to elicit a T cell response (Holder et al. 2012) and substantial expression of proteins from the endogenous retrovirus HERV-K has been demonstrated in villous and extravillous cytotrophoblast (Kämmerer et al. 2011). In the context of the functional relevance to this type 1 interferon signalling, interferon-α is known to be a significant antiangiogenic factor, resulting in downregulation of

proangiofactors including VEGF (Denny et al. 2007). This can result in impairment of vascular remodelling in the case of lupus patients, and it has been suggested that this interferon response can be localised to the decidua resulting in malplacentation and pregnancy complication (Andrade et al. 2015). Together this data may implicate decidual T cells in a role that modulates further vascularisation as pregnancy progresses.

Interestingly, our microarray results also revealed substantial upregulation of the CLIC1 gene in decidual CD4+ effector T cells. This chloride channel protein has garnered much attention recently within solid-tumors where its expression is associated with metastasis and invasion (Lu et al. 2015; Zhao et al. 2015; Tian et al. 2014). CLIC1 is a metamorphic protein that can shift between two or more stable conformations and its expression is increased by hypoxia where it acts to regulate cell function through the ROS-mediated p38 MAPK signalling pathway (Zhao et al. 2015; Averaimo et al. 2010). It is known that the placenta develops in a hypoxic environment, and that Hypoxia Inducible Factors (HIFs) play a key role in modulating placental cell function under low oxygen conditions (Patel et al. 2010). Our work identifies a new role for CLIC1 in reproductive T cell immunology and demonstrates that more research will be required to establish its physiological function in decidual CD4+ effector memory T cells. Of note CLIC3, which is another member of the CLIC family, has been shown to be important in pre-eclampsia, where increased levels at the syncytiotrophbast layer result in disruption of membrane potential and downstream apoptosis (Murthi et al. 2012).

Transcription of the LGALS1 gene which encodes Galectin-1 was upregulated in CD4+ T-cells. Galectin-1 is expressed by both endometrial and uterine NK cells during

pregnancy (Koopman et al. 2003), and is involved in generation of tolerogenic dendritic cells (Ilarregui et al. 2009). Intracellular expression of galectin 1 is also observed in activated T cells (Blaser et al. 1998; Fuertes et al. 2004) where it acts to increase sensitivity to the action of extracellular Galectin-1, in which it activates downstream apoptosis (Deák et al. 2015). In contrast, decidual CD8+ T cells upregulate galectin-9, a ligand of Tim-3 which is involved in both chemo-attraction and apoptosis (Rabinovich & Toscano 2009) as well as inhibition of chronic inflammation (Zhu et al. 2005). Expression of Galectin-9 by T regulatory cells promotes stability of an immunosuppressive phenotype (Wu et al. 2014) and its expression by decidual CD8+ T cells may therefore increase effector cell regulation following interaction with Tim-3 (Deák et al. 2015). Through Gene Set Enrichment Analysis, we also discovered other immune markers that have not been fully examined in the context of pregnancy and fetal allo-antigen responses. Immune checkpoint protein, TIGIT and death receptor ligand TRAIL could play essential roles in T cell modulation at the maternal-fetal interface given their similar mechanisms to well-studied immune proteins in pregnancy, such as PD-1 and FAS-L respectively.

To address the subject of whether our microarray data represents a general pattern of upregulated genes in tissue in comparison to blood we performed a database search of genes found to represent upregulated pathways in decidual T cells from our microarray data (Response to Interferon and Response to Virus) against the Molecular Signature Database. We were unable to find similar upregulated lists of genes in other tissues from the database of 4872 experimental data. This gives us increased confidence that our data demonstrates a gene signature specific to the decidual environment, rather than that of T cells that have infiltrated tissues.

The transcriptomic analysis of decidual effector CD4+ and CD8+ T cells has revealed several highly novel mechanisms at work within the maternal-fetal interface. With further investigation these could prove to play a previously unidentified, yet critical, role in the maintenance of healthy pregnancy.

Chapter 7.
General Discussion and Conclusions

The human placenta is a key interface at which the maternal immune system interacts with the allogeneic fetus. Despite the evolution of mechanisms of fetal tolerance including MHC class I and Class II restriction on fetal trophoblast and involvement of tolerogenic uterine T regulatory cells and uterine NK cells, it has been widely reported that immune dysregulation during pregnancy has detrimental effects. Indeed, fetal cells are able to cross the placenta into maternal circulation, and during pregnancy the maternal immune system is able to elicit a T cell response to fetal antigens (Lissauer et al. 2012). Our research has characterised CD4+ and CD8+ memory subsets at the maternal-fetal interface as well as maternal PBMCs and cord blood populations, and have shown that highly differentiated effector memory T cells are enriched in the decidua. Intracellular cytokine profiling also demonstrates that decidual T cells have increased expression of cytotoxic cytokine IFNγ. Despite the presence of this activated and potentially cytotoxic profile, we also have provided evidence that the decidua has increased populations of CD4+ T regulatory cells levels relative to maternal PBMCs. In addition to this regulatory cell population at the maternal-fetal interface, we demonstrated that decidual effector memory T cells had increased expression of immune checkpoint proteins PD-1, Tim-3, Lag-3 and CTLA-4, suggesting a mechanism of exhaustion in this subset of cells. Given the presence of activated T cells at the decidua, we examined T cell fetal specificity and discovered that CD8+ T cells that were specific to the male HY antigen in pregnancies with male fetuses were significantly increased in the decidua. Additionally, following mixed lymphocyte reaction assay using cord blood as a source of fetal antigen, we demonstrated that decidual T cells had an increased proliferative and cytotoxic expression of granzyme B relative to maternal PBMC. Following depletion of CD4+ T regulatory cells, this proliferative

and cytotoxic response was significantly enhanced. Lastly, through the application of a microarray of CD4+ and CD8+ effector memory cells derived from decidua and matched maternal PBMCs, we were able to determine a novel transcriptomic response to type 1 interferon, and further clarified pathways of both activation and exhaustion that were eluded to in our flow cytometry analysis.

The presence of large numbers of potentially fetal-reactive effector T cells found in the decidua of healthy pregnancies at term has a particular consequence in the role of allo-reactive associated disorders such as V.U.E. It is now recognised that V.U.E. may result from the recognition of fetal tissue by the humoral and cellular components of the maternal adaptive immune system (Tamblyn et al. 2013). The clinical expression of V.U.E. includes fetal growth restriction, preterm birth and recurrent pregnancy loss. In these cases the histology is characterized by a heavy maternal inflammatory cell infiltrate of T cells and macrophages with a predominant CD8+ profile (Redline 2007). This is associated with focal inflammation of fetal villi, and it is noteworthy that this profile is present to a modest extent in many normal pregnancies. The evolutionary balance of fetal-specific T cell responses may be delicately poised to permit successful reproduction in the great majority of cases, whilst avoiding intensive local or systemic maternal immune suppression.

Previous studies have established that differentiated T cell populations exist in healthy decidua at term (Tilburgs et al 2010). However, to date, a comprehensive phenotype of both CD8 and CD4 T cells, combined with functional, regulatory, specificity and novel transcriptional profiles provides a solid foundation of work that can be taken

further in order to identify T cell-modulated pathways in pregnancy complications. Despite V.U.E being detected in healthy pregnancy, our study did not detect any cases of V.U.E in any of our sample collection. Therefore an important next step would be to assess T cell phenotypes in pregnancies affected by various pathological complications that are also correlated with V.U.E. Pathologies that could be easily targeted in a clinical setting would be women experiencing either pre-eclampsia or gestational diabetes. Presence of V.U.E. has been correlated with instances of preeclampsia (Tamblyn et al 2013) and despite its association with poor decidualisation during 1st trimester, it is nevertheless important to elucidate how effector T cell populations differ in cases of pre-eclampsia and how they may contribute to destructive villitis towards term of pregnancy. Previous studies have shown that women with gestational diabetes have higher T cells of an activated phenotype (CD69+ and HLA-DR+), and reduced CTLA-4 expression (Pendeloski et al. 2015) in their peripheral blood. This gives credence to the study of decidual T cells in the context of such pregnancy complications, and how it may affect the placental environment and fetal tolerance.

The role of T cells at the maternal-fetal interface is an often overlooked area of research, with potentially great implications for the onset of various pregnancy pathologies. Historically, leukocyte populations at the decidua are studied in the context of tissue remodelling, the invasion of trophoblast of the uterine wall and the role that NK cells and macrophages have on this during the first trimester. However, our research has demonstrated that in the third trimester of healthy pregnancy, the maternal-fetal interface has highly enriched populations of functional, differentiated and fetal specific T cells, suggesting that this immune population could play a role in multiple pregnancy

complications. A critical next step would be to comprehensively assess the T cell phenotype during the 1st and 2nd trimester of healthy pregnancy. Despite evidence of lower total T cells counts in the placenta at these time points, it would be of significant interest to track how activated and fetal specific T cell populations accumulate throughout gestation.

A limitation of this study is the narrow perspective of the role of T regulatory cells have within the decidua. Despite demonstrating that there is an increased proportion in decidua, and that after depletion in an MLR setting resulted in increased proliferation of effector T cells, it should not be assumed that their function is based entirely on recognition of fetal antigen. Indeed, there is an emerging role of T regulatory cells in maintenance of metabolism and tissue repair. Feuerer et al. (2009) demonstrated in murine models, that T regulatory cells from abdominal fat (visceral adipose tissue), were transcriptionally distinct from lymphoid organ T regulatory cells and were significantly reduced in insulin resistant mouse models of obesity. Additionally loss and gain of function studies influenced the inflammatory environment of adipose tissue and response to insulin. In vitro experiments demonstrated the effect of IL-10 on cultured adipocytes and its function on reducing synthesis of inflammatory mediators and insulin response. In relation to tissue repair, Burzyn et al. (2013) in murine models, described a phenotypically distinct population of T regulatory cells that accumulate to sites of skeletal muscle injury coinciding with a switch of M1 macrophages to tissue repair M2 macrophages. A critical factor in these skeletal muscle T regulatory cells was expression of amphiregulin, which functions to improve muscle repair. It is likely that T regulatory cells located at the maternal-fetal interface perform a multitude of homeostatic functions, beyond that of

limiting antigen specific responses, including maintenance of localised cellular metabolism and assisting tissue repair during placental growth. It should be noted however that human research into these specific alternate roles of T regulatory is limited, especially in pregnancy.

One focus of this study was HY specific T cells in the decidua; however, this greatly limits the potential response to other mHAGs inevitably present on fetal matter, including the trophoblast. In addition to HY proteins expressed on placental EVT cells (KDM5D, DDX3Y, and RPS4Y1) autosomal mHAGs HMHA1, KIAA0020, and BCL2A1 were also shown to be expressed (Holland et al 2012). Moreover, HMHA1, which is also highly expressed on syncytiotrophoblast has been shown to elicit an antigen-specific CD8+ T cell response in haematopoietic stem cell transplantation (van Halteran et al 2009). However, as with many arguments over whether decidual T cell are able to directly respond to fetal antigen, syncytiotrophoblast cells do not express any MHC molecules and, therefore, lack the capacity to directly present mHag peptides to T cells. It is hypothesised that despite these antigens being processed and presented to T cells, subsequently, these T cells will be unable to respond in an allo-reactive nature. However, there are several caveats to this assumption which may explain how cytotoxic damage of the placenta arises, especially in the case of V.U.E. Even though the syncytiotrophoblastic layer is considered a barrier between the maternal tissue and intra-placental layers, this does not discount the presence of focal damage or gaps due to shedding of layers during infiltration and expansion of the placenta or upregulation of adhesion molecules (Redline 2007, Tamblyn et al 2013). Consequently, this may allow T cells to enter the placental tree and, thereby, elicit further responses. Specifically, in the case of CD8+ T cells, they will be able to interact

not only with HLA-C expressing extravillous trophoblast, but also non-trophoblastic stromal bodies including endothelial cells, fibroblasts and fetal derived macrophage Hofbauer cells. CD4+ T cells would also be able to interact with MHC class II expressing Hofbauer cells and help prolong a cytotoxic response. Our research has demonstrated consistently that T cells with cytotoxic potential are situated in the decidua, and are fetal specific, even in a healthy pregnancy. This gives significant weight to the hypothesis that pathologies like V.U.E. are a result of a breakdown of the immunomodulatory mechanism. Indeed, as demonstrated there are multiple targets for this potential breakdown, which could be carried forward for further investigation, such as the significance of expression of multiple immune checkpoint proteins present on decidual T cells.

The presence of fetal-reactive effector T cells at the decidua are likely to have been primed following systemic transfer of fetal cells and placental debris into the maternal circulation, which may involve either local or systemic presentation within lymphoid tissue. It should be noted that the HLA-peptide multimers used in this study included the HLA alleles of HLA-A2 and HLA-B7, and these proteins are not expressed on fetal syncytiotrophoblast or extravillous trophoblast (EVT). As such, it remains possible that anatomical limitations may serve to largely limit the potential interaction between fetal-specific T cells and fetal tissue. In this regard, it will be important in future studies to investigate the presence and function of fetal-specific cells restricted by HLA-C and HLA-G, which are the only polymorphic HLA molecule expressed on EVT. Indeed, in humans, HLA-C mismatched pregnancies were shown to induce CD4+ T regulatory cells in decidual tissue, whereas HLA-C-matched pregnancies did not (Tilburgs et al 2009). It was suggested that these findings indicated that paternal MHC/HLA-C molecules were specifically recognized

by maternal leukocytes, however HLA-C specific dextramer was not applied. Despite this, our research provides some of the first evidence of CD8+ specific to HY antigen being present at the maternal-fetal interface. Investigation into whether MHC/HLA-C specific CD8+ T cells play a clinical role in recurrent miscarriages and V.U.E, and the role of T regulatory in managing this allo-reactive response will be a critical next step in understanding the mechanism of T cell tolerance to the allogeneic fetus.

One argument against the relevance of the presence of T cells at the decidua, is that they are merely transient or trafficked non-specifically to an area undergoing significant tissue remodelling. The focal nature of V.U.E. contradicts this mechanism, as a non-specific infiltration would result in a diffuse pattern of infiltration. Our research also demonstrates T cell specific to fetal antigen have an increased proliferatory response to fetal cells, suggesting mechanisms of antigen presentation and directed trafficking. In order to clarify specific trafficking of decidual T cells and allo-antigenic responses, further research is needed to analyse the TCR clonality of the decidual T cell. The presence of reduced clonotypes at the maternal fetal interface would suggest increase in specific T cell trafficking rather than a diffuse broad populations. Additionally, TCR analysis could be performed following MLRs involving maternal T cells responders and fetal cell effectors. Reduced clonality of proliferative T cells would suggest specific antigenic responses to fetal antigens.

An exciting and extremely novel finding of our research is the discovery, through transcriptomic analysis, of an upregulated response to Type 1 interferon. This pathway is significantly upregulated in effector memory T cells at the decidua and is something

previously unrecognized in T cell function at this site. The fact that this pathway is upregulated in the absence of known viral infection, suggests that this immune response is associated with classical viral infection and may be a unique identifier of T cell allo-reactivity at the maternal-fetal interface. As discussed in Chapter 6, we can only speculate at this point which antigen or effector elicits this type 1 interferon response. As previously mentioned, the possibility of endogenous retroviral proteins on the surface of trophoblast such as syncytin 1, may be presented as an additional mHAG in the adaptive immune response. Further weight to this argument is provided by the pathology of V.U.E. resembling an inflammatory response to similar villitis conditions of known pathogenic origin such as CMV associated chronic villitis. An important next step would be to isolate and analyse decidual macrophages, and to look for similar associated pathways following transcriptomic analysis. Given their significant role in antigen presentation, and the high numbers present in cases of V.U.E., establishing whether a similar response to interferon is present during healthy pregnancy would present an alternative pathway towards fetal allo-reactivity.

The presence of activated, antigen experienced T cells at the decidua in healthy pregnancy reinforces the emerging concept that T cells play an important role at the maternal-fetal interface and that fetal-specific recognition is carefully modulated by multiple checkpoint proteins and T regulatory cells. Dysregulation of this immune homeostasis has substantial implications for the development of healthy pregnancy. Taking into account the research undertaken in this project, Figure 7.1 outlines the various immunological pathways that may occur at the maternal-fetal interface. Further research

into this unique microenvironment and the how it is regulated will lead to a better understanding of similar allo-reactive immune mechanisms in other clinical settings.

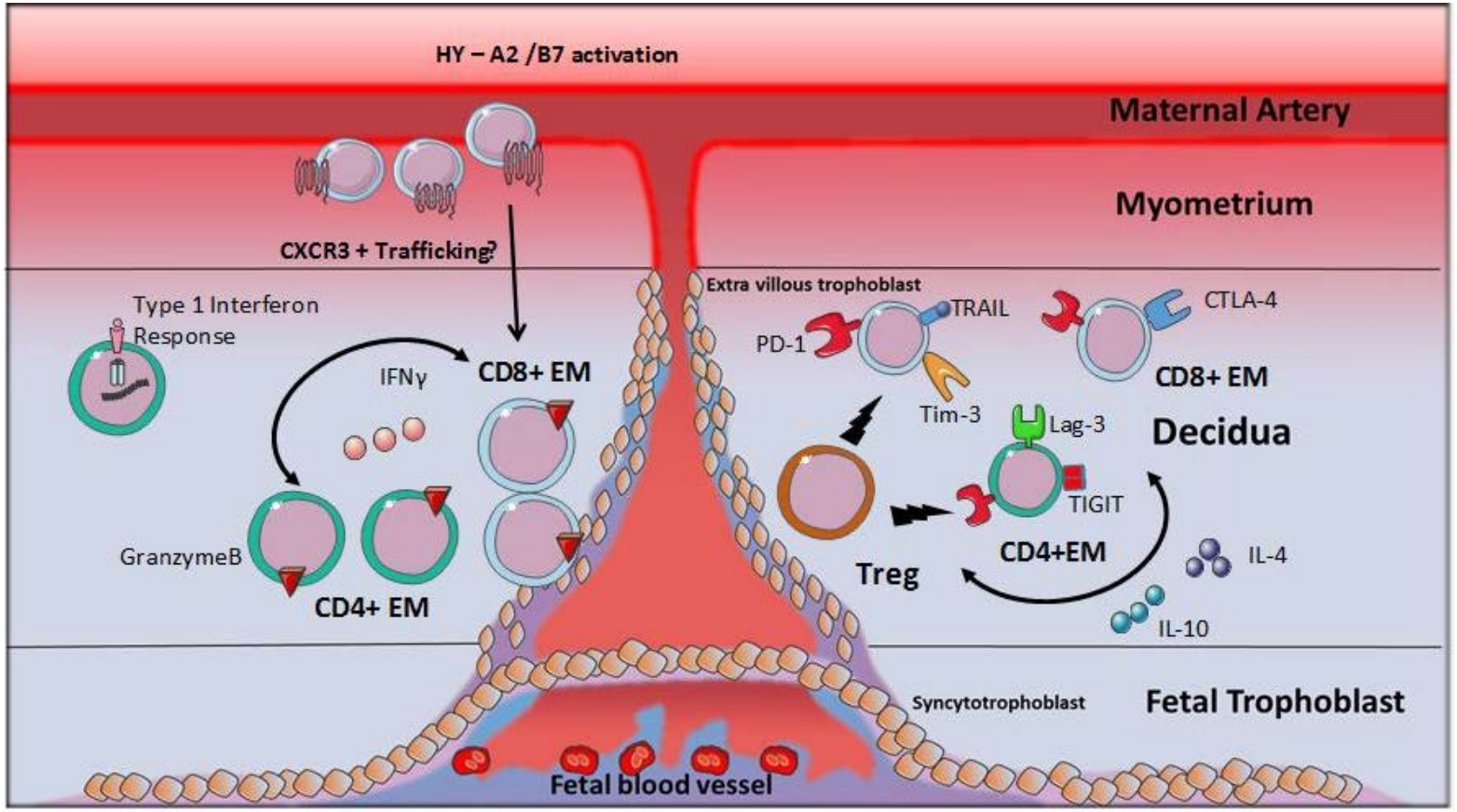

Figure 7. 1.Summary of T cell interaction with maternal-fetal interface in healthy pregnancy

Our project has demonstrated that HY specific T cells are present in the decidua, and these are potentially trafficked to this site from the periphery. Activated effector memory CD4+ and CD8+ express increased levels of IFNγ and upon stimulation with fetal antigen, granzyme B. Additionally there is an enhanced response to Type 1 interferon in decidual T cells. Tempering this potentially alloreactive response, CD4+ T regulatory cells are increased at the decidua, along with increased expression of immune checkpoint proteins PD-1, Tim-3, Lag-3 and CTLA-4 by effector T cells. Increased expression of IL-4 and IL-10 also contribute to this modulatory response, along with evidence of expression of TIGIT and TRAIL.

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Appendices 1.

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Decidual T cells exhibit a highly differentiated phenotype and demonstrate potential fetal-specificity and a strong transcriptional response to interferon.

Decidual T Cells Exhibit a Highly Differentiated Phenotype and Demonstrate Potential Fetal Specificity and a Strong Transcriptional Response to IFN

Richard M. Powell,*[,1] David Lissauer,[†,1] Jennifer Tamblyn,[†,‡] Andrew Beggs,[§] Philip Cox,[¶] Paul Moss,*[,2] and Mark D. Kilby[†,‡,2]

Immune tolerance during human pregnancy is maintained by a range of modifications to the local and systemic maternal immune system. Lymphoid infiltration is seen at the implantation site of the fetal–maternal interface, and decidual NK cells have been demonstrated to facilitate extravillous trophoblast invasion into maternal decidua during the first trimester, optimizing hemochorial placentation. However, although there is considerable T cell infiltration of the maternal decidua, the functional properties of this T cell response remain poorly defined. We investigated the specificity and regulation of $CD4^+$ and $CD8^+$ T cells obtained from human third trimester decidua and demonstrated that decidual $CD4^+$ and $CD8^+$ T cells exhibit a highly differentiated effector memory phenotype in comparison with peripheral blood and display increased production of IFN-γ and IL-4. Moreover, decidual T cells proliferated in response to fetal tissue, and depletion of T regulatory cells led to an increase in fetal-specific proliferation. HY-specific T cells were detectable in the decidua of women with male pregnancies and were shown to be highly differentiated. Transcriptional analysis of decidual T cells revealed a unique gene profile characterized by elevated expression of proteins associated with the response to IFN signaling. These data have considerable importance both for the study of healthy placentation and for the investigation of the potential importance of fetal-specific alloreactive immune responses within disorders of pregnancy. *The Journal of Immunology*, **2017, 199: 3406–3417.**

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