

# THE BIOLOGICAL AND CLINICAL SIGNIFICANCE OF THE MATERNAL IMMUNE RESPONSE TO FETAL ANTIGENS

by

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# Abstract

Tolerance of the semi-allogeneic fetus presents a significant challenge to the maternal immune system.

The effect of pregnancy on maternal cellular immunity was established by assessing maternal effector and regulatory T-cell subsets during human pregnancy. This demonstrated that an increase in maternal peripheral regulatory T-cells or a shift from a Th1 to Th2 phenotype was not a requirement for normal pregnancy. We also determined the profound impact of maternal Cytomegalovirus seropositivity on maternal T- cell dynamics.

T-cells with specificity for fetal epitopes have been detected in women with a history of pregnancy but it has been thought that such fetal specific cells were deleted during pregnancy. We identified, using MHC-peptide multimers, fetal-specific CD8 T-cells in half of all pregnancies. The fetal-specific response increased during pregnancy and persisted in the post natal period. Fetal-specific cells demonstrated an effector memory phenotype and retained functional potential. These data show that the development of a fetal-specific adaptive cellular immune response is a normal consequence of human pregnancy.

Women with recurrent miscarriage were found to have abnormal T-cell function, with increased IFN $\gamma$  and IL-17 production. Fetal specific T-cells were also detected in this cohort and progesterone attenuated their function, which may have therapeutic implications.

# Dedication

For Sam and Jacob

# Acknowledgements

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# List of abbreviations

Throughout the text the term “T cell” has been used to describe “T lymphocyte”

ANOVA	Analysis of variance
APC	Antigen presenting cell
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
CD	Cluster of differentiation
CLIP	MHC class II-associated invariant chain peptide
CM	Central memory
CMV	Cytomegalovirus
Ct	Cycle threshold
CTACK	Cutaneous T-cell attracting chemokine (CCL27)
CTL	Cytotoxic T lymphocyte
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
ELISA	Ezyme linked immunosorbancy assay
EM	Effector memory
EMRA	Effector memory 'RA' positive revertant
Fab	Fragment antigen binding
FAM	6-carboxy-fluorescein
FAS	CD95
FCS	Fetal calf serum
FIDS	FIDSYICQV (Phenylalanine-Isoleucine-Aspartic acid-Serine-Tyrosine-Isoleucine-Cysteine-Glutamine-Valine)
FISH	Fluorescent in situ hybridisation
FITC	Fluorescein isothiocyanate
FOXP3	Forkhead box P3

Fractalkine	CX3CL1
FSC	Forward scatter
GCSF	Granulocyte colony-stimulating factor
GE	Genome equivalents
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HGF	Hepatocyte growth factor
HLA	Human leukocyte antigen
IDO	Indolamine 2,3-dioxygenase
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IP-10	Interferon gamma-induced protein 10 (CXCL10)
ITAC	Interferon-inducible T cell alpha chemoattractant (CXCL11)
IV	Intravenous
KIR	Killer cell Immunoglobulin-like Receptor
LIF	Leukaemia inhibitory factor
MCP-1	Monocyte chemotactic protein-1 (CCL2)
MFI	Median fluorescent intensity
MGB	Minor Groove Binder probes
MHC	Minor Histocompatibility Antigen
MIG	Monokine induced by interferon (CXCL9)
MIP-1 $\alpha$	Macrophage inflammatory proteins 1alpha (CCL3)
MIP-1 $\beta$	Macrophage inflammatory proteins 1beta (CCL4)
MMP	Matrix metalloproteinases
mPR	Membrane Progesterone Receptor
NK	Natural-Killer Cell
Ova	Ovalbumin
P4	Progesterone
PAPCs	Pregnancy-associated progenitor cells
PBMC	Peripheral blood mononuclear cells

PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
PDL-1	Programmed cell death 1 ligand 1 (PD-L1)
PE	Phycoerythrin
PHA	Phytohemagglutinin
PMA	Phorbol Myristate Acetate
PN	Postnatal
Q-PCR	Quantitative real-time polymerase chain reaction
RANTES	Regulated on activation, normal T expressed and secreted (CCL5)
RM	Recurrent miscarriage
Rn	Normalised reporter
RNA	Ribonucleic acid
RPM	Revolutions per minute
RPMI	Roswell Park Memorial Institute medium
RT-PCR	Reverse transcriptase polymerase chain reaction
SAPE	Streptavidin-PE
SDF-1	Stromal cell-derived factor-1 (CXCL12)
SDS	Sodium Dodecyl Sulfate
SSC	Side scatter
T cell	T lymphocyte
TAP	Heterodimeric transporter associated with antigen processing
TCR	T cell receptor
Th	T-helper cell
TMB	3,3'-5,5'-tetramethylbenzidine
TNF	Tumour necrosis factor
Treg	Regulatory T cell
VEGF	Vascular endothelial growth factor

# **Chapter 1.**

## **Introduction**

## **1.1 T lymphocyte development and immunity**

### **1.1.1 Introduction to the cellular immune system**

The immune system is “an organization of cells and molecules with specialised roles in defending against infection” (Delves and Roitt 2000). The immune system consists of two fundamental arms, the “innate” and “adaptive”. Innate responses are the most evolutionarily ancient forms of host defence. This requires recognition of pathogens through germ line encoded, invariant receptors which have evolved specificities to detect common, conserved features of pathogenic organisms. The innate effector cells include neutrophils, monocytes and macrophages which are important phagocytic cells, mast cells, basophils and eosinophils which can release inflammatory mediators, and natural killer cells which can target cells which are “non self” through cytotoxic actions and the activation of cell death through apoptosis (Murphy, Travers et al. 2008). The innate immune system provides rapid responses. However, the diversity of pathogenic organisms, the rapidity with which they can evolve to evade recognition and the limitations on the diversity of receptors which can be encoded in this way (the human genome is limited to less than 25,000 protein coding genes (IHGSC 2004)) means that the protection provided by the innate immune system can be overcome by many organisms.

The acquired or adaptive immune system utilises a different approach to overcome this. The T cell and B cell receptors are not germ-line encoded but are generated somatically, as the cell develops, through a remarkable process which endows each cell with a unique receptor ( $10^{15}$  different T cell receptors may be generated) (Davis and Bjorkman 1988; Arstila, Casrouge et al. 1999).

A subsequent process of selection is required to remove those cells with receptors which may be harmful (recognise self) or useless. Cells which subsequently recognise their antigen, and receive the required additional (co-stimulatory) signals are then activated and replicate. This clonal expansion takes time, and thus an adaptive response initially takes days to develop. However, a significant number of clonally expanded cells can persist after the antigen has been eliminated and this enables subsequent encounters to be more rapid and effective, the phenomenon of immunological memory (Tanchot, Lemonnier et al. 1997).

Lymphocytes when viewed under a light or electron microscope are small, featureless cells with few organelles, scant cytoplasm and condensed chromatin, hence they appear relatively inactive. As recently as the 1960s these cells were described in textbooks as having no function. Yet, we now recognise these cells as being absolutely central to the adaptive immune system and remarkable in their functions and complexity. T lymphocytes, named due to their lineage commitment and development occurring in the thymus, are responsible for the cell mediated responses of the adaptive immune system. T lymphocytes will be the focus of this thesis, thus their development and functions will now be examined in more detail.

### **1.1.2 Antigen recognition by T lymphocytes**

T cells recognise their antigen through the interaction of the T cell receptor (TCR) with a peptide fragment which has been mounted on the major histocompatibility complex (MHC) and displayed upon the cell surface.

The T cell receptor is a heterodimer, structurally similar to the Fab component of an immunoglobulin molecule, composed of 2 polypeptide chains linked by a disulphide bond

(Davis and Bjorkman 1988). T cells with a TCR composed of an alpha and a beta chains will be the subject of this thesis and introduction, and I will not be describing the minority gamma-delta T cell population. The alpha and beta chain each consists of a variable (V) and a constant region (C) (Fig. 1.1.2.1). The diversity of the TCR stems from the process of VJ (alpha chain) or VDJ (beta chain) segment rearrangement that occurs to form complete variable domain exons, much like the process for the formation of immunoglobulin light chains. The extreme diversity of the TCR is due to the combinatorial diversity due to the numerous gene segments which can be combined and so called “junctional diversity” due to nucleotide changes introduced during the process of gene segment rearrangement (Krangel 2009).

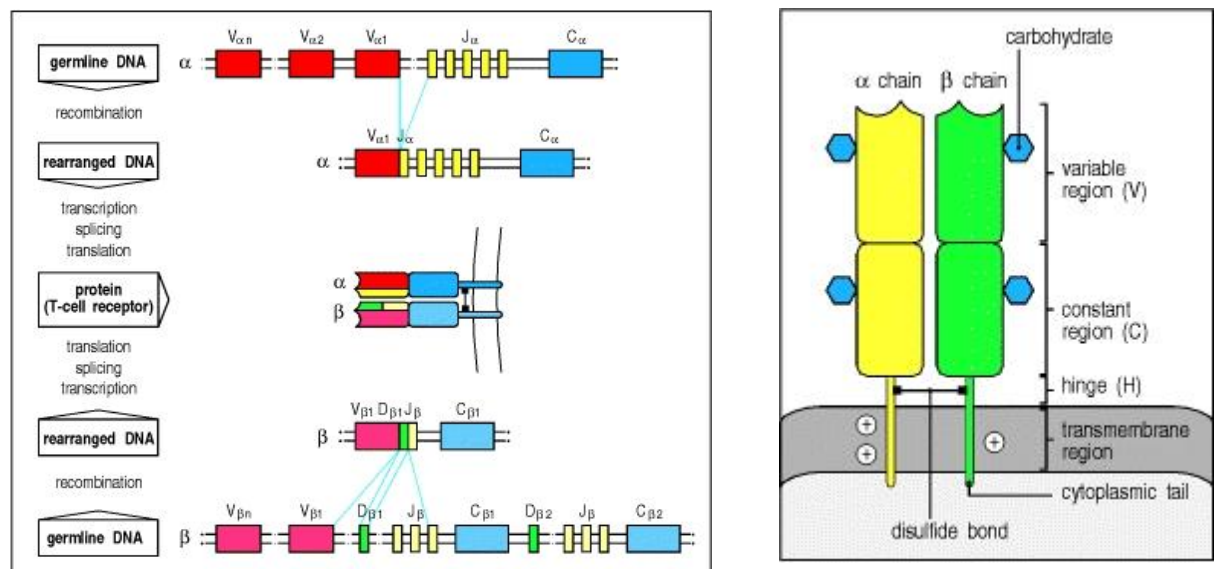


Fig 1.1.2.1 T cell receptor generation.

Rearrangement of the TCR genes involves the joining of gene segments by somatic recombination. Transcription and splicing of the VJ exon and the VDJ exon of the alpha and beta chain respectively is followed by transcription and splicing to the C region. This results in mRNA that is translated to form either the alpha or beta chain, which then forms a heterodimer (Figure reproduced with permission from (Murphy, Travers et al. 2008)

The TCR has a crucial co-receptor, either CD8 or CD4, separating T cells into 2 major classes. Which of these co-receptors is present is functionally important as it determines whether the TCR interacts with MHC class I or class II. CD8 binds the  $\alpha 3$  domain of MHC class I (Gao, Tormo et al. 1997), whereas CD4 binds to  $\beta 2$  domain of the MHC class II molecule (Wang and Reinherz 2002).

MHC class I is found on almost all nucleated cells. The class I antigen presentation pathway provides a mechanism for displaying a sample of peptides from the cell's internal proteome on the cell's surface. These proteins are cleaved by the proteasome and transported into the endoplasmic reticulum by the TAP transporter (Cresswell, Ackerman et al. 2005). Thus CD8 T cells, recognising peptides in the context of MHC class I are able to survey the hosts cell's proteome for the presence of viral and other foreign or tumour associated proteins (Fig. 1.1.2.2).

MHC class II is constitutively active on 'professional' antigen presenting cells such as B cells, dendritic cells and macrophages. Peptides processed and presented in MHC class II are derived from proteins within endosomal compartments (Watts 2004). These are therefore predominantly proteins which have been internalised by the antigen presenting cell, thus CD4 T cells are surveying peptides derived from exogenous proteins (Fig. 1.1.2.2). Though it is becoming increasingly clear that endogenous cytoplasmic antigens can also access this pathway via several mechanisms (Dengjel, Schoor et al. 2005; Zhou, Li et al. 2005; Schmid, Pypaert et al. 2007).

It is now recognised that antigen presenting cells can also present exogenous antigens to CD8 T cells, in a process known as "cross-presentation" (Groothuis and Neefjes 2005; Bevan 2006), via several cellular mechanisms (Guermonprez, Saveanu et al. 2003;



Houde, Bertholet et al. 2003; Shen and Rock 2006; Burgdorf, Kautz et al. 2007). This enables CD8 T cells to respond to antigens which would otherwise not access the class I processing pathway (Fig. 1.1.2.3).

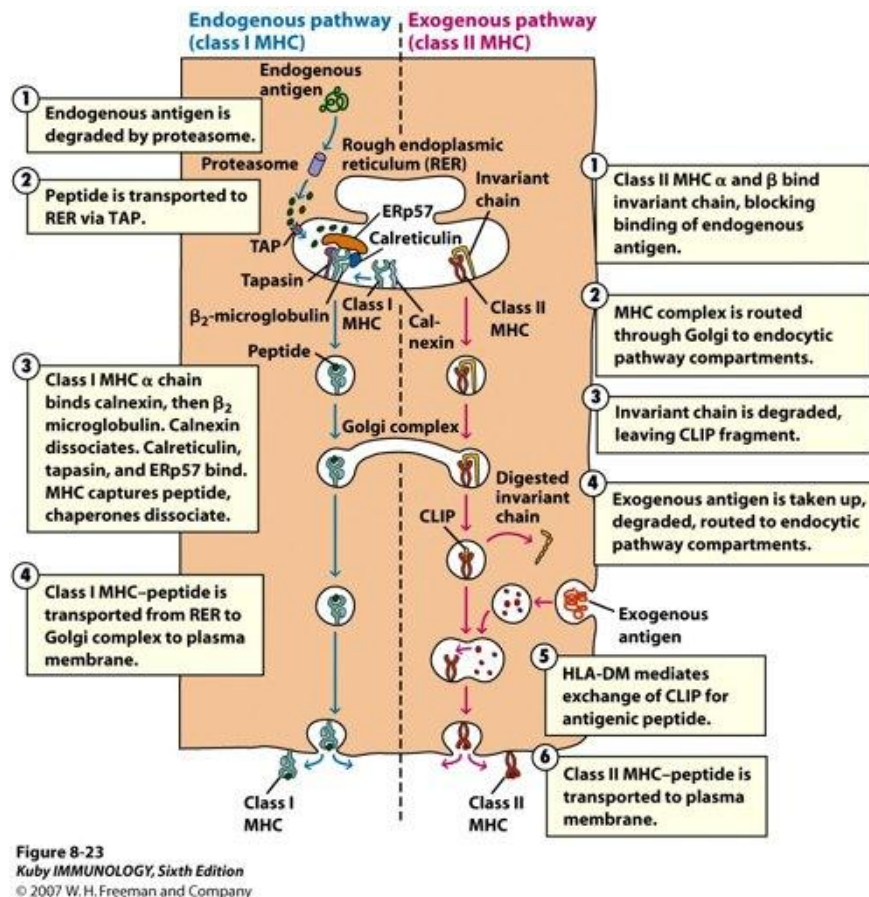


Figure 1.1.2.2 The MHC class I and MHC II processing pathways.

MHC class I pathway involves the presentation of endogenous peptide, via proteasome degradation and entry into the endoplasmic reticulum via the TAP transporter. Peptide is loaded into the MHC class I molecule, with the aid of the peptide-loading complex including calreticulin, ERp57, and tapasin. MHC class II processing instead enable presentation of exogenous peptides. The MHC class II molecule is assembled with the aid of the chaperone protein the "invariant chain" which is then degraded to leave only the CLIP fragment, bound to the binding groove. HLA-DM exchanges CLIP for an exogenous peptide in the endosome (Reproduced with permission (Kindt 2007)).

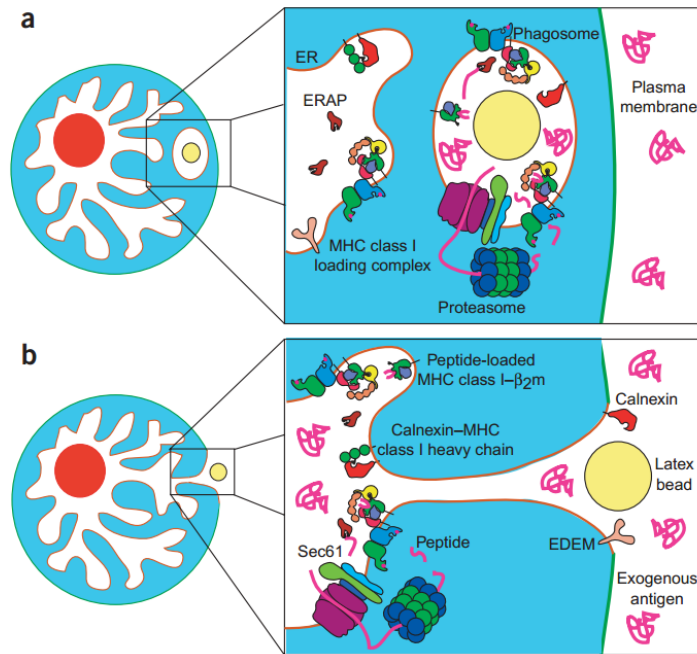


Figure 1.1.2.3 Mechanisms of cross presentation.

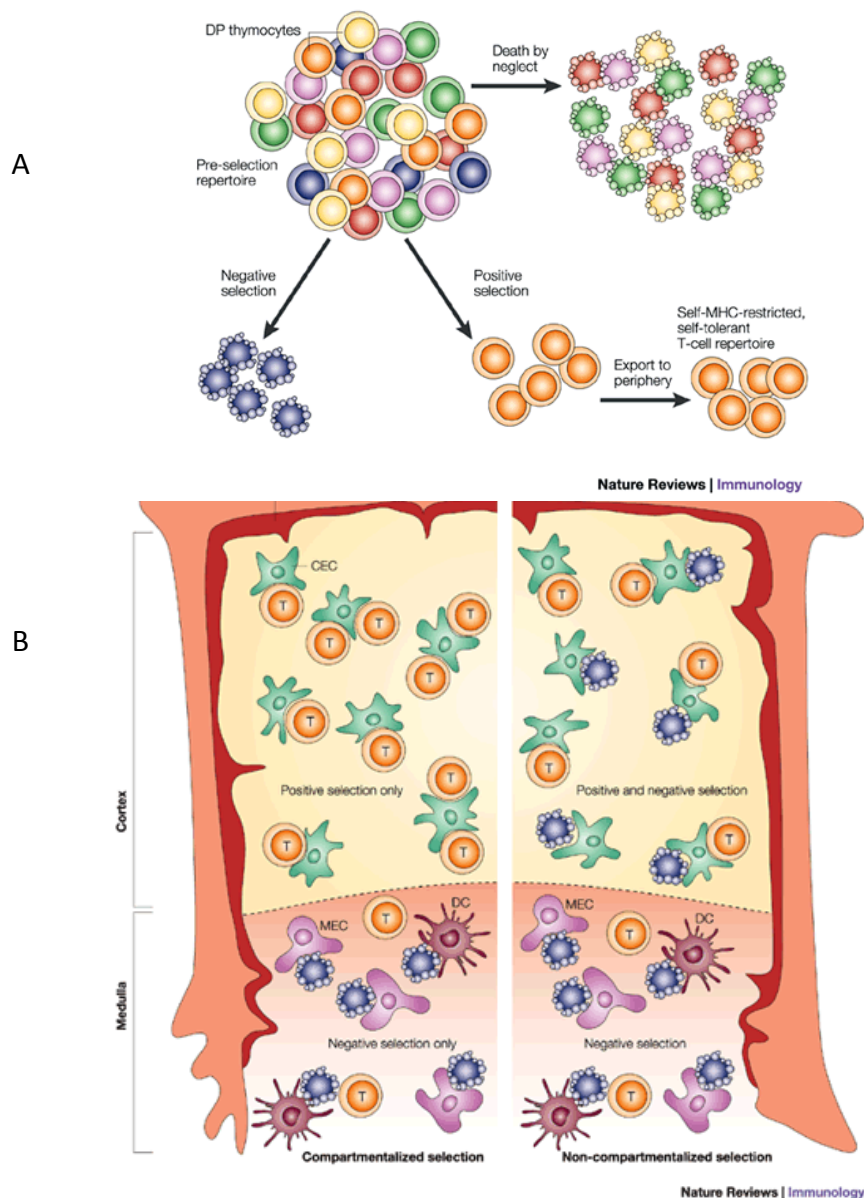
Two models of cross-presentation. The first in which the phagosome is self contained including the proteasomes and the peptide loading complex. (a) The second in which external proteins have direct access to the endoplasmic reticulum by transient continuities or by regulated mechanisms (b). (ER=Endoplasmic reticulum, ERAP=ER Aminopeptidase, EDEM=ER (Endoplasmic Reticulum) Degradation Enhancing Alpha-Mannosidase-Like). Reproduced with permission from (Ackerman and Cresswell 2004).

### 1.1.3 T cell development

T cell production and development predominantly takes place in the specialised environment of the thymus, where T cells generated from bone marrow derived precursor cells have migrated and undergo development and selection.

The antigen specificity of a T cell is determined early in its development by gene segment rearrangement as previously described (section 1.1.2). However, the random nature of the TCR generated requires a secondary process to test the unique TCR for its

ability to recognise antigen appropriately. This involves a process of positive selection, by which only T cells which are able to recognise antigen in the context of self MHC molecules are given survival signals. Similarly, negative selection ensures that lymphocytes which bind self antigens too strongly and could therefore recognise self peptide and trigger autoimmune disease are deleted. These processes are responsible for most T cells undergoing apoptosis and not surviving to become part of the mature T lymphocyte pool. The molecular details of the selection process are beyond the scope of this introduction, but are comprehensively reviewed elsewhere (Petrie 2002; Palmer 2003; Starr, Jameson et al. 2003). This process is summarised in Figure 1.1.3.



*Figure 1.1.3 T cell selection in the thymus.*

*CD4+CD8+ double positive (DP) thymocytes have an unselected T cell receptor (TCR) repertoire. High affinity TCR self-peptide-MHC interaction leads to apoptosis (negative selection), low affinity interactions lead to differentiation into CD4+ or CD8+ single positive (SP) thymocytes (positive selection). (a) Thymic cortex contains epithelial antigen-presenting cells (APCs) responsible for positive selection. The epithelial and dendritic cells of the medulla mediate negative selection (left panel), alternatively the selection may not be strictly compartmentalised in this way (right panel). (CEC, cortical epithelial cell; MEC, medullary epithelial cell). Figures reproduced from (Palmer 2003) with permission.*

#### **1.1.4 T cell activation and anergy**

To become activated a T cell must have a T cell receptor that recognises a peptide-MHC complex. However, this is not sufficient for activation of a naïve T cell. For this a second “co-stimulatory” signal is required. These signals are provided by professional antigen presenting cells (Itano and Jenkins 2003). The best characterised co-stimulatory molecules are the B7 family, which interact with molecules such as CD28 family proteins on the T cell (Murphy, Travers et al. 2008). The requirement for co-stimulation is a fundamental mechanism to protect from autoimmunity as antigen presenting cells are induced to upregulate costimulatory proteins by interactions with the innate immune system and cytokines which are triggered by pathogens (Brightbill, Libraty et al. 1999). The co-stimulatory signal is required for the production of IL-2. TCR engagement in the absence of co-stimulation leads to a state of anergy. These anergic cells are unable to produce IL-2 and subsequently cannot respond to antigenic stimulation, even in the presence of co-

stimulation. However, this anergic state can be reversed by exposure to IL-2 (Schwartz 2003).

After this initial interaction and T cells have proliferated and differentiated into effector cells the requirement for co-stimulation is lost and exposure to antigen alone is sufficient for activation. This is important for the function of T cells, such as the necessity for CD8 T cells to respond to viral infection of any cell type, not only professional antigen presenting cells (London, Lodge et al. 2000).

#### **1.1.5 CD4 T cell effector subsets and function**

The CD4 T-helper cell immune response is highly heterogeneous, with the development of distinct subsets of T helper cells based on their cytokine production profiles. The previous paradigm was that there were two distinct subsets of T helper cells, Th1 and Th2 in both mice and humans (Mosmann, Cherwinski et al. 1986; Del Prete, De Carli et al. 1991). Th1 cell differentiation is driven by the transcription factors STAT 4 and T-bet (Rengarajan, Szabo et al. 2000). Th1 cells are characterised by the production of cytokines such as IFN  $\gamma$  and TNF $\alpha$ , with the function of phagocyte activation and the induction of opsonising and complement activating antibodies, mediating predominantly cell mediated immunity against intracellular pathogens. Th2 cell development is instead under the control of the transcription factors STAT-6 AND GATA3 and their responses are characterised by cytokines such as IL-4 and IL-5, IL-9 and IL-13 and a humoral immune response function important for defence against helminthes but also allergic disease (Mosmann, Cherwinski et al. 1986; Rengarajan, Szabo et al. 2000; Piccinni 2006). Early studies suggested that CD4 T

cells were programmed irreversibly to go down one of these two lineages, with the mutually exclusive cytokine production profiles of either Th1 or Th2 cells. However, we now recognise that T helper cell lineages retain considerable scope for plasticity (Wei, Wei et al. 2009). An important factor in lineage determination is the cytokine environment early in the immune response, for example, the presence of IL-4 promoting Th2 differentiation (Abbas, Murphy et al. 1996).

The paradigm was further eroded by the recent recognition of a 3<sup>rd</sup> distinct subset of effector T helper cells, called Th17 cells (Bettelli, Carrier et al. 2006). This third subset of effector cells are functionally distinct from Th1 and Th2 cells, characterised by the secretion of IL-17 and the transcription factor ROR  $\gamma$ t. Differentiation factors have been identified including TGF  $\beta$  and IL-6 or IL-21. This has been an important finding as Th17 cells are important in many models of autoimmune disease (Cua, Sherlock et al. 2003) and also are implicated in a wide range of human diseases (Korn, Bettelli et al. 2009). The physiological role of these cells is thought to be defence against fungal infections, such as *Candida*. Little is yet known about the potential function of these cells during normal or pathological pregnancy. There has however been early reports that these cells are found more commonly in the decidua than in peripheral blood, with no difference observed at different gestations in peripheral blood (Nakashima, Ito et al. 2010). They may be more frequent in women with pre-eclampsia (Santner-Nanan, Peek et al. 2009) and increased in frequency in the decidua of women with inevitable abortion (Nakashima, Ito et al. 2010) and in women with recurrent miscarriage (Liu, Wu et al. 2011).

T helper cell effector subsets can be differentiated as described by their cytokine producing function and transcription factors, but also by their chemokine receptor

expression. CCR5 and CXCR3 expression dominate in CD4 T cells with a TH1 cytokine expression profile, while CCR3, CCR4 and CCR6 predominate in cells with a Th2 profile (Sallusto, Lenig et al. 1998; Appay, van Lier et al. 2008). CCR6 expression is similarly associated with IL-17 but not IFN  $\gamma$  producing cells, while co-expression of CCR6 and CXCR3 defines a more heterogeneous population that produce IL-17 and IFN  $\gamma$  (Acosta-Rodriguez, Rivino et al. 2007; Annunziato, Cosmi et al. 2007).

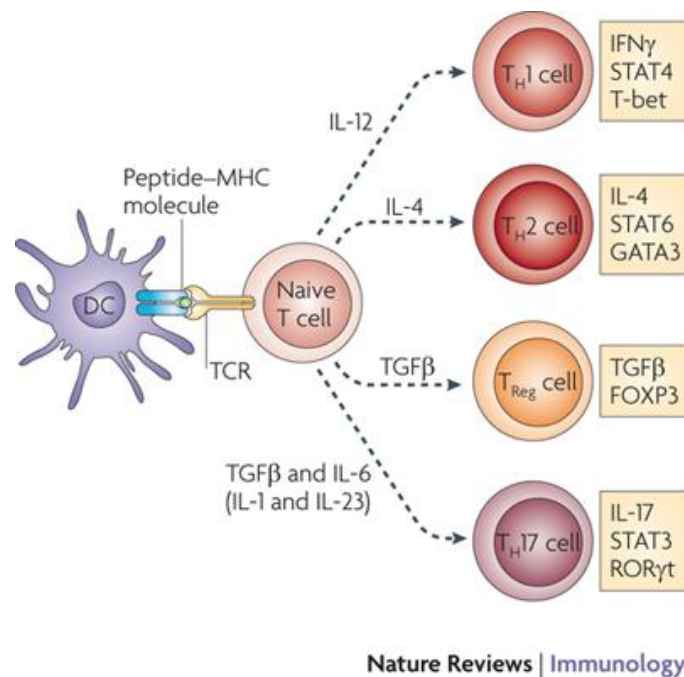


Figure 1.1.5 CD4 T cell subsets.

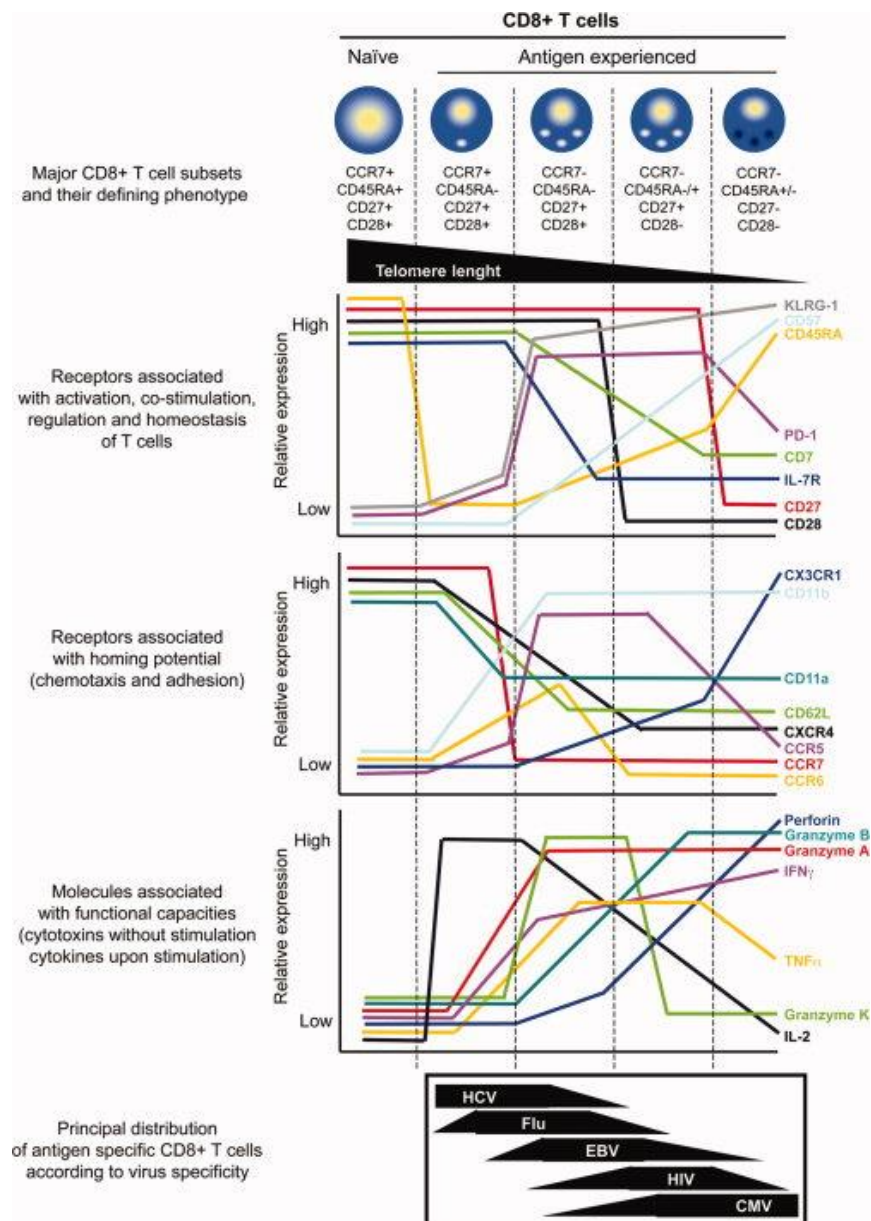
Following activation a naive CD4 T cells can differentiate into 3 effector T helper (TH) subsets; TH1, TH2, TH17 and regulatory T (T Reg) cells. The local cytokines are important in controlling this polarisation (examples of which are illustrated above) and this is then under the control of distinct transcription factors. (FOXP3, forkhead box P3; GATA3, GATA-binding protein 3; IFN $\gamma$ , interferon- $\gamma$ ; TCR, T cell receptor). Figure reproduced from (Zou and Restifo 2010) with permission.

### 1.1.6 CD8 T cells differentiation

CD8<sup>+</sup> T lymphocytes also demonstrate considerable heterogeneity with marked differences in the expression of molecules such as cytokines, chemokines and co-stimulatory molecules being observed during cell differentiation (Lanzavecchia and Sallusto 2005; Appay, van Lier et al. 2008) (Summarised in Figure 1.1.6). Due to the focus of this thesis on CD8 T cell differentiation and phenotype I will discuss this in more detail. CD8 T cells can be separated into four major subsets on the basis of two surface markers. CD45RA, which is the high molecular weight isoform of the CD45 common lymphocyte marker, and the chemokine receptor CCR7 (Sallusto, Lenig et al. 1999). These subsets are termed the naïve (N; CCR7+CD45RA+), central memory (CM; CCR7+CD45RA-), effector memory (EM; CCR7-CD45RA-) and highly differentiated 'CD45RA revertant' effector memory populations (EMRA; CCR7-CD45RA+). Naïve and central memory cells both express CCR7 which is crucial for their ability to circulate through secondary lymphoid tissue. In contrast, the loss of CCR7 and expression of alternative chemokine receptors allows EM and EMRA subsets to migrate to peripheral tissues. The CD45RA isoform of CD45 is expressed on naïve T cells but is spliced to CD45RO following T cell activation. CD45RA can then be re-expressed in subpopulations of memory cells and this EMRA pool is considered as a more highly differentiated subset which has undergone extensive cell division and exhibits reduced replicative potential and short telomeres. However, EMRA cells do retain strong ex-vivo cytolytic capacity with constitutive expression of perforin and granzyme (Appay, van Lier et al. 2008). The additional surface marker CD57 is also increased on this population and its expression is inversely proportional to telomere length (Brenchley, Karandikar et al. 2003).



Further subdivision of CD8 memory subsets on the basis of expression of the co-stimulatory and pro-survival molecules CD27 and CD28 can also yield extra information. In particular, within the effector memory subset (CCR7-CD45RA-) four further subsets can be defined based on their CD27 and CD28 expression. These functionally distinct subsets are termed EM1 (27+28+), EM2 (27+28-), EM3 (27-28-), and EM4 (27-28+). This classification is useful as assessment of CTL function shows that EM1 cells share features with CM cells whilst EM3 cells are functionally more similar to EMRA cells (Romero, Zippelius et al. 2007).



*Figure 1.1.6 CD8 T cell memory phenotype.*

*Circulating CD8 T cell subsets are here shown on the basis of the commonly used surface markers CD45RA, CCR7, CD27 and CD28. The varying expression of a variety of other cell surface receptors and intracellular molecules, associated with activation, costimulation, regulation, homing potential, and function are shown.*

*Figure reproduced from (Appay, van Lier et al. 2008) with permission.*

### **1.1.7 T cell peripheral tolerance and regulatory T cells**

During T cell development the process of thymic (central) deletion of autoreactive T cells is important. However, it is incomplete. There are self antigens which T cells would not be exposed to in the thymus and a host of foreign, yet innocuous antigens, which will be encountered by the host in future, but are not pathogenic. Therefore mechanisms of peripheral tolerance exist to enable the immune system to further regulate its responses. These are of particular importance in relation to maternal tolerance of the allogenic fetus in reproductive biology, and the relevance of these mechanisms in reproductive biology specifically will be covered in more detail later.

A subset of CD4 T cells, termed regulatory T cells (T regs) have an important role in the negative regulation of CD8 and CD4 T cells, mediating responses to autoantigens and in the control of T cell responses to foreign antigens (Tang and Bluestone 2008). T regs suppress immune responses through a variety of mechanisms targeting both dendritic cells and effector cells. These include the release of inhibitory cytokines such as IL-10, IL-35 and TGF $\beta$  (Collison, Workman et al. 2007; Joetham, Takeda et al. 2007), metabolic disruption including the CD25 mediated uptake of IL-2 (Pandiyan, Zheng et al. 2007), and adenosine

receptor mediated immunosuppression (Deaglio, Dwyer et al. 2007), contact mediated mechanisms via CTLA-4 (Qureshi, Zheng et al. 2011) and even cytotoxicity of effector cells (Grossman, Verbsky et al. 2004).

T regs can develop in the thymus, termed “natural” T regs. In which case they have a diverse TCR repertoire for self antigens. T regs can though also be “induced” from effector T cells in the periphery, and therefore have a more restricted TCR specificity. However, the markers used to identify these subtypes of T regs are the same (reviewed in (Sakaguchi, Miyara et al. 2010)).

The identification of molecular markers to identify regulatory T cells in humans has been a challenge. Early characterisation of regulatory T cells on the basis of CD4+CD25+ cells (Somerset, Zheng et al. 2004) is inadequate due to the upregulation of CD25 on activated effector T cells. Though the identification is improved by strategies which focus on the CD25 bright population (Fontenot, Rasmussen et al. 2005).

The transcription factor FOXP3 is recognised as a key regulator of T reg development and function and is probably the best marker available for regulatory T cells (Hori, Nomura et al. 2003), but unfortunately this is also not totally specific to regulatory cells as it can also be expressed on activated, non regulatory CD4 T cells (Morgan, van Bilsen et al. 2005; Gavin, Torgerson et al. 2006; Wang, Ioan-Facsinay et al. 2007).

Identification of further markers, in particular surface markers to enable the isolation of these cells with strategies which do not kill the cell, is ongoing. Currently the most useful of these is the IL-7 receptor, or CD127, which is down regulated on T regs. The literature reports a strong correlation between FOXP3 expression and the CD4+CD25+CD127- population (Liu, Putnam et al. 2006; Seddiki, Santner-Nanan et al. 2006).

Mechanisms other than regulatory T cells also serve to regulate T cell responses in the periphery. These can be broadly classified into ignorance, death by deletion and functional unresponsiveness. Peripheral ignorance can occur when the access of the T cell to the antigen is restricted either by physical separation or by restricting presentation of the antigen to T cells (Srinivasan and Frauwirth 2009). Death by deletion can occur either as a consequence of incomplete T cell activation (Redmond and Sherman 2005) or due to the presence of FasL on tissues which is able to delete activated T cells (Kauma, Huff et al. 1999). Finally, functional unresponsiveness can occur via several mechanisms. Anergy (defective proliferation and IL-2 production) can be induced due to incomplete T cell activation, with the absence of co-stimulation (Schwartz 2003). It may also be induced by high antigen doses (Redmond, Marincek et al. 2005) or high numbers of antigen specific T cells (Rocha, Grandien et al. 1995; Guillaume, Tuosto et al. 2003). “Activation-induced non-responsiveness” is a state of reversible quiescence that follows rapid T cell proliferation (Deeths, Kedl et al. 1999; Tham and Mescher 2001). Finally, T cell exhaustion, as demonstrated in chronic viral infections occurs and may be associated with upregulation of PD-1 (Day, Kaufmann et al. 2006; Petrovas, Casazza et al. 2006) and IL-10 (Brooks, Trifilo et al. 2006).

The regulation of CD8 T cell responses in the periphery is perhaps particularly critical due to the presence of MHC class I on all cells and the potent cytotoxic function of CD8 T cells. The requirement for CD4 T cell help in the generation of many CD8 responses (Bennett, Carbone et al. 1998; Ridge, Di Rosa et al. 1998) or for the generation of memory responses (Janssen, Lemmens et al. 2003) is well described. More recently it has become apparent that CD4 T cells may provide a role as “gate-keepers” regulating the tissue infiltration of CD8 T cells, even at the effector stage (Nakanishi, Lu et al. 2009).

A fascinating recent development is our recognition that exposure to antigens as a fetus in utero, such as non inherited maternal antigens (NIMA) which cross the placenta, may generate a tolerogenic response, mediated by regulatory T cells, that persists till adulthood. This is a novel mechanism which can shape the developing immune system (Mold, Michaelsson et al. 2008).

## **1.2 Maternal immunological adaptation to pregnancy**

### **1.2.1 Historical perspective**

Peter Medawar first recognised in 1953 the immunological paradox presented by pregnancy, with the mother carrying a haploidentical fetus, without rejection (Medawar 1953). He proposed three reasons for this apparent maternal tolerance of the fetus.

- (1) Anatomical separation of the mother and fetus
- (2) A form of antigenic immaturity of the fetus
- (3) Immunological “inertness” of the mother towards the fetus

In the subsequent 60 years it has become clear that none of these explanations are valid, but that the true picture is far more complex and dynamic and that despite our advancing understanding much still remains unknown.

1) Successful pregnancy is not a consequence of physical separation as the human haemochorial placental structure bathes the placental villi in maternal blood and ensures

intimate cellular contact between the mother and placenta (Moffett and Loke 2006). Furthermore, it is now recognized that prenatally there is the transfer of fetal cells and non-cellular material into the maternal circulation (Lo, Lo et al. 1996; Bianchi, Williams et al. 1997) and that fetal cells may even persist lifelong in the maternal circulation (Gammill, Guthrie et al. 2010) and tissues (O'Donoghue, Chan et al. 2004) a phenomenon termed “fetal microchimerism”.

2) What are now classic experiments performed with skin and fetal tissue allografts demonstrated that fetal tissues were antigenically mature and could elicit a potent immune response (Woodruff 1985) (reviewed in (Chaouat, Petitbarat et al. 2010)).

3) Murine models (Tafari, Alferink et al. 1995; Jiang and Vacchio 1998) and human work from our own group and others (Verdijk, Kloosterman et al. 2004; Piper, McLarnon et al. 2007) has demonstrated that the maternal immune system is aware of fetal antigens and actively responds. The details of this response will be covered in some detail in section 1.5.1 and 1.5.2.

It is now recognised that successful pregnancy is an active immunological process. Both maternal and fetal immune systems are functional (Marchant, Appay et al. 2003; Mold, Michaelsson et al. 2008), are immunologically aware of antigen from the other (Tafari, Alferink et al. 1995; Piper, McLarnon et al. 2007; Mold, Michaelsson et al. 2008) and multiple overlapping processes appear important in enabling successful pregnancy. Interactions of the mother and fetus cannot be viewed as simply analogous to a grafted organ, but as a unique immunological scenario.

Indeed, the traditional view of the immune system being solely a harmful threat to the developing fetus is now also being eroded, with the role of lymphocytes in the establishment of the uterine vasculature increasingly recognised.

### **1.2.2 The maternal fetal interface**

It is important to recognise that the unique placental interface means maternal fetal interactions are quite unlike those seen with a surgical graft.

Placental structure varies widely between species. The gross anatomy is strikingly different between murine and human placenta with the placental villi in humans formed into multiple cotyledons, then grouped into a single disc. However a mouse placenta has a single cotyledon, termed a “discoid” placenta. A similarity, is that in both murine and human placenta the endometrium and maternal vessels are eroded, bathing the placental villi in blood, so there is direct contact between maternal blood and the trophoblast surface, this arrangement is termed haemochorial. However, mice have three trophoblast cell layers, 2 syncytial layers plus the spongiotrophoblast layer but in humans there is a single syncytial layer and an underlying trophoblast layer. The invasiveness of the trophoblast into the decidual stroma and arteries is also far more pronounced in human pregnancy (Rossant and Cross 2001).

The predominant cell type which forms the placenta are from the trophoblast lineage. These cells are derived from the trophectoderm and are therefore not a somatic cell, but are extraembryonic in origin and have many unique characteristics. In humans the

placental villi are composed of fetal vessels surrounded by the cytotrophoblast and this is then encased by the syncytiotrophoblast layer. The other trophoblast type is extravillous trophoblast. Extravillous trophoblast invades deeply into the uterine wall during early pregnancy. These cells degrade the muscular walls of uterine arteries and endovascular trophoblast cells actually replace the vessel endothelium resulting in transformation of the spiral arteries and increased blood flow (Pijnenborg, Bland et al. 1983). Villous and syncytiotrophoblast are thought to lack any MHC expression (Tilburgs, Scherjon et al.). However, extravillous trophoblast do express HLA C, HLA-E, HLA-F and HLA-G. Of these only HLA C is highly polymorphic. HLA C and HLA E are important NK cell ligands and both can potentially also act as ligands for T cells (Heinzel, Grotzke et al. 2002; Tilburgs, Scherjon et al. 2009). Interestingly, activated and regulatory CD4 T cells are increased in HLA-C mismatched pregnancies (Tilburgs, Scherjon et al. 2009), though the mechanism for this may be indirect as CD4 T cells are not known to interact with HLA-C directly.

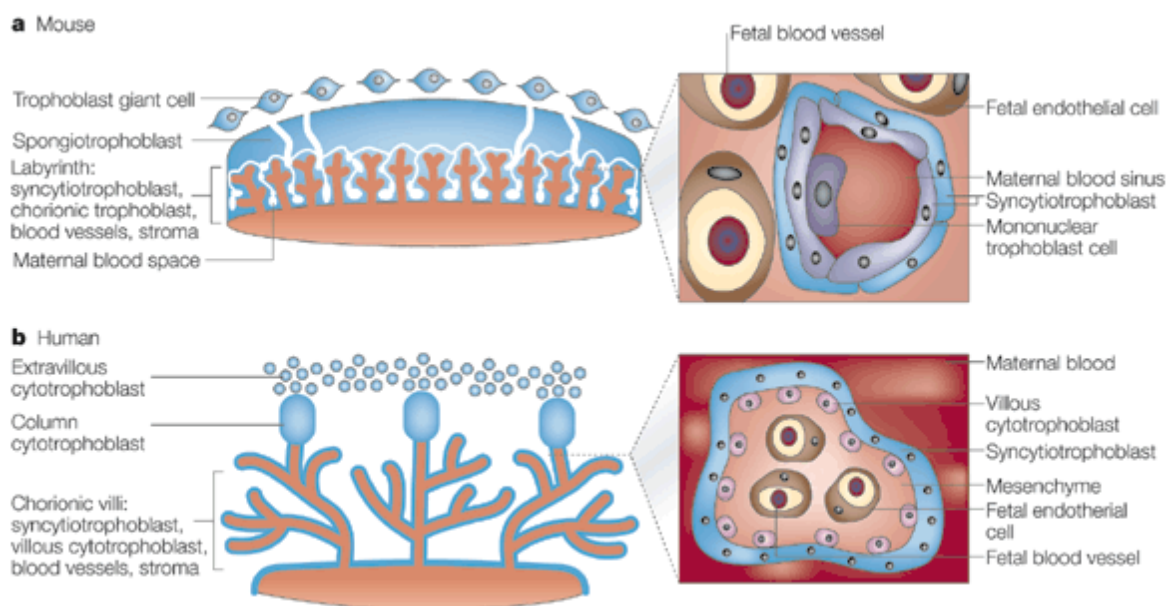


Figure 1.2.2.1 Structure of the mouse (a) and human (b) placenta



*Inset is a cross-section through a chorionic villus contrasting these structures. Trophoblast derived structures are in blue, with mesoderm derived structures in orange. Figure reproduced from (Rossant and Cross 2001) with permission.*

### **1.2.3 Role of CD4 regulatory T cells**

Regulatory T cells are thought to have an important role in the modulation of alloimmune responses in pregnancy.

Murine models have demonstrated that in the absence of T regs syngenic but not allogenic pregnancy can proceed normally (Aluvihare, Kallikourdis et al. 2004; Darrasse-Jeze, Klatzmann et al. 2006). Using an HY antigen based murine model system antigen specific T regs were generated during pregnancy and the depletion of T regs was found to lead to rejection of male fetuses (Kahn and Baltimore 2010). Murine abortion prone models have also been associated with deficient natural T regs and adoptive transfer of T regs can prevent fetal rejection (Zenclussen, Gerlof et al. 2005; Schumacher, Wafula et al. 2007).

Human studies have been somewhat variable and limited by the difficulties in identification of human T regs (see section 1.1.6) and differences in the techniques utilised. Early work suggested an increase in T regs in peripheral blood in human pregnancy (Somerset, Zheng et al. 2004). However, more recently studies using further phenotypic markers such as FOXP3 have failed to show an elevation in peripheral blood T regs during pregnancy (Tilburgs, Roelen et al. 2008) but a clearer picture may be emerging in which there is the recruitment of fetal antigen specific T regs to the maternal fetal interface during pregnancy (Heikkinen, Mottonen et al. 2004; Sasaki, Sakai et al. 2004; Tilburgs, Roelen et al. 2008). The relative contribution of paternal antigens or hormonal mechanisms to changes in

T regs during human pregnancy remains unclear (Prieto and Rosenstein 2006; Mjosberg, Berg et al. 2007).

#### **1.2.4 Role of NK cells**

The most prevalent lymphocytes in the decidua at the time of implantation are NK cells. These are a unique population of uterine NK cells (Koopman, Kopcow et al. 2003) with phenotypic and functional characteristics distinct from peripheral blood NK cells. Uterine NK cells are typically CD56<sup>bright</sup> with low levels of CD16, CD69+ and CD45RO+, with low cytolytic but high cytokine secretory activity (Manaster and Mandelboim 2010).

Due to the cytotoxic potential of NK cells in peripheral blood it was hypothesised initially that NK cells may be associated with pathological pregnancy and recurrent miscarriage. Case control studies reported an association between the numbers of peripheral NK cells (Kwak, Beaman et al. 1995; Ntrivalas, Kwak-Kim et al. 2001; Yamada, Morikawa et al. 2003) and uterine NK cells (Clifford, Flanagan et al. 1999; Quenby, Bates et al. 1999; Tuckerman, Laird et al. 2007) or NK cell activity (Aoki, Kajiura et al. 1995; Shakhar, Ben-Eliyahu et al. 2003) and recurrent miscarriage. However, other studies have reported no such association (Emmer, Nelen et al. 2000; Souza, Ferriani et al. 2002; Wang, Li et al. 2008) and a recent systematic review of the existing evidence does not confirm the measurement of peripheral or uterine NK cells as having clear prognostic value (Tang, Alfirevic et al. 2011). Therapeutic approaches to treat recurrent miscarriage with glucocorticoids have been attempted on the basis of the ability of these agents to suppress NK cell activity (Thum,

Bhaskaran et al. 2008), but the results of a placebo controlled trial of this approach are awaited to see if this is efficacious (Tang, Alfievic et al. 2009).

More recently, the recognition that uterine NK cells have low cytotoxic potential but a high cytokine secretory activity and do not appear to have cytotoxic activity against trophoblast (King, Birkby et al. 1989) despite high levels of intracellular perforin and granzyme (Koopman, Kopcow et al. 2003; Vacca, Pietra et al. 2006) has started to alter our understanding of their role. It is now hypothesised that uterine NK cells rather than being necessarily pathogenic are actually an important part of the mechanism enabling extravillous trophoblast invasion and the conversion of maternal spiral arteries (Li, Charnock-Jones et al. 2001; Moffett and Loke 2004; Lash, Schiessl et al. 2006). NK deficient mice were noted to have reduced placental size and reduced transformation of the spiral arteries, with increased fetal loss (Guimond, Wang et al. 1998) and similar effects were confirmed in other NK knockout strains (Barber and Pollard 2003). In this context allorecognition mediated by the interaction of NK cells receptors with HLA molecules on the extravillous trophoblast is part of the process of controlling trophoblast invasion. HLA-C is highly polymorphic and therefore most likely to be important in paternal allorecognition (King, Allan et al. 2000).

#### **1.2.5 Th1/Th2 cytokine regulation**

A change in the Th1-Th2 balance towards a Th2 dominated cytokine profile during pregnancy has been proposed as an important mechanism for maternal tolerance of the fetus (Lin, Mosmann et al. 1993; Wegmann, Lin et al. 1993; Piccinni, Beloni et al. 1998). With the generation of Th2 cytokines at the decidual interface proposed to favour maternal-fetal

tolerance and successful pregnancy (Lin, Mosmann et al. 1993; Piccinni, Beloni et al. 1998), with a Th1 dominated pattern with elevated IFN $\gamma$ , TNF $\alpha$ , IL-2 and IL-6 seen during miscarriage thought to be associated with rejection (Lin, Mosmann et al. 1993; Hill, Polgar et al. 1995; Marzi, Vigano et al. 1996; Raghupathy, Makhseed et al. 1999; Jenkins, Roberts et al. 2000; Zenclussen, Blois et al. 2003). Th2 type cytokines were even found to rescue pregnancy in murine abortion prone models (Chaouat, Assal Meliani et al. 1995). These findings seemed to help explain the clinical observations of the amelioration of rheumatoid arthritis during pregnancy – a Th1 disease, but the propensity to disease flares in systemic lupus erythematosus, considered a more Th2 dominated problem. This experimental evidence, clinical utility of the concept, and its simplicity mean that it was rapidly adopted by scientists and clinicians.

However, more recently this paradigm has been called into question. In particular, how critical Th2 cytokines are to successful pregnancy is unclear in the light of IL-10 (White, Johansson et al. 2004) or IL-4, IL-10 knockout mice (Svensson, Arvola et al. 2001) and even IL-4, IL-5, IL-9, IL-13 knock out mice still having normal pregnancy (Fallon, Jolin et al. 2002). Even the early seminal papers contained hints that the story may be more complex, with anti IL-10 antibodies not effecting pregnancy outcome, though IL-10 was shown important in murine abortion prone models (Chaouat, Assal Meliani et al. 1995). The very existence of a Th2 bias in human pregnancy has even been challenged (Vince and Johnson 1996), and peripheral TH1/Th2 cytokine changes do not appear to be profound or always reproducible and the variability in cytokine levels between individuals appears larger than the changes induced by pregnancy (Kraus, Sperling et al. 2010). It is increasingly clear that whatever the changes in established pregnancy implantation events are outside this Th1/Th2 paradigm with many Th1 cytokines crucial for this process (Stewart, Kaspar et al. 1992; Bilinski,

Roopenian et al. 1998; Ashkar, Di Santo et al. 2000), reviewed in (Chaouat 2007). Furthermore, as discussed previously the increasing knowledge of Th cell subsets means that new distinct subsets such as Th17 are now recognised as important (Bettelli, Carrier et al. 2006) and need to be considered alongside the traditional classification. Also, many T cell cytokines do not fit neatly into the simple Th1/Th2 dichotomy (Zourbas, Dubanchet et al. 2001).

### **1.2.6 Immunomodulation by progesterone**

Progesterone a C21 steroid hormone, is essential for the establishment and maintenance of pregnancy. In addition to its endocrine effects it has been suggested to have important immunomodulatory roles, influencing the maternal fetal immune interaction during pregnancy and miscarriage (Arck, Hansen et al. 2007; Szekeres-Bartho, Halasz et al. 2009). In particular, effects of progesterone on maternal cytokine production and NK cell function have been reported. This is of particular interest due to the therapeutic use of progesterone in women with recurrent miscarriage (Haas and Ramsey 2008) and preterm labour (Rode, Langhoff-Roos et al. 2009).

Many studies of the effects of progesterone have been conducted in the context of the prevailing Th1/Th2 paradigm at that time, and therefore must be assessed in that context.

Peripheral blood lymphocytes from women with recurrent miscarriage when activated in the presence of progesterone have been shown to produce less IFN  $\gamma$  and TNF  $\alpha$

and increase IL-4 and IL-6 production, interpreted as a reduction in the Th1/Th2 ratio. This effect could be blocked by the progesterone receptor antagonist mifepristone (Raghupathy, Al Mutawa et al. 2005). Progesterone has also been shown using a T cell clone model to switch established Th1 clones to a Th2 phenotype and favour the development of Th2 type clones (Piccinni, Giudizi et al. 1995). The application of progesterone to lymphocytes induces the production of progesterone induced blocking factor (PIBF). PIBF has been shown to have wide ranging effects including increased production of Th2 type cytokines in patients with pathological pregnancies (Raghupathy, Al-Mutawa et al. 2009) via the Jak/STAT pathway (Kozma, Halasz et al. 2006). Progesterone can also act directly and via PIBF to modulate NK cell activity (Szekeres-Bartho, Kinsky et al. 1990; Arruvito, Giulianelli et al. 2008).

Evidence for the presence of conventional nuclear progesterone receptors in lymphocytes is in general conflicting, and may be only present in lymphocytes following activation and during pregnancy (Szekeres-Bartho, Szekeres et al. 1990; Mansour, Reznikoff-Etievant et al. 1994; Bamberger, Else et al. 1999; Polgar, Barakonyi et al. 1999). However, novel membrane progesterone receptors have been identified (reviewed in (Thomas 2008)) and transcripts for mPR $\alpha$  and mPR $\beta$  have been demonstrated in T lymphocytes (Dosiou, Hamilton et al. 2008). Non genomic effect of progesterone have also been demonstrated to operate on lymphocytes via the rapid inhibition of voltage gated and Ca<sup>2+</sup> activated K<sup>+</sup> channels (Ehring, Kerschbaum et al. 1998).

### **1.2.7 Other maternal immunomodulatory mechanisms**

A wide range of other mechanisms have been proposed that may contribute to maternal tolerance of the fetus during pregnancy (Table 1.2.7). The relative contribution of all these different factors is hard to determine. The evolutionary pressure for successful reproduction and the need to avoid singular mechanisms of tolerance which could be usurped by pathogens will mean that the mechanisms of fetomaternal tolerance will necessarily be numerous, overlapping and include redundancy.

*Table 1.2.7 Additional proposed mechanisms of maternal-fetal tolerance*

Mechanism	Description	Reference
Tryptophan depletion	IDO degrades tryptophan and is expressed on trophoblast, inhibiting T cell activation which is tryptophan dependent	(Munn, Zhou et al. 1998) (Mellor, Sivakumar et al. 2001)
Complement regulation	The complement regulatory protein crry is essential in murine pregnancy to avoid complement deposition at the maternal-fetal interface	(Xu, Mao et al. 2000)
Leukaemia inhibitory factor (LIF)	LIF is a requirement for implantation and reduced or absent levels are associated with pregnancy loss	(Stewart, Kaspar et al. 1992) (Piccinni, Beloni et al. 1998)
Programmer death ligand -1 (PDL-1)	PDL-1 is an inhibitory co-stimulatory molecule expressed by trophoblast which can inhibit T cell proliferation and is pro-apoptotic. PD-1 antibody blockade leads to abortion of allogenic but not syngenic fetuses.	(Guleria, Khosroshahi et al. 2005)

### **1.3 Transfer of fetal antigen into the maternal circulation during pregnancy and fetal microchimerism**

#### **1.3.1 Fetomaternal cellular traffic and fetal microchimerism**

During normal pregnancy, there is a bidirectional passage of cells across the placenta, both from the fetus to the mother and vice versa (Lo, Lo et al. 1996). It has been appreciated for many years that fetal cells such as trophoblasts and fragments of syncytiotrophoblast circulate in the maternal circulation during pregnancy (Douglas, Thomas et al. 1959).

However, it has only become accepted more recently, that cells of fetal origin can be detected within the mother's circulation (Bianchi, Zickwolf et al. 1996) and tissues (O'Donoghue, Chan et al. 2004), decades after pregnancy. This persistence of relatively low numbers of fetal cells in the mother's circulation is termed 'fetal microchimerism' (Liegeois, Escourrou et al. 1977). A chimera is an organism in which there are two or more different populations of genetically distinct cells that have originated from separate zygotes. The term originates from the mythological chimera, which was a monstrous creature made up of the body of a lion, the head of a goat and the tail of a serpent (Bazopoulou-Kyrkanidou 2001).

### **1.3.2 Detection of fetal cells in the maternal circulation**

Fetal cells are found at very low frequency in the maternal circulation and their identification requires a sensitive methodology. One strategy commonly adopted to facilitate the identification of fetal cells is the utilisation of the Y chromosome as a surrogate to identify cells of male origin in a female host. It is then presumed that male cells are derived from previous male children and are fetal in origin (Bianchi, Zickwolf et al. 1996; Lo, Lau et al. 2000). Techniques used include fluorescent in-situ hybridisation (FISH) with chromosome-specific probes for markers such as the Y chromosome to microscopically identify fetal cells. Using this technique, fetal cells can be seen in samples of maternal blood



at a frequency of less than 20 fetal cells per ml of maternal blood (Krabchi, Gadji et al. 2006). However, this is limited by the difficulty of screening the large number of maternal cells necessary to detect rare fetal cells. Therefore several strategies have been used to enrich the fetal cells before FISH analysis. These include sorting cells by flow cytometry or magnetic beads and columns to enrich populations of fetal nucleated erythrocytes, trophoblast cells and fetal leukocytes (Bianchi, Zickwolf et al. 1996; Lim, Tan et al. 2001; Guetta, Gordon et al. 2003).

DNA amplification using the polymerase chain reaction (PCR) provides a sensitive technique to identify and quantify the presence of fetal cells in the maternal circulation during pregnancy. Quantitative PCR has also shown that the number of fetal cells in the maternal circulation is small during normal pregnancies, with a mean of one fetal nucleated cell per ml of maternal venous blood (Bianchi, Zickwolf et al. 1996). Subsequent studies identified that following elective first trimester termination of pregnancy, approximately 500,000 nucleated fetal cells might transfer into the maternal circulation (Bianchi, Farina et al. 2001). It is therefore clear that a pregnancy does not need to continue beyond the first trimester for there to be a significant transfer of fetal cells to the mother. It is presumed that this fetomaternal transfer would also occur in early miscarriage, but this has not yet been well studied. However, the particularly large fetomaternal transfusion that is seen after a surgical termination of pregnancy might be partly due to mechanical disruption of the fetomaternal barrier during the procedure.

The use of quantitative Taqman real-time PCR enables precise quantification of the transfer of fetal cells into the maternal circulation and minimises the risks of contamination because it is carried out in a closed system. A majority of studies now use this technology to

quantify the presence of Y chromosome DNA as a measure of male fetal cell exchange. The single-copy gene SRY (Lo, Lau et al. 2000; Zimmermann, El-Sheikhah et al. 2005; Zhong, Holzgreve et al. 2006) or the more sensitive multi-copy DYS14 gene (Lambert, Lo et al. 2002; Adams, Lambert et al. 2003; Zimmermann, El-Sheikhah et al. 2005; Zhong, Holzgreve et al. 2006) are both commonly detected as markers for the Y chromosome.

Free fetal DNA is present in the maternal plasma during pregnancy and this increases with advancing gestation and is easier to detect than nucleated fetal cells (Lo, Lau et al. 2000). When plasma is examined for free fetal DNA 292 to 443 genome equivalents are found per ml (Lo, Corbetta et al. 1997; Lo, Lau et al. 2000).

Longitudinal studies have been carried out during pregnancy that clearly show increases in the level of free fetal DNA throughout pregnancy, with a particularly marked increase over the last 8 weeks of pregnancy (Lo, Tein et al. 1998). However, following delivery, free fetal DNA is cleared very rapidly from maternal plasma, becoming undetectable after just one day. This rapid clearance implies that to maintain the high levels of fetal DNA found in maternal plasma during pregnancy, there must be a large liberation of free fetal DNA into the maternal circulation. In fact, it has been estimated that over 2000 copies of fetal DNA must be liberated into the maternal circulation every minute to maintain this steady state.

### **1.3.3 Persistence of fetal cells**

The persistence of fetal cells in the maternal circulation and tissues was first demonstrated after human pregnancy in 1996 when it was demonstrated using PCR for sequences on the Y chromosome that male haematopoietic stem cells, presumed to be of fetal origin, could be detected in healthy women up to 27 years after the birth of a male child (Bianchi, Williams et al. 1997). Subsequent research with fluorescent probes specific for the Y chromosome, has demonstrated persistent fetal cells in a wide range of maternal tissues. These include skin (Ohtsuka, Miyamoto et al. 2001), liver (Stevens, McDonnell et al. 2004), thyroid (Klitschar, Schwaiger et al. 2001; Srivatsa, Srivatsa et al. 2001; Klitschar, Immel et al. 2006), intestine (Johnson, Nelson et al. 2001), lymph node (Khosrotehrani, Johnson et al. 2004) and bone (O'Donoghue, Chan et al. 2004). It therefore appears that fetal cells are widely distributed within the body and become incorporated into a range of differentiated cell types.

Murine studies have demonstrated that fetal cells are present in all previously pregnant mice and that these cells are detectable in a range of tissues (Khosrotehrani, Johnson et al. 2005). In humans, fetal microchimerism has also been consistently shown to be common even after normal pregnancy (Adams, Lambert et al. 2003; Loubiere, Lambert et al. 2006) or after pregnancy loss (Khosrotehrani, Johnson et al. 2003), but the exact proportion of women in whom this occurs, or the factors that are responsible are difficult to determine. This might be due in part to the diverse range of methodologies utilised and inconsistency in factors such as the quantity of DNA examined or the nature of the target gene.

Some of the findings of these studies can appear difficult to explain. For example, 21% of women who did not report any previous male pregnancies were found to harbour

low levels of male, presumed fetal, cells in their circulation. When the pregnancy history of these women was examined, both the prevalence and level of male microchimerism were greater in women with a history of induced abortion (Yan, Lambert et al. 2005). However, some women in whom persistent male cells were found reported no previous known pregnancies. A possible explanation may be that such women have suffered a subclinical pregnancy loss. It has been shown from longitudinal studies that around 24% of pregnancies detectable by sensitive HCG assays result in early pregnancy losses before the pregnancy is clinically detected (Wang, Chen et al. 2003). However, microchimerism might also result from non-pregnancy-related sources such as blood transfusion or transplantation. The mother might also have been a twin herself, either from a recognised twin pregnancy or as a result of the 'vanishing twin' phenomenon might have been part of a twin pregnancy that was never recognised. Finally, it has also been suggested that microchimeric cells can originate from older siblings, having been transferred to the mother during pregnancy and then in a future pregnancy transferred from the mother to a subsequent child (Yan, Lambert et al. 2005).

The precise identity of the fetal cells which give rise to the persistent fetal cell population remains unclear but the term 'pregnancy-associated progenitor cells' (PAPCs) was proposed to describe the presumed fetal stem cell population transferred during pregnancy. PAPCs have the capacity for engraftment in the maternal host and lead to multilineage differentiation and generation of a persistent fetal cell population. It has been suggested that there could be the transfer during pregnancy of an embryonic-like stem cell that could then differentiate into circulating, lineage-specific stem cells. It is, however, also possible that the established plasticity of bone-marrow-derived stem cells could allow circulating haematopoietic stem cells to transdifferentiate into another lineage (Rossi 2004).

In the mouse, fetal stem cells with lymphoid progenitor capacity have been shown to transfer into the maternal circulation. They developed into functional T and B cells in the maternal host. T cells of fetal origin even underwent selection in the maternal thymus (Khosrotehrani, Leduc et al. 2008). In humans, fetal haematopoietic and mesenchymal stem cells have been identified in maternal blood during and after pregnancy (Bianchi, Williams et al. 1997; O'Donoghue, Choolani et al. 2003; O'Donoghue, Chan et al. 2004). It has also been possible to culture pure fetal mesenchymal stem cells from maternal blood after termination of pregnancy and differentiate these cells into bone and fat. In a striking piece of work, the bone marrow of women was examined many years after their last pregnancy and mesenchymal stem cells of male, presumed fetal, origin could be found in all the women examined who had previous sons (O'Donoghue, Chan et al. 2004).

#### **1.3.4 Fetal microchimerism and autoimmune disease**

The clinical similarities of autoimmune diseases such as systemic sclerosis with chronic graft-versus-host disease (Jaffee and Claman 1983; Chosidow, Bagot et al. 1992) led to the suggestion that an association may exist between fetal microchimerism and autoimmune disease. Other circumstantial factors that support this concept are the female predominance of many autoimmune diseases and the peak incidence in women of childbearing age. If this theory is true, then diseases that have been traditionally considered as autoimmune processes might in fact have an alloimmune component (Nelson 1996) with the maternal immune response to persistent fetal antigens implicated.

Studies have in particular reported that fetal microchimerism is more prevalent in the peripheral blood mononuclear cells, skin lesions and other organs of women with systemic sclerosis compared with controls (Artlett, Smith et al. 1998; Nelson, Furst et al. 1998; Ichikawa, Kotake et al. 2001; Johnson, Nelson et al. 2001; Lambert, Lo et al. 2002). Furthermore, the chimeric cells detected were more likely to be dendritic cells or B cells, which are cell types with an important role in disease pathogenesis (McNallan, Aponte et al. 2007). In women with systemic sclerosis, T cells of fetal origin have also been isolated and these appear to have a Th-2-oriented profile, which is again consistent with the nature of this disease (Scaletti, Vultaggio et al. 2002). However, even in healthy controls, it was clear that fetal microchimerism was present, and other studies have not definitively identified a correlation between systemic sclerosis and the presence or magnitude of fetal microchimerism (Murata, Nakauchi et al. 1999; Gannage, Amoura et al. 2002; Selva-O'Callaghan, Mijares-Boeckh-Behrens et al. 2003). An additional finding was of increased HLA class II compatibility between patients with scleroderma and their children compared with controls (Nelson, Furst et al. 1998). It has been suggested that HLA compatibility is associated with increased microchimerism by allowing fetal cells to cross the placenta undetected. An alternative hypothesis is that it might allow increased engraftment of fetal material by minimising the magnitude of the alloreactive immune response. A range of other autoimmune diseases has also been examined for their association with fetal microchimerism. I have reviewed this elsewhere (Lissauer, Piper et al. 2009), detailing the current evidence on the association between fetal microchimerism and primary biliary sclerosis (Rubbia-Brandt, Philippeaux et al. 1999; Tanaka, Lindor et al. 1999; Corpechot, Barbu et al. 2000; Invernizzi, De et al. 2000; Jones 2000; Schoniger-Hekele, Muller et al. 2002; Stevens, McDonnell et al. 2004), autoimmune thyroid disease (Klitsch, Schwaiger et

al. 2001; Srivatsa, Srivatsa et al. 2001; Ando, Imaizumi et al. 2002; Imaizumi, Pritsker et al. 2002; Ando and Davies 2003; Badenhop 2004; Prummel, Strieder et al. 2004; Prummel and Wiersinga 2004; Renne, Ramos et al. 2004; Walsh, Bremner et al. 2005; Klintschar, Immel et al. 2006), sjogrens syndrome (Mijares-Boeckh-Behrens, Selva-O'Callaghan et al. 2001; Toda, Kuwana et al. 2001; Aractingi, Sibilia et al. 2002; Endo, Negishi et al. 2002; Giacomelli, Matucci-Cerinic et al. 2002; Kuroki, Okayama et al. 2002) and systemic lupus erythematosus (Johnson, McAlindon et al. 2001; Abbud, Pavarino-Bertelli et al. 2002; Mosca, Curcio et al. 2003; Khosrotehrani, Mery et al. 2005; Stevens, Tsao et al. 2005; Kremer, Koopmans et al. 2006; Stevens 2006; Kremer, Koopmans et al. 2007; Kremer, Koopmans et al. 2007; Kremer, Koopmans et al. 2008). Evidence of an association remains the most robust for systemic sclerosis. However, many studies have been limited by inadequate controls and a paucity of clinical information for alternative causes of fetal microchimerism other than live children. These include early pregnancy loss, termination of pregnancy, blood transfusion and the mother having been a twin herself.

Whether the role of fetal cells is an integral component of the pathogenesis of the disease or whether the finding of fetal cells in affected tissues, or at increased frequency in blood, is a reflection of the recruitment or mobilisation of fetal cells as part of the inflammatory process, remains controversial.

### **1.3.5 Fetal microchimerism and recurrent miscarriage**

Recurrent miscarriage, which is defined as three or more consecutive miscarriages, is a common problem. There are many causes of recurrent miscarriage (reviewed in (Rai and

Regan 2006), but in many couples no cause is identified, even after detailed investigation. In a proportion of these, immune-mediated mechanisms might play a role. The association between certain HLA types and recurrent miscarriage seems particularly strong in those women who have an initial successful pregnancy but then go on to suffer recurrent miscarriages. This is termed secondary recurrent miscarriage, and it might be this group for whom immunological causes are most important (Kruse, Steffensen et al. 2004). It has been established that T cell immunity might develop during pregnancy against minor histocompatibility antigens from the Y chromosome (Piper, McLarnon et al. 2007). Therefore, it has been suggested that the birth of a boy in the first pregnancy predisposes to subsequent recurrent miscarriage because of immunisation against Y chromosome antigens. Indeed, epidemiological evidence from Dutch cohorts with secondary recurrent miscarriage have found that a male first child is associated with a reduced chance of having another successful pregnancy in women with secondary recurrent miscarriage (Christiansen, Pedersen et al. 2004; Nielsen, Andersen et al. 2008). The suggested mechanism for this is that the initial male pregnancy primes the maternal immune system and initiates a response to antigens including those encoded by the Y chromosome. In subsequent pregnancies, T-cell-mediated responses to these antigens are therefore suggested to cause immune recognition of the developing fetus, resulting in miscarriage. This hypothesis is further supported by a recent finding in women with secondary recurrent miscarriage. If these women have a class II HLA type known to be able to present epitopes from the Y chromosome, this is associated with a reduction in chances of a subsequent live birth, but this effect is not seen in women with these HLA types who had a daughter (Nielsen, Steffensen et al. 2009). This hypothesis has not yet been supported by in vitro evidence of alterations in the immune response to fetal antigens in recurrent miscarriage patients. This is



an important area for further research, because if such an effect was demonstrated, it would enable novel therapeutic strategies to be pursued.

### **1.3.6 Fetal microchimerism and cancer**

It has been suggested that fetal microchimerism or the immune response to persistent fetal cells could protect a mother against future malignancy. Conversely, persistent fetal cells recruited to sites of tumour development may in fact have a role in tumour pathogenesis. Maternal T cells exposed to fetal antigen during pregnancy can be primed during this exposure and have been shown to be functional and able to kill cells expressing these antigens (Piper, McLarnon et al. 2007). It is well recognised from experience with bone marrow transplantation that maternal T cells specific for tumour-related minor histocompatibility antigens play an important role in eliminating tumour cells in what is termed the 'graft-versus-tumour' effect. Some minor histocompatibility antigens are only expressed on haematopoietic or tumour cells and these are termed tumour-specific minor histocompatibility antigens. One such antigen, HA-1, has been shown to induce a maternal response following pregnancy (Verdijk, Kloosterman et al. 2004; van Halteren, Jankowska-Gan et al. 2009). Priming of maternal T cells against these tumour-related antigens could provide a mechanism by which pregnancy could enhance immune tumour surveillance and protect a mother from future cancer.

In this context, it is fascinating that there is epidemiological evidence that fetal microchimerism may indeed be associated with a protective effect against malignant disease. A previous history of pregnancy is, in the long term, a protective factor against the

future development of breast cancer and this effect is generally presumed to operate through hormonal mechanisms. However, Gadi and co-workers compared levels of fetal microchimerism in a cohort of women with breast cancer and a well-matched control cohort. They demonstrated that the cohort with breast cancer had a significant reduction in both the presence and quantity of fetal microchimeric cells (Gadi, Malone et al. 2008). It is possible that this potentially protective mechanism could either be due to the fetal cells themselves being immunologically active and eliminating tumour cells, or because, as suggested above, an increased level of fetal microchimerism is associated with an increased maternal cellular immune response against fetal and tumour antigens.

The presence of persistent fetal cells has also been investigated in a range of tumours that have arisen during pregnancy or in tumours from women who had previous pregnancies (Cha, Khosrotehrani et al. 2003; Nguyen Huu, Oster et al. 2007; Cirello, Recalcati et al. 2008; O'Donoghue, Sultan et al. 2008; Dubernard, Oster et al. 2009; Nguyen Huu, Oster et al. 2009). Dubernard demonstrated the frequent presence of fetal-derived cells in tumour stroma of breast carcinoma associated with pregnancy. They suggest that the unusual properties of fetal cells, such as the presence of HLA-G, which assists in immune evasion, could influence breast tumour pathogenesis (Dubernard, Aractingi et al. 2008). Further work with a murine model of pregnancy-associated breast tumours demonstrated that high-grade tumours contained significantly more fetal cells (Dubernard, Oster et al. 2009).

## **1.4 Immunology of recurrent miscarriage**

### **1.4.1 Recurrent miscarriage**

Miscarriage is the loss a fetus before viability, normally defined as 24 weeks gestation (Rai and Regan 2006). Miscarriage is the commonest complication of pregnancy, effecting 15% of all clinically recognized pregnancies and up to 50% of all conceptions (Wilcox, Weinberg et al. 1988).

Recurrent miscarriage (RM) is commonly defined as 3 or more consecutive pregnancy losses. With this definition recurrent miscarriage occurs in 1% of couples (Rai and Regan 2006). It can be further classified as primary or secondary in origin. Primary recurrent miscarriage occurs in those who have lost all previous pregnancies and have no live births. Secondary recurrent miscarriage is when couples have had at least one successful pregnancy (Pandey, Rani et al. 2005).

Recurrent miscarriage seems to be a distinct clinical entity, when compared with sporadic miscarriage. Evidence in support of this is that the incidence of recurrent miscarriage is higher than would be expected by chance alone (0.34%) (Regan 1991) and tends to occur even in a fetus with a normal karyotype (Sullivan, Silver et al. 2004) and a woman's risk of miscarriage is directly related to the outcome of previous pregnancies (Risch, Weiss et al. 1988; Regan, Braude et al. 1989). For example, the risk of subsequent miscarriage is 24% after 2 miscarriages, increasing to 30% after 3 losses and 40% after four miscarriages (Regan, Braude et al. 1989).

Recurrent miscarriage has been attributed to a range of causes. These known causes include chromosomal (Rubio, Simon et al. 2003), genetic (Takakuwa, Adachi et al. 2003), structural (Salim, Regan et al. 2003), infective (Matovina, Husnjak et al. 2004), endocrine (Craig, Ke et al. 2002; Prummel and Wiersinga 2004), antiphospholipid syndrome (Rai, Regan et al. 1995) and thrombophilic disorders (Preston, Rosendaal et al. 1996). These causes, their investigation and management have been recently reviewed (Pandey, Rani et al. 2005; Rai and Regan 2006) and are the subject of national clinical guidelines (Regan, Backos et al. 2011).

However, in more than 50% of cases, despite investigation, the cause remains unknown. The majority of couples are therefore labelled as having unexplained recurrent miscarriages (Rai and Regan 2006).

#### **1.4.2 Immunology of recurrent miscarriage**

The natural extension of the many mechanisms proposed to regulate maternal tolerance of the fetus is that failure of these processes leads to miscarriage.

The lack of an identifiable cause of recurrent miscarriage in a majority of women (Rai and Regan 2006) means there has been a great deal of research to identify underlying immunological causes. Immunomodulatory therapies are in use (Table 1.4.2.2), despite poor evidence for their efficacy and guidelines suggesting they should not be used outside of clinical trials (Regan, Backos et al. 2011).

Many of the mechanisms proposed to regulate maternal tolerance of the fetus have been reported to be dysfunctional in women with recurrent miscarriage. Which of these findings are causative mechanisms and which are the result of multiple miscarriages is difficult to determine from the available evidence. Many of these studies are also limited by small cohorts, highly variable methodologies and difficulties with the matching of cases and controls.

In particular it has been reported that those women most likely to have an immunological cause for their recurrent miscarriages are those who have “secondary” recurrent miscarriage. These are women who have had a liveborn pregnancy and then have subsequent miscarriages. It is suggested that this may be because the initial pregnancy is a sensitising event that triggers an immunological response which is detrimental to future pregnancies. It is in this cohort that the HLA-DR3 allele has been found to have a much higher association (Kruse, Steffensen et al. 2004). It has also been suggested in some immunotherapy studies that the therapeutic effect of intravenous immunoglobulin is restricted to this group of women (Christiansen, Pedersen et al. 2002).

Table 1.4.2.1 Immunological abnormalities identified in recurrent miscarriage

Mechanism	Alteration in recurrent miscarriage	Reference
Th1/Th2 shift	Women with recurrent miscarriage have been characterized as having a Th1 shifted T cell response to placental antigens and mitogen stimulated peripheral blood lymphocytes.	(Hill, Polgar et al. 1995; Piccinni, Beloni et al. 1998; Raghupathy, Makhseed et al. 1999; Raghupathy, Makhseed et al. 2000; Makhseed, Raghupathy et al. 2001)
NK cells	Recurrent miscarriage has been associated with higher rates of uterine NK cells. Furthermore, a correlation is suggested between high uterine NK cells and poor outcome. However, peripheral blood NK cell counts do not appear to correlate with uterine NK cell numbers, and evidence of the correlation between peripheral blood NK cells and outcome has been inconsistent.	(Aoki, Kajiura et al. 1995; Kwak, Beaman et al. 1995; Lachapelle, Miron et al. 1996; Clifford, Flanagan et al. 1999; Quenby, Bates et al. 1999; Emmer, Nelen et al. 2000; Ntrivalas, Kwak-Kim et al. 2001; Wang, Li et al. 2008; Tang, Alfirevic et al. 2011)
Regulatory T cells	Regulatory T Cells have been suggested to be reduced in frequency in recurrent miscarriage in peripheral blood and in the decidua.	(Sasaki, Sakai et al. 2004; Yang, Qiu et al. 2008; Jin, Chen et al. 2009)
Th17 Cells	A single paper was recently published reporting increased Th17 cells in the peripheral blood and decidua of women with recurrent miscarriage.	(Wang, Hao et al. 2010)

Table 1.4.2.2 Immunomodulatory therapies for recurrent miscarriage

Therapy	Rationale	Evidence of efficacy	References
Progesterone	Progesterone has been suggested to induce a Th1 to Th2 shift and alter NK cell mediated cytotoxicity (See section 1.2.6)	Meta-analysis of trials showed no effect of progesterone on miscarriage rate for women with sporadic miscarriage, but subgroup analysis showed there may be benefit in women with recurrent miscarriage. Multicenter PROMISE trial ongoing.	(Haas and Ramsey 2008; Coomarasamy, Truchanowicz et al. 2011)
Intravenous immunoglobulin therapy	IV immunoglobulin is established therapy for autoimmune disorders, with diverse immunological effects. It may act by inhibiting activated NK cells, suppression of T cell and B cell activity, promoting a Th1 to Th2 shift or depletion of harmful antibodies. Which, if any, of its actions have a role in promoting tolerance to the fetus is unknown.	Whilst controversial most systematic reviews of the evidence do not suggest its use is beneficial in recurrent miscarriage, some have suggested efficacy in secondary recurrent miscarriage. It is not recommended in national guidelines.	<i>Mechanisms of action:</i> (Kazatchkine and Kaveri 2001; Omwandho, Gruessner et al. 2004; Bayry, Negi et al. 2011) <i>Efficacy:</i> (Hutton, Sharma et al. 2007; Ata, Tan et al. 2011)
Anti TNF $\alpha$ therapy	TNF $\alpha$ , an important Th1 cytokine is hypothesised to cause miscarriage and induce other Th1 cytokines. Monoclonal antibody therapy capable of blocking TNF $\alpha$ is efficacious in the context of autoimmune disease such as rheumatoid arthritis.	Use of anti TNF $\alpha$ therapies is associated with increased risk of infection. Safety in pregnancy is not well established. Insufficient evidence of safety or efficacy means use is not recommended in the UK outside of trials.	(Winger and Reed 2008; Clark 2010)
Lymphocyte immunotherapy	Hypothesis is that recurrent pregnancy is a alloimmune response to paternal antigens. Therapeutic attempts to induce tolerance to paternal antigens include; paternal cell immunisation, third party leukocytes and trophoblast membrane immunisation.	A Cochrane review of 16 trials (12 of paternal cell immunisation, 641 patients) did not show any beneficial effect of therapy over placebo.	(Porter, LaCoursiere et al. 2006)
Glucocorticoids	Glucocorticoids have been shown to reduce NK cell activity. Glucocorticoid use is thus based on the hypothesis that NK cell activation is harmful in pregnancy.	Randomised controlled trial underway in women with raised uterine NK cell levels to evaluate efficacy. However, prognostic importance of raised NK cells not established.	(Tang, Alfirevic et al. 2009; Tang, Alfirevic et al. 2011)

### **1.4.3 Immunity to HY antigens in recurrent miscarriage**

An initial report from single large centre observed that a firstborn boy rather than a girl was more frequent in women with secondary recurrent miscarriage. A firstborn boy was also associated with a decreased risk of subsequent live birth in the 14 year follow-up period (Christiansen O. Hum Repro 2004; 19:2946).

Subsequently, a further cohort from the same centre again demonstrated that the male sex of the firstborn child was poor prognostic factor in women with secondary recurrent miscarriage. Logistic regression showed that a firstborn boy was comparable in its prognostic significance to the number of previous miscarriages (Nielsen Fert Steril, 2008;89:907). This potential detrimental immune response was confirmed by a large epidemiological study using the Danish Birth Registry, which found that the risk of stillbirth was increased by 12% after delivery of boys compared with girls (Nielsen, Mortensen et al. 2010).

It was hypothesised that this is due to a dysfunctional maternal immune response to male antigens. In particular male minor histocompatibility antigens were proposed due to their known importance in transplantation (section 1.5.3) and the recognition that these responses are induced by pregnancy (section 1.5.1).

HY cellular immune responses have not previously been directly studied in women with recurrent miscarriage. However, it has been noted that women the an HLA class II type known to be able to present recognised class II minor histocompatibility antigens a firstborn boy is a poor prognostic factor, whereas for these women a firstborn girl was not. There was



a dose effect with two HY presenting class II types having a poorer prognosis than a single HY presenting class II type. This further implicates that an HY mediated immune response may have a role in these women. Due to the prevalence of MHC class I types known to present HY antigens to CD8 T cells the same type of analysis would not be informative for CD8 T cells (Nielsen Hum Mol Gen 2009; 18:1684).

## **1.5 Maternal cytotoxic T cell response to fetal antigen**

### **1.5.1 CD8 T cell responses to fetal antigen following pregnancy**

We have previously described the intimate cellular contact between mother and fetus. This is modulated at the decidual interface by mechanisms which would reduce cellular recognition of fetal antigens at this site such as attenuated HLA class I expression on trophoblast. However, it is now also recognized that fetal cells and non-cellular material are transferred into the maternal circulation prenatally (Lo, Lo et al. 1996; Bianchi, Williams et al. 1997) and that some fetal cells can then persist lifelong in the maternal circulation (Gammill, Guthrie et al. 2010) and tissues (O'Donoghue, Chan et al. 2004). This phenomenon is "fetal microchimerism". This transplacental 'cellular traffic' means that maternal-fetal immunological interactions can also occur at a peripheral interface (Taglauer, Adams Waldorf et al. 2010). In murine models it has been described how fetal antigens can accumulate in peripheral and central lymph nodes draining the uterus (Erlebacher, Vencato et al. 2007) and be cross presented by maternal antigen presenting cells (Seavey and

Mosmann 2006). These antigens can then be cross-presented by maternal or even potentially by fetal microchimeric antigen presenting cells, such as monocytes and macrophages (Seavey and Mosmann 2006; Erlebacher, Vencato et al. 2007; Moldenhauer, Diener et al. 2009; Moldenhauer, Hayball et al. 2010). Alternatively, dendritic cells which have scavenged fetal antigen may traffic to lymph nodes (Kim J Exp Med 2010; antigen persistence and the control of local T cell memory by migrant respiratory dendritic cells after acute viral infection). It is therefore possible that fetal antigens could be presented to the maternal immune system in the periphery, away from the potential immunological constraints of the placental interface.

Immune recognition of fetal tissue by maternal T cells may be direct, by engagement with fetal antigen-presenting cells, or indirect, through interaction with fetal-derived peptides presented on the surface of maternal cells (Lissauer, Piper et al. 2009). It has also been suggested that fetal T cells that have crossed into the maternal circulation, even if present in only relatively small numbers, may be directly involved in recognition of maternal antigen. Though any effects by fetal T cells directly would be modulated by the small numbers of such cells involved (Summarised in Fig. 1.5.1). Murine studies have supported that indirect pathways of fetal antigen presentation may be the most important (Erlebacher, 2007).

Study of the maternal responses to antigen derived from the Y chromosome offers a convenient surrogate for maternal responses against the fetus. It has been demonstrated clearly in humans that CD8 maternal T cells specific for fetally derived antigens can be isolated from peripheral blood of women post partum (James, Chai et al. 2003; Verdijk, Kloosterman et al. 2004; Piper, McLarnon et al. 2007). In particular, HLA-peptide tetramers

containing immunogenic epitopes derived from the *SMCY* gene on the Y chromosome have been used to isolate maternal CD8 T cells which are specific for fetal antigens. Responses were detected in 37% of women with a history of previous pregnancy and women who had had two or more sons demonstrated an HY-specific response in 50% of cases. T cells specific for fetal antigen are rare and their detection is facilitated by short term T cell culture to allow cell expansion in vitro. However, they have also been detected directly “ex-vivo” from maternal blood either using magnetic selection (Piper, McLarnon et al. 2007) or cell sorting techniques (Verdijk, Kloosterman et al. 2004). These HY-specific CD8 T cells are functional and produced IFN- $\gamma$  following stimulation with either peptide or whole male cells in vitro. HY-specific T cell clones are able to kill male cells expressing the appropriate HLA allele, whereas female cells were not recognised (Piper, McLarnon et al. 2007).

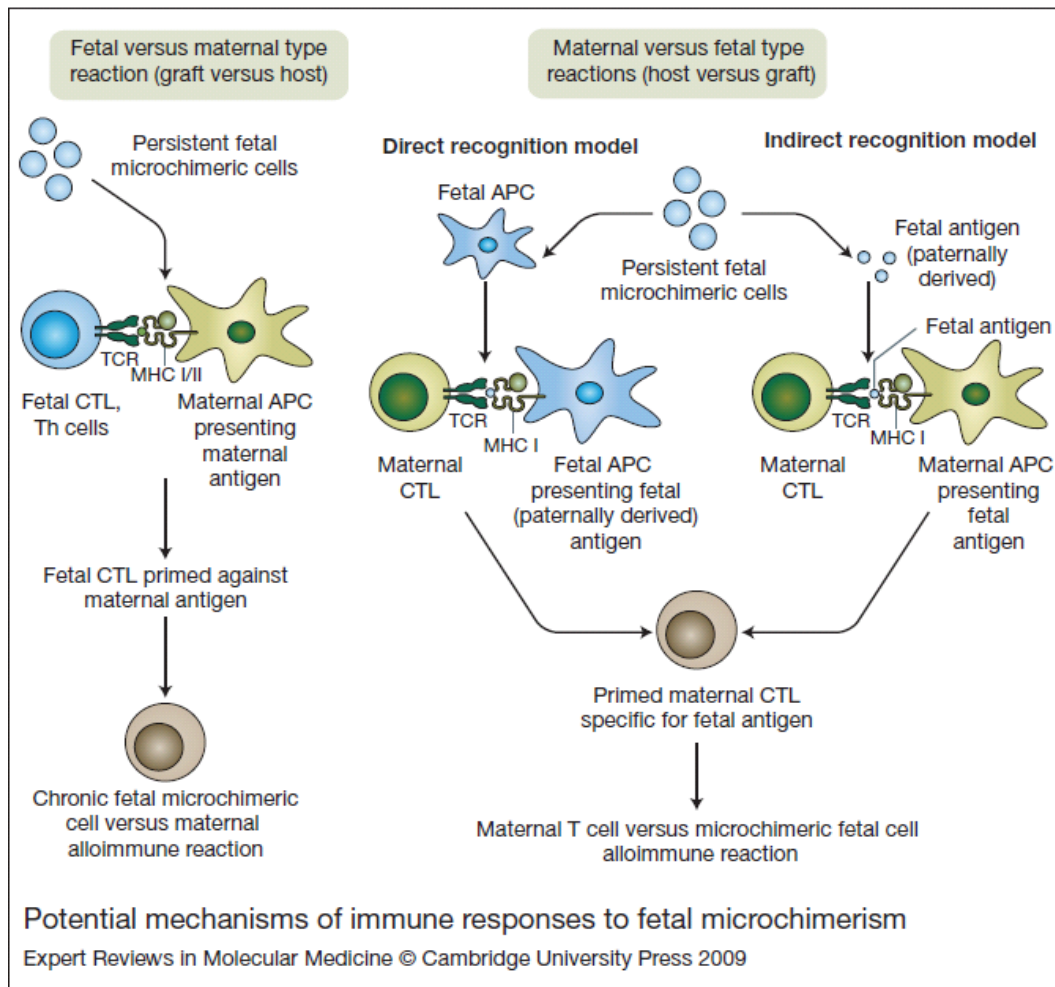


Figure 1.5.1 Potential mechanisms of maternal cellular immune response to fetal antigen.

Persistent fetal cells include immunologically active cells such as cytotoxic T lymphocytes and T-helper cells. These may respond directly towards maternal cells due to variation in major or minor histocompatibility antigens. This can be considered a fetal v maternal reaction, analogous to graft v host disease. An alternative mechanism is that the maternal lymphocytes will be primed against fetal antigens. These can be presented directly by persistent fetal antigen presenting cells. Alternatively, the fetal antigen may be presented by an indirect pathway, with fetal minor histocompatibility antigens processed by maternal antigen presenting cells. (CTL=cytotoxic T lymphocyte, Th= T-helper cell, APC=Antigen presenting cell, TCR=T cell receptor, MHC=Major histocompatibility complex) Figure reproduced from (Lissauer, Piper et al. 2009) with permission.

### **1.5.2 CD8 T cell responses to fetal antigen during pregnancy**

Whilst the maternal priming against fetal minor histocompatibility responses after pregnancy is now recognised (section 1.5.1) no studies have examined the development of a maternal CD8 T cell response to fetal minor-histocompatibility antigens during human pregnancy. There have though been several murine models investigating maternal cellular responses against fetal antigens, including responses against minor antigens. The findings from these have been variable and sometimes contradictory, but overall suggest that there is initial proliferation of fetal specific T cells, demonstrating maternal “awareness” of fetal antigen (Tafari, Alferink et al. 1995) but that these cells are hypo-responsive (Tafari, Alferink et al. 1995; Jiang and Vacchio 1998; Vacchio and Hodes 2003) with TCR and co-receptor down regulation (Tafari, Alferink et al. 1995; Zhou and Mellor 1998; Mellor, Zhou et al. 1999). Several murine models demonstrate deletion of the fetal specific T cells, though this is incomplete (Tafari, Alferink et al. 1995; Jiang and Vacchio 1998; Vacchio and Hodes 2003), and this deletion may be FAS/FASL dependent (Vacchio and Hodes 2005). However, other models have not reported fetal specific T cell deletion (Zhou and Mellor 1998; Norton, Fortner et al. 2010) or loss of function (Norton, Fortner et al. 2010). Due to their particular relevance to this thesis the existing murine studies of maternal cellular responses against fetal minor-histocompatibility antigens have been summarised (Table 1.5.2).

Whilst these murine models have the benefit of enabling fetal specific cells to be easily tracked and measured in a range of tissues, and have been important in furthering our understanding of the potential mechanisms they also have serious limitations due to the anatomical and immunological differences between murine and human pregnancy

(Chatterjeehasrouni and Lala 1979). For example murine trophoblast expresses MHC class I in the latter half of gestation (Jaffe, Jeannotte et al. 1990; Mattsson, Mattsson et al. 1992; Jiang and Vacchio 1998; Erlebacher, Vencato et al. 2007). In transgenic murine models, the supra-physiological numbers of fetal specific T cells are also likely to alter the nature of the immune response observed (Badovinac, Haring et al. 2007). Interestingly, in James et al. where tetramers were used to observe HY specific T cells in a more physiological scenario, after pregnancy in multiparous mice, an accumulation of HY specific T cells, with a memory phenotype was observed (James, Chai et al. 2003). Similarly, the antigens used in these models are varied in expression levels (Moldenhauer, Hayball et al. 2010), antigen processing (Ovalbumin is cross presented particularly effectively) (Erlebacher, Vencato et al. 2007) and trophoblast expression (Moldenhauer, Hayball et al. 2010), which may account for further variability.

Table 1.5.2 Murine models of maternal cellular responses against fetal minor histocompatibility antigens during pregnancy

Antigen	Control of antigen expression	Maternal T cells subset studied	Lymphocyte receptor transgene	Summary of findings	Ref.
HY	Endogenous	CD8	Anti HY TCR transgene	Antigen specific T cell expansion and then clonal deletion, deletion persisted post-partum, deletion appeared Fas/Fas-L dependent Remaining HY specific T cells hyporesponsive, decreased proliferation and cytotoxicity, even in the presence of IL-2. Hyporesponsiveness is not TCR or co-receptor downregulation dependent. No effect of HY specific T cells on litter size	(Jiang and Vacchio 1998)
HY	Endogenous	CD8	Anti HY TCR transgene	Maternal CD28/B7 interactions are needed for clonal deletion and induced hyporesponsiveness The hyporesponsive cells demonstrate reduced proliferation and cytotoxicity but no change in IFN $\gamma$ production.	(Vacchio and Hodes 2003)
HY	Endogenous	CD8	Anti HY TCR transgene	Placental expression of FasL, interacting with maternal Fas expression is necessary and sufficient for clonal deletion and also for hyporesponsiveness of fetal specific T cells Maternal expression of FasL is not crucial	(Vacchio and Hodes 2005)
HY	Endogenous	CD8	Anti HY TCR transgene	HY antigen specific T cells are functional during pregnancy and retain proliferative and cytotoxic capacity, HY specific T cells present at maternal-fetal interface Fetal expression of TCR specific alloantigen does not lead to T cell deletion No effect of HY specific T cells on litter size	(Norton, Fortner et al. 2010)
Ovalbumin (Ova)	Transgenic (cell transfer model)	CD8 CD4	Ova specific TCR transgene	Ova specific cells proliferated in secondary lymphoid organs in response to exposure to fetal antigen Presentation was indirect, by maternal APCs Fetal specific T cells undergo clonal deletion and defective priming Presence of fetal specific T cells did not induce fetal loss.	(Erlebacher, Vencato et al. 2007)

### **1.5.3 Clinical significance of maternal T cell response to fetal antigen in transplantation**

Maternal immunological priming during pregnancy has important implications for both the clinical practice of transplantation and for our understanding of transplantation biology. It is recognised that female donors are associated with a higher risk of graft-versus-host disease in the setting of stem cell transplantation and that this risk is particularly high when the donor has a history of pregnancy (Flowers, Pepe et al. 1990; Kollman, Howe et al. 2001). Graft-versus-host disease that occurs in the setting of HLA-matched bone marrow transplantation results from the activity of donor T cells that recognise minor histocompatibility antigens presented by the host tissues, thereby causing an immune response to these tissues (Mutis, Gillespie et al. 1999). In both murine models and human pregnancy, it has been shown that pregnancy can prime the maternal T cell immune response to minor histocompatibility antigens from paternal genes (James, Chai et al. 2003; Verdijk, Kloosterman et al. 2004; Piper, McLarnon et al. 2007; van Halteren, Jankowska-Gan et al. 2009). This observation thus provides a biological explanation for the effect of parity on increasing the incidence of graft-versus-host disease. More direct evidence has been provided by the correlation between HY specific T cells, identified by tetramer staining, and the severity of graft versus host disease (Mutis, Gillespie et al. 1999).

A crucial corollary of this effect is related to the observation that in bone marrow transplantation for malignant disease, donor T cell responses are also directed against the tumour itself. This is termed the 'graft-versus-tumour' effect, and is clearly demonstrated by the effectiveness of donor lymphocyte infusion. In this technique, patients who have relapsed after allografting are given an infusion of lymphocytes from the original donor to



induce a potent immune-mediated 'graft-versus-tumour' effect (Dazzi and Goldman 1999). This understanding of graft-versus-leukaemia explains why multiparous female donors are associated not only with an increased risk of graft-versus-host disease, but also with a reduced risk of leukaemia relapse (Gratwohl, Hermans et al. 2001; Randolph, Gooley et al. 2004; Gahrton, Iacobelli et al. 2005). It is therefore possible that a greater understanding of the priming of the maternal immune system during pregnancy will assist in optimal selection of donors for transplantation, with the aim of optimising graft-versus-tumour responses while minimising the graft-versus-host effect. For example, while HLA is ubiquitously expressed there are other fetal antigens which are only expressed on particular tissue types (such as HA-1 on haemopoietic cell)(Cai, Lee et al. 2004), or so called "onco-fetal proteins" which are embryonically expressed proteins only found in fetal development or cancer (Goodyear, Piper et al. 2005). It may be that responses to these antigens (a pregnancy induced response to HA-1 has been demonstrated (van Halteren, Jankowska-Gan et al. 2009)) may, due to their tissue or tumour specificity, have even greater importance in providing a focussed graft versus tumour response.

The potential for these effects to be translated to solid organ transplantation is also now being actively investigated. In renal transplantation, it has been suggested there may be responses by the female host against minor histocompatibility antigens if male kidneys are transplanted into females. These could potentially worsen graft survival through chronic rejection (Gratwohl, Dohler et al. 2008).

## 1.6 Cytomegalovirus (CMV)

### 1.6.1 Cytomegalovirus

Human cytomegalovirus (CMV) or HHV-5, is a  $\beta$  herpesvirus. It is the largest of the known herpes viruses, with a 230 kb, double stranded, linear genome (Fields, Knipe et al. 2007). Initial infection of an immunocompetent individual is usually asymptomatic, or results in a self limiting mononucleosis like illness. However, severe disease can occur if the host is immunocompromised such as following stem cell or solid organ transplantation, in patients with HIV infection or in the context of congenital infection (Gandhi and Khanna 2004).

An important property of CMV, in common with other herpesviruses, is its ability to establish lifelong persistence after initial infection. CMV can remain latent throughout the lifetime of the host, with sporadic reactivations being controlled by a cell-mediated immune response. Unlike other herpesviruses where the molecular biology of this latency is well established in CMV infection many questions remain unanswered. CMV is difficult to study in this context due to the extremely low frequency of virally infected cells found in healthy latently infected individuals (Taylor-Wiedeman, Sissons et al. 1991; Larsson, Soderberg-Naucler et al. 1998). Therefore, the cell types responsible for harbouring persistent infection in humans, or if there is a specific latency associated gene expression profile such as found in Epstein Barr Virus or if this is actually lytic infection but with only very low levels of virus production remains unclear (reviewed in (Sinclair and Sissons 2006)).

In addition to secondary infection occurring due to the reactivation of persistent infection an unusual characteristic of cytomegalovirus is that secondary reinfection, termed

“superinfection” can also occur (Boppana, Rivera et al. 2001) when one individual is reinfected with multiple genotypes (Meyer-König, Ebert et al. 1998) despite high titre neutralizing antibody responses and high frequency CMV specific T cell responses. This may be related to the evasion of CD8 T cell responses by MHC class I downregulation (Hansen, Powers et al. 2010) and is a major challenge for the development of vaccination strategies.

The prevalence of infection varies widely between populations. Prevalence increases with age and is higher in lower socio-economic groups. In women of reproductive age seropositivity is over 90% in developing countries but less than 50% in many western countries (Kenneson and Cannon 2007; Kaye, Miles et al. 2008).

#### **1.6.2 Congenital cytomegalovirus infection**

CMV infection is an important cause of infectious morbidity in the neonate (Fowler, Stagno et al. 2003) and vaccination against CMV is considered an urgent priority (Stratton K 2000) and while progress is being made (Pass, Zhang et al. 2009) no effective vaccine yet exists.

In a sero-negative mother the risk of primary infection during pregnancy is 0.1-4.1% and these women carry a 40% risk of passing on the infection to the fetus (Fowler, Stagno et al. 1992; Kenneson and Cannon 2007). Unfortunately, around 10% of infected infants will have symptoms at birth (Adler, Nigro et al. 2007). Problems include very severe infection or cytomegalic inclusion disease (CID) which causes significant mortality and long term handicap in many survivors. In other infants infection may be initially asymptomatic but can

later lead to the development of neurological impairments such as hearing loss and visual loss (Malm and Engman 2007).

In women with infection prior to pregnancy transmission of CMV to the fetus can still occur, but it only occurs in 0.5-2% percent of these women (Fowler, Stagno et al. 2003). Though this percentage is low, because of the large proportion of women who have previous CMV infection the overall occurrence of transmission through this route is significant and is thought to account for around half of all infected fetuses (Ornoy and Diav-Citrin 2006). In these fetuses the presence of prior immunity seems to reduce the risk of severe infection, but sequelae such as hearing loss are still seen in 5% of those fetuses infected (see table 1.6.2). Cases have also demonstrated that severe disease does occur in some fetuses despite the mother having serological evidence of previous immunity.

*Table 1.6.2 Sequelae of Cytomegalovirus primary and secondary infection*

<b>Sequelae</b>	<b>Primary Infection *</b>	<b>Recurrent Infection *</b>
Sensorineural hearing loss	15 (18/120)	5 (3/56)
Bilateral hearing loss	8 (10/120)	0 (0/56)
Speech threshold >60dB	8 (9/120)	0 (0/56)
IQ <70	13 (9/68)	0 (0/32)
Chorioretinitis	6 (7/112)	2 (1/54)
Other neurological sequelae	6 (8/125)	2 (1/64)
Death	2 (3/125)	0 (0/64)
<b>Any sequelae</b>	<b>25 (31/125)</b>	<b>8 (5/64)</b>

\* Percentage (number of sequelae/total number evaluated).

From (Fowler, Stagno et al. 1992).

A number of antiviral drugs are active against CMV and are currently used in the treatment of CMV disease in immunocompromised individuals (Biron 2006). Early experience with maternal oral valaciclovir to treat intrauterine CMV infection has shown that therapeutic concentrations of the drug can be achieved in the maternal and fetal compartments, with a decrease in viral load of fetal blood (Jacquemard, Yamamoto et al. 2007). This is currently the subject of a randomized controlled trial in symptomatic intrauterine cytomegalovirus infection.

### **1.6.3 T cell responses to CMV**

Cytomegalovirus elicits a potent response from all arms of the host immune system. Humoral immunity is rapidly established, hence IgG sero-positivity remains the standard test of prior infection (Adler, Nigro et al. 2007). However, once infection has occurred the cellular immune response appears to be most important, with both CD4, CD8 T cells and NK cells playing a role (Moss and Khan 2004). In particular CD8+ T lymphocytes appear to be critical. A lack of CD8+ T lymphocytes following transplantation has been correlated with CMV disease (Li, Greenberg et al. 1994). Moreover, the adoptive transfer of CMV specific CD8+T cell clones has led to the restoration of viral-specific immunity in immunocompromised individuals (Walter, Greenberg et al. 1995).

CMV appears to be a very immunodominant antigen and CMV specific T cells can occupy a significant proportion of the T cell repertoire in sero-positive individuals (Khan, Hislop et al. 2004; Sylwester, Mitchell et al. 2005). These responses are not only sustained but appear to increase over time, coming to dominate up to 50% of the repertoire of elderly

donors and are often clonally restricted (Khan, Shariff et al. 2002). This expansion is termed “memory inflation” (Karrer, Sierro et al. 2003). This dominance of the repertoire by CMV specific T cells has been suggested to be detrimental to the host with the finding in CMV sero-positive pregnant women of a relative increase in late differentiated CD8<sup>+</sup> T cells with a loss of CD27 and CD28 expression being a pattern that is considered a marker of immune senescence and part of the ‘immune risk phenotype’. Indeed, CMV sero-positivity and elevated antibody titre appear, at least in the elderly, to be associated with immunosenescence and excess mortality (Roberts, Haan et al. ; Derhovanessian, Larbi et al. 2009; Pawelec, Derhovanessian et al. 2009; Strandberg, Pitkala et al. 2009).

The phenotype of CMV specific T cells has been the subject of much study. CMV specific T cells are characteristically of a late differentiated phenotype. This is characterised by low expression of CCR7, low expression of the co-stimulatory molecules CD27 and CD28, a large number of highly differentiated effector memory which are CD45RA revertant, high CD57 expression, and high perforin expression (Fig. 1.1.6)(reviewed in (Moss and Khan 2004)).

#### **1.6.4 T cell response to CMV during pregnancy**

Due to the clinical importance of primary CMV infection during pregnancy considerable work has characterised the maternal cellular immune response to primary CMV infection during pregnancy. The study of CMV infection is a good illustration of how pregnancy does to not prevent the initiation of a strong cellular immune response to new infections (Constantin, Masopust et al. 2007). Furthermore, in primary CMV infection it has

been shown that the nature of the maternal T cell response is important in influencing the potential for viral transmission to the fetus (Lilleri, Fornara et al. 2007). A number of characteristics of the immune response have been proposed to be important in determining transmission although many have been identified in relatively small studies and there is little consensus on the critical components of the immune response that play the predominant role. The percentage of CMV-specific CD8 T cells that re-express the marker CD45RA is reduced in mothers who transmit infection to the fetus and this may reflect higher levels of viraemia in the mother (Lilleri, Fornara et al. 2008). Interestingly, differences have been observed in the maturation of the CMV-specific CD4+ and CD8+ T cell response after maternal infection compared to that seen following solid organ transplantation. Both the rate of expansion, and the simultaneous development of CD4 and CD8 CMV-specific T cells, have been shown to be enhanced during pregnancy (Lilleri, Zelini et al. 2009).

How the changing immunological milieu of pregnancy alters an existing T cell response to CMV is not well characterised. Viral reactivation can occur in pregnancy and as discussed previously this may have important clinical consequences. It is therefore important to better understand the dynamics of the T cell immune response during pregnancy in those with prior immunity to CMV. Currently it has been reported that in women with recurrent infection transmission in utero and the early neonatal period is associated with viral excretion into breast milk, and the genital tract. Higher rates of viral excretion are associated with higher CMV IgG titres and a higher frequency of CMV specific CD4 T cells. Interestingly, in the mother markers of T cell activation (Ki67 and CD38) were more frequent in those that transmitted CMV to their fetus or neonate (Kaye, Miles et al. 2008).

Fetal CMV specific T cell responses have also been detected in infected fetuses. Some studies have suggested that fetal CMV specific T cell responses to antigens from the protein pp65 are less frequent than in adults, also the ability of fetal CD8 T cells to produce IFN  $\gamma$  in response to activation via TCR cross-linking appears reduced and this may have an important impact on the ability of a fetus to mount an effective response to cytomegalovirus (Pedron, Guerin et al. 2007). However, overall human fetuses seem remarkably able to mount a CD8 T cell response to CMV, that appears phenotypically similar in nature to an adult response, with the phenotype of CMV specific T cells maturing during the course of infection in a similar fashion to that found in adults, with memory T cells produced with the loss of the co-stimulatory molecules CD28 and CD27 and a reversion to a C45RA positive state (Hermann, Truyens et al. 2002; Marchant, Appay et al. 2003). The magnitude of the response has even been suggested to be sufficient to significantly skew the overall T cell phenotype of infected fetuses compared to healthy controls (Marchant, Appay et al. 2003; Elbou Ould, Luton et al. 2004).

## **1.7 Detection of antigen specific T cell responses**

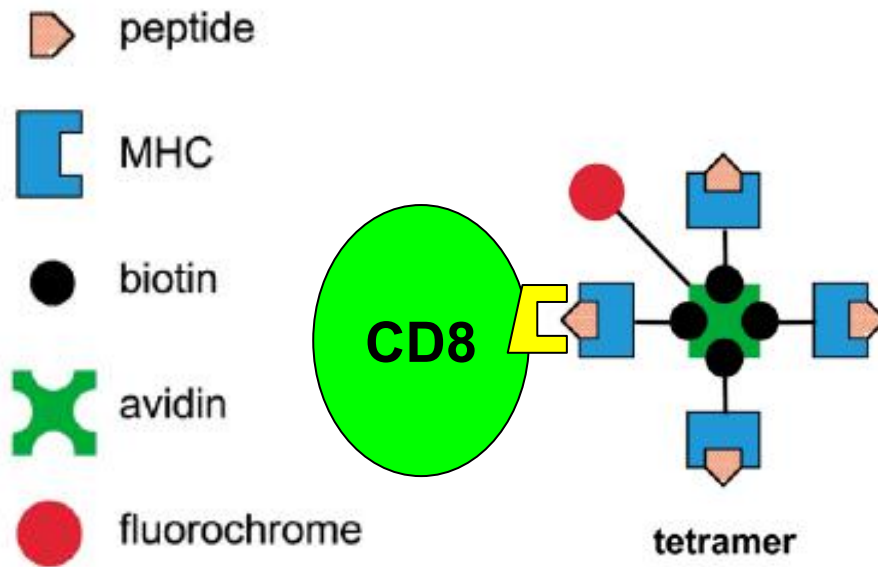
The weak interaction of the TCR with the peptide MHC complex is inevitable as the TCR has been selected in the thymus for its ability to only bind a self peptide/MHC complex weakly, to avoid self reactive T cells. The TCR/peptide-MHC interaction lasts for only seconds at physiological temperatures, posing a challenge for the identification of antigen specific T cells.



This problem was solved in 1996, with the development and use of MHC-peptide multimers to identify antigen specific T cells (Altman, Moss et al. 1996). The multimers enable the low affinity of the TCR/peptide-MHC complex interaction to be overcome. This occurs as with a multimer the probability that all monomeric interactions simultaneously dissociate is very small and the avidity of the interaction thus far exceeds the combined avidities (Laugel, Boulter et al. 2005), and the result is binding of sufficient duration to enable the use of MHC-peptide multimers for the cell surface staining.

The original and most common peptide-MHC multimer in use is a 'tetramer'. This construct utilises avidin to bind 4 biotinylated TCRs resulting in a tetrahedral structure (Fig. 1.7). However, higher order multimers are now also widely available (Reviewed in (Bakker and Schumacher 2005). We utilised Dextramer reagents (Immudex, Denmark) which consist of a dextran backbone upon which are complexed >10 peptide-MHC molecules. These higher order complexes have a longer interaction half-life than tetramers. We hypothesised that this may facilitate the identification of HY associated minor histocompatibility antigens, as tumour derived and autoimmune epitopes are thought to have a lower interaction affinity than viral derived epitopes (Cole, Pumphrey et al. 2007).

## Tetramer



## Dextramer

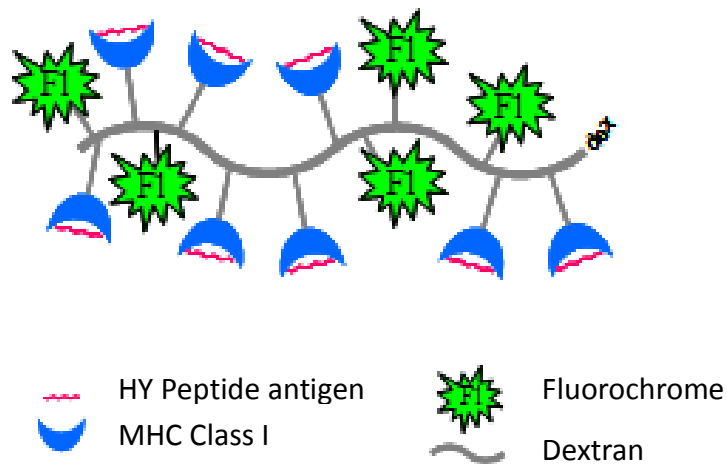


Figure 1.7 Comparison of tetramer and dextramer structures.

The tetramer is a multimer of 4 peptide-MHC complexes formed through the binding of 4 biotinylated MHC molecules to an avidin molecule. A dextramer contains multiple peptide-MHC complexes (>10) which are arranged along a dextran backbone. Both multimers also have fluorochromes bound to enable flow cytometric visualisation. (Dextramer figure reproduced with permission from Immudex, Denmark)

## 1.8 Aims of thesis

- 1) To establish how pregnancy modulates maternal cellular immunity
  - a. Specifically, by conducting an assessment of maternal T cell effector and regulatory subsets during pregnancy and the postnatal period and by establishing how maternal Cytomegalovirus seropositivity may influence this
- 2) To investigate maternal cellular immunity to fetal antigen during pregnancy and the postnatal period
  - a. Specifically, to use MHC-peptide multimer technology to detect fetal specific CD8 T cells in the maternal circulation and study their dynamics during pregnancy and the postnatal period.
  - b. Furthermore, to characterise the phenotype and function of fetal specific T cells during pregnancy.
- 3) To investigate maternal cellular immunity in women with a history of recurrent miscarriage
  - a. Specifically, to determine if these women have differences in their T cell biology and if fetal specific T cells can be detected in these women.
  - b. To establish whether T cell function can be modulated by progesterone.

# **Chapter 2.**

## **Materials and methods**

## 2.1 Media, and solutions

Wash media	RPMI 1640 (Gibco BRL) 100U/ml Penicillin (Gibco BRL) 100 µg/ml Streptomycin (Gibco BRL)
General media	RPMI 1640 (Gibco BRL) 100U/ml Penicillin (Gibco BRL) 100 µg/ml Streptomycin (Gibco BRL) 2mM Glutamine (Gibco BRL) 10% Fetal Calf Serum (SBS Biologicals)
Freezing media	Fetal Calf Serum (SBS Biologicals) 10% DMSO (Sigma)
CsA media	RPMI 1640 (Gibco BRL) 100U/ml Penicillin (Gibco BRL) 100 µg/ml Streptomycin (Gibco BRL) 2mM Glutamine (Gibco BRL) 10% Fetal Calf Serum (SBS Biologicals) 2 µg/ml Cyclosporin A (Sandoz, UK)
MACS buffer	1 x PBS (University of Birmingham) 0.5% Bovine serum albumin (Sigma) 2mM EDTA (Sigma) Sterile filtered

Peptide line media	RPMI 1640 (Gibco BRL) 100U/ml Penicillin (Gibco BRL) 100 µg/ml Streptomycin (Gibco BRL) 2mM Glutamine (Gibco BRL) 10% Human serum (HD Supplies)
T cell cloning media	RPMI 1640 (Gibco BRL) 100U/ml Penicillin (Gibco BRL) 100 µg/ml Streptomycin (Gibco BRL) 2mM Glutamine (Gibco BRL) 10% Human Serum (HD Supplies) IL-2 (100 U/ml final conc.) (Chiron) IL-4 (5 ng/ml final conc.) (R&D Systems) IL-7 (5 ng/ml final conc.) (Peprotech)
T cell clone maintenance media	RPMI 1640 (Gibco BRL) 100U/ml Penicillin (Gibco BRL) 100 µg/ml Streptomycin (Gibco BRL) 2mM Glutamine (Gibco BRL) 10% Human Serum (HD Supplies) IL-2 (100 U/ml final conc.) (Chiron)
ELISA coating buffer	1xPBS (University of Birmingham) 0.1M Na <sub>2</sub> HPO <sub>4</sub> (Sigma) adjust to pH 9 with 0.1 M NaH <sub>2</sub> PO <sub>4</sub>

ELISA blocking buffer 1x PBS (University of Birmingham)

1% Bovine serum albumin (Sigma)

0.05% Tween 20 (Sigma)

ELISA Wash buffer 1x PBS (University of Birmingham)

0.05% Tween 20

ELISA Stop buffer 0.5M H<sub>2</sub>SO<sub>4</sub>

TBE Buffer 89nM Tris HCL

89nM Boric Acid

3mM EDTA

## **2.2 Subjects**

### **2.2.1 Ethical approval**

The study was approved by the South Birmingham Research Ethics Committee (08/H1207/94). All samples were obtained with written, informed consent. Human tissues were used and stored in compliance with the requirements of the Human Tissue Act (2004).

### **2.2.2 Healthy pregnancy cohort**

Between February 2009 and September 2010 healthy pregnant women (n=134) were recruited from antenatal clinics at Birmingham Women's Hospital, United Kingdom. Women with medical or obstetric problems, recurrent miscarriage or prior blood transfusion were

excluded from analysis. The women had a median age of 31 (Range 18-46). They were 71% white, 20% Asian and 9% of other ethnicity. The median parity was 1 (Range 0-5) and the median number of miscarriages was 0 (Range 0-2). 57% were found to be Cytomegalovirus IgG seropositive.

Venous blood samples were obtained between 10 weeks gestation and delivery and post natal samples were obtained within the first 6 weeks after delivery. 20ml of heparinised blood and 5ml of blood anticoagulated with EDTA was obtained at each time point. In some donors multiple samples were collected. In each person a maximum of 6 samples were obtained. 1 sample each trimester, 1 sample in labour and 2 postnatal samples.

The number of samples and the timepoint in pregnancy from which they were obtained are detailed in the results of each individual assay and in the figure legends.

### **2.2.3 Recurrent pregnancy cohort**

Between October 2009 and January 2011 women with recurrent miscarriage were recruited from the recurrent miscarriage clinic at Birmingham Women's Hospital, United Kingdom (n=68). Women with previous blood transfusion, twin siblings, or transplant recipients were excluded. The median age was 36 (Range 22-44) and 84% of these women were of white ethnicity, 10% were Asian ethnicity and 6% were of other ethnicities. The median parity was 1 (Range 0-3). The median number of miscarriages was 3.7 (Range 3-10) and 62% of women were Cytomegalovirus IgG seropositive. An age and parity matched control cohort (n=65) was recruited from staff members at Birmingham Women's Hospital. There was no significant differences between the recurrent miscarriage and control cohort in



any characteristic except for the number of previous miscarriages, which in the control cohort was a median of 0 (Range 0-1) ( $p < 0.0001$  by T-test).

A venous blood sample was obtained in clinic, these women were not pregnant at the time of venepuncture. Standard investigations were conducted to rule out known causes of recurrent miscarriage (testing for antiphospholipid antibodies, pelvic ultrasound, thrombophilia screening) (Regan, Backos et al. 2011) and women with an identified cause were excluded from further analysis. 20ml of heparinised blood and 5ml of blood anticoagulated with EDTA was collected.

#### **2.2.4 Isolation of peripheral blood mononuclear cells (PBMCs)**

Peripheral blood mononuclear cells for flow cytometry and cellular assays were isolated from heparinised blood samples under sterile conditions by density gradient centrifugation. Samples obtained overnight from women in labour were kept at room temperature and separated the next morning (within 12 hours). Whole blood was diluted 1:1 with “wash media” mixed well and then carefully layered onto lymphoprep (Nycomed, Sweden). This was centrifuged at 758g, at room temperature, for 30 minutes with no brake. The buffy layer was removed with a transfer pipette. Cells were resuspended in “wash media” to a volume of 50ml and centrifuged at 1800RPM for 10 minutes, with brake applied. Cells were washed 1 further time in “wash media” at 1500RPM, for 10 minutes, with brake applied. For the final wash cells were resuspended in “general media” and spun at 1200RPM, for 10 minutes, with brake applied. Cells were counted with a haemocytometer. Cells were either used immediately for assays or cryopreserved, as per section 2.9.1.

### **2.2.5 Preparation of plasma and PBMC for DNA extraction**

Peripheral blood mononuclear cells to be used for DNA extraction and plasma were isolated from a blood sample anti-coagulated with EDTA. This blood was layered undiluted onto lymphoprep (Nycomed, Sweden) which was centrifuged at 758g, at room temperature, for 30 minutes with no brake. The plasma was removed with a transfer pipette and aliquoted into 1.8ml cryovials (Nunc, Thermo scientific) which were then stored at -80 °c. The buffy layer was removed with a transfer pipette, then washed twice in “wash media” at 1500RPM for 10 minutes. The cells were then transferred into a cryovial (Nunc, Thermo scientific), and the cell pellet stored at -80°c until used for DNA extraction.

## **2.3 Production of HLA peptide tetramers**

### **2.3.1 Generation of HLA peptide tetramers**

Class I HLA-peptide tetramers were synthesised using standard methods (Altman et al. 1996). HLA-A\*0201 heavy chain and B2M proteins were kindly provided by Mrs E Manoli. These were then refolded around the FIDSYICQV peptide (Alta Biosciences, UK) over a 48 hour period, being continually stirred at 4°C in a refold buffer containing 400mM L-Arginine, 100mM Tris.HCL pH8.0, 2mM EDTA, reduced glutathiones, oxidised glutathiones and 0.1mM PMSF. 30mg heavy chain, 5mg peptide and 10mg B2M were used for a 500ml refold. The refold solution was then concentrated using a miniplate device (Amicon “Stir Cell”, model 8400) using a Millipore 10,000 MW filter and then buffer exchanged using PD-10 Sephadex G-25 M desalting columns (Amersham pharmacia, UK) into the biotinylation reaction buffer (100mM Tris.HCL pH 7.5, 5mM MgCl<sub>2</sub>, 20mM NaCl, 0.1mM PMSF). Biotinylation was carried

out overnight at room temperature in a final volume of 2ml containing 5mM ATP, 5mM D-biotin and BirA enzyme. Complexes were then purified by fast protein liquid chromatography (FPLC) using gel filtration and ion exchange columns (Amersham pharmacia, UK).

Protein concentration of the refold was then estimated. Briefly, a standard curve was constructed with known concentrations of BSA as standards, in triplicate alongside the refolded proteins. 100 microlitres of Bio-Rad protein assay reagent (Bio-Rad) was added to each well, and after 10 minutes at room temperature the absorbances were then read at 595nm using a platereader. The standard curve was then used to assess the protein concentrations of the refold fractions. Biotinylation was then also confirmed by a biotinylation ELISA. Briefly, this involved serial dilutions of the refold being added to a 96 well plate, and left for 1 hour at 37<sup>0</sup>C. Now the proteins had adhered the plate was thoroughly washed with PBS containing 0.05% Tween 20 (Sigma). 100 microl of a 1/1000 dilution of extravidin-peroxidase conjugate (Sigma, UK) in PBS with 0.1% BSA was added to each well, binding only to biotinylated proteins. This was incubated for 15mins at room temperature and then again thoroughly washed in PBS with 0.05% Tween 20 (Sigma, UK). 100microlitre of TMB substrate solution (3,3'-5,5'-tetramethylbenzidine liquid substrate system, Sigma, UK) was added to each well. Blue reaction indicated biotinylated proteins. Monomers known to be biotinylated were then stored at -80 °C until required.

Finally, prior to use, tetrameric complexes were made by the addition of PE-conjugated streptavidin (Molecular probes, Invitrogen) in a molar ratio of 1:4 to the biotinylated monomer over 2 days in 10 separate aliquots. This is the equivalent of adding

0.312 micrograms of streptavidin PE to each 1 microgram of monomer. Following this the tetramers were kept at 4 °C in the dark, and used within 4 weeks of tetramerisation.

## **2.4 Identification, expansion and phenotyping of fetal antigen specific T cells**

### **2.4.1 Expansion of fetal specific T cells in a peptide pulsed CD8 T cell line**

Freshly isolated or thawed PMBC according to the experiment were resuspended after washing in 1ml of serum free “wash media”. 15micrograms/ml of the appropriate peptide was then added. All peptides were dissolved in DMSO and kept at -20 °c in a stock concentration of 1mg/ml) The peptide was incubated for 1 hour, at 37°c in a 5% CO<sub>2</sub> environment and the cells agitated every 15 minutes. Cell were then resuspended in “peptide line media”. IL-7 (Peprotec, UK) was added to make a final concentration of 25ng/ml and IL-15 (Peprotec, UK) to make a final concentration of 2ng/ml. The cells were seeded in a 24 well plate at 2-3x10<sup>6</sup> cells per ml, with 1 ml added to each well. After 3-5 days 1ml of additional “peptide line media” with 100units/ml of IL-2 (Chiron Ltd, UK) was added to each well. Thereafter, cells were fed by removing 1ml of media and replacing with 1ml of “peptide line media” containing IL-2 100 units/ml after 3-4 days on 2-3 occasions. The line was then used after 10-14 days. No additional rounds of peptide stimulation or additional antigen presenting cells were used.

### **2.4.2 HLA-peptide tetramer staining**

For tetramer staining cells were washed and resuspended in 100 microliters of MACS buffer. They were then wrapped in foil and incubated for 15 minutes in a waterbath at 37 °c

with the tetramer. Each tetramer refold was titrated against controls to optimise reaction concentrations. The tetramer was then washed off in MACS buffer and further staining conducted on ice to minimise tetramer internalisation.

#### **2.4.3 HLA-peptide dextramer staining**

For staining cells with dextramer (Immudex, Denmark) the staining conditions were different. Staining was carried out at room temperature, in the dark for 20 minutes. Dextramers similarly required titration against positive and negative controls to optimise reaction conditions.

#### **2.4.4 Antigen specific T cell enrichment with autoMACS pro magnetic selection**

Antigen specific T cells were enriched by an automated magnetic cell sorting method using anti-phycoerythrin (anti-PE) magnetic beads attached to the phycoerythrin flouochrome on the tetramer or dextramer. Selection used the AutoMACS Pro machine (Miltenyi Biotec, Germany). The AutoMACS Pro (Miltenyi Biotec, Germany) was maintained in a clean room, which was UV irradiated between uses. The machine probes were cleaned with ethanol prior to each selection and the selection columns changed as per manufacturers instructions. Sterile MACS buffer and MACS rinse (Miltenyi Biotec, Germany) was prepared for each set of selections. Cells already stained with tetramer or dextramer buffer as previously described, and then thoroughly washed with MACS buffer, were pelleted in a FACS tube. The supernatant was removed as completely as possible and the cells then resuspended in 80 µl of MACS buffer. 20 microliters of anti-PE beads (Miltenyi Biotec, Germany) were then added. This was incubated in the dark at 4°C for 20 minutes. The

beads were washed off with 2 washes in MACS buffer at 4°C. The cells then were resuspended in 500 microliters of MACS buffer. 50 microliters was removed to test the cell frequency pre selection. The FACS tube was then placed in the autoMACS cooled rack. The “POSSELD” program was used to provide a double positive selection programme and select cells at high purity. Positive and negative fractions were collected after the procedure was complete for further analysis by flow cytometry, or cloning or both procedures, as required.

#### 2.4.5 Fetal specific T cell detection and phenotyping by flow cytometry

Following dextramer/tetramer staining the cells were washed and either stained with the appropriate antibody panel for the detection of fetal specific T cells, or in ex-vivo experiments the fetal specific T cell phenotyping panel was used. Antibody staining was carried out at 4 °C, in the dark for 20 minutes.

*Table 2.4.5.1 Antibody panel for detection of fetal specific T cells*

Laser	Filter	Antibody	Clone	Conjugate
488 nm	575/26	Dextramer		PE
	610/20	Propidium Iodide		
	695/40	CD14 <sup>c</sup>	RM052	ECD
		CD19 <sup>c</sup>	J3-119	ECD
		CD28 <sup>d</sup>	L293	PerCP-Cy5.5
405 nm	450/50	CD8 <sup>e</sup>	OKT8	Pacific Blue
	525/50	CD3 <sup>d</sup>	SK7	AmCyan

Table 2.4.5.2 Antibody panel for fetal specific T cell phenotyping

Laser	Filter	Antibody	Clone	Conjugate
488 nm	530/30	CCR7 <sup>a</sup>	150503	FITC
	575/26	Dextramer		PE
	610/20	Propidium Iodide		
		CD14 <sup>c</sup>	RM052	ECD
		CD19 <sup>c</sup>	J3-119	ECD
	695/40	CD28 <sup>d</sup>	L293	PerCP-Cy5.5
405 nm	450/50	CD8 <sup>e</sup>	OKT8	Pacific Blue
	525/50	CD3 <sup>d</sup>	SK7	AmCyan
633 nm	660/20	CD57	HCD57	APC
	730/45	CD45RA <sup>b</sup>	HI100	AF700
	780/60	CD27 <sup>e</sup>	O323	APC-AF750

a=R&D Systems, b=Biolegend, c=Beckman Coulter, d=Becton Dickinson, e=eBioscience

Flow cytometric examination of fetal specific T cells utilised strategies to optimise flow cytometric examination of rare cell populations, as previously described (Chattopadhyay, Melenhorst et al. 2008). A meticulous gating strategy was utilised to exclude aberrant binding events. Briefly, singlet cells were identified on a FSC-A vs FSC-H plot and lymphocytes gated on the basis of standard light scatter properties. A “dump” channel used to exclude dead cells (using PI), monocytes (CD14) and B-cells (CD19). T lymphocytes were then selected by CD3 expression. Finally, dextramer “positive” cells were visualised against CD8 expression. Proportions of dextramer “positive” cells are given as a percent of CD8 positive cells.

A negative control dextramer was used that contained an irrelevant peptide. This enabled levels of background staining to be determined, if present. Any 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. A positive result was only considered with dextramer staining 10x above background.

“Fluorescence minus one” (FMO) controls were used to determine gating thresholds. Phenotypic gating was initially carried out by reference to the dextramer negative cell population, where populations could be clearly delineated due to the larger cell populations, with the same gating template then applied to the dextramer positive population.

## **2.5 Cloning procedures**

### **2.5.1 Cloning by dextramer selection and enrichment followed by limiting dilution**

Cloning mix was prepared. Allogenic PBMCs were obtained from 3 healthy lab donors or 3 apheresis “cones” provided by the National Blood Service. These were stimulated overnight with PHA at 5 microgram/ml and then washed thoroughly 4 times prior to pooling and irradiating the cells. These were used at  $10^6$  cells/ml.  $10^5$  irradiated LCLs/ml were also added. These cells were resuspended in “cloning media”, this formed the “cloning mix”.

Cells from the peptide lines, established to expand antigen specific T cells as previously described, were harvested on day 10-14, washed and stained with dextramer and then the antigen specific T cells selected with the autoMACS Pro as previously described. Samples were taken pre and post selection for flow cytometric analysis to monitor the frequency of the antigen specific T cells pre and post selection. The post selection flow cytometric analysis was also used to determine the appropriate cell density and dilutions required for cloning. The remaining cells were seeded into 96 well plates at a dilution of 0.3



cells per well in 100 microliters of “cloning mix” per well. The plates were incubated at 37 °c in a 5% CO<sub>2</sub> atmosphere for 7 days, after which 100 µlitres of “clone maintenance media” was added. After day 14 wells were inspected for outgrowth visually. Any wells which were expanded were transferred into a 24 well plate with 2ml of cloning mix, prepared as previously but the only cytokine added was IL-2. These cultures were initially screened by dextramer prior to further functional assessment.

### **2.5.2 Maintenance of T cell clones**

T cell clones were fed every 3-4 days with T cell clone “maintenance media”. When cells became confluent cells were split into additional wells. Every 2-4 weeks cells were restimulated with “cloning” mix as previously described, however, only IL-2 was added. Following restimulation the cells were not used for at least 10 days.

## **2.6 Assessment of T cell clones**

### **2.6.1 Interferon gamma ELISA**

T cell function and specificity was assessed through the ability of T cell clones to release IFN  $\gamma$  when co-cultured with antigen specific target cells. Antigen specificity was confirmed with a range of targets; female A2 positive LCL loaded with HY peptide, male LCLs (HY positive with endogenous antigen processing), female A2 positive LCL loaded with CMV specific (irrelevant) peptide and A2 negative LCL loaded with HY peptide. Further negative controls of LCL alone and T cell alone were also used. Targets were peptide loaded as required and seeded in triplicate in 96 well round bottom plates at a density of  $3 \times 10^4$  target

cells/well. T cell clones were washed well and added at  $3 \times 10^3$  cells per well in a total volume of 200  $\mu$ l per well of “peptide line media” supplemented with IL-2 at 25 units/ml. These were incubated for 16 hours at 37 °C in a 5% CO<sub>2</sub> environment. The plate was then centrifuged and the supernatant harvested and either assayed immediately or frozen at -80 °C prior to the IFN  $\gamma$  ELISA.

For the IFN  $\gamma$  ELISA a MAXIsorp plate (NUNC, Denmark) was coated with anti-human IFN  $\gamma$  antibody (Pharmingen) diluted in “coating buffer” at 2  $\mu$ g/ml and left at 4 °C for 16 hours. The wells were then blocked with 200ml/well of blocking buffer and left at room temperature for 2 hours. Meanwhile, IFN $\gamma$  control standards were prepared ranging from 2000pg/ml to 31.25 pg/ml as serial 1:1 dilutions, plus a well of culture medium alone. All standards were run in triplicate. The plates were then washed 3 times in “wash buffer” and then either 50  $\mu$ l of standard or test supernatant was added to each well, in triplicate. The plate was then sealed and a further 3 hour incubation at room temperature performed. The plates were washed a further 4 times prior to the addition of 50  $\mu$ l/well of biotinylated anti-IFN  $\gamma$  antibody (2  $\mu$ g/ml in blocking buffer). Following a 1 hour incubation at room temperature the plates were washed 4 times. 50  $\mu$ l of extra-avidin peroxidase (Sigma) (diluted 1/1000 in blocking buffer) was then added and incubated for a further 30 minutes. A final series of 5 washes was then performed. 100  $\mu$ l TMB substrate solution (3,3'-5,5'-tetramethylbenzidine liquid substrate system, Sigma, UK) was added to each well and left at room temperature in the dark for 20 minutes. To stop the reaction 100  $\mu$ l per well of stop buffer was used. The plates were read on an absorbance plate reader at 450 nm. The IFN  $\gamma$  for the test supernatants was calculated by reference to the standard curve.

### **2.6.2 Chromium release cytotoxicity assay**

T cell clones were tested for cytotoxicity using a standard <sup>51</sup>chromium release assay (Piper, McLarnon et al. 2007). Clones were tested after day 20 and more than 10 days after restimulation with buffy and LCL. Antigen specificity was as before demonstrated by using several targets; female A2 positive LCL loaded with HY peptide, male LCLs, female A2 positive LCL loaded with CMV specific peptide and A2 negative LCL loaded with HY peptide. Further negative controls of LCL alone and T cell alone were also used. All targets were labelled with 100 µCi of Na<sub>2</sub><sup>51</sup>CrO<sub>4</sub> (Amersham Pharmacia, UK) for 1 hour prior to pulsing with the appropriate peptide for a further hour. Targets were then washed twice, counted and plated out at 2,500 cells per well in a 96 well U bottomed plate. T cells were added at 5:1 and 1:5 effector to target ratio, and to calculate maximum lysis 100 µl of 1% SDS was used. After 4 hour incubation at 37°C in a 5% CO<sub>2</sub> environment plates were briefly spun (2 minutes at 2000RPM). 100 µl of supernatant was harvested from each well and lysis determined using a top counter (Cobra II auto-gamma, Packard). Lysis values were calculated using the equation:  $100 \times (\text{test release} - \text{spontaneous release}) / (\text{maximal release} - \text{spontaneous release})$ . All assays were conducted in triplicate.

### **2.6.3 Immunomodulation of progesterone assays**

The assessment of the effect of progesterone on T cell clone function utilised the technique of IFN $\gamma$  ELISA following antigen specific stimulation as previously described (section 2.6.1). This was however modified by the addition of further reaction conditions in which the T cell clone was co-cultured as previously with HY peptide loaded LCL or male cells but with the

addition of varying amount of progesterone (P4, Sigma). Progesterone was added at 100  $\mu$ M, 20 $\mu$ M, 0.2 $\mu$ M and vehicle (DMSO) alone at the commencement of the 16 hour co-culture . All reactions were conducted in triplicate.

#### **2.6.4 Imagestream analysis of T cell clones**

Cells were stained with HY dextramer (HLA\*0201 FIDSYICQV, PE, Immudex) or negative control dextramer (HLA\*0201, Immudex) and CD8 (PE-CY5, Abcam). Cells were kept on ice and run on the ImageStream multispectral imaging flow Cytometer (Amnis, USA). Images were analyzed using IDEAS image analysis software (Version 4.0, Amnis). 1,000 cells were collected in each sample, and single stained compensation controls were used to compensate fluorescence between channel images on a pixel-by-pixel basis using IDEAS software off-line compensation.

Gating identified single cells on the basis of aspect ratio versus area on the brightfield channel and focused cells selected by gating on a gradient RMS histogram on the brightfield channel. Gating thresholds were set by visual examination of individual cells. Compensation and analysis settings were kept constant between positive and negative control samples.

## **2.7 Cytomegalovirus assays**

### **2.7.1 CMV IgG ELISA**

CMV IgG sero-positivity was determined using a CMV IgG Enzyme Immunoassay test kit (BioCheck Inc, USA) as per the manufacturer's instructions. Briefly, the plasma samples, cut-off calibrator, negative and positive controls were prepared in a 1:40 dilution. Once diluted, 100µl of each was then dispensed to CMV antigen coated micro-titre wells. The wells were sealed and incubated at 37°C for 30 minutes. Wells were washed 4 times with wash buffer (1/10 dilution). 100µl of enzyme conjugate (Horesradish peroxidase labelled anti-human IgG conjugate) was added per well and incubated at 37°C for 30 minutes. Wells were again washed 5 times. 100µl of TMB reagent (Tetramethylbenzidine) was dispensed to each well, which were incubated in the dark at 37°C for 15 minutes. At the end of the incubation period, 100µl of stop solution (1M HCl) was added and the wells were read at 450nm with a microwell reader. Interpretation of the ELISA followed the manufacturer's directions. Each O.D. value was divided by the O.D. value of the cut-off calibrator to obtain a CMV IgG index. A CMV IgG index above 1.0 was positive for CMV and below 0.9 was considered negative. If the CMV IgG index was between 0.91-0.99 the test was equivocal and the sample repeated.

### **2.7.2 Real time quantitative PCR for identification of CMV DNA**

A Taqman real time PCR assay was utilised to enable detection and quantification of cytomegalovirus copy number from maternal peripheral blood. Amplification of the CMV UL132 gene (GenBank Accession AY446894) was performed using the Applied Biosciences Gene Expression Assay (Pa03453400\_s1, Applied Biosciences) according to the

manufacturers instructions. DNA extracted from cultured CMV (strain AD169) was quantified spectrophotometrically (Nanodrop, Thermo Scientific) and the DNA concentration calculated. 10 fold dilutions were prepared ranging from  $10^7$  to 10 copies per reaction. These standards were included in duplicate in every assay and a standard curve constructed from which the viral load for patient samples could be extrapolated. These standard curves demonstrated the assay was sensitive to less than 10 copies per reaction and linear though a large range. In each assay samples were prepared in duplicate with 2 positive controls from patients with known viraemia, 2 negative controls from CMV IgG negative patients and 2 water controls in each assay. DNA amplification was carried out on the PE-ABI 7500 sequence detection system. After activation of the uracil-N-glycosylase (2 min at 50°C) and Amplitaq Gold (10 min at 95°C), amplification was carried out over 40 cycles (15s at 95°C, 60 s at 60°C). A fluorescence threshold value was determined as 10 standard deviations above the mean of the background fluorescence emission for all wells between cycle 3 and 15. Fluorescence less than this threshold after 40 cycles was determined to be a negative result.

### **2.7.3 Identification and phenotyping of CMV specific T cells**

6 MHC-peptide tetramers were utilised to identify CMV specific T cells. HLA-A\*0101 tetramers incorporating the pp65 residue YSEHPTFTSQY and pp50 residue VTEHDTLLY. HLA-A\*0201 tetramers incorporating the pp65 residue NLVPMVATV. HLA-B\*0702 tetramers incorporating the pp65 residues RIPHERNGFTVL and TPRVTGGGAM and HLA-B801 tetramers incorporating the IE-1 residue ELKRKMIYM.

Thawed PBMCs were incubated with the appropriate tetramer at 37°C for 15 minutes. After washing with MACS buffer cells were incubated with a 10 colour antibody panel, staining was carried out at 4°C for 20 minutes (Table 2.7.3).

Table 2.7.3. CMV specific T cell phenotype antibody panel

Laser	Filter	Antibody	Clone	Conjugate
488 nm	530/30	CCR7 <sup>a</sup>	150503	FITC
	575/26	CD57 <sup>b</sup>	HCD57	PE
	610/20	Propidium Iodide		
		CD14 <sup>c</sup>	RM052	ECD
		CD19 <sup>c</sup>	J3-119	ECD
	695/40	CD28 <sup>d</sup>	L293	PerCP-Cy5.5
	780/60	CD38 <sup>d</sup>	HIT2	PE-Cy7
405 nm	450/50	CD8 <sup>e</sup>	OKT8	Pacific Blue
	525/50	CD3 <sup>d</sup>	SK7	AmCyan
633 nm	660/20	Tetramer		APC
	730/45	CD45RA <sup>b</sup>	HI100	AF700
	780/60	CD27 <sup>e</sup>	O323	APC-AF750

a=R&D Systems, b=Biolegend, c=Beckman Coulter, d=Becton Dickinson, e=eBioscience

## 2.8 Non antigen specific immunological assays

### 2.8.1 T regulatory cell assays

T regulatory cells were identified on the basis of CD4, CD25, CD127 and FOXP3 using multiparameter flow cytometry. Cells were thawed as per protocol, and stained at 4°C in the dark with the following antibodies; CD127 FITC (e-bioscience), CD25 PC5 (Beckman Coulter), CD8 PB (e-bioscience), CD4 PC7 (e-bioscience), CD3 APC-CY7 (Biolegend). Fixation and permeabilization was carried out with the eBioscience Fixation/Permeabilization kit (e bioscience, cat. 00-5123) according to the manufacturer's instructions. A rat serum blocking

step was used (15 minute incubation at 4°C, 2% solution) and intracellular staining was then conducted. Intracellular staining for FOXP3 used the anti-human FOXP3 PE antibody (ebioscience, 12-4776-435) and a duplicate tube was stained with the isotype control rat IgG2a PE isotype (ebioscience, 12-4321-73).

### **2.8.2 Intracellular cytokine staining for IL-17 and IFN $\gamma$ and the surface chemokines CXCR3 and CCR4.**

Multiparameter flow cytometry was used to simultaneously examine CD4+ and CD8+ T cells for the intracellular expression of the cytokines IFN  $\gamma$  and IL-17, and the surface chemokines CXCR3 and CCR4. PBMC's were thawed, with all timepoints from a single donor analysed simultaneously.

For each assay cells were split into a stimulated sample and an unstimulated control sample. Surface chemokine receptor data was obtained from the unstimulated sample as stimulation led to downregulation of the chemokine receptors. Surface staining was initially carried out with CXCR3, PE (TG1/CXCR3, Biolegend) and CCR4 Pe-Cy-7 (1G1, BD). Surface staining was conducted for 20minutes at 4°C in the dark. Cells were then washed twice and resuspended in "peptide line media". The samples to be stimulated were then incubated with PMA (25ng/ml, Sigma) ionomycin (400ng/ml, Sigma) for 1 hour at 37°C, 5% CO<sub>2</sub>. The golgi blocker monensin (2 $\mu$ M, Sigma) was added to both the stimulated and unstimulated samples. The cells were then incubated as before for a further 5 hours and then kept at 4°C overnight. The cells were then washed in PBS. Surface staining with CD14 ECD (RMO52, BC),



CD3 APC-Cy7 (HIT3a, Biolegend), CD8 Amcyan (RPA-T8, BD) was carried out at 4°C in the dark for 20 minutes. Cells were then washed and samples were split into 2 tubes, with 1 tube for the use of appropriate isotype controls. For all tubes cells were fixed with 2% paraformaldehyde at room temperature for 10 minutes and then permeabilised with 0.5% saponin at room temperature for 5 minutes. Intracellular staining was then conducted with IL17A Pacific Blue (BL168, Biolegend) and IFN- $\gamma$  AF700 (4S.B3, Biolegend) or appropriate isotype controls for 30 minutes at room temperature prior to a final wash in MACS buffer.

### **2.8.3 Plasma cytokine and chemokine quantification by luminex**

A multiplex fluorescent bead-based immunoassay (Panomics, Italy) was used to analyse plasma cytokine, chemokine and chemokine receptor levels. This was a 38 plex assay, but the concentrations of MIP-3a, MCP-3, IL-22 and sRANKL were outside the range of the standard curves provided and therefore could not be analysed further. The factors analysed were; FGF-basic, GCSF, GM-CSF, HGF, IFN gamma, IL-1 beta, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-13, IL-15, IL-17a, IL-17f, IP-10, ITAC (CXCL11), MCP-1 (CCL2), MCP-3, MIG (CXCL9), MIP-1a (CCL3), MIP-1b (CCL4), RANTES (CCL5), TNF $\alpha$ , TNF $\beta$ , VEGF, Fractalkine (CX3CL1), IL-21, IL-23, SDF-1 (CXCL12), CTACK (CCL27), MMP-3, MMP-9.

The assay was conducted according to the manufacturer's instructions (Panomics, Italy). Briefly, standards were prepared as directed. Plasma samples were thawed, vortexed and spun briefly to remove any potential debris. The 96 well filter plate provided was pre-wet with reading buffer and this was then removed by vacuum aspiration using a vacuum manifold (Affymetrix, UK). The plate was then prepared with the appropriate antibody beads which were added at 50  $\mu$ l per well and then washed once with wash buffer. 25  $\mu$ l of

standard or sample was then added per well, the plate sealed and left for 1 hour at room temperature on a plate shaker at 500RPM. The solution was then removed by filtration and washed 3 further times. The biotinylated detection antibody was then added at 25µl per well. The plate again sealed and incubated at room temperature for 30 minutes on a plate shaker at 500RPM. The solution was removed by filtration and the plate washed 3 times. Finally, 50 µl of SAPE (streptavidin-PE) was added per well. The plate again sealed and incubated at room temperature for 30 minutes on a plate shaker at 500RPM. This was washed 3 times. Prior to reading the plate 120 µl of reading buffer was added to each well, the plate sealed and incubated for 5 minutes on a plate shaker at 500RPM. Samples were analysed on the Luminex 100 (Luminex, USA). The machine was calibrated and checked with xMAP Reporter calibration microspheres (Luminex) and xMAP classification and reporter control microspheres (Luminex). Offline analysis was conducted using StarStation Luminex software (version 3.0).

## **2.9 General Procedures**

### **2.9.1 Freezing of cells for long term storage**

Cells were resuspended in “general media” and pelleted by centrifugation at 300g for 5 minutes, the supernatant was carefully removed from the pellet and the cells resuspended in the residual media. The cells were then cooled on ice for 10 minutes. Cells were then resuspended in “freezing media”, also pre-cooled on ice, in a dropwise manner, while being continually agitated, to a final volume of 1ml per vial to be stored. The solution was then transferred to pre-cooled cryovials (Nunc, Thermo Scientific). These were placed in a “Mr Frosty” (Freezing container, Nalgene®, C1562-1EA, Sigma) in a -80 freezer to control cooling

to 1°C per minute. After 12 hours cells were then transferred to liquid nitrogen for longterm storage.

### **2.9.2 Recovery of cells from long term storage**

Cells were defrosted in a waterbath at 37 °C for no longer than 2 minutes. Thawed cells were transferred into 10ml of “general media” and centrifuged at 300g for 5 minutes. The cells were then washed again in “general media” prior to counting using Trypan blue (Sigma) to check viability. Cells were then maintained at 37°C in a 5% CO<sub>2</sub> atmosphere.

### **2.9.3 Generation of B95.8 EBV transformed lymphoblastoid cell lines (LCLs)**

$5 \times 10^6 - 1 \times 10^7$  PBMC from the donor were pelleted and resuspended in 2.5ml of B95.8 tissue culture supernatant (kindly supplied by Dr C Shannon-Lowe, University of Birmingham). These were incubated for 1 hour at 37 °C in a 5% CO<sub>2</sub> environment, and gently agitated every 15 minutes. After this the cells were washed twice in “general media”. Cells were then resuspended in 2ml CSA media and plated into 2 wells (1ml per well) of a 48 well plate. These were incubated at 37 °C in a 5% CO<sub>2</sub> environment. 1ml CSA media added on day 7. After day 14 days they were fed twice weekly with GM media and when cell clumps were forming they were expanded into 24 well plates. When established they were transferred into T25 flasks.

### **2.9.4 Maintenance of LCLs**

LCLs were kept at 37 °C in a 5% CO<sub>2</sub> environment and maintained by feeding every 3-4 days with general media, and splitting 1 in 2 as required.

## **2.10 Molecular biology**

### **2.10.1 DNA extraction from PBMCs**

DNA was extracted from PMBCs using the DNeasy Blood and Tissue Kit (Qiagen). Briefly,  $5 \times 10^6$  cells were resuspended in sterile PBS with 20  $\mu$ l of proteinase K. This was mixed with 200  $\mu$ l of the supplied “AL” buffer and incubated at 56 °C for 10 minutes. 200  $\mu$ l of ethanol was then added and the sample transferred into a DNeasy mini spin column. This was centrifuged at 6000g for 1 minute, the flow through discarded and the column washed through with AW1 and twice with AW2. After the final wash the column membrane was completely dried with a 3 minute spin at 20,000g. The DNA was then eluted in 100  $\mu$ l of buffer AE into a nuclease-free eppendorf (Eppendorf). DNA was stored at -20 °C.

### **2.10.2 DNA extraction from maternal plasma**

DNA was extracted from maternal plasma for the assessment of free fetal DNA. This was done using the QIAamp DNA Blood Mini Kit (Quiagen) as per the manufacturer’s directions. DNA was extracted from 400  $\mu$ l of plasma, and eluted into 40 $\mu$ l of eluent.

### **2.10.3 HLA typing**

HLA typing was carried out by 2 methods:

**HLA typing by flow cytometry –**

When the only information required was if the donor was HLA-A\*0201 positive or negative, then this was determined by flow cytometry using the anti human HLA-A\*02 antibody BB7.2 (kindly provided by Mr G Ryan). This was carried out on whole blood from venous or capillary samples if rapid HLA typing was required, or on PBMCs.

#### **HLA typing by PCR –**

To enable use of the appropriate CMV tetramers the HLA type had to be more fully defined. HLA types A\*01, A\*02, B\*07, B\*08, and B\*035 were determined by a PCR based method as previously described (Bunce, O'Neill et al. 1995).

A master mix (70.87µl TDMH, 35µl nuclease-free water, 2uM of APC control primers (ATGATGTTGACCTTTCCAGGG) and (TTCTGTAACCTTTTCATCAGTTGC) and 3.75 units of Taq polymerase (Bioline)) 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 various primer combinations, as per table 2.10.3.1. A thermal cycler was used to for the following heating programme; 60 seconds (95.0°C), 5 cycles of 25 seconds (94.0°C), 45 seconds (70.0°C), 45 seconds (72.0°C) followed by 21 cycles of 25 (96.0°C), 50 seconds (65.0°C), 45 seconds (72.0°C) followed by 4 cycles of 25 seconds (96.0°C), 60 seconds (55.0°C) and 120 seconds (72.0°C), then 72.0°C for 5 minutes before being held at 10°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. The gels

were run for approximately 40 minutes at 120V and then visualised using an ultraviolet transilluminator.

*Table 2.10.3 – Primers for HLA typing (Alta Biosciences, UK)*

<b>HLA</b>	<b>Sense primer sequence</b>	<b>Anti-sense primer sequence</b>	<b>Final concentration (uM)</b>
A1	5-CGACGCCGCGAGCCAGAA	5-AGCCCGTCCACGCACCG	3.3
A2	5-GTGGATAGAGCAGGAGGGT	5-CCAAGAGCGCAGGTCCTCT	3.3
B7	5-GGAGTATTGGGACCGGAAC	5-TACCAGCGCGCTCCAGCT	3.3
B7	5-GAGCCTGCGGACCCTGCT	5-TACCAGCGCGCTCCAGCT	3.3
B8	5-GACCGGAACACACAGATCTT	5-CCTCCAGGTAGGTAGGCTCTGTC	7.7
B8	5-GACCGGAACACACAGATCTT	5-CCGCGCGCTCCAGCGTG	7.7
B35	5-GGAGTATTGGGACCGGAAC	5-GCCATACATCCTCTGGATGA	3.3
B35	5-GCCGCGAGTCCGAGGAC	5-GCGCAGGTTCCGCAGGC	3.3
B35	5-GACCGGAACACACAGATCTT	5-GAGGAGGCGCCCGTCG	3.3
		5-GGAGGAAGCGCCCGTCG	
Positive Control	5-TGCCAAGTGGAGCACCCAA	5-GCATCTTGCTCTGTGCAGAT	3.3
No DNA Control	5-TGCCAAGTGGAGCACCCAA	5-GCATCTTGCTCTGTGCAGAT	3.3

#### **2.10.4 Real time PCR for fetal (male) cells by DYS14**

A Taqman real time PCR assay for the DYS14 gene was used to detect and quantify male DNA, relative to total cellular DNA, in maternal peripheral blood and plasma. This assay was based on previously described protocols (Zimmermann, El-Sheikhah et al. 2005; Zhong,

Holzgreve et al. 2006). Amplification of DYS14 and beta-globin was performed separately as single-plex reactions. The primer and probe sequences were as follows;

DYS14 forward: 5'-GGG CCA ATG TTG TAT CCT TCT C-3'

DYS14 reverse: 5'-GCC CAT CGG TCA CTT ACA CTT C-3'

DYS14 probe FAM-MGB probe : 5'-TCT AGT GGA GAG GTG CTC-3'

Beta-globin forward: 5'-GTG CAC CTG ACT CCT GAG GAG A -3'

Beta-globin reverse: 5'-CCT TGA TAC CAA CCT GCC CAG 3'

Beta-globin probe VIC – MGB probe: 5'-AAG GTG AAC GTG GAT GAA GTT GGT GG -3'

(Primers obtained from Alta Bioscience, UK, Probes from Applied Biosystems)

The DYS14 assay was performed in 25 µl containing 12.5 µl of Taqman Universal master mix (PE Biosystems), 0.3 µl of forward and reverse DYS14 primers (300 nM each), 0.25 µl of FAM-labeled DYS14 probe (100 nM), 6.65 µl of water, and 5 µl of test DNA. The B2M assay was also performed in 25 µl containing 12.5 µl of Taqman Universal master mix (PE Biosystems), 0.3 µl of forward and reverse B2M primers (300 nM each), 0.25 µl of VIC-labeled B2M probe (100 nM), 6.65 µl of water, and 5 µl of test DNA.

DNA amplification was carried out on the PE-ABI 7500 sequence detection system. After activation of the uracil-N-glycosylase (2 min at 50°C) and Amplitaq Gold (10 min at 95°C), amplification was carried out over 40 cycles (15s at 95°C, 60 s at 60°C). A fluorescence threshold value was determined as 10 standard deviations above the mean of the background fluorescence emission for all wells between cycle 3 and 15. Fluorescence less than this threshold after 40 cycles was determined to be a negative result.

Standard curves for DYS14 and beta-globin were produced using DNA extracted from male PBMCs and quantified spectrophotometrically (Nanodrop, Thermo Scientific) and the DNA concentration calculated based on each cell containing 6.6pg DNA. 10 fold dilutions were prepared ranging from  $10^7$  to 1 genomic equivalents per reaction. These standards were included in duplicate in every assay and a standard curve constructed from which the copy number for patient samples could be extrapolated. These standard curves demonstrated the assay was sensitive to 1 copy per reaction and linear to at least  $10^7$  copies. In each assay samples were prepared in duplicate with 2 male positive controls, 2 negative controls from women with no previous sons and 2 water controls in each assay.

## **2.11 Flow cytometry**

### **2.11.1 Instrument**

Flow cytometry was conducted on an LSRII flow cytometer (BD Biosciences). The instrument was set-up with a blue laser (488nm), violet laser (405nm) and red laser (633nm). Filters were as follow: 488nm; 695/40, 610/20, 530/30, 780/60, 660/20, 575/26, 480/10. 405nm; 605/12, 560/20, 450/50, 655/8. 585/15, 525/50; 633nm; 780/60, 660/20, 730/45.

### **2.11.2 Compensation and acquisition**

The cytometer was calibrated daily with cytometer setup and tracking beads (BD Biosciences). Single colour compensation control beads (BD Biosciences) were included for each experiment and offline automated compensation (FACS DIVA version 6.1.3) was used. The offline compensation set-up was specified so the unstained tube was not used to



obtain the negative fluorescence, but instead negative and positive fluorescence was obtained separately for each single colour compensation.

## **2.12 Statistical analysis**

Statistical analysis was performed using GraphPad Prism version 5 (GraphPad Software). To determine differences between two groups, a non parametric Mann-Whitney test was performed. When analysing more than 2 groups a non-parametric Kruskal–Wallis test was used, with a Dunn post-test to compare all groups. Other tests are specified when used. The null hypothesis was rejected at a p value of  $<0.05$ .

## **Chapter 3.**

# **Modulation of maternal cellular immunity during human pregnancy**

### 3 Introduction

To understand the maternal cellular immune response to fetal antigens one is first required to appreciate how the maternal cellular immune system overall is modulated by the unique immunological environment of pregnancy. Whilst there has been much written on this topic (Section 1.2) the continual improvement in the immunological techniques and of our understanding of T cell biology means that this field of the maternal T cell response to pregnancy is constantly changing and requires re-evaluation.

I studied modulation during pregnancy of the major T cell subsets, including Th17 subsets and regulatory T cells. Th17 cells were initially characterised in 2006 but as yet little is known about their modulation during gestation or their role in pregnancy. Improvements in techniques to identify and classify regulatory T cells meant that a reassessment of this subset during pregnancy with state of the art and historical methods was particularly important.

We also studied how the maternal immunological changes that occur in women with “latent” CMV infection modulates the CD8<sup>+</sup> T cell repertoire and the changes during healthy gestation. Cytomegalovirus (CMV) infects the majority of the human population and establishes lifelong persistence. Furthermore, cytomegalovirus is a very immunodominant antigen, which can lead to the oligoclonal expansion of differentiated T cells and has been shown outside of pregnancy to have a profound effect on the T cell repertoire. Also, primary CMV infection and, less commonly, secondary infection during pregnancy can cause fetal disease and morbidity. The balance between maternal immune competence and viral pathogenicity is thus delicately poised and clinically important. This section of the chapter relating to CMV was recently published (Lissauer, Choudhary et al. 2011).

### 3.1 T cell effector subsets during pregnancy and the postnatal period

T cell effector subsets in maternal peripheral blood during uncomplicated human pregnancy were evaluated through a combination of intracellular staining and the assessment of surface chemokines.

Intracellular staining for the cytokines IFN  $\gamma$  and IL-17 was performed on CD4 T cells, following stimulation with PMA-Ionomycin, to define the T cell effector subsets TH1 and TH-17 respectively. Simultaneously the intracellular expression of IFN  $\gamma$  and IL-17 on CD8 T cells was determined (See section 5.6). A representative example of the gating strategy used is shown (Fig 3.1.1 a). Due to the downregulation of CD4 upon stimulation it was necessary for CD4 T cells to be defined on the basis on CD3+CD8<sup>-</sup> expression, whilst CD8 T cells were defined as CD3+CD8<sup>+</sup> (Fig 3.1.1 b). Finally, IFN  $\gamma$  was visualised against IL-17 and isotype controls were used to determine gating thresholds, as shown (Fig. 3.1.1 c).

Th17 cells (defined by flow cytometric markers as CD3+CD8-IL17+) were characterised in a cohort of women, with normal pregnancies, with samples obtained in each trimester and the postnatal period (n=71) and the finding presented in a cross-sectional manner (Fig. 3.1.2 a). For a subset of 17 women in whom multiple samples were prospectively obtained the results have been analysed longitudinally (Fig. 3.1.2 b). Cross sectional analysis does not demonstrate any significant difference in the proportion of TH17 cells throughout gestation. However, longitudinal analysis (i.e prospective evaluation in the same subjects with changing gestation) reveals that there was a significant elevation in the frequency of Th17 cells in the 1<sup>st</sup> trimester, compared to the 2<sup>nd</sup> trimester ( $p < 0.05$ ). In 71% of the donors the level of Th17 cells fell between the 1<sup>st</sup> and 2<sup>nd</sup> trimester.

The findings for Th1 cells (CD3+CD8-IFN  $\gamma$ +) were analysed using a similar strategy. Cross sectional analysis demonstrated no significant difference in the frequency of TH1 cells across gestation (Fig. 3.1.3 a) and similarly no differences were observed when longitudinal analysis of changes in TH1 frequency was carried out within individuals (Fig. 3.1.3 b).

The ideal definition of Th2 cells would have been a functional characterisation based on the production of Th2 cytokines, but technical difficulties with this meant that Th1 and Th2 subsets were further defined by the surrogate markers of the surface chemokine receptors CXCR3 (Th1) and CCR4 (Th2). Gating to identify CD4 T cells was carried out as before (Fig. 3.1.1 a,b), but on an unstimulated sample, as stimulation led to the down-regulation of surface chemokine receptors. CXCR3 and CCR4 expression were then visualised and gating thresholds determined by FMO controls (Fig. 3.1.4a). The expression of these chemokine receptors was analysed by 2 methods. In Fig. 3.1.4 b the percentage of cells classified as Th1 or Th2 on the basis of being CXCR3+ or CCR4+ is illustrated by gestation. In Figure 3.1.5 the expression level of the markers is quantified by MFI. There was no statistically significant difference seen across gestation and the postnatal period in the percent or level of expression of these chemokine receptors. There was a trend towards a reduction in the percent of CD4 T cells expressing CXCR3 but this was not statistically significant (4.5% to 2.6%).

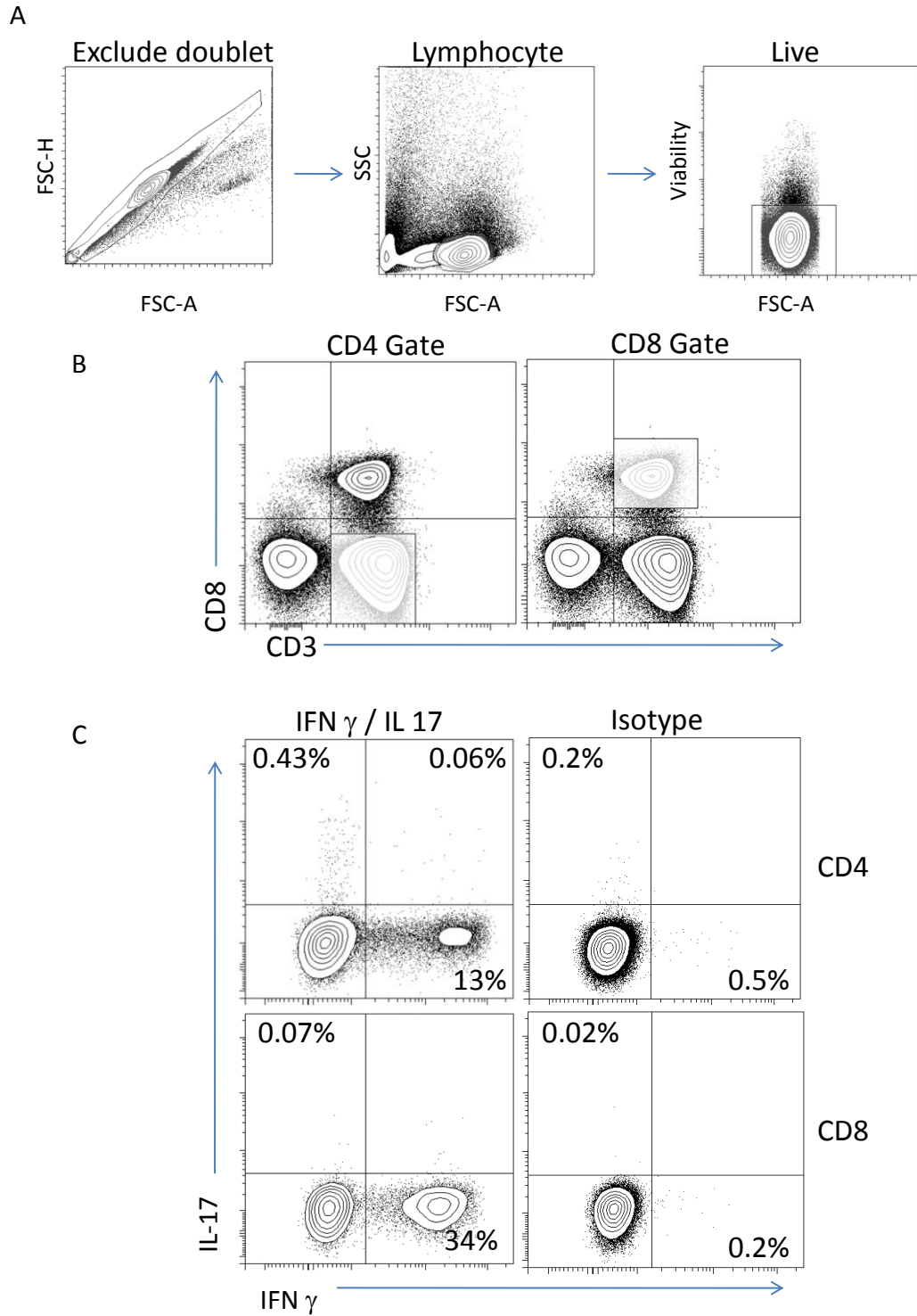


Figure 3.1.1. The gating strategy for the identification of TH1 and Th17 cells.

Gating involved the exclusion of doublets, gating of lymphocytes on the basis of their forward and side scatter properties and then the exclusion of dead cells (a). CD8 T cells and CD3+CD8- T cells were then selected for further examination (b). Finally IFN  $\gamma$  and IL-17 production were visualised for the CD8 and CD3+CD8- gates, alongside appropriate isotype controls (c).

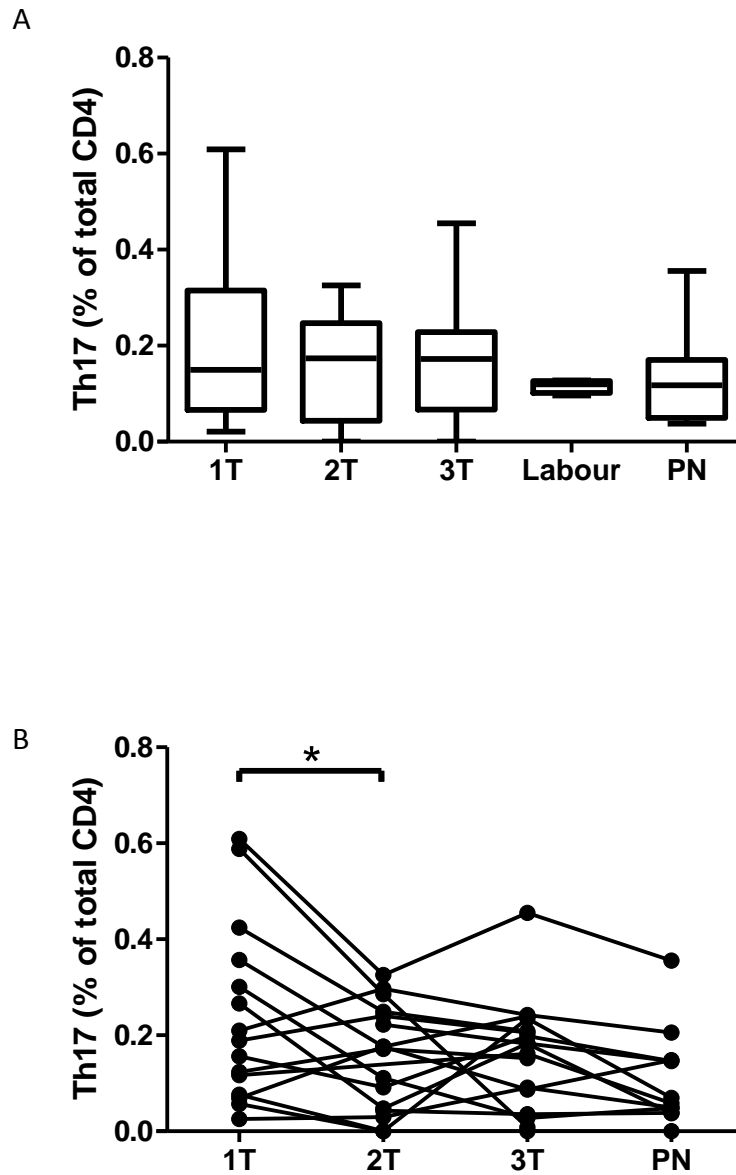
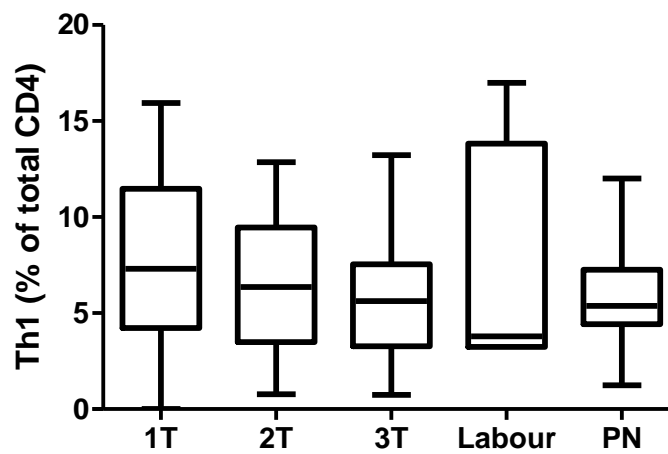


Figure 3.1.2 Th17 cells during pregnancy and the postnatal period.

The percentage of TH17 cells (CD3+CD8-IL17+) is illustrated in a cross-sectional cohort ( $n=71$ ) of women bled during pregnancy in the 1<sup>st</sup> trimester (1T), 2<sup>nd</sup> trimester (2T), 3<sup>rd</sup> trimester (3T), in labour and the post natal period (a). In a subset of women ( $n=17$ ) responses were followed longitudinally each trimester and in the postnatal period (b). (Lines represent median, boxes represent inter-quartile range and whiskers range,  $*=p<0.05$ , by repeated measures ANOVA with Tukey post-hoc test)

A



B

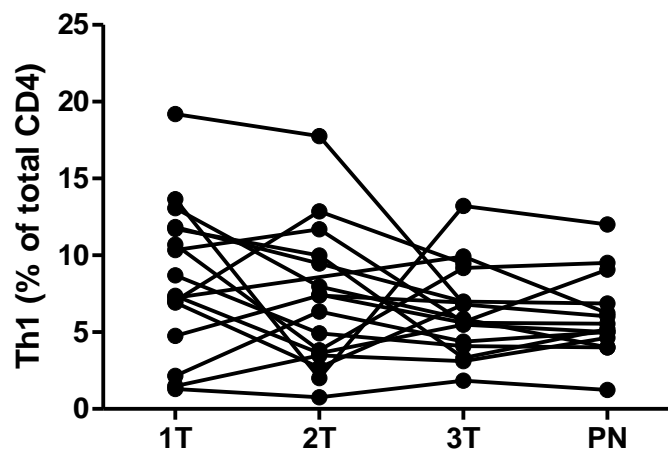


Figure 3.1.3 Th1 cells during pregnancy and the postnatal period.

The percentage of TH1 cells (CD3+CD8-IFN  $\gamma$ +) is illustrated in a cross-sectional cohort ( $n=71$ ) of women bled during the 1<sup>st</sup> trimester (1T), 2<sup>nd</sup> trimester (2T), 3<sup>rd</sup> trimester (3T), in labour and the post natal period (PN). In a subset of women ( $n=17$ ) responses were followed longitudinally with the individual rebled each trimester and in the postnatal period (b) (Lines represent median, boxes represent inter-quartile range and whiskers range, analysis by repeated measures ANOVA with Tukey post-hoc test, no statistically significant changes in Th1 % were seen by gestation ).



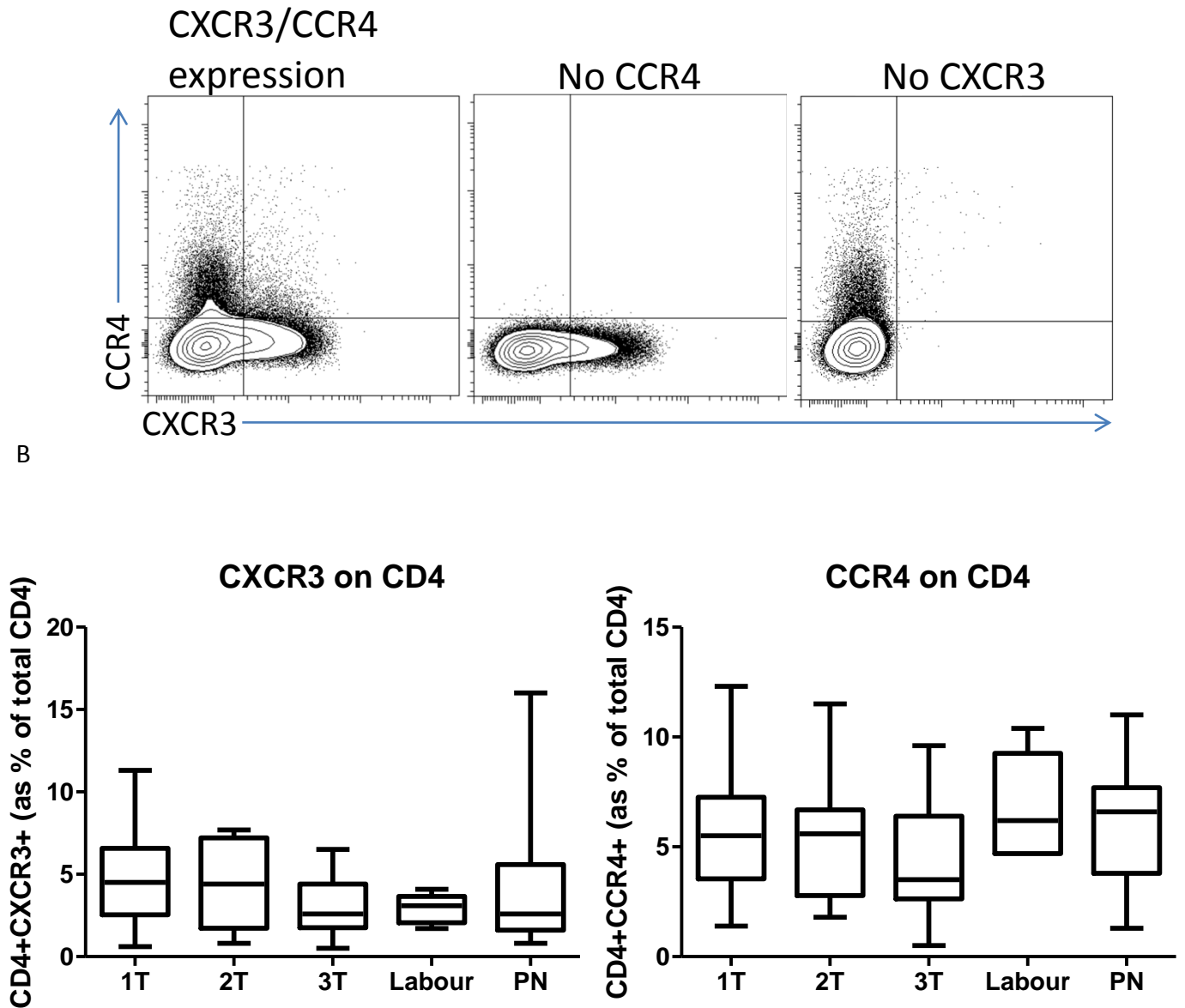


Figure 3.1.4 Th1 and Th2 cells during pregnancy and the postnatal period classified by surface chemokine receptor expression.

The percentage of TH1 and Th2 cells, classified on the basis of CXCR3 and CCR4 chemokine surface expression respectively is illustrated. The gating thresholds were determined by FMO controls as demonstrated. (a) Analysis of a cross-sectional cohort of 74 women bled during the 1<sup>st</sup> trimester (1T), 2<sup>nd</sup> trimester (2T), 3<sup>rd</sup> trimester (3T), in labour and the post natal period (PN) was conducted. (Lines represent median, boxes represent inter-quartile range and whiskers range)



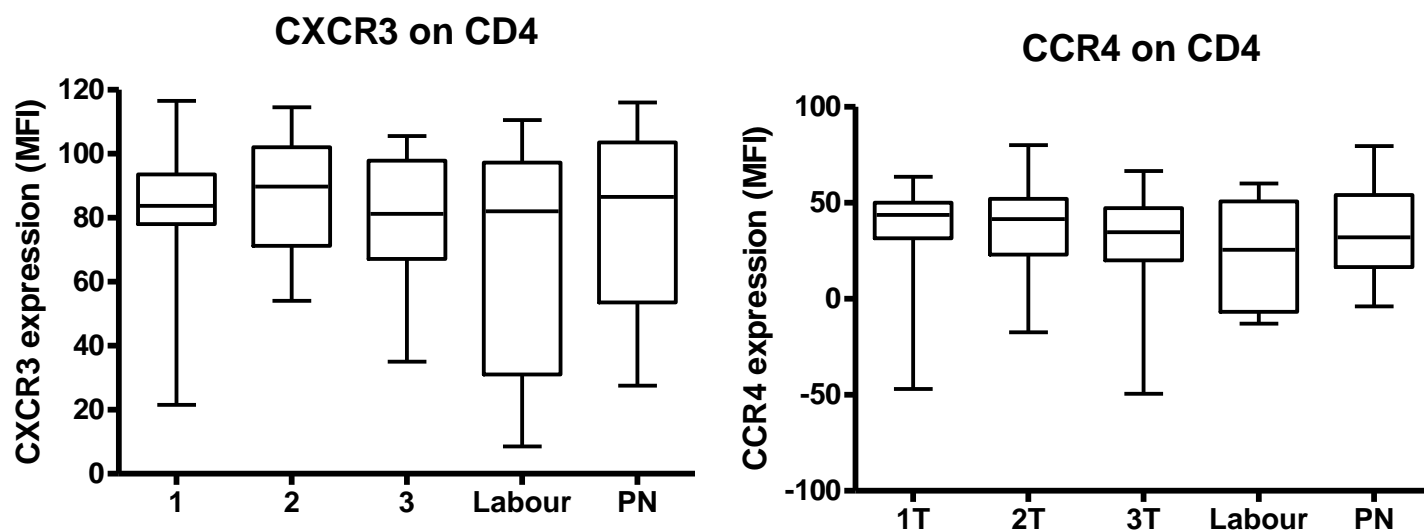


Figure 3.1.5 Chemokine receptor expression assessed by median fluorescent intensity

The expression of the chemokines CXCR3 and CCR4 on CD4 (CD3+CD8-) T cells was also assessed by median fluorescent intensity (MFI). In women (n=74) during the 1<sup>st</sup> trimester (1T), 2<sup>nd</sup> trimester (2T), 3<sup>rd</sup> trimester (3T), in labour and the post natal period (PN). (Lines represent median, boxes represent inter-quartile range and whiskers range)

### **3.2 Regulatory T cell characterisation during pregnancy and the postnatal period**

We sought to identify changes in regulatory T cells in maternal peripheral blood during human pregnancy. The identification of regulatory T cells (T regs) was done with the most robust current methodology, involving a combination of markers to identify T regs as; CD3+CD4+CD25+CD127-FOXP3+ cells. However, the reproductive immunology literature has, as the understanding of T reg biology advanced over time, previously used a variety of other means of identifying T regs during pregnancy. We therefore also examined and contrasted these previous systems, such as classification on the basis of CD3+CD4+CD25+ and CD3+CD4+CD25<sup>high</sup>, with and without the measurement of FOXP3. The flow cytometric gating strategy to define these subgroups is illustrated in Figure 3.2.1.

T regs in maternal peripheral blood, characterised with the most robust strategy available (CD3+CD4+CD25+CD127-FOXP3+) did not differ in frequency during pregnancy compared to a non-pregnant age and parity matched control cohort (median 2.25%, n=46 v median 2.37%, n=18, p=0.74) (Fig. 3.2.2 a). If this data was examined by trimester of pregnancy, rather than pooling all timepoints in pregnancy, it again showed no significant difference at any gestation (Fig. 3.2.4 a). Prospective longitudinal analysis was also conducted in a subgroup of donors who underwent repeat venesection at multiple timepoints within the pregnancy (n=14 in all 3 trimesters, n=6 postnatally) and this enabled analysis of any changes during gestation within individual donors, confirming there was no significant difference in peripheral T reg numbers during pregnancy (3.2.2 b).

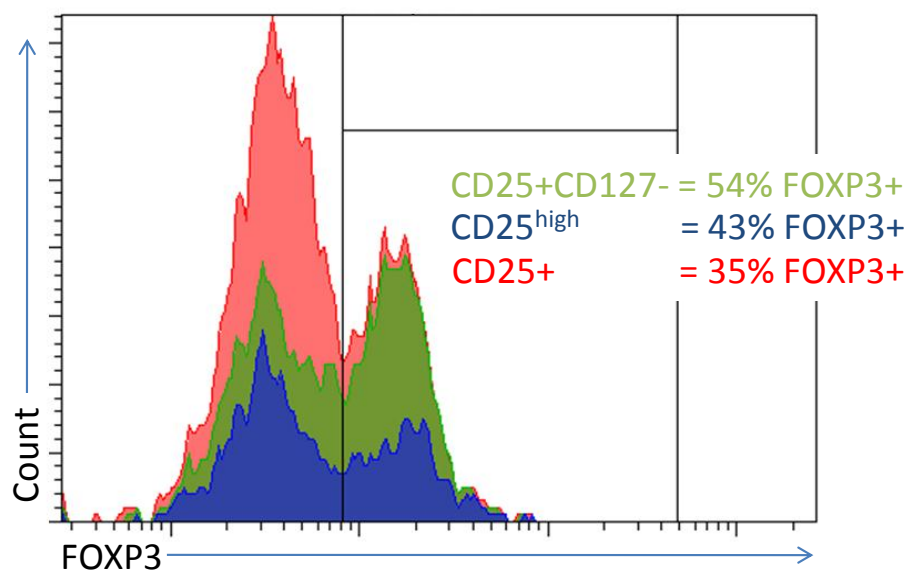
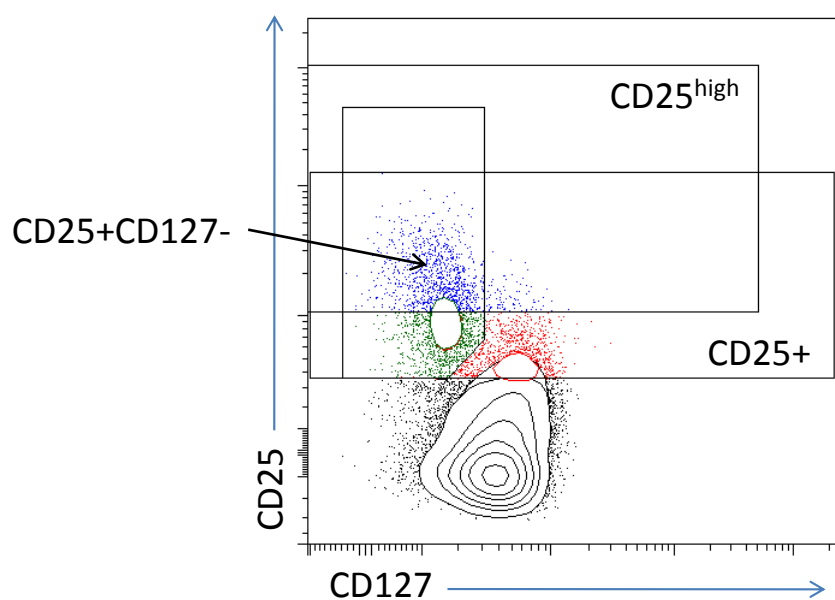
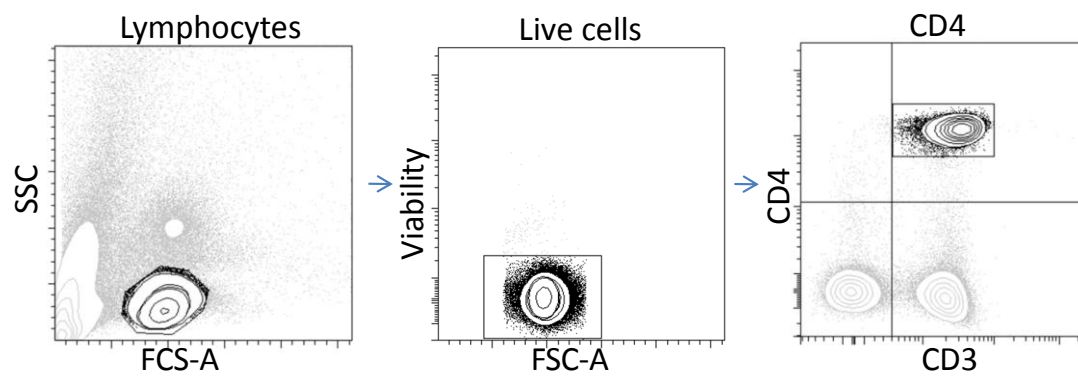
Due to the importance of the transcription factor FOXP3 as the key T reg transcription factor and regulator of T reg development and function we measured what proportion of CD4 T cells were FOXP3 positive throughout gestation (Zheng and Rudensky 2007). Again there was no difference in the proportion of FOXP3 expressing CD4 T cells during pregnancy compared to controls (6.8 v 5.7,  $p=0.2398$ ) (Fig. 3.2.3 a). There was also no difference in frequency if the pregnancy timepoints were grouped by each trimester (Fig. 3.2.3 b), or if a longitudinal analysis of individual patients was conducted (Fig. 3.2.3 c).

We then sought to examine the frequency and distribution of T regs by other methods used historically and reported in the reproductive immunology literature (Section 1.2.3). We thus contrasted the frequency of T regs in each trimester and the postnatal period as CD25<sup>high</sup>FOXP3<sup>+</sup> (Fig. 3.2.4 b), CD25<sup>+</sup>FOXP3<sup>+</sup> (Fig. 3.2.4 c), CD25<sup>+</sup>CD127<sup>-</sup> (Fig. 3.2.5 a), CD25<sup>high</sup> (Fig. 3.2.5 b) and CD25<sup>+</sup> (Fig. 3.2.5 c). For none of these classifications was a statistically significant difference in the frequency of T regs seen during gestation, or comparing during and after pregnancy. There may have been a trend towards an increase in CD25<sup>+</sup>FOXP3<sup>+</sup> cells in the 2<sup>nd</sup> and 3<sup>rd</sup> trimester and in CD25<sup>+</sup> cells in the 3<sup>rd</sup> trimester and labour, but these were not statistically significant, or of a large magnitude. The consistency of these markers throughout pregnancy and the postnatal period was in fact a notable feature considering the variability in the percentage of T regs seen between different healthy individuals.

The different systems that have been used for classifying T regs were contrasted directly in Figures 3.2.4 d and 3.2.5 d. Clearly the larger the number of markers used to define the subset, and the more stringent the classification the smaller the percentage of cells classified as T regs.

Finally, the proportion of cells which were FOXP3 positive was examined in the CD3+CD4+CD25+CD127- (Fig. 3.2.6 a), CD3+CD4+CD25high (Fig. 3.2.6 b) and CD3+CD4+CD25+ (Fig. 3.2.6 c) subsets throughout gestation and the postnatal period. Whilst there was no significant difference in the proportion of FOXP3 in any of these subsets during these different time points of gestation, in all subsets there was a trend for the proportion of FOXP3 positive cells to be lower during labour.

FOXP3 expression is the best single surrogate marker available for regulatory function of T cells (Zheng and Rudensky 2007). In the absence of functional assessment analysis of the proportion of cells found by each method to be FOXP3+ therefore demonstrates the stringency of the different methods of regulatory T cell classification (Fig. 3.2.6 d). It can be clearly seen that with the use of both CD25 and CD127 to classify CD4 cells a majority will be FOXP3 positive, but using only CD25+ as a marker this falls to a median of 31%.



*Figure 3.2.1 Flow cytometric characterisation of CD4 regulatory T cells.*

*CD4 T cells were identified on the basis of gating on small lymphocytes based on their forward and side scatter properties, excluding dead cells with a viability dye and then gating on CD3+CD4+ cells. T regs were then characterised by several methods to include those used previously in the reproductive immunology literature. Using the visualisation of CD25 against CD127 the CD25+CD127- population could be readily identified. The CD25+ population was also gated separately, alongside the CD25<sup>high</sup> subset, as shown. The percentage of FOXP3 positive cells in all these populations was then determined.*



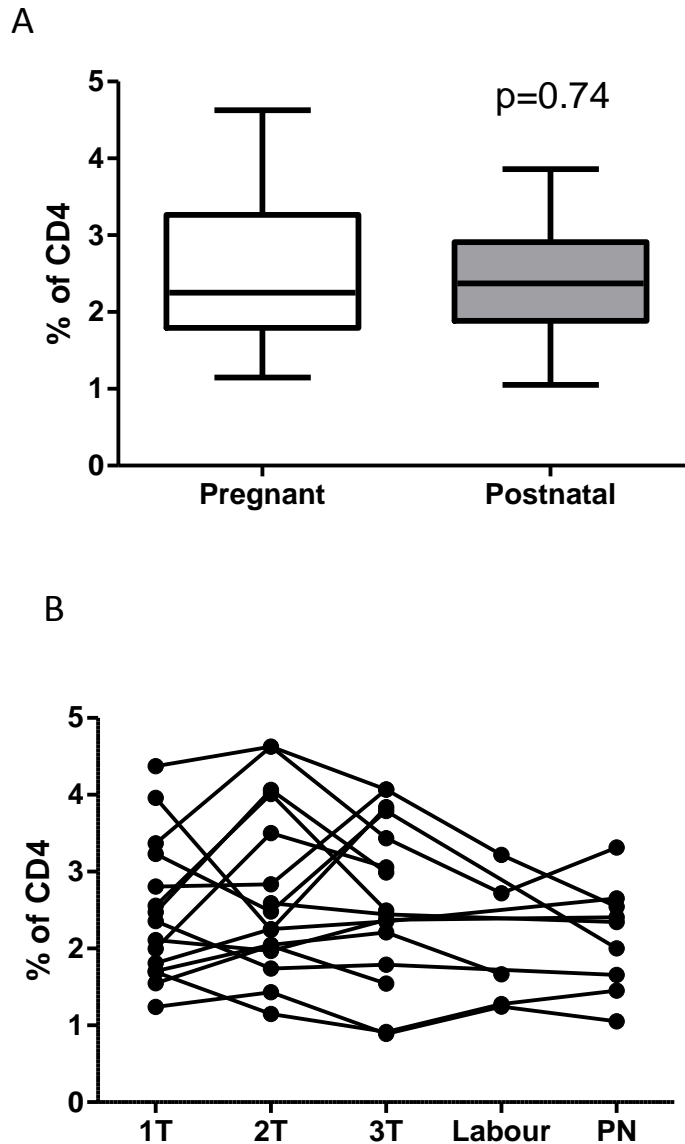


Figure 3.2.2 Characterisation of CD4+CD25+CD127-FOXP3+ regulatory T cells as a percent of total CD4 T cells throughout gestation.

The frequency of regulatory T cells defined on this basis are compared during ( $n=46$ ) and after ( $n=18$ ) pregnancy, with the results from all gestations of pregnancy pooled to see if there is any overall effect observed.

(a) A subset of donors were followed longitudinally at multiple time points within the pregnancy ( $n=16$  during pregnancy,  $n=9$  also bled postnatally) (b). 1<sup>st</sup> trimester (1T), 2<sup>nd</sup> trimester (2T), 3<sup>rd</sup> trimester (3T), in labour and the post natal period (PN) (Analysis by Mann-Whitney (a) and Kruskal-Wallis with Dunn post-hoc test (b))

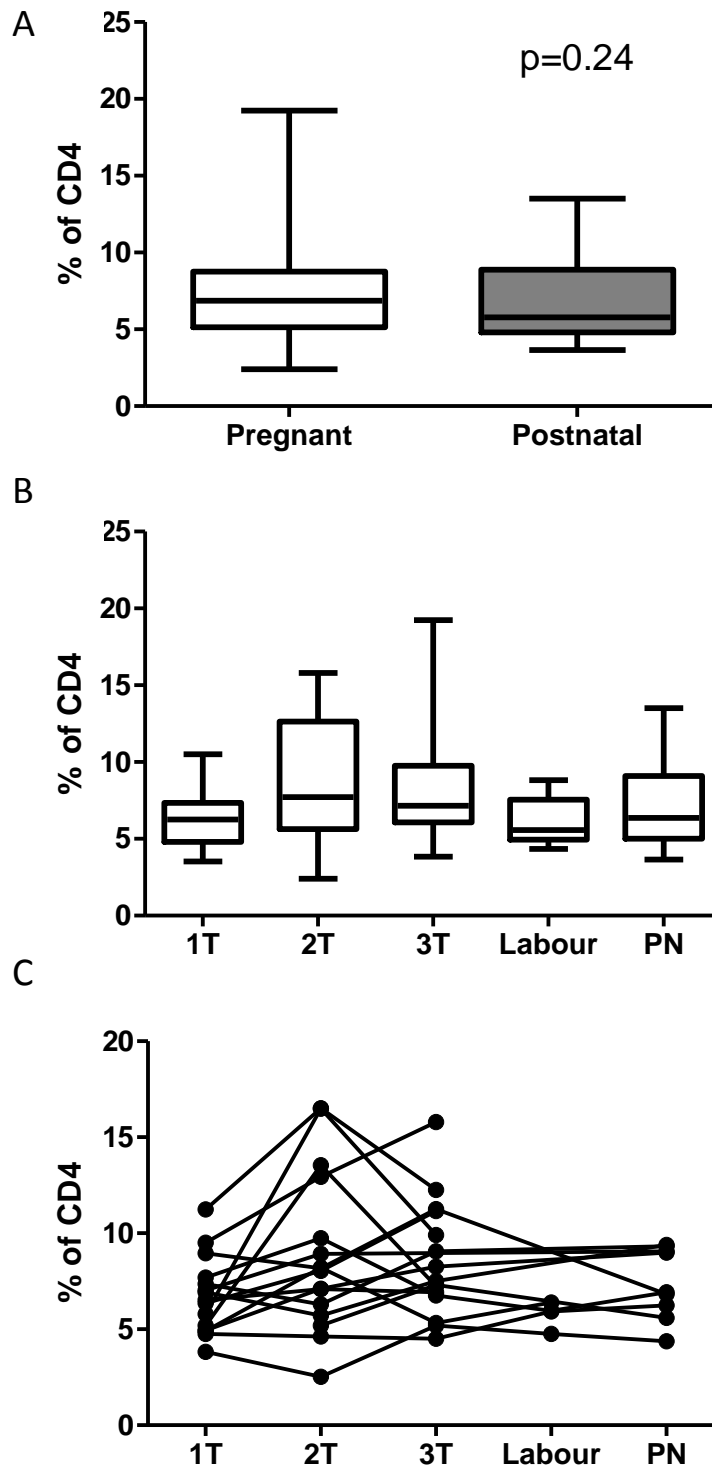


Figure 3.2.3 Characterisation of CD4+FOXP3+ T cells as a percent of total CD4 T cells throughout gestation.

The frequency of FOXP3 positive cells are compared during (n=46) and after (n=18) pregnancy, with the results from all gestations of pregnancy pooled to see if there is any overall effect observed. (a) This is also presented separated by results from the 1<sup>st</sup> trimester (1T)(n=16), 2<sup>nd</sup> trimester (2T)(n=15), 3<sup>rd</sup> trimester (3T)(n=15), in labour (n=5) and the post natal period (PN)(n=12) (b). A subset of donors were followed longitudinally at multiple time points within the pregnancy (n=16 during pregnancy, n=9 postnatally) (c). (Analysis by Mann-Whitney (a), Kruskal-Wallis with Dunn post-hoc test (b))

Figure 3.2.4 Characterisation of FOXP3 expressing T regs across gestation

The percentage of T regs as a percent of total CD4 T cells, classified in 3 ways are demonstrated. These are CD4+CD25+CD127-FOXP3+ cells (a) CD4+CD25<sup>high</sup>FOXP3+ cells (b) and CD25+FOXP3+ cells (c). Each subset is characterised across the 1<sup>st</sup> trimester (1T)(n=16), 2<sup>nd</sup> trimester (2T)(n=15), 3<sup>rd</sup> trimester (3T)(n=15), in labour (n=5) and the post natal period (PN)(n=12). Boxes represents interquartile range and whiskers represent maximum and minimum values. A summary plot then compares all three subsets simultaneously (d). In this plot the medians are displayed, with the whisker representing the quartile below the median.

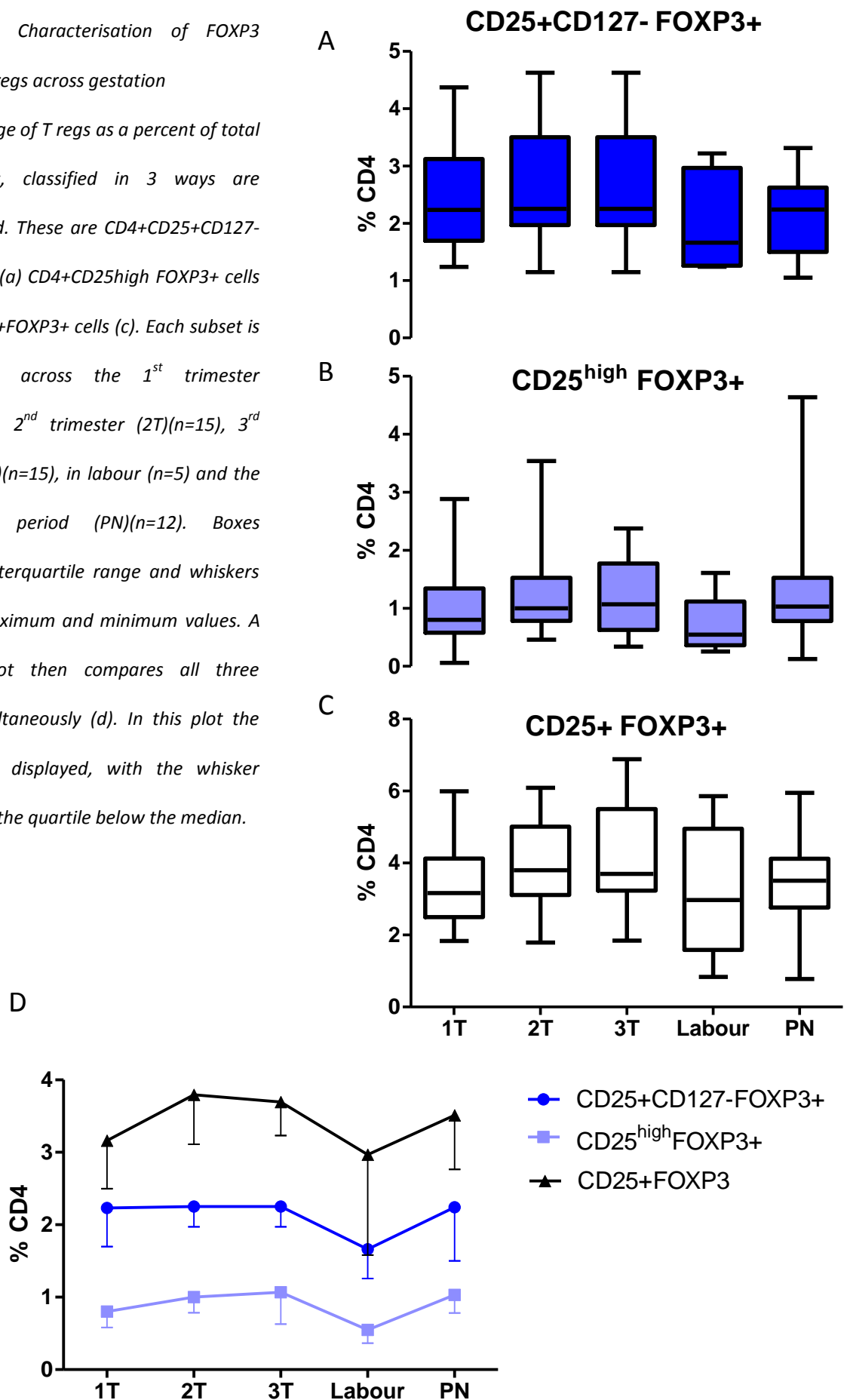


Figure 3.2.5 Characterisation of T regs on the basis of CD25 and CD127 across gestation

Putative T regs, as a percentage of CD4 T cells, classified in 3 ways are demonstrated. These are CD4+CD25+CD127- cells (a) CD4+CD25<sup>high</sup> cells (b) and CD25+ cells (c). Each subset is characterised across the 1<sup>st</sup> trimester (1T)(n=16), 2<sup>nd</sup> trimester (2T)(n=15), 3<sup>rd</sup> trimester (3T)(n=15), in labour (n=5) and the post natal period (PN)(n=12). Boxes represents interquartile range and whiskers represent maximum and minimum values. A summary plot then compares all three subsets simultaneously (d). In this plot the medians are displayed, with the whisker representing the quartile below the median.

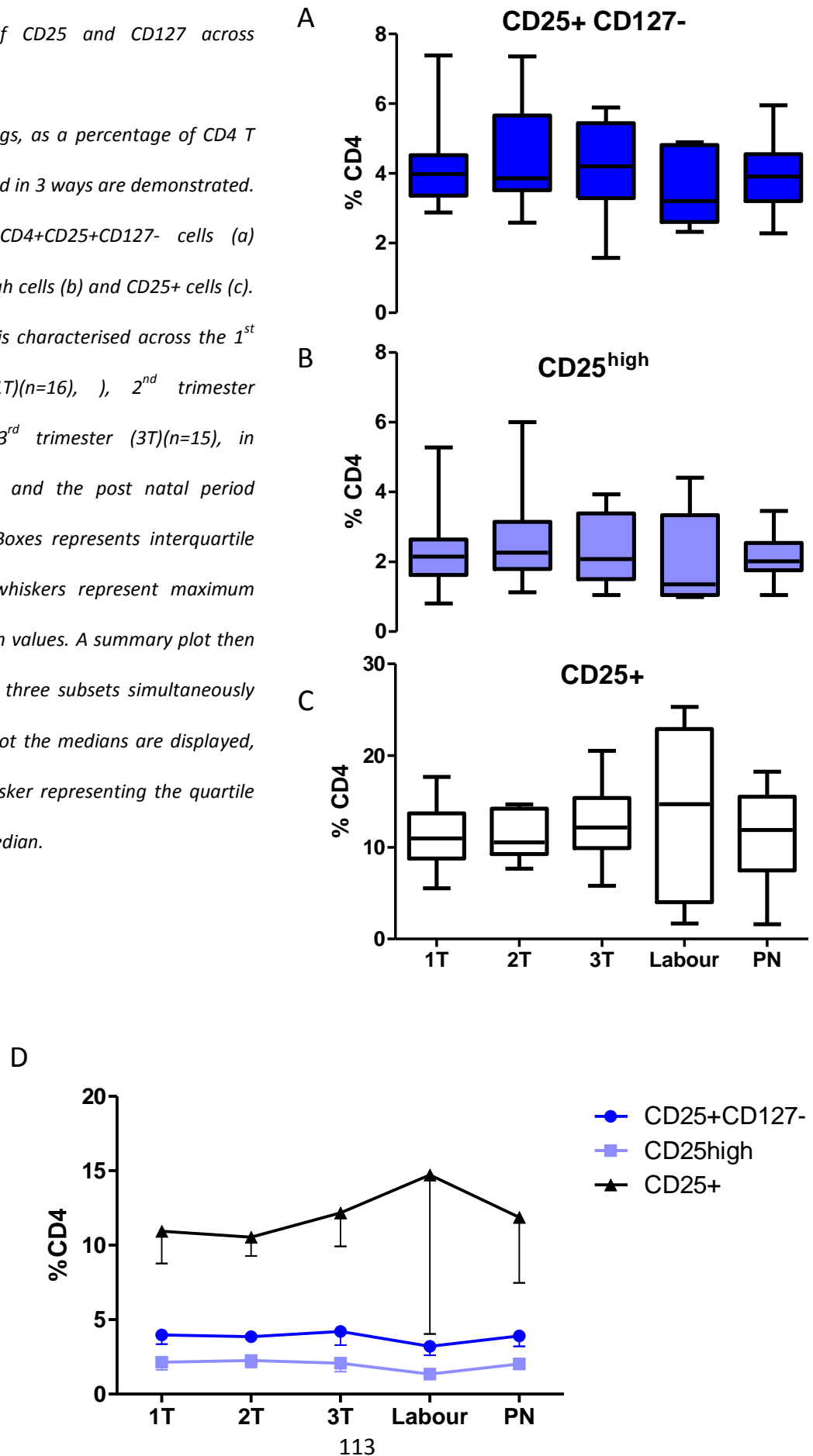
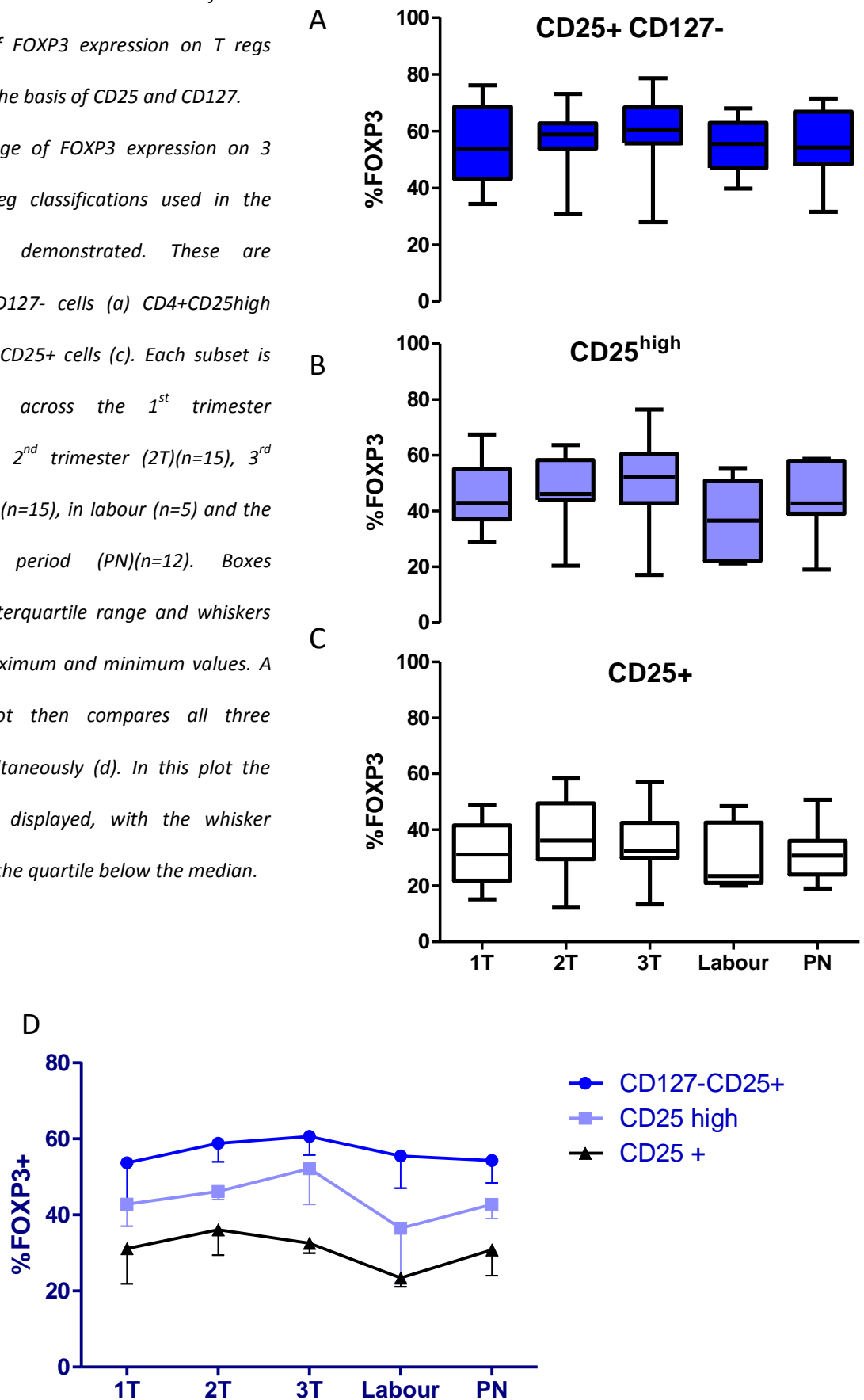


Figure 3.2.6 Characterisation of the proportion of FOXP3 expression on T regs classified on the basis of CD25 and CD127.

The percentage of FOXP3 expression on 3 putative T reg classifications used in the literature is demonstrated. These are CD4+CD25+CD127- cells (a) CD4+CD25<sup>high</sup> cells (b) and CD25+ cells (c). Each subset is characterised across the 1<sup>st</sup> trimester (1T)(n=16), 2<sup>nd</sup> trimester (2T)(n=15), 3<sup>rd</sup> trimester (3T)(n=15), in labour (n=5) and the post natal period (PN)(n=12). Boxes represent interquartile range and whiskers represent maximum and minimum values. A summary plot then compares all three subsets simultaneously (d). In this plot the medians are displayed, with the whisker representing the quartile below the median.



### 3.3 T cell activation during pregnancy

CD38 is an exonuclease and is often used as a marker of T lymphocyte activation and proliferation. We observed an increase in CD38 expression during pregnancy (Fig. 3.3.1 a). This was independent of cytomegalovirus sero-status (Fig. 3.3.1 b). In particular, the increased levels of CD38 were observed during the 3<sup>rd</sup> trimester and postnatal period ( $p < 0.05$ ) (Fig. 3.1.1 a). The use of multiparameter flow cytometry allowed us to further characterise CD38 expression on CD8<sup>+</sup> T cell subsets. CD38 expression was specifically increased in the EMRA subset and there was no statistically significant increase in naïve, effector memory or central memory subsets (Fig. 3.1.1 c). The expression level of CD38 increased by 2.5 fold on TEMRA cells from the first to the third trimester ( $p < 0.01$ ) and this effect was stable during the early post natal period (1<sup>st</sup> trimester vs early postnatal  $p < 0.001$ ).

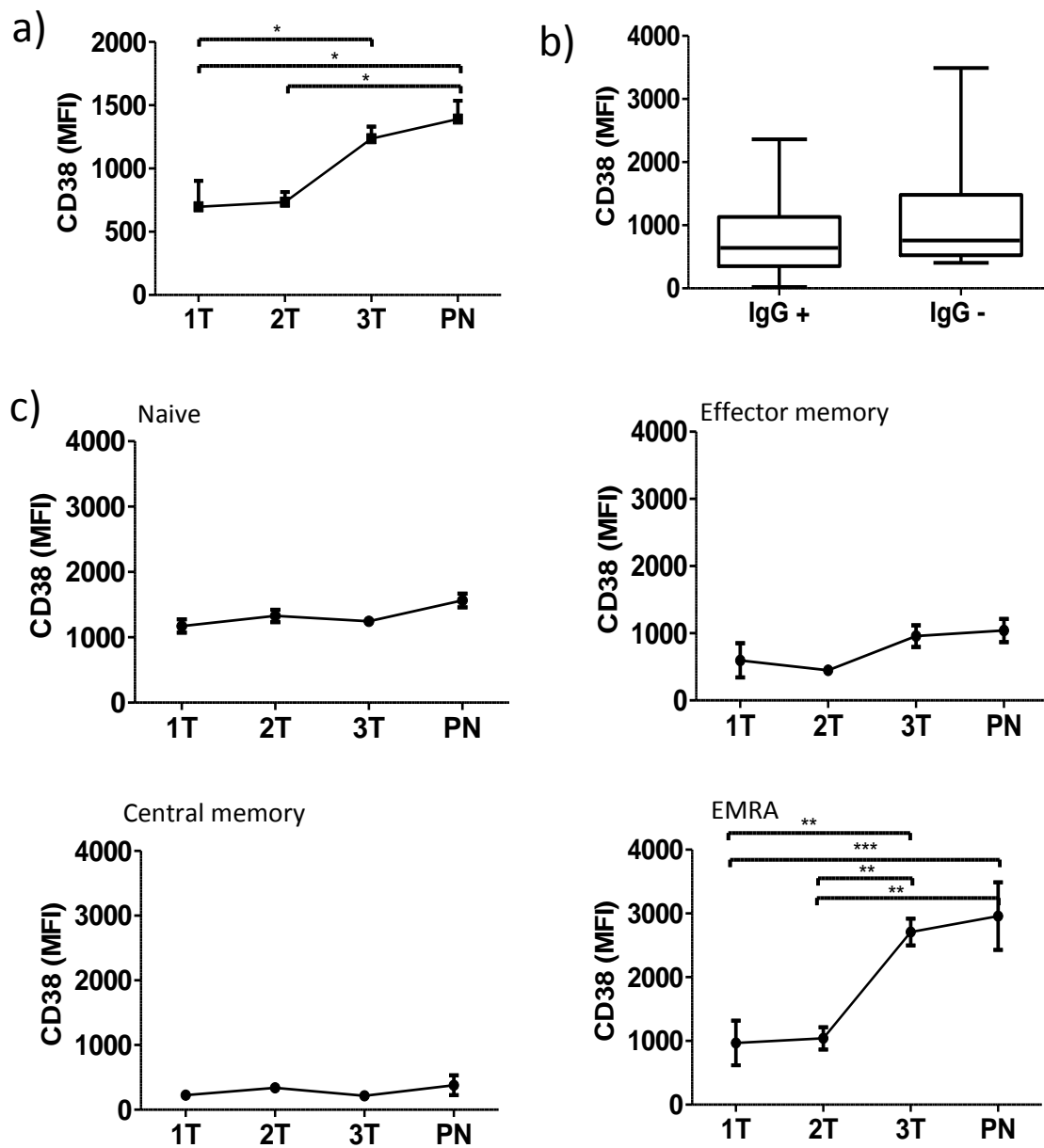


Figure 3.3.1. T cell activation measured by CD38 expression across gestation.

T cell activation increased during gestation, independent of CMV serostatus. Changes in the expression of CD38 on CD8+ T cells was investigated during gestation and the early postnatal period (n=87) (a) CD38 expression on CD8+ T cells was compared between CMV IgG sero-positive (n=39) and sero-negative (n=21) individuals during pregnancy. (b) CD38 expression on CD8+ T cells was further characterised by its expression on naïve (CD45RA+CCR7+), effector memory (CD45RA-CCR7-), central memory (CD45RA-CCR7+) and highly differentiated effector memory (EMRA, CD45RA+CCR7-) T cells (c). (T1=1<sup>st</sup> trimester, T2=2<sup>nd</sup> trimester, T3=3<sup>rd</sup> trimester, PN=Postnatal, statistical analysis by Kruskal-Wallis \* denotes  $p < 0.05$ , \*\* denotes  $p < 0.01$ , \*\*\* denotes  $p < 0.001$ )

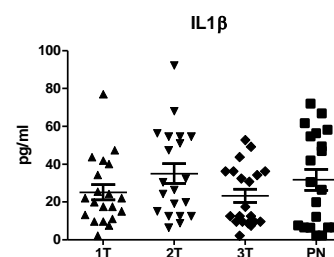
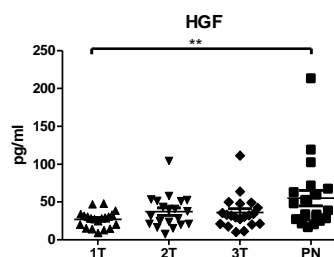
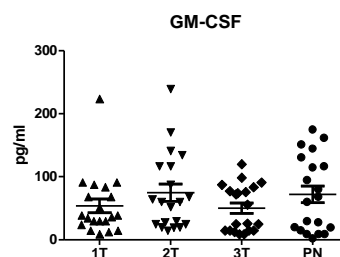
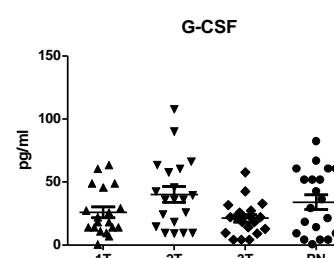
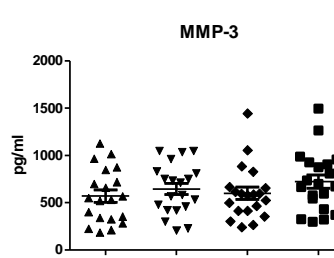
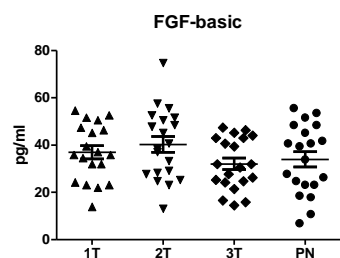
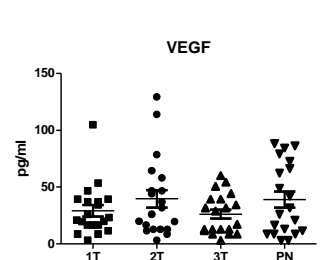
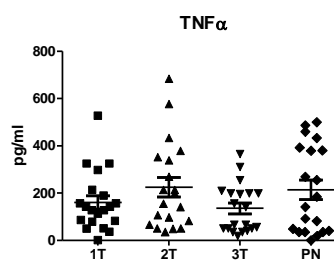
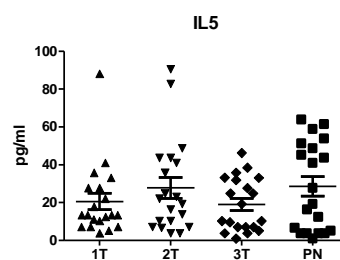
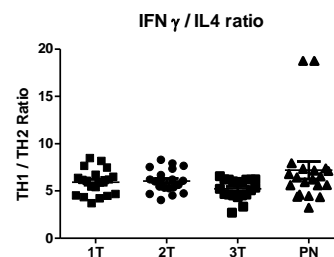
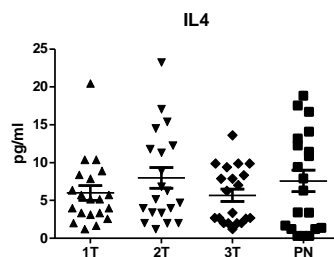
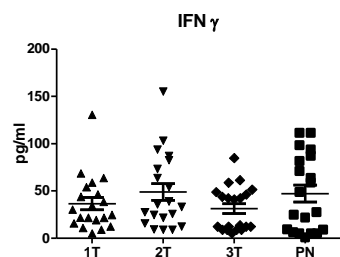
### **3.4 Maternal plasma cytokine, chemokine and chemokine receptors levels during human pregnancy**

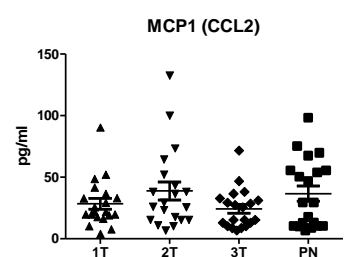
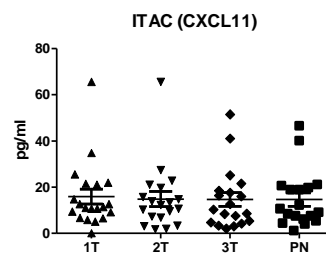
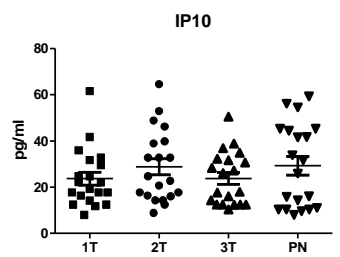
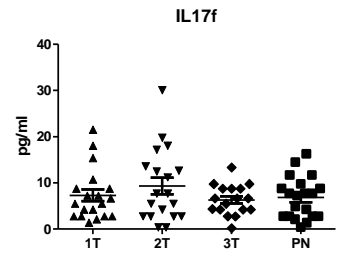
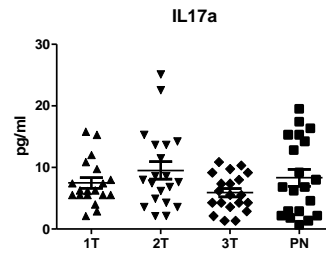
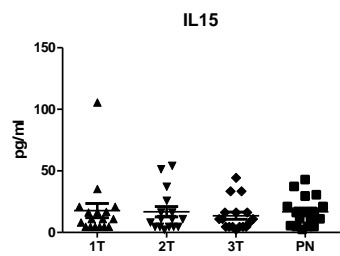
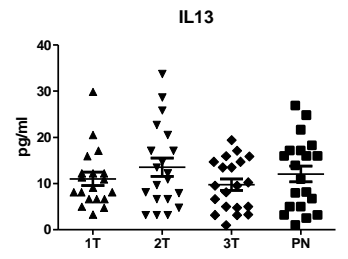
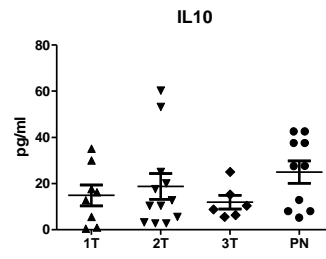
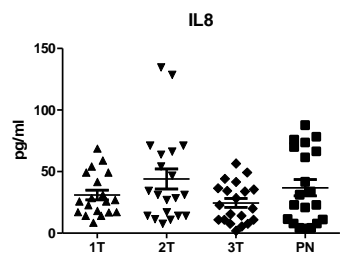
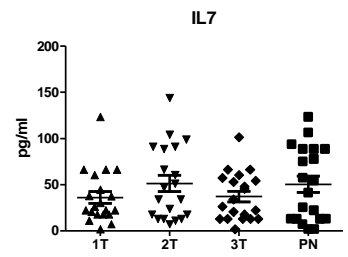
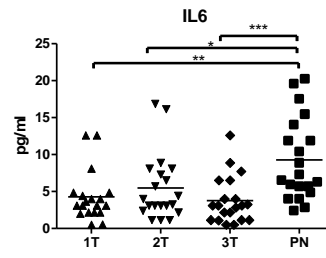
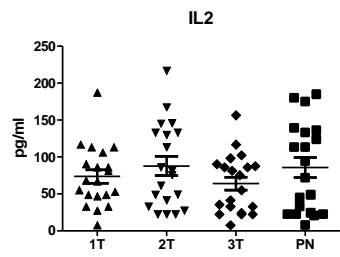
We investigated if there were alterations in the plasma level of cytokines or chemokines within maternal plasma across pregnancy and the postnatal period. In particular we wanted to investigate if the observed cellular changes in Th17 cells were associated with circulating IL-17 or related chemokines and if global changes in Th1 and Th2 cytokines were observed as pregnancy progressed.

For this measurement of plasma cytokines a cohort of women were selected for study who had plasma samples collected prospectively in each trimester and the early postnatal period (< 1 week postnatal). Therefore a total of 4 samples per participant were collected. (CMV IgG+ n=10 participants, 40 samples, CMV IgG- n=10 participants, 40 samples).

This data demonstrates the wide variation in the serum levels of these cytokines and chemokines between individuals. Interestingly, the differences between individuals, demonstrated by the wide spread of the data, were far greater than differences observed in the mean changes across the three trimesters of pregnancy. The only factors which showed statistically significant changes across gestation were hepatocyte growth factor (HGF) and IL6. These were found to be significantly elevated in the postnatal period.







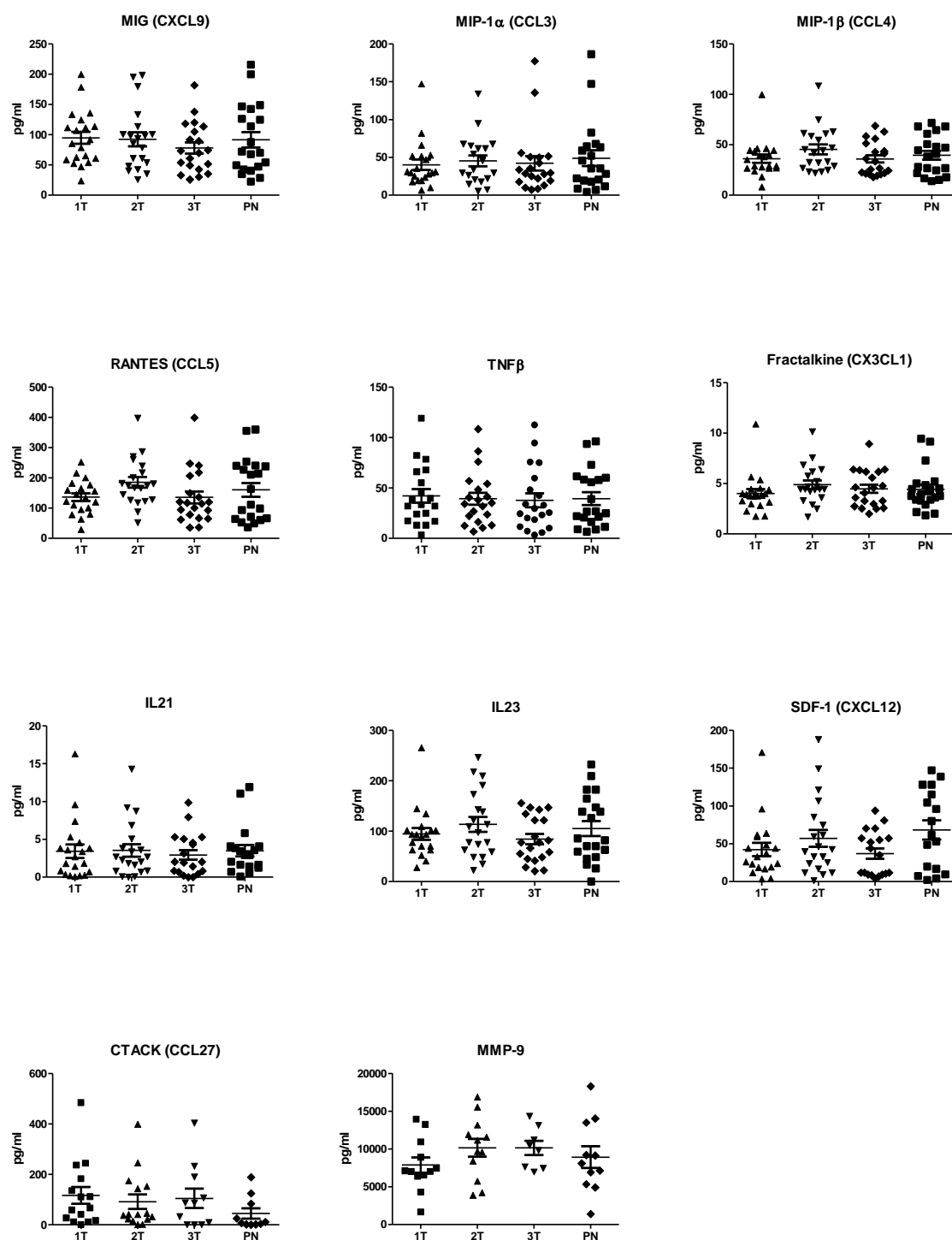


Figure 3.4.1 Plasma cytokines and chemokines during gestation and the postnatal period

34 plasma factors were measured by a luminex multiplex bead assay. Results are displayed for each trimester and the postnatal period. Values were omitted if they fell outside of the standard curve. (T1=1<sup>st</sup> trimester, T2=2<sup>nd</sup> trimester, T3=3<sup>rd</sup> trimester, PN=Postnatal, \* denotes  $p < 0.05$ , \*\* denotes  $p < 0.01$ , \*\*\* denotes  $p < 0.001$ )

### **3.5 Effect of CMV IgG sero-positivity on maternal T cell phenotype during pregnancy**

CD8<sup>+</sup> T cells were identified by a rigorous gating strategy (Fig. 3.5.1) and then classified into four functionally distinct memory subsets on the basis of expression of CD45RA and CCR7. These comprise naive (N, CD45RA<sup>+</sup>CCR7<sup>+</sup>), central memory (CM, CD45RA<sup>+</sup>CCR7<sup>+</sup>), effector memory (EM, CD45RA<sup>+</sup>CCR7<sup>-</sup>) and highly differentiated 'revertant' CD45RA<sup>+</sup> effector memory subsets (EMRA CD45RA<sup>+</sup>CCR7<sup>-</sup>) (Fig. 3.5.2 a). Flow cytometric analysis was used to determine the relative proportion of CD8<sup>+</sup> T cells within these subsets in peripheral blood samples from healthy pregnant women. These data were then related to the presence of CMV-specific antibody in order to determine how 'latent' CMV infection modified the CD8<sup>+</sup> T cell repertoire. Example contour plots from two CMV IgG sero-positive and two CMV IgG seronegative donors are illustrated in Figure 3.5.2 b and c.

CMV sero-positivity was associated with a dramatic reduction in the proportion of naive CD8<sup>+</sup> T cells which represented a median of only 23% in the CMV sero-positive group compared to 46% in CMV sero-negative donors ( $p=0.0024$ ) (Fig 3.5.2 d). The proportion of memory CD8<sup>+</sup> T cells is therefore increased from 54% in CMV sero-negative women to 77% in CMV sero-positive donors and it was of interest to determine the pattern of distribution in individual CD8<sup>+</sup> memory subsets.

CMV infection did not significantly alter the proportion of effector memory or central memory CD8<sup>+</sup> T cells (Fig 3.5.2 e,f). However, the proportion of 'revertant' CD45RA<sup>+</sup> effector cells was markedly increased in the CMV sero-positive group to a median of 38% compared to only 17% in CMV sero-negative women ( $p=0.0002$ ) (Fig 3.5.2 g). The expression

of the anti-apoptotic and co-stimulatory molecules CD27 and CD28 was also examined on the CD8 repertoire. In CMV sero-positive women there was a significant reduction in expression of both CD27 (median 59% v 87%,  $p < 0.000$ )(Fig 3.5.2 h) and CD28 (median 47% v 82%,  $p < 0.001$ ) on CD8+ T cells (Fig 3.5.2 i). In contrast, expression of CD57 which is a marker of late T cell differentiation was greatly increased in sero-positive pregnant women (median 44% v 19%,  $p < 0.001$ )(Fig 3.5.2 j).

CMV infection is associated with an increase in memory cells in healthy donors and it was therefore of interest to observe that there was no increase in the proportion of CD45RA-CCR7- effector cells in CMV sero-positive pregnant women. In order to investigate this observation in more detail we studied the expression of CD27, CD28 and CD57 on CD8+ T cells within this subset. CD27 and CD28 expression can be used to classify effector cells into 4 functionally distinct memory subsets (Romero, Zippelius et al. 2007) termed EM1 (CD27+CD28+), EM2 (CD27+CD28-), EM3 (CD27-CD28-), and EM4 (CD27-CD28+) (Fig 3.5.3 a). A representative contour plot of these populations, obtained after gating on viable CD3+CD8+CD45RA-CCR7- cells, is shown in Fig. 3.5.3 b. Expression of CD27 and CD28 is lost during CD8+ T cell expansion and differentiation, with EM1 being the least differentiated subset and EM3 representing the most differentiated compartment. This is confirmed by our analysis of CD57 expression on the four groups with highest expression of this marker being seen on EM3 cells (Fig 3.5.3 c).

When effector memory cells were classified into these four subsets and compared according to CMV serostatus, clear differences could be demonstrated between CMV sero-positive and sero-negative pregnant women (Fig 2.5.3 d). The CD45RA-CCR7- subset within CMV sero-positive women exhibited a markedly more differentiated pattern, as

demonstrated by an increase in the EM3 subset from 3% in sero-negative donors to 15% in the CMV seropositive group ( $p < 0.0001$ ). In contrast the size of the EM1 subset, which shares functional similarities with central memory cells but is resident within tissue rather than secondary lymphoid organs, was reduced in sero-positive donors (median 52% v 72%,  $p < 0.0001$ ). The EM4 subset was also increased in CMV sero-positive women (median 13% v 20%,  $p = 0.0002$ ) whereas the proportion of EM2 cells was similar in both groups (median 9% v 12%,  $p = 0.75$ ).

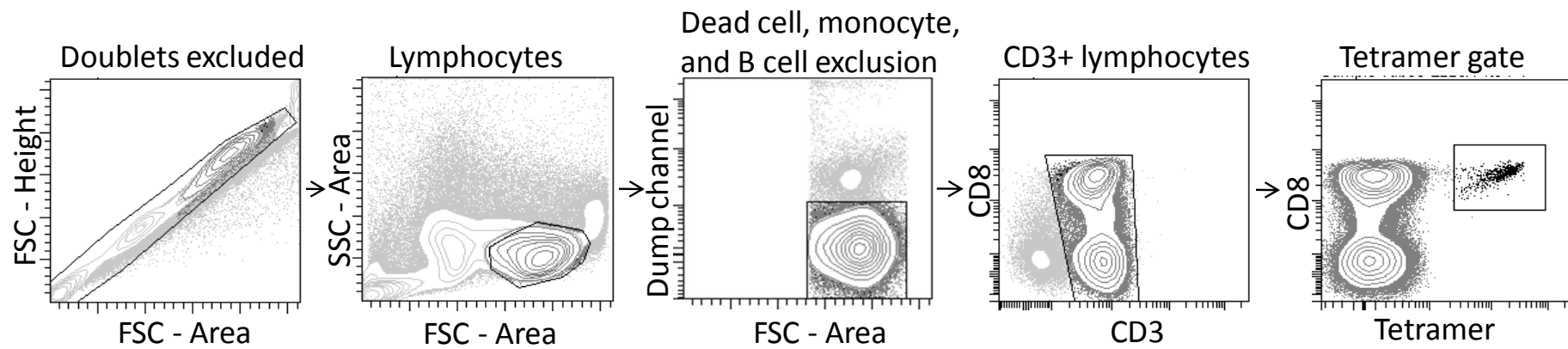
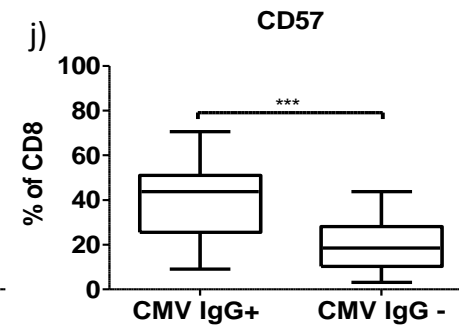
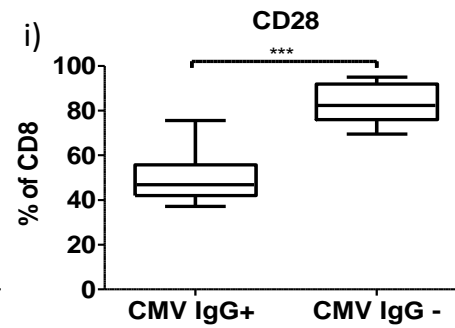
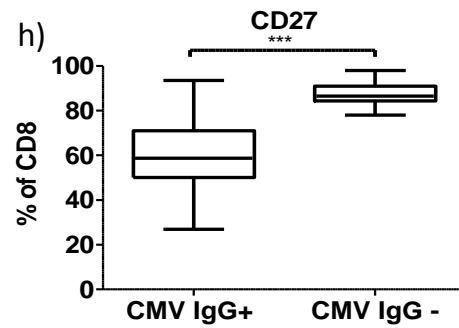
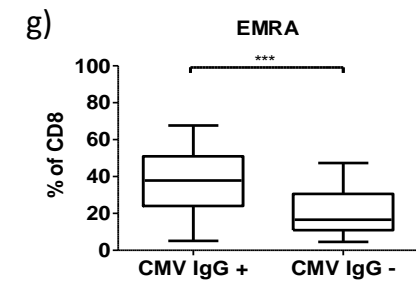
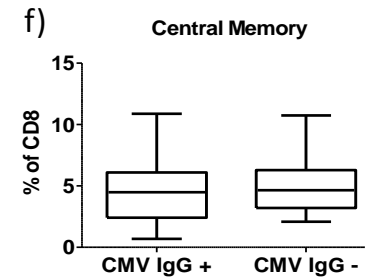
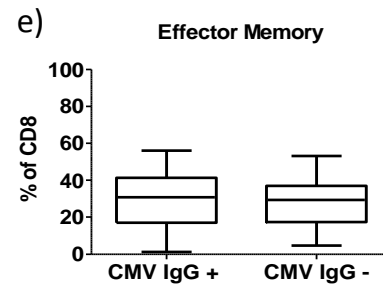
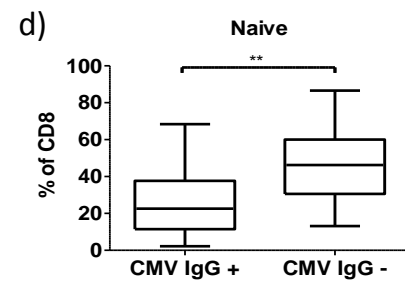
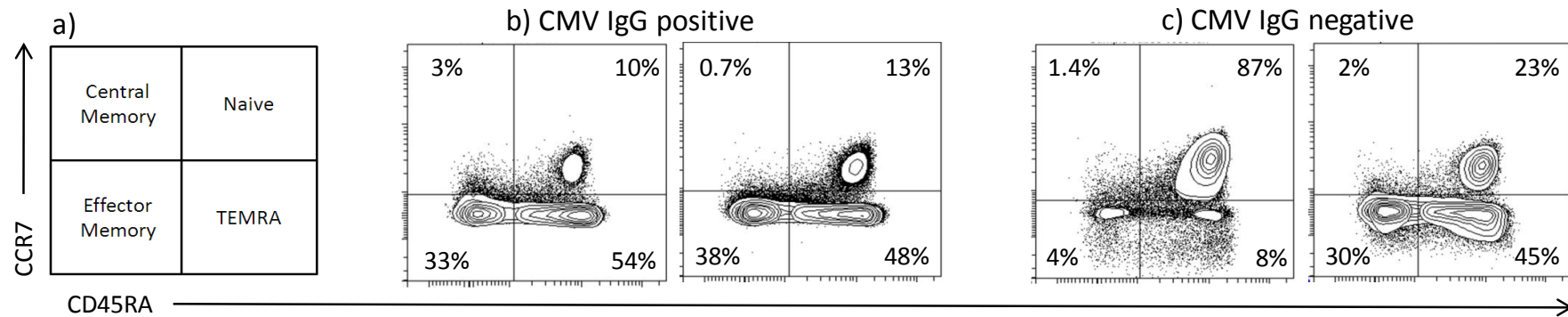


Figure 3.5.1. Gating strategy for CMV specific CD8 T cells.

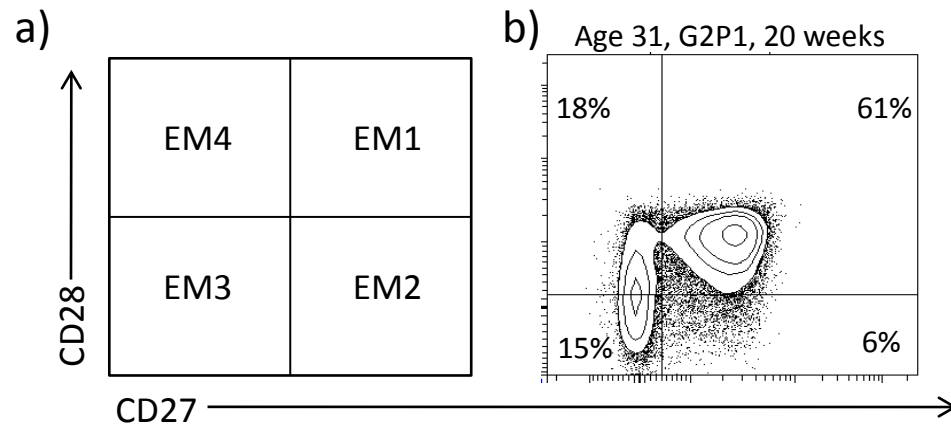
Contour plot of gating strategy involving: exclusion of doublets and other irregularly shaped cells, gating of lymphocytes on the basis of forward and side-scatter properties, a dump channel to exclude dead cells, monocytes and B cells, then selection of CD3+ lymphocytes. In this example, tetramer positive cells are then displayed against CD8. The black box gate highlights tetramer positive CD8+ T cells.



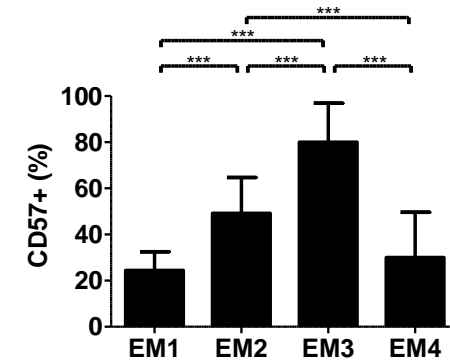


*Figure 3.5.2 T cell differentiation in CMV sero-positive and seronegative women.*

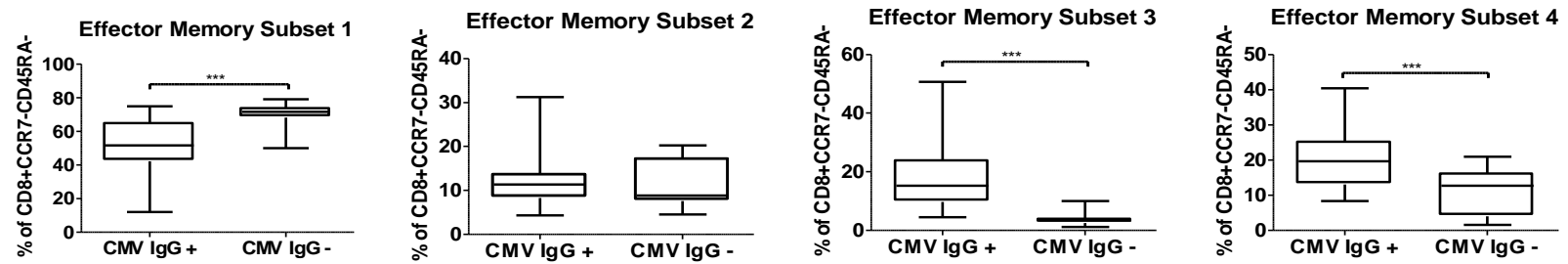
*CD8<sup>+</sup> T cells can be separated into naïve, central memory, effector memory and highly differentiated effector cells on the basis of CCR7 and CD45RA expression (a). 2 representative contour plots from CMV IgG positive (b) and CMV IgG negative (c) donors show the CD8<sup>+</sup> T cell phenotype on the basis of CCR7 and CD45RA expression. Summary data comparing the CD8 T cell phenotype in CMV IgG sero-positive (n=39) and sero-negative (n=21) donors during pregnancy are displayed illustrating naïve (CD45RA<sup>+</sup>CCR7<sup>+</sup>) (d) effector memory (CD45RA<sup>+</sup>CCR7<sup>-</sup>) (e) central memory (CD45RA<sup>-</sup>CCR7<sup>+</sup>) (f) and highly differentiated effector memory (EMRA, CD45RA<sup>+</sup>CCR7<sup>-</sup>) subsets (g). Percentage of CD3<sup>+</sup>CD8<sup>+</sup> T-cells positive for CD27 (h), CD28 (i) and CD57 (j) were also compared between CMV IgG sero-positive and sero-negative individuals. (Box represents interquartile range and whiskers represent maximum and minimum values, \*\* denotes  $p < 0.01$ , \*\*\* denotes  $p < 0.001$ )*



c) CD57 expression in effector memory subsets



d)



*Figure 3.5.3 Effector memory subsets in CMV sero-positive pregnant women.*

*Effector memory (CD45RA-CCR7-) cells can be further defined on the basis of CD27 and CD28 expression into 4 functionally distinct effector memory subsets. (a) Following gating on CD3+CD8+CCR7-CD45RA- cells a representative contour plot is shown. (b) The percentage of CD57+ cells in the 4 effector memory subsets is illustrated. (c). The frequency of the 4 effector memory subsets as a percentage of the overall effector memory cells (CD3+CD8+CD45RA-CCR7-) in CMV IgG sero-positive (n=39) and sero-negative (n=21) donors during pregnancy (d). (Boxes represent interquartile range, whiskers indicate minimum and maximum values, \*\*\* denotes  $p < 0.001$ )*

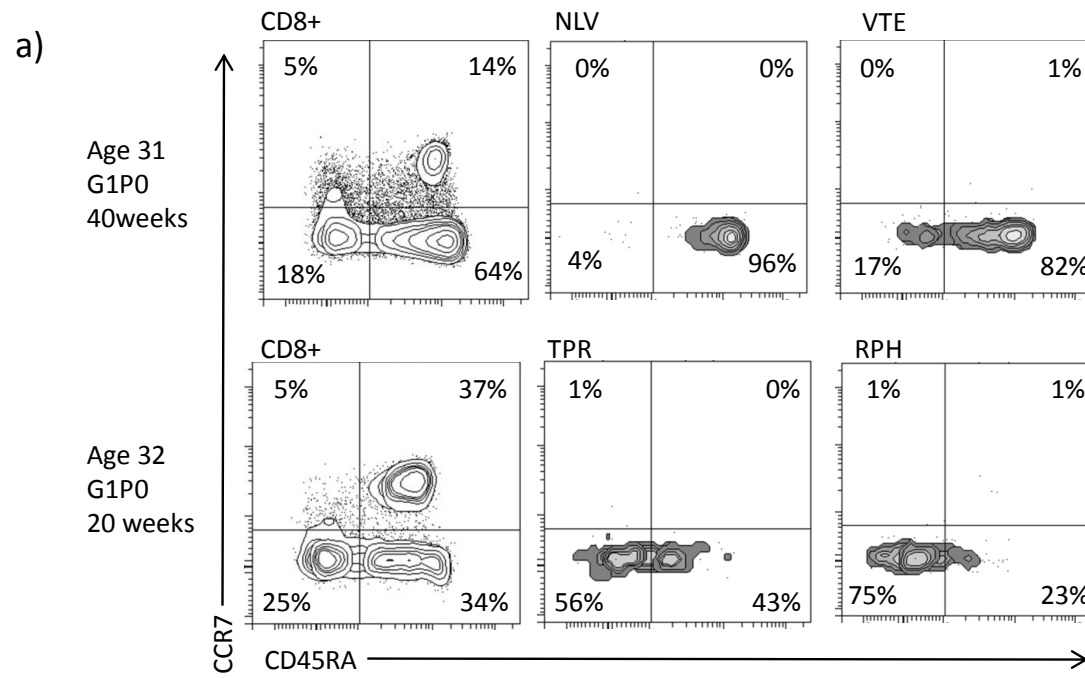
### 3.6 CMV specific T cells during pregnancy

As CMV serostatus was found to have such a marked effect on the global CD8+ T cell repertoire of pregnant donors, it became of interest to see if the effects were due to expansion of CMV-specific CD8+ T cells or reflected a more general effect on all CD8+ T cells. HLA-peptide tetramers containing immunodominant CMV epitopes were therefore used to identify and phenotype CMV-specific T cells within the study cohort. Six tetramers were available and example contour plots contrasting the CMV-specific T cell phenotype for 4 epitopes in two donors in comparison with the global CD8+ T cell phenotype are shown in Fig 3.6.1 a. As expected, CMV-specific CD8 T cells did not exhibit a naïve phenotype and central memory cells were also rarely observed. Significant heterogeneity of phenotype was observed both between different individuals and in relation to individual epitopes. However the overall phenotype of CMV-specific T cells correlated precisely with the changes observed in the global CD8+ cell repertoire in CMV sero-positive women. In particular, the CMV-specific cells were largely focussed in the more highly differentiated CD45RA+ effector subset and expressed high levels of CD57 with frequent loss of CD27 and CD28 (Fig. 3.6.1 b). Thus the more differentiated CD8+ T cell repertoire seen in CMV sero-positive pregnant women is due to expansion of CMV specific T cells

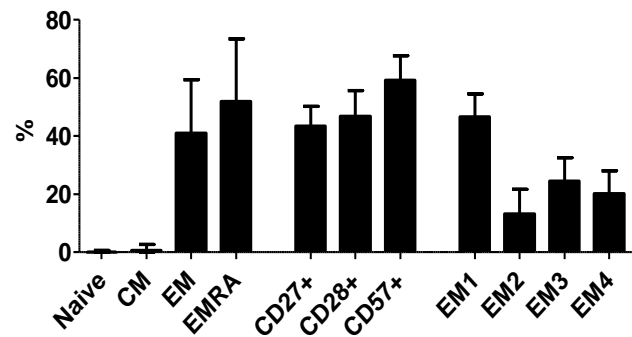
The unique and dynamic immunological environment of pregnancy raised the question as to how the CMV-specific immune response may be modulated in relation to gestation. Samples were therefore obtained longitudinally from each trimester and the postnatal period in five donors and these were studied in a prospective analysis. The frequency of CMV-specific T cells peaked in the 3<sup>rd</sup> trimester in four cases, and in three of

these the CMV-specific T cell response doubled between the first and third trimesters (Fig 3.6.1 c). Interestingly, this increase returned to baseline by six week postnatally. The CMV-specific immune response is known to increase following episodes of viral reactivation (Cwynarski, Ainsworth et al. 2001) and we therefore used a sensitive quantitative real time PCR assay to look for evidence of subclinical viraemia. However, no evidence of CMV reactivation was detected within the peripheral blood mononuclear cells of any women included in the study (data not shown).

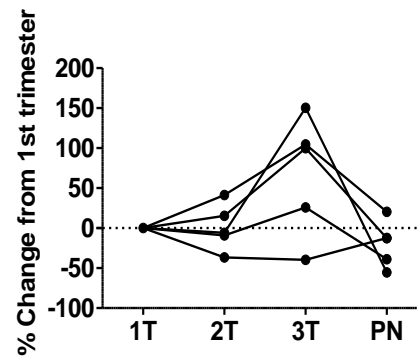
When the CMV-specific immune response was measured by cross sectional analysis of the whole patient cohort in each trimester, no significant differences were seen in relation to gestation (Fig 3.6.1 d).



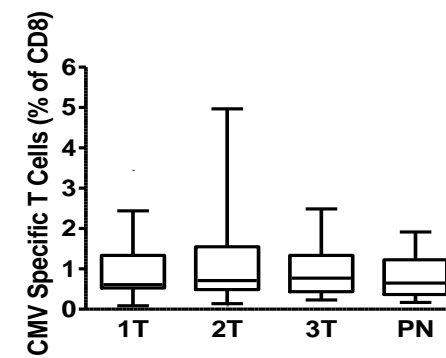
b) Cytomegalovirus specific T cell phenotype



c)



d)



*Figure 3.6.1 Identification and phenotype of CMV specific T cells during gestation.*

*The more differentiated T cell repertoire of CMV sero-positive women is due to the phenotype of CMV specific T cells. Representative examples from 2 nulliparous individuals at 40 and 20 weeks gestation are shown. The left hand panels illustrates the overall CD8+ T cell phenotype. The other panels demonstrate CMV specific T cells for 4 different epitopes identified by HLA-peptide tetramers containing the CMV epitopes (NLV, VTE, TPR and RPH). (a) The phenotype of CMV specific T cells is shown, with the overall expression of CD27, CD28, CD57 and the effector memory subsets. (n=110, bars demonstrate median and error bars show interquartile range) (b) A longitudinal examination of the frequency of CMV specific T cells as a percent of total CD8+ T cells (n=5) (c) Cross sectional analysis of the frequency of CMV specific T cells as a percentage of CD8+ T cells in each trimester and the postnatal period. (n=55, boxes demonstrate interquartile range and whiskers maximum and minimum values) (d) (CM=Central memory, EM=Effector memory, EMRA= Highly differentiated effector, T1=1<sup>st</sup> trimester, T2=2<sup>nd</sup> trimester, T3=3<sup>rd</sup> trimester, PN=Postnatal)*

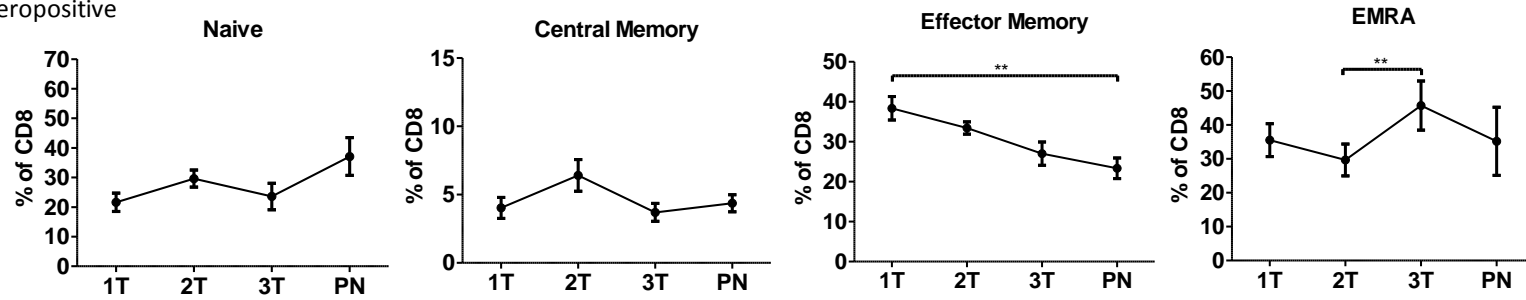
### **3.7 Effects of CMV IgG seropositivity on T cell dynamics during normal pregnancy**

Examination of how the phenotype of the global CD8<sup>+</sup> T cell repertoire changed with advancing gestation and in puerperium was performed. The differences between sero-positive and sero-negative individuals in naive and EMRA proportions during pregnancy previously described (Fig. 3.5.2) remain statistically significant when each trimester is compared. Therefore, we divided the cohort into CMV sero-positive and CMV sero-negative groups and contrasted these two populations. Within CMV sero-positive donors there was a marked increase in the percentage of 'revertant' CD45RA<sup>+</sup> effector cells which rose to a peak value of 46% in the third trimester (Fig 3.7.1 a), with a corresponding reduction in the proportion of effector memory cells throughout gestation. In CMV sero-negative women this effect was not observed and in these individuals there were no statistically significant changes in phenotype during gestation (Fig 3.7.1 b).

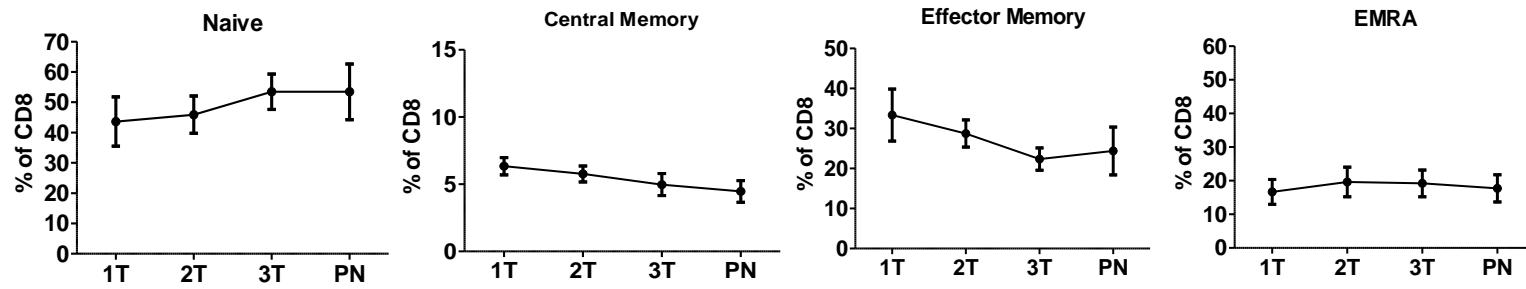
The distinct pattern of changes during gestation in CMV sero-positive women was reflected in significant changes in the phenotype of the CMV-specific T cell pool during pregnancy. The major observation was a marked shift in the proportion of CMV-specific memory cells from CD45RA<sup>-</sup> effector memory to a 'revertant' CD45RA<sup>+</sup> memory profile. This pattern was observed primarily between the second and third trimesters with effector memory cells falling from a median of 58% to 32% ( $p < 0.05$ ) and EMRA increasing from a median of 35% to 65% ( $p < 0.01$ ) (Fig 3.7.1 c).



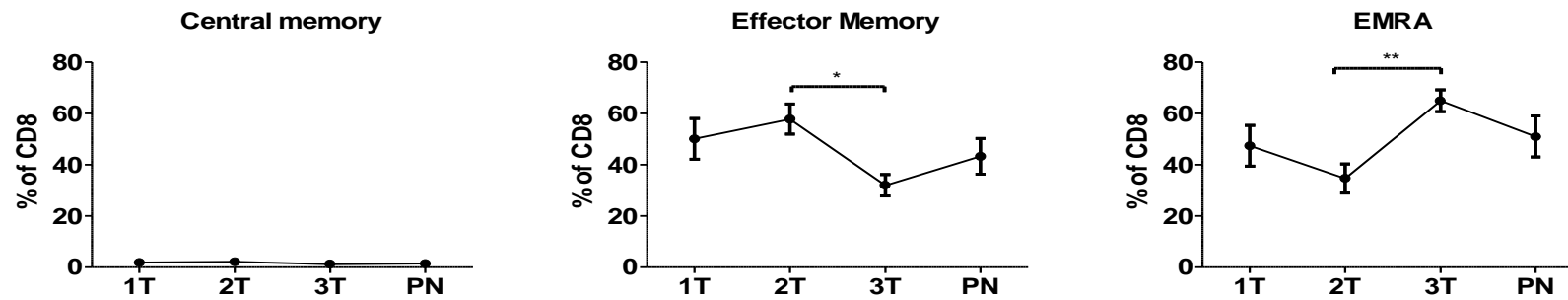
a) CMV seropositive



b) CMV seronegative



c) CMV specific CD8+ T cells



*Figure 3.7.1. Changes in the global T cell phenotype, and CVM specific T cell phenotype across gestation.*

*During gestation there are changes in the global T cell phenotype in CMV sero-positive women due to CMV specific T cells. The frequency of naïve (CCR7+CD45RA+), Central Memory (CM, CCR7+CD45RA-), effector memory (EM, CCR7-CD45RA-) and highly differentiated effector memory (EMRA, CCR7-CD45RA+) cells as a percentage of total CD8+ T cells is illustrated in CMV sero-positive (n=55) (a) and sero-negative (n=32) (b) women during pregnancy and the postnatal period. Changes in the frequency of CMV specific CD8+ T cells identified by HLA-peptide tetramers containing immunodominant CMV epitopes are shown in the central memory, effector memory and highly differentiated effector memory cell subsets as a percentage of total CD8+ T cells in CMV sero-positive women (c). (T1=1<sup>st</sup> trimester, T2=2<sup>nd</sup> trimester, T3=3<sup>rd</sup> trimester, PN=Postnatal \* denotes  $p<0.05$ , \*\* denotes  $p<0.01$ , error bars indicate standard error)*

### **3.8 Effect of CMV IgG sero-positivity on maternal plasma cytokine and chemokine levels during human pregnancy**

As profound changes had been observed within the distribution of CD8<sup>+</sup> memory subsets in relation to CMV serostatus we proceeded to investigate if this was associated with an alteration in the level of cytokines, chemokines or growth factors within maternal plasma. However, multiplex analysis of thirty four factors did not show any significant differences in plasma concentration between CMV sero-positive and CMV sero-negative women (Table 3.8).

Table 3.8 CMV serostatus does not alter maternal plasma cytokine and chemokine levels

	CMV IgG+				CMV IgG-				p
	Median	Interquartile Range			Median	Interquartile Range			
IFN gamma (pg/ml)	31.8	( 12.4	- 59.0	)	30.8	( 12.4	- 63.8	)	0.651
TNF $\alpha$ (pg/ml)	141.7	( 51.3	- 213.6	)	124.3	( 64.2	- 304.4	)	0.725
TNF $\beta$ (pg/ml)	41.2	( 23.6	- 62.6	)	24.7	( 11.5	- 39.2	)	0.011
IL-1 $\beta$ (pg/ml)	24.3	( 9.7	- 45.2	)	19.9	( 11.9	- 44.6	)	0.836
IL-2 (pg/ml)	73.8	( 31.0	- 113.4	)	65.5	( 35.3	- 114.3	)	0.732
IL-4 (pg/ml)	5.7	( 2.0	- 8.7	)	4.3	( 2.7	- 10.4	)	0.661
IL-5 (pg/ml)	17.1	( 7.1	- 32.9	)	17.8	( 7.1	- 37.1	)	0.580
IL-6 (pg/ml)	4.0	( 2.4	- 7.4	)	4.0	( 2.7	- 6.9	)	0.832
IL-7 (pg/ml)	34.2	( 13.2	- 59.8	)	30.4	( 17.9	- 66.5	)	0.563
IL-8 (pg/ml)	25.8	( 13.5	- 43.0	)	29.2	( 14.2	- 55.5	)	0.321
IL-10 (pg/ml)	0.0	( 0.0	- 10.4	)	2.8	( 0.0	- 13.5	)	0.156
IL-13 (pg/ml)	11.6	( 6.7	- 16.3	)	9.6	( 5.0	- 16.0	)	0.623
IL-15 (pg/ml)	11.1	( 4.8	- 16.4	)	4.8	( 2.4	- 12.4	)	0.018
VEGF (pg/ml)	26.1	( 12.5	- 40.1	)	25.0	( 12.8	- 50.2	)	0.449
MCP-1 (CCL2) (pg/ml)	26.6	( 14.8	- 38.3	)	24.6	( 13.0	- 44.3	)	0.802
MIP-1 $\alpha$ (CCL3)(pg/ml)	29.6	( 19.2	- 48.0	)	39.1	( 21.5	- 60.0	)	0.240
MIP-1 $\beta$ (CCL4)(pg/ml)	38.2	( 26.9	- 51.6	)	33.0	( 23.9	- 46.6	)	0.528
RANTES (CCL5) (pg/ml)	123.0	( 91.6	- 209.1	)	157.5	( 93.5	- 218.4	)	0.436
CTACK (CCL27) (pg/ml)	12.6	( 0.0	- 85.2	)	4.4	( 0.0	- 72.0	)	0.756
MIG (CXCL9) (pg/ml)	76.6	( 55.8	- 113.8	)	89.7	( 51.3	- 125.1	)	0.467
ITAC (CXCL11) (pg/ml)	11.7	( 5.6	- 19.0	)	10.1	( 6.3	- 21.3	)	0.773
SDF-1 ( CXCL12) (pg/ml)	38.7	( 11.3	- 63.9	)	34.2	( 11.9	- 65.5	)	0.791
Fractalkine (CX3CL1) (pg/ml)	4.4	( 3.3	- 4.9	)	4.0	( 3.1	- 5.8	)	0.985
MMP-3 (pg/ml)	668.3	( 449.0	- 910.4	)	568.2	( 403.5	- 718.1	)	0.089
MMP-9 (pg/ml)	2111.1	( 0.0	- 9581.8	)	4820.1	( 0.0	- 9301.8	)	0.759
FGF-basic (pg/ml)	38.3	( 24.7	- 45.5	)	32.9	( 24.6	- 46.6	)	0.651
G-CSF ( pg/ml)	25.9	( 10.8	- 48.9	)	22.2	( 13.0	- 42.7	)	0.691
GM-CSF (pg/ml)	51.4	( 16.0	- 86.5	)	46.0	( 24.1	- 91.2	)	0.644
il17a (pg/ml)	6.2	( 3.6	- 9.1	)	7.3	( 4.3	- 12.8	)	0.294
il17f (pg/ml)	7.5	( 2.8	- 9.8	)	5.5	( 2.8	- 8.8	)	0.383
ip10 (pg/ml)	23.5	( 12.9	- 36.4	)	20.3	( 14.7	- 35.4	)	0.923
il21 (pg/ml)	3.2	( 0.8	- 4.8	)	2.0	( 0.8	- 3.8	)	0.919
il23 (pg/ml)	78.0	( 63.0	- 134.5	)	89.6	( 48.5	- 144.8	)	0.694

Maternal plasma was analysed for 34 cytokines and chemokines. CMV IgG sero-positive and sero-negative individuals were compared. Statistical analysis was by Mann-Whitney test, with the Bonferroni post-hoc correction for multiple tests. A value of <0.0019 was considered statistically significant at an alpha level of 0.05.

### 3.9 Discussion

#### ***Modulation of the Th17 cell subset during pregnancy does not appear to be an essential requirement for normal pregnancy***

Th17 cells are an inflammatory effector subset, with an important role physiologically in the defence against fungal infections and as part of the pathogenesis in numerous disease processes (Korn, Bettelli et al. 2009). Since their recognition as a distinct effector subset there has been an enormous effort to characterise the development and role of these cells (section 1.1.5). However, little is known about their potential role in reproductive immunology and it is important that the Th1/Th2 paradigm, much quoted in the reproductive immunology literature, must be re-assessed on the basis of this 3<sup>rd</sup> distinct subset (Raghupathy, Makhseed et al. 1999; Piccinni 2006; Chaouat 2007). We investigated the frequency of Th17 cells throughout normal pregnancy. We hypothesised that there may be a shift away from the inflammatory Th17 subset during pregnancy as a mechanism of maternal tolerance of the allogenic fetus and the increased prevalence of fungal infections such as candida during pregnancy was circumstantial evidence in support of such an effect. When the frequency of Th17 cells was examined in each trimester no significant difference was noted across each trimester and the postnatal period. The only other report of Th17 cells during pregnancy only conducted cross-sectional observations and, as in our analysis of this type, they found no significant differences across gestation (Nakashima, Ito et al. 2010).

When a longitudinal analysis was conducted of the same individuals at multiple timepoints during pregnancy there was found to be a statistically significant elevation in the proportion of Th17 cells in the periphery during the 1<sup>st</sup> trimester. Whilst this was a

statistically significant observation, the biological significance of this is less clear, as a reduction in Th17 cells between the 1<sup>st</sup> and 2<sup>nd</sup> trimester was not seen in all women and these were small absolute differences. Furthermore, the lack of a significant difference in Th17 cell numbers between pregnancy and the postnatal period, even when examined in a longitudinal cohort, suggests that a reduction in Th17 cells in the periphery is not an important part of maternal immunological tolerance of the allogenic fetus.

It is interesting to speculate what the physiological reasons may be that could explain the possible finding of elevated Th17 cells in the 1<sup>st</sup> trimester, as this seems initially to be counter to the traditional paradigm of pregnancy being a Th2 state. However, it is increasingly recognised that implantation is essentially an inflammatory process and many Th1 cytokines are crucial for this process (Stewart, Kaspar et al. 1992; Bilinski, Roopenian et al. 1998; Ashkar, Di Santo et al. 2000) (Reviewed in (Chaouat 2007)). Our observations may therefore be part of this phenomenon with Th17 cells potentially also involved in the inflammatory events of early pregnancy. In support of this interpretation is the recent finding by Nakashima et al. that Th17 cells are found at a higher frequency in the decidua than in the peripheral blood (Nakashima, Ito et al. 2010).

Future studies would ideally include human decidual tissue to determine the frequency of Th17 cells at the maternal fetal interface. A limitation of our current study is that we cannot be sure that this increased frequency of Th17 cells in the peripheral blood is associated with homing of these cells to the maternal fetal interface, and an increased frequency of cells at this site. With our current findings raising the possibility that Th17 cells may have a role in the events of early pregnancy such as implantation and placentation it would also be fascinating to extend this work to study cohorts with pathological

pregnancies, such as implantation failure and malplacentation associated with fetal growth restriction.

***A Th1 to Th2 shift was not demonstrated in maternal peripheral blood T cells or plasma cytokine levels***

The traditional paradigm that pregnancy was associated with a Th1 to Th2 shift is increasingly being re-examined. The limitations of this model of T cell function are now clear with the addition of a 3<sup>rd</sup> distinct T cell subset, the discovery of cytokines which do not fit into this simple dichotomy and our recognition of T cell subset plasticity (section 1.1.5). Furthermore, the recognition that knocking out Th2 cytokines does not inhibit reproduction and that the events of early pregnancy are associated with Th1 type responses means an increasing number of caveats are needed when considering the validity of this hypothesis (section 1.2.5).

Th1 cells were also identified functionally through IFN  $\gamma$  production by intracellular cytokine staining. We also examined changes in the surface chemokine receptors CXCR3 and CCR4 (Appay, van Lier et al. 2008) which some have associated with the Th1 and Th2 subsets. Though clearly these chemokine receptors are imperfect surrogates for Th1 and Th2 subsets caution is therefore important when extrapolating from this part of the analysis. Furthermore, a wide range of Th1 and Th2 cytokines were measured in maternal plasma, though again it must be remembered that the levels of cytokines found in the plasma may not reflect activity at the tissue level and specifically at the maternal-fetal interface.

However, if Th1 cells were classified by IFN  $\gamma$  production using intracellular staining, or by surface chemokine receptor expression, no reduction in Th1 cells was seen across gestation, even with longitudinal analysis within individuals.

Interestingly, the lack of a difference across gestation found by intracellular staining was also demonstrated when the maternal plasma levels of Th1 and Th2 cytokines were observed. In this longitudinal cohort no reduction in Th1 cytokines, elevation in Th2 cytokines or decrease in the Th1/Th2 ratio was observed in maternal plasma. This suggests that measured in peripheral blood, during normal human pregnancy, a reduction in Th1 cells or an associated Th1 to Th2 shift cannot be readily detected.

It must be recognised that our relatively small sample size could have failed to detect a subtle change and studies have used alternative techniques such as the assessment of cytokines secreted into the supernatant after stimulation and that this could account for differences in our findings. However, bearing in mind these limitations our study benefited from longitudinal analysis which means that we could clearly demonstrate that a reduction in Th1 cells during gestation in the periphery is not consistently observed.

***Regulatory T cells (T reg) in maternal peripheral blood are not elevated during normal pregnancy***

Regulatory T cells (section 1.1.7) have been suggested to have an important role in maternal tolerance of the allogenic fetus in both murine and human pregnancy (section 1.2.3). We studied regulatory T cells alongside the effector subsets to build a comprehensive picture of changes in maternal peripheral cellular immunity across gestation. The current



“gold standard” flow cytometric method for the identification of T regs is through multiparameter flow cytometric identification of CD4+CD25+CD127-FOXP3+ cells. We therefore used this classification for T regulatory cells, but in addition we gathered additional data to simultaneously enable analysis with all the various flow cytometric strategies for T reg identification used previously in the reproductive immunology literature. This enabled direct comparison between these approaches and we could identify if the phenotypic classification of T regs altered the result obtained.

We found that there was no significant difference in the frequency of T regs identified on the basis of the full CD3+CD4+CD25+CD127-FOXP3+ phenotype during pregnancy, including by prospective longitudinal analysis. The same was found when the less stringent classification of CD4+CD25<sup>high</sup> cells, or CD4+CD25+ cells was used. Similarly, whether FOXP3 staining was included or not in the gating strategy we still found no significant difference in T regs across gestation. The more stringent the identification technique the lower the frequency of T regulators cells found, as false positive events were increasingly excluded. Interestingly, though not statistically significant there was a trend for CD4+CD25+ cells to be more common in the 3<sup>rd</sup> trimester and during labour, which may be because CD25 is up-regulated by T cell activation in cells which are not regulatory in function (Fontenot, Rasmussen et al. 2005). This phenomenon may explain why earlier studies which identified T regs solely on the basis of CD25 expression found an increase in these cells during pregnancy (Somerset, Zheng et al. 2004).

However, even in recent studies, using similar techniques and cell markers to identify T regs there have been conflicting findings reported. Some studies have reported similar findings to ours (Tilburgs, Roelen et al. 2008) and do not identify any increase in peripheral T

regs during normal pregnancy. Whilst others report a statistically significant increase in T regs during pregnancy (Santner-Nanan, Peek et al. 2009). Reconciling these divergent results is difficult biologically but may be due to experimental differences and the classification of what we understand as a T reg cell. There are still technical challenges which exist in the rigorous identification of T regs. In particular there is no agreed method for deciding on the gating thresholds to differentiate CD25 bright and CD25 dim cells. Similarly differences in antibody clones used for FOXP3 staining and even subtle methodological differences such as altering the means by which the cells are permeabilised and isotype controls can have profound effects on the results obtained (Grant, Bourcier et al. 2009; Law, Hirschhorn et al. 2009). The strength of our study is the longitudinal analysis which enables differences in T reg frequency within individuals to be measured over time. Thus we can conclude with some confidence that despite using a wide range of methods to identify T regs, within individuals an increase in peripheral T regs during gestation is not an absolute requirement for normal pregnancy.

### ***Highly differentiated CD8 T cells are activated in the third trimester of pregnancy***

CD38 is a molecule with diverse functions, acting as both a NADase/ADPR cyclase enzyme and a cell surface receptor which associates with the TCR/CD3 complex. In T cell biology it is commonly used as a marker of cell activation and we have studied it in this context (Malavasi, Deaglio et al. 2006). During pregnancy there was a significant increase in expression of CD38 on maternal CD8 T lymphocytes. This overall effect was predominantly due to increased expression in the EMRA subset. This effect was not influenced by previous CMV infection. This finding confirms previous studies suggesting a gestational increase in T

cell activation (Mikyas et al., 1997; Truong et al., 2010), and our own findings of a trend to increasing CD25 expression on CD4 T cells in the 3<sup>rd</sup> trimester. However, previously the T cell subsets involved or the relationship with previous cytomegalovirus infection had not been described. Understanding the expression of CD38 during pregnancy is important as high levels of CD38 expression are observed on decidual T cells (Abadia-Molina et al., 1996; Ho et al., 1996) and is an important determinant of prognosis in HIV infection (Froebel et al., 2000; Liu et al., 1997). Our findings reveal that CMV sero-positivity does not appear to be responsible for the pregnancy-related increase in CD38 on CD8 T cells and suggests that this may therefore either be a hormonal effect, such as that seen to regulate levels of CD38 on the myometrium during pregnancy (Dogan et al., 2006) or be related to peripheral T cell activation by alloimmune fetal antigen.

***Cytomegalovirus sero-positivity dramatically alters the maternal CD8 T cell repertoire and leads to the accumulation of highly differentiated memory cells during human pregnancy***

These data demonstrate that CMV sero-positivity has a dramatic effect on the CD8+ T cell repertoire during pregnancy. CMV IgG sero-positive women have a more differentiated peripheral CD8+ T cell phenotype with a 50% reduction in the proportion of naïve T cells and a significant increase in EMRA cells. In addition, even though the proportion of effector memory (EM) cells was not increased in the CMV sero-positive group there was a marked shift from the less differentiated EM1 to the more differentiated EM3 subset, characterised by the loss of the co-stimulatory molecules CD27 and CD28. This work has confirmed that these effects were directly due to the accumulation of CMV-specific T cells through the use of six HLA-peptide tetramers containing immunodominant CMV epitopes which revealed

that the global changes were reflected in the phenotype of the CMV-specific T cells. Furthermore, CMV sero-positivity was shown to cause a difference in the immunological changes observed in the maternal immune system in response to pregnancy. In the sero-positive group there was a large increase in the proportion of CMV-specific EMRA cells within the peripheral blood during the third trimester of pregnancy. This effect altered the overall CD8<sup>+</sup> T cell phenotype in CMV sero-positive women but no change was seen in sero-negative women. 'Latent' CMV infection in sero-positive women therefore alters not only the overall CD8<sup>+</sup> repertoire but also the dynamics of the CD8 T cell response during pregnancy. We also noted an increase in T cell activation during gestation, predominantly due to increases in CD38 expression within the EMRA subset and this was independent of CMV infection.

Our group and others (Hooper et al., 1999; Looney et al., 1999; Chidrawar et al., 2009) have demonstrated CMV infection results in a more highly differentiated T cell phenotype within peripheral blood and this is most clearly observed in the elderly. However, the influence of CMV infection on the CD8 T cell repertoire has not been investigated in the context of pregnancy. We have demonstrated that this effect extends to the pregnant population and whilst we do not believe this effect is unique to pregnancy it has been shown to be an important consideration in this patient group. There is an extensive reproductive immunology literature regarding the role of CD8<sup>+</sup> T cells within maternal blood and decidua during pregnancy, but this has not accounted for CMV sero-status as a potentially important confounding factor. Rates of CMV infection are highly variable within different cohorts and our data reveal that CMV infection status is an important consideration when comparing CD8 T cell memory subsets and differentiation between different pregnant cohorts.

The finding in CMV sero-positive pregnant women of a relative increase in late differentiated CD8<sup>+</sup> T cells with a loss of CD27 and CD28 expression is a pattern that is considered a marker of immune senescence and part of the 'immune risk phenotype'. Indeed, CMV sero-positivity and elevated antibody titre appear, at least in the elderly, to be associated with excess mortality (Roberts, Haan et al. ; Derhovanessian, Larbi et al. 2009; Pawelec, Derhovanessian et al. 2009; Strandberg, Pitkala et al. 2009). It is not yet clear if these detrimental effects occur only at the extremes of age or if their presence in a pregnant cohort could also influence maternal health or pregnancy outcome.

Pre-eclampsia is an important and common disorder that is associated with increased maternal and perinatal mortality and morbidity. The aetiology remains unknown, though malplacentation is thought to be an early part of the pathogenesis (Steegers, von Dadelszen et al.). In the later pathogenesis of the disease endothelial dysfunction (Roberts, Taylor et al. 1989) and systemic inflammation (Weiss, Goldsmith et al. 2009) are important features. Through their influence on these processes infection (Xie, Hu et al. ; Heine, Ness et al. 2003; von Dadelszen, Magee et al. 2003; Goulis, Chappell et al. 2005) and chronic inflammation (Kunnen, van Doormaal et al.; Xiong, Buekens et al. 2006) have been associated with preeclampsia.

Recent analyses have identified that CMV sero-positivity is associated with a 1.5 fold relative risk for the development of pre-eclampsia (Xie, Hu et al. 2010) though these findings require confirmation in larger cohorts. Cytomegalovirus is able to infect endothelial cells and a detrimental effect of chronic CMV infection has been demonstrated on endothelial cell function in healthy donors (Grahame-Clarke, Chan et al. 2003) with induction of

hypertension also being observed in a murine model of CMV infection (Cheng, Ke et al. 2009). It is currently unknown if the immunological profile of CMV sero-positive women influences the ability of the maternal immune system to correctly regulate trophoblast invasion or if CMV infection induces endothelial dysfunction (Kovacs, Hegedus et al. 2007; Pereira and Maidji 2008). Furthermore, the altered dynamics of the immune response in sero-positive women may alter the risk of pre eclampsia in these women and this warrants further investigation.

The relative reduction in naive lymphocytes and accumulation of CMV-specific clonal expansions has been proposed to have a detrimental impact on the ability of the host to respond to infections (Akbar and Fletcher, 2005). In support of this it has been observed that in CMV sero-positive elderly individuals (Trzonkowski et al., 2003) and in those with accumulations of CD8<sup>+</sup> CD28<sup>-</sup> negative cells there is an inverse correlation with responsiveness to vaccination (Goronzy et al., 2001; Saurwein-Teissl et al., 2002). As the same phenotypic changes in the T cell repertoire have been observed during pregnancy further investigation is warranted to identify whether CMV sero-positivity is detrimental to responses to infection or vaccination in a pregnant cohort.

In sero-positive women in the 3rd trimester there was a significant increase in effector CTL (CD45RA<sup>+</sup>CCR7<sup>-</sup>) compared to the 2nd trimester, which was not observed in sero-negative women and was due to CMV specific CTL. This demonstrates that infection history can alter the maternal immunological response to pregnancy. We appreciate that this experimental design cannot determine if the change was caused by increased mobilisation of EMRA cells or due to a change in phenotype with a re-expression of CD45RA on circulating EM cells. It has been shown that the immunological response to exercise is

similarly effected by the previous infection history and that exercise preferentially mobilises CMV specific EMRA cells into the peripheral blood and this is thought to be driven by catecholamines (Turner et al., 2010). A potential explanation for the effect we observed during pregnancy could similarly be through a hormonally driven mechanism that alters T cell mobilisation or differentiation. An alternative explanation is that, although CMV was not detectable in peripheral blood of these women during pregnancy, local reactivation during pregnancy is common, with virus excretion into breast milk found in most sero-positive women (Hamprecht et al., 2001). The increase in the tissue-homing EMRA population in late pregnancy could therefore be a response to this local reactivation during late pregnancy.

Prospective longitudinal analysis also revealed that the proportion of CMV specific T cells overall peaked within the 3rd trimester of pregnancy in a majority of individuals. This was not observed with a cross sectional analysis due to the high variability between individuals in the magnitude of their CMV specific CD8+ T cell response. This effect was not due to systemic viral reactivation or reinfection as sensitive quantitative PCR demonstrated that this was not accompanied by CMV viraemia. Changes in CMV specific T-cell mobilisation or a response to localised viral reactivation could again be responsible.

It is interesting that these profound changes in CD8 T cell phenotype were seen without significant differences being observed in plasma cytokines, chemokines and chemokine receptors when sero-positive and sero-negative individuals were compared. Thus, while the cellular phenotypic changes are important and demonstrate altered cellular differentiation, it may be that in the context of latent infection, without antigenaemia due to viral reactivation, the differences in effector cell secretory functions in-vivo are less pronounced. Further studies investigating directly the modulation of T cell cytotoxicity and

secretory functions in response to CMV antigens during pregnancy would enable this to be better defined.

## **Conclusion**

A detailed examination of all 3 effector T cells subsets and regulatory T cells was conducted in maternal peripheral blood throughout gestation and the postnatal period. Longitudinal analysis suggested an elevation in Th17 cells in the 1<sup>st</sup> trimester of normal pregnancy and whilst the biological significance is unclear it does suggest that Th17 cells do not have the detrimental role to pregnancy that we hypothesised and modulation of Th1 7 cells measured in the periphery did not seem to be essential for normal pregnancy.

We did not observe a significant Th1 to Th2 shift in the peripheral T cell phenotype or plasma cytokines. One strength of our study was that longitudinal analysis of changes within individuals showed clearly that a Th1 to Th2 shift measurable in the periphery was not required for successful pregnancy. Similarly, we detected no global increase in regulatory T cells in maternal peripheral blood during normal pregnancy, again suggesting that peripheral changes in the proportion of T regs in peripheral blood cannot be considered essential to normal pregnancy.

We also examined the impact of previous CMV infection on the CD8 T cell repertoire during pregnancy. The effect of previous CMV infection was dramatic with a more differentiated CD8 T cell memory phenotype in women with previous CMV infection. CMV status must therefore be considered whenever investigating CD8 T cell memory subsets and differentiation between different pregnant cohorts. How these profound immunological



changes during pregnancy that are induced by previous CMV infection alter maternal health or pregnancy related outcomes now require further investigation.

## **Chapter 4.**

# **Detecting a maternal cellular immune response to fetal antigens during pregnancy**

## 4.1 Introduction

The presence of fetal minor histocompatibility antigen specific T cells after human pregnancy has been established (Verdijk, Kloosterman et al. 2004; Piper, McLarnon et al. 2007; van Halteren, Jankowska-Gan et al. 2009) (section 1.5.1).

However, no studies have investigated the presence or characteristics of fetal minor histocompatibility antigen specific T cells during the course of normal human pregnancy. Murine models investigating this have been variable in their findings (Table 1.5.2). However, in general they have demonstrated maternal awareness of fetal antigen, with initial proliferation but then deletion of fetal antigen specific T cells and those fetal specific T cells which remain are relatively hyporesponsive (section 1.5.2). Lack of consensus of the evidence from murine models and the important differences between murine and human pregnancy (Fig. 1.2.2.1) mean this remains an important question to be addressed. Understanding this aspect of the maternal fetal immunological relationship has implications for our understanding of the overall immunology of normal pregnancy, pregnancy related disease and also applications for transplantation and cellular therapy.

MHC-peptide multimers enable minor histocompatibility antigen specific T cells to be characterised (Altman, Moss et al. 1996) (Section 1.8.1). The weak nature of the TCR/pMHC interaction means that multimeric forms of pMHC are required to provide the binding avidity sufficient for their use for cell staining. Dextramers consist of multiple (>10) pMHC complexes on a dextran backbone. We hypothesised that this higher order multimer may be beneficial by enhancing the detection of low affinity T cells. In the context of a male fetus T cells specific for HY derived epitopes have been used to study fetal specific T cells.

## **4.2 Comparison of tetramers and dextramers for the identification of fetal specific T cells**

The use of HY (FIDS) specific T cell clones enabled the direct comparison of the staining properties of HY-HLA\*0201 tetramers and dextramers. The specificity of the T cell clones used was confirmed by cytotoxicity and cytokine release assays (as described in section 2.6).

The Dextramer reagent stained the T cell clone more brightly, allowing easier differentiation between positive and negative populations. This is illustrated by the higher MFI during staining of the HY specific T cell clone seen with dextramer compared to tetramer staining. The dextramer staining was also observed to result in less non-specific binding and thus a better defined positive population. The dextramers were titrated from 10 µl per test as this was the recommended concentration in the manufacturers protocol, with bright staining still maintained at 1.25 µl per test. The protein concentration per test was not provided by the manufacturer. The tetramers were constructed with standard methods and the protein concentration calculated by a modified Bradford assay. Tetramer was titrated from 10 mg per test, in excess of the normal concentration of tetramer required per test.

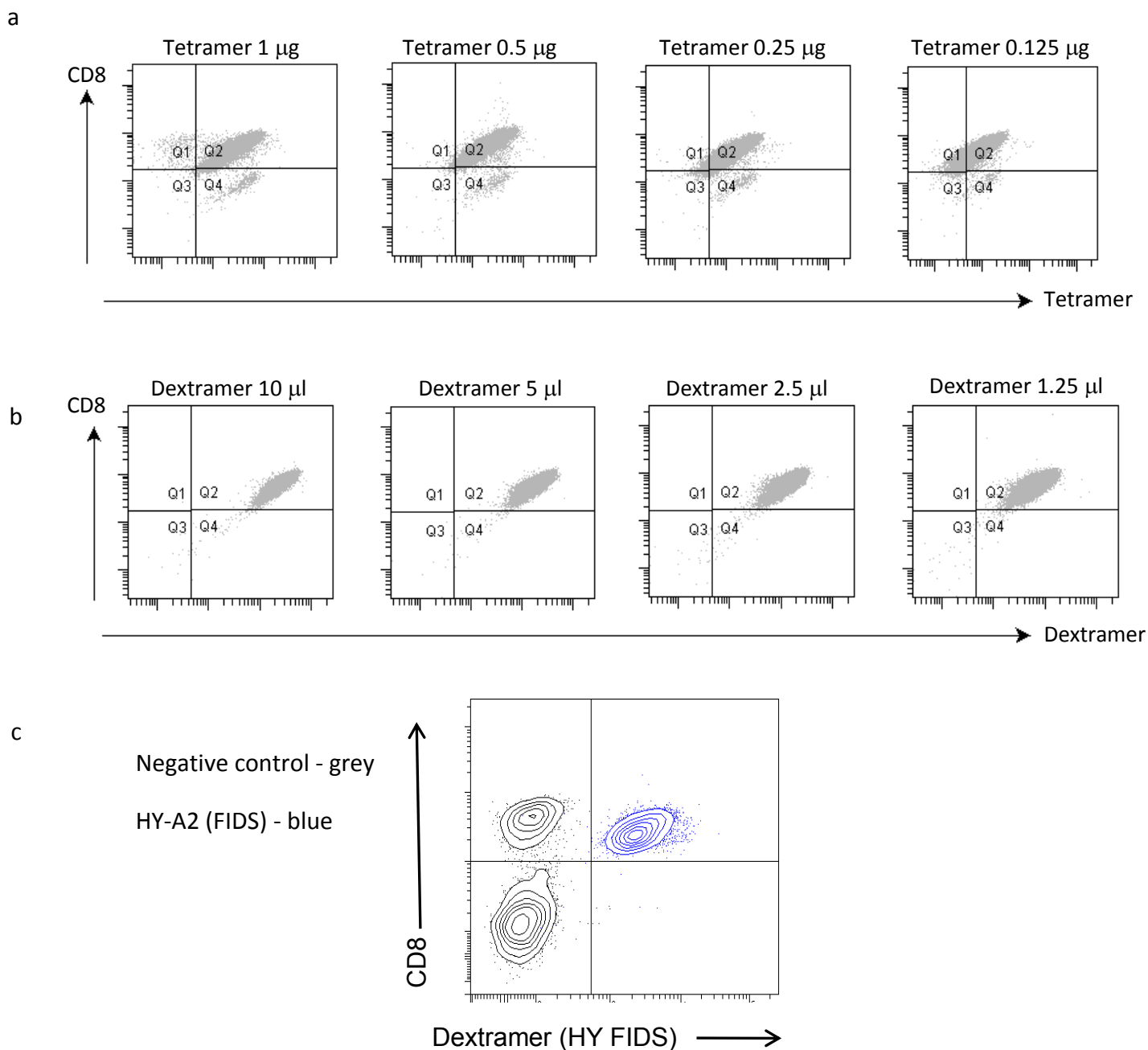


Figure 4.2.1 Comparison of tetramer and dextramer staining.

An HY specific T cell clone was used to compare staining with the peptide-MHC tetramer for the HY-A2 epitope (FIDS) (a) and with the higher order multimer “dextramer” for the same epitope (b). These were both titrated over a ten fold range. The staining of an HY specific T cell clone with the dextramer (FIDS) (blue) superimposed on staining of polyclonal non HY specific T cells with the same dextramer (FIDS) (grey) demonstrates the clear population separations obtained with these negative and positive controls (c)

### 4.3 Detection of fetal specific T cells during and after pregnancy

HLA-peptide dextramers were used to identify CD8 T cells specific for the HLA\*0201-restricted HY minor histocompatibility antigen FIDSYICQV in the blood of women with a male pregnancy during and after pregnancy (n=42 during pregnancy and n=42 after pregnancy).

The rarity of antigen specific T cells in this context necessitated a rigorous flow cytometric gating strategy (Lissauer, Choudhary et al. 2011), to minimise aberrant binding events. This gating strategy is illustrated in Figure 4.3.1. This strategy is contrasted with the use of a conventional gating strategy (a lymphocyte gate determined by forward and side scatter properties and a CD8<sup>+</sup> gate) for the same data (Fig. 4.3.2). The final plot demonstrates that non-specific binding events have not been excluded as well by an approach using only the gating of lymphocytes in the conventional manner by their forward and side scatter properties. Back-gating to identify the characteristics of these false positive events illustrates that this is predominantly due to the inadequate exclusion of monocytes, NK cells, dead cells, and cell aggregates with the traditional gating strategy.

Representative examples show the detection of HY-specific CD8 T cells in maternal blood during pregnancy (Fig. 4.3.3 a) and in post-partum samples (Fig. 4.3.3 b). These donors have also been selected to demonstrate examples in the quartile above (Fig. 4.3.3 a) and below (Fig. 4.3.3 b) the median. The specificity of dextramer staining was illustrated by the lack of staining of CD8-negative T cells and the low background staining using the negative control dextramer. HY-specific T cell responses were not detected in women without a history of a male pregnancy (n=8). The efficiency of the magnetic selection can be observed to depend on the pre-selection frequency of the HY specific T cells.

Fetal-specific T cells were detected in 50% of women during pregnancy with a male fetus and this was comparable to the frequency of detection of 45% in the cohort with previous pregnancy history (50% v 45%,  $p=0.57$ ) (Fig. 4.3.4 a).

We then examined the magnitude of the HY specific T cell response. The frequency of HY specific T cells as a percentage of the total CD8 cells is compared in donors in whom a response had been observed. The median magnitude of the HY-specific response during pregnancy was 0.043% of the total CD8+ T cell pool and this was also similar to the value measured after pregnancy (0.043% v 0.029%,  $p=0.86$ ) (Fig. 1d).

A subgroup analysis was conducted to further examine if the number of previous male pregnancies altered the frequency of detection of HY specific T cells. It was observed that multiple previous male pregnancies increased the frequency of detection of HY -specific T cells but this did not reach statistical significance (1 male v >2 male  $p=0.15$ ). The analysis of only donors during or after pregnancy did not make a significant difference to the findings (Fig. 1e).

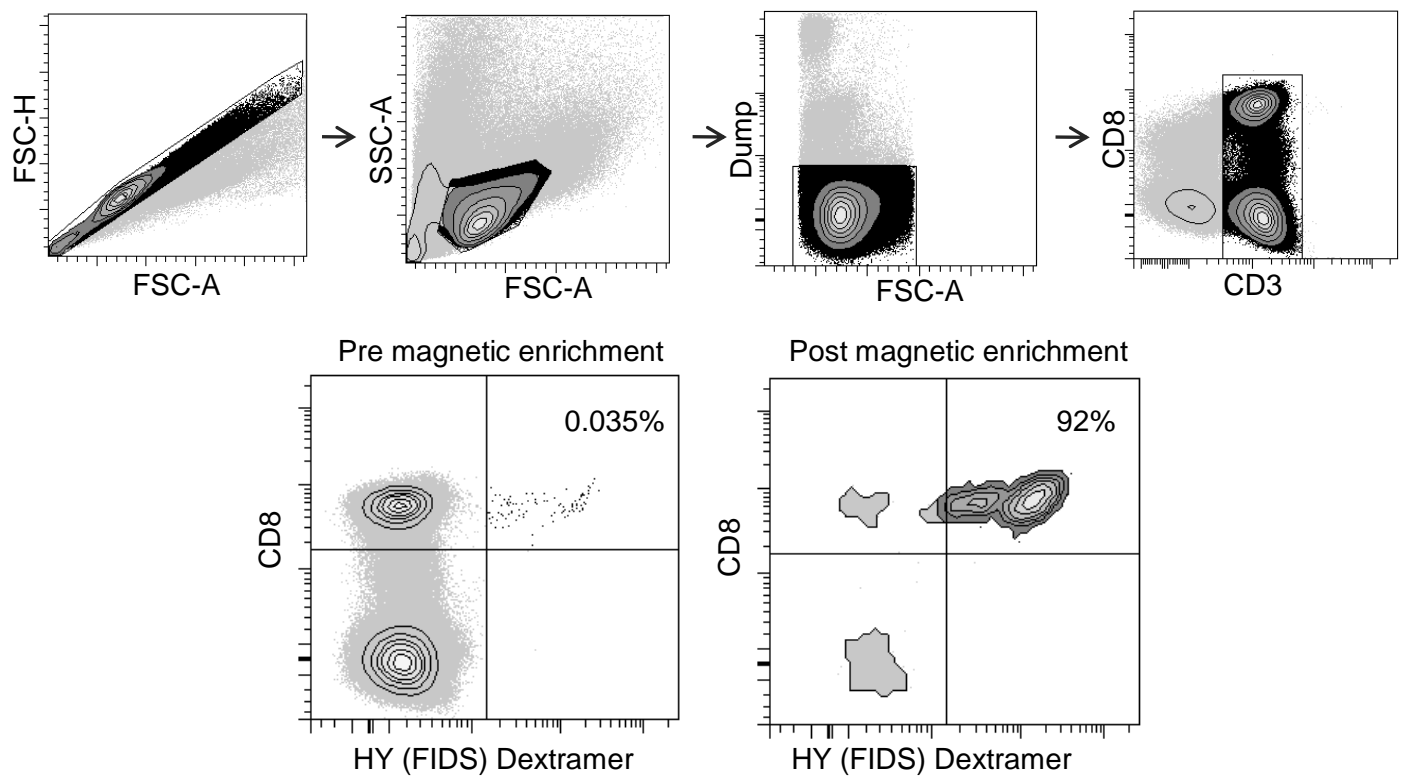


Figure 4.3.1. Gating strategy for identification of HY specific CD8 T cells.

Singlet cells were identified on a FSC-A vs FSC-H plot and small lymphocytes gated on the basis of standard light scatter properties. A "dump" channel was used to exclude dead cells , monocytes and B-cells. T lymphocytes were then selected by CD3 expression. Finally, dextramer "positive" cells were visualised against CD8 expression. Proportions of dextramer "positive" cells are given as a percent of CD8 positive cells.



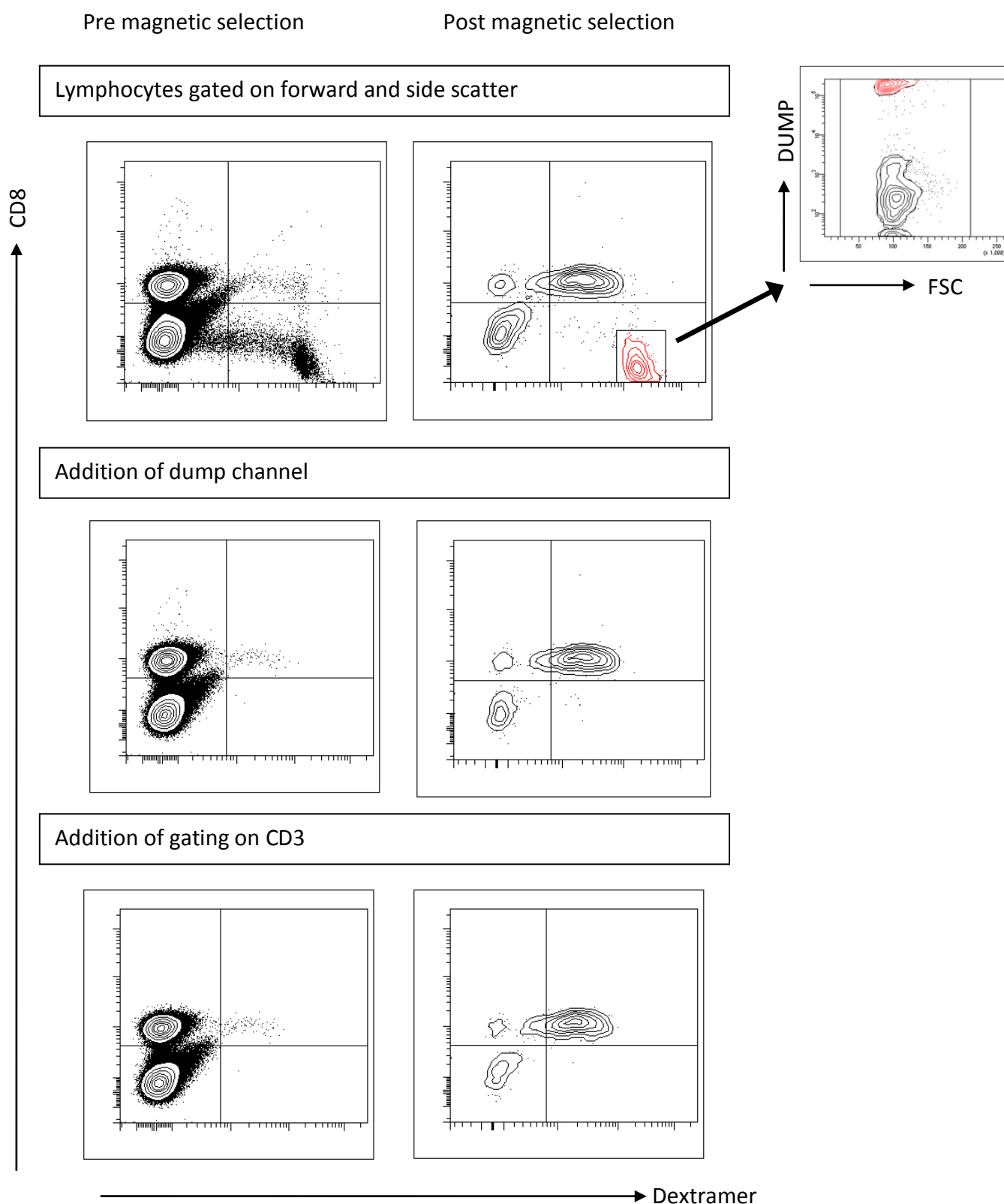


Figure 4.3.2 Importance of a rigorous gating strategy.

The uppermost panels illustrate gating conducted on the basis of conventional forward and sidescatter properties, which identifies small lymphocytes prior to the evaluation of CD8+ dextramer+ cells. A large population of dextramer positive CD8 negative events which are therefore non specific background staining can

*be seen in both the pre and post magnetic selection samples. The middle panels illustrates how the addition of the dump channel; which incorporates a viability dye (propidium iodide), CD14 and CD19 to exclude dead cells B cells and monocytes, substantially reduces this background staining. The bottom panels illustrate how specificity is further improved by the addition of CD3 to define T cells prior to gating on the CD8 subset.*

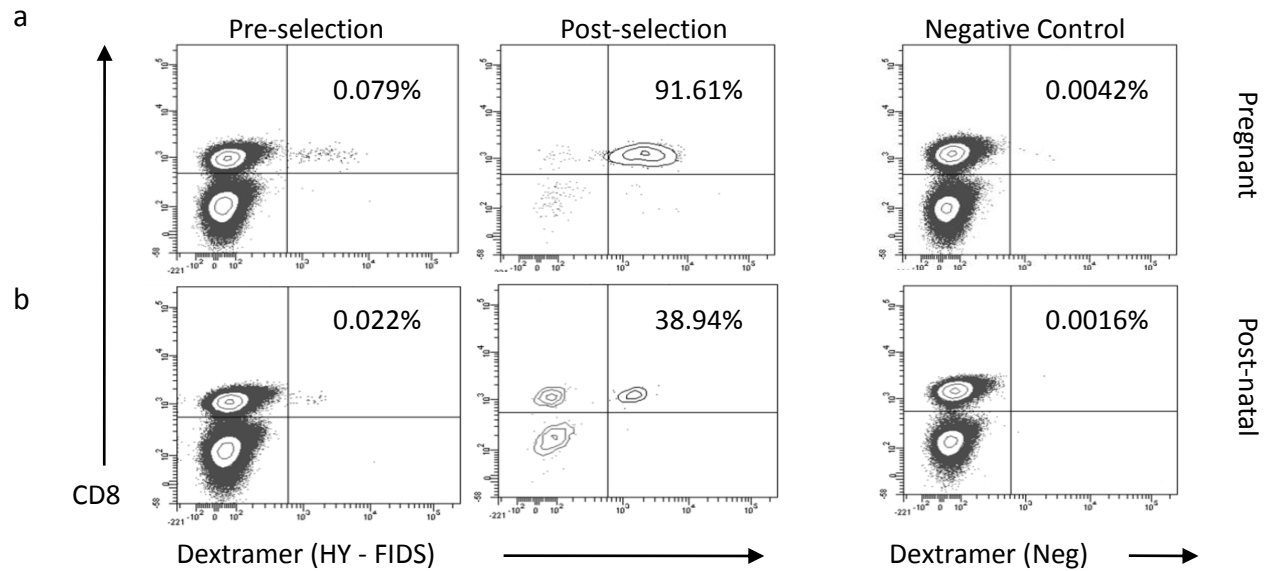


Figure 4.3.3 Detection of fetal specific T cells.

Flow cytometry contour plots illustrate the detection using MHC-peptide dextramers of fetal (HY minor histocompatibility antigen – FIDS) specific CD8 T cells in a women with a male fetus, following 10 day peptide line expansion, in representative women during (a) and after (b) normal pregnancy. The left hand column shows the frequency of fetal specific T cells prior to magnetic enrichment. The central column demonstrates magnetic enrichment of fetal specific T cells and the right hand column a negative control using an HLA matched dextramer with a negative control peptide. Percentages represents fetal specific T cells as a % of CD8 T cells.

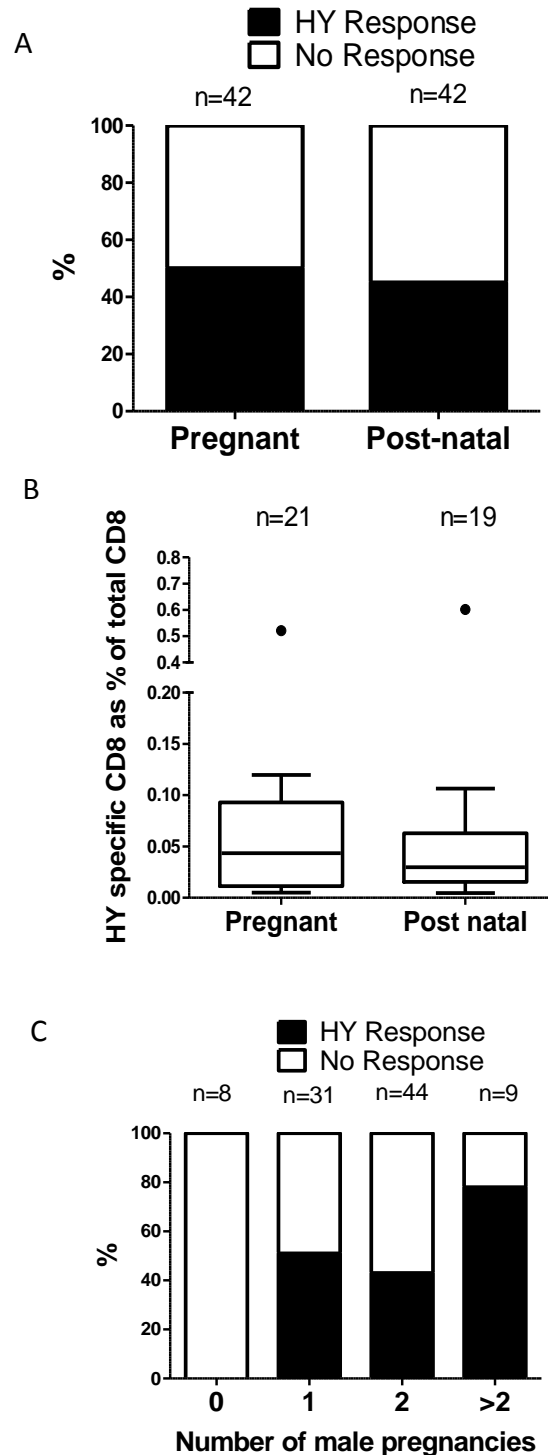


Figure 4.3.4 The rate of detection of fetal specific T cells.

The detection of fetal specific T cells is illustrated in women during (n=42) and after (n=42) pregnancy with a male fetus (a). In women in whom a fetal specific T cell response was detected the frequency of fetal specific T cells as a percent of total CD8 T cells is compared during (n=21) and after (n=19) pregnancy (b). The rate of detection of fetal specific T cells with a woman's total number of male pregnancies (including current pregnancy) is shown (1 male n=31, 2 male n=44, >2 male n=9). A negative control with no male pregnancies (n=8) is included (c).

#### 4.4 Dynamics of fetal specific T cells during gestation

The dynamics of the fetal-specific CD8+ T cell response during pregnancy was then examined in detail. HY-specific T cell responses could be detected in 28% of women from as early as the 1<sup>st</sup> trimester of pregnancy in those with a male fetus (Fig. 4.4.1). The proportion of women in whom an HY response was detected increase during pregnancy but this did not reach statistical significance ( $p=0.1057$ ). However, when the magnitude of the HY-specific CD8+ T cell response was examined, a correlation was seen between the frequency of HY-specific T cells and gestational age ( $p=0.03$ ). The median frequency of the HY-specific T cell response was 0.035% in women at 10 weeks of pregnancy and increased to 0.085% at term (Fig. 4.4.1b).

In order to investigate this further, an additional cohort of 20 HLA\*0201 pregnant women with male fetuses were recruited for a prospective study. Blood samples were taken in each trimester, during labour and in the postnatal period. Longitudinal analysis of 11 women in whom a response was seen confirmed that the frequency of HY-specific T cells increased between the 1<sup>st</sup> and the 3<sup>rd</sup> trimester and the 1<sup>st</sup> trimester and the post-natal period (Fig. 4.4.2). There was no significant difference between the frequency of HY specific T cells in late pregnancy, in labour and in the early post natal period.

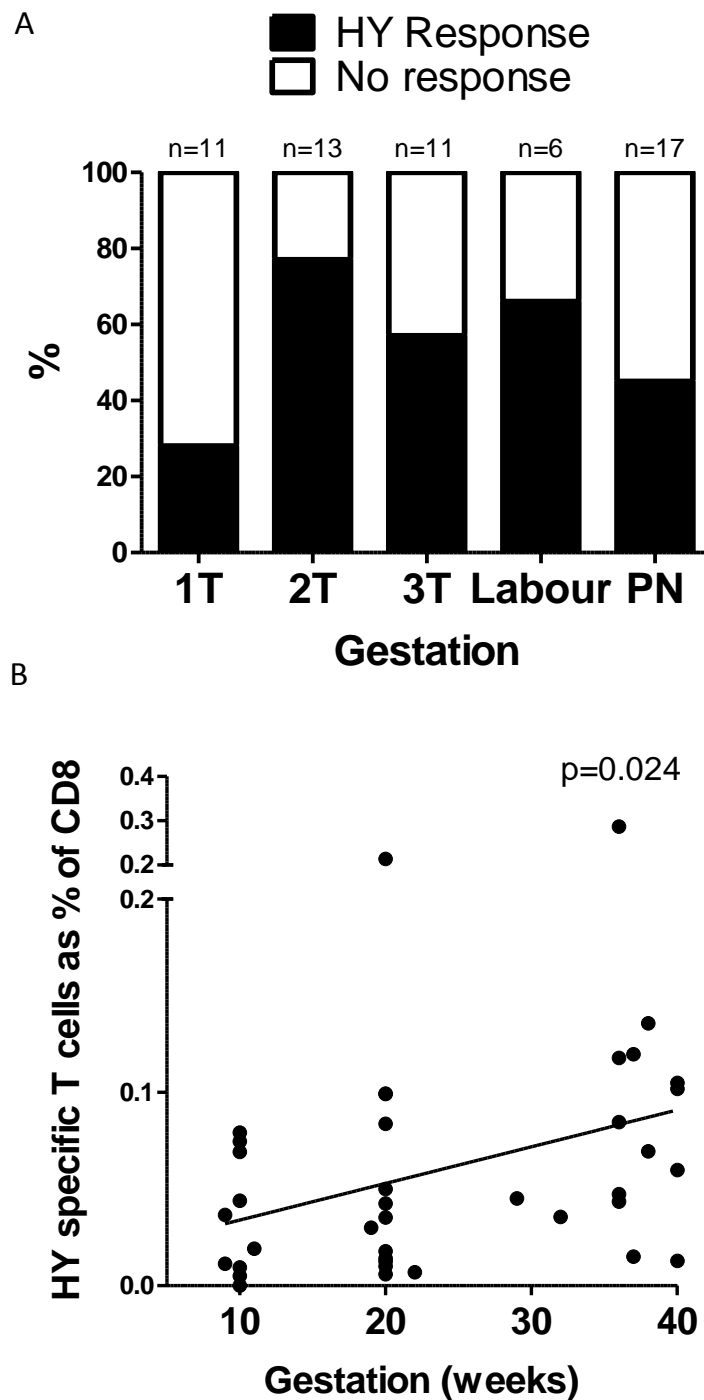


Figure 4.4.1. Rate of detection of fetal specific T cells across gestation.

MHC-peptide dextramers were used to detect fetal specific CD8 T cells following 10 day peptide line expansion of fetal (HY minor histocompatibility antigen – FIDS) antigen specific T cells at different gestational ages. This is demonstrated in a cross sectional analysis of women with a male pregnancy in the 1<sup>st</sup> trimester (1T) (n=11), 2<sup>nd</sup> trimester (2T)(n=12), 3<sup>rd</sup> trimester (3T)(n=11), during labour (n=6) or within the puerperium (PN)(n=17) (a). In the same cohort, in women in whom a fetal specific T cell response was detected (n=39) the magnitude of response is correlated with gestational age in weeks during pregnancy (b)

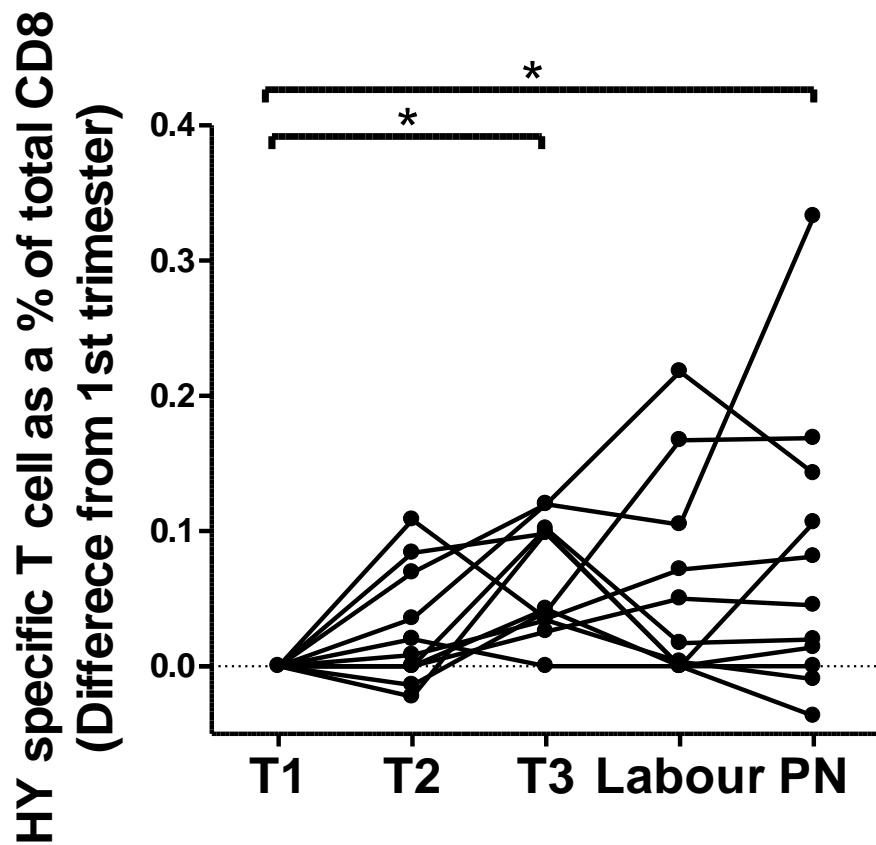


Figure 4.4.2 Longitudinal assessment of the rate of fetal specific T cells across gestation.

In a separate, prospectively recruited, longitudinal cohort, of women with a male fetus, the frequency of fetal specific T cells is examined in simultaneously assessed frozen specimens collected throughout pregnancy and the puerperium (n=11). The percentage of fetal specific T cells relative to the response in the 1<sup>st</sup> trimester is plotted for each individual. \* illustrates a p value of <0.05 by ANOVA with Tukey post hoc test (c).

## 4.5 Phenotypic characterisation of fetal specific T cells

In order to determine the phenotype of HY-specific CD8<sup>+</sup> T cells within peripheral blood we used dextramers to identify cells directly *ex vivo* and used multiparameter flow cytometry to examine the expression of markers associated with CD8<sup>+</sup> T cell differentiation. The same gating strategy was utilised as shown in Figure 4.3.1. CD8<sup>+</sup> T cells can be classified into four functionally distinct subsets on the basis of expression of CD45RA and CCR7. These comprise naive (N, CD45RA<sup>+</sup>CCR7<sup>+</sup>), central memory (CM, CD45RA<sup>-</sup>CCR7<sup>+</sup>), effector memory (EM, CD45RA<sup>-</sup>CCR7<sup>-</sup>) and highly differentiated 'revertant' CD45RA<sup>+</sup> effector memory subsets (EMRA CD45RA<sup>+</sup>CCR7<sup>-</sup>) (Fig. 4.5.1 a).

Further subdivision of CD8<sup>+</sup> memory subsets on the basis of expression of the co-stimulatory molecules CD27 and CD28 was also performed. In particular, within the effector memory subset (CCR7<sup>-</sup>CD45RA<sup>-</sup>), four further subsets can be defined based on their CD27 and CD28 expression. These functionally distinct subsets are termed EM1 (27<sup>+</sup>28<sup>+</sup>), EM2 (27<sup>+</sup>28<sup>-</sup>), EM3 (27<sup>-</sup>28<sup>-</sup>), and EM4 (27<sup>-</sup>28<sup>+</sup>) (Fig 4.5.1 b).

The T cell phenotype of HY specific T cells in a donor during pregnancy is illustrated (Fig. 4.5.1 c and d). HY specific T cells are shown as a density plot in black, superimposed on a grey contour plot illustrating the overall CD8 T cell phenotype. Figure 4.5.1c demonstrates 98% of the fetal specific T cells are memory T cells, with 45% effector memory and 51% more highly differentiated effector memory, re-expressing CD45RA. The contrast of this phenotype with the overall CD8 T cell phenotype is demonstrated with the overall CD8 T cell repertoire consisting of 15% naive T cells, 16% effector memory and 67% EMRA (Fig 4.5.1 c). Similarly, when the effector memory T cells are examined in more detail on the basis of



CD27 and CD28 expression the HY specific T cells are less well differentiated than the overall CD8 T cell phenotype with a higher proportion of EM1 T cells (86% v 43%) which is accounted for by a reduction in the most highly differentiated EM3 subtype (2 v 39%) (Fig 4.5.1 d).

The memory phenotype and effector memory subsets of 10 donors during pregnancy was then examined. HY specific T cells we found to have a memory phenotype, with a significantly reduced proportion of naive T cells compared to the overall CD8 T cell repertoire ( $p=0.0039$ ) (Fig. 4.5.2). The median memory phenotype (Fig 4.5.2a) illustrates that overall HY specific T cells are of an effector memory phenotype, with some of the effector cells re-expressing CD45RA. However, between individuals the memory phenotype appeared variable and with the small numbers of donors it was not possible to correlate this with pregnancy history or gestation (Fig. 4.5.2b)

The frequency of the EM1 subset was significantly higher in the HY specific T cells than in the global CD8 T cell population ( $p=0.039$ ). Thus the HY specific effector memory cells are less well differentiated than the overall CD8 T cell phenotype. (Fig 4.5.3 a and c) However, the effector memory subsets again appeared to be variable between individuals (Fig 4.5.3b). CD57, a surface marker which characterises highly differentiated cells, with low replicative potential and short telomeres was also measured. HY specific T cells were found to have a low expression of CD57, in keeping with the other indicators that these are not highly differentiated (data not shown).

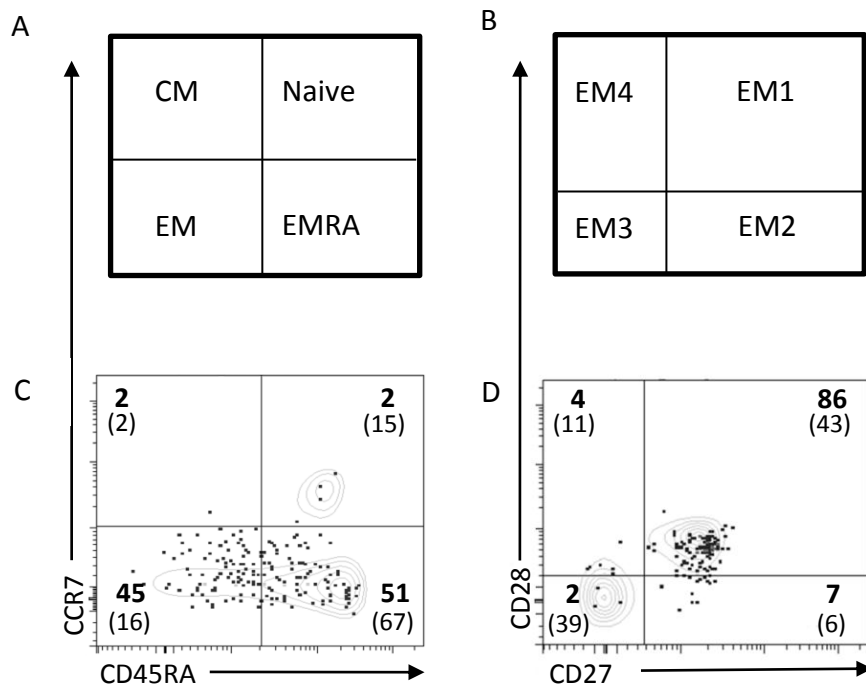


Figure 4.5.1 Ex-vivo identification of HY specific T cells.

A similar gating strategy was used for the ex-vivo identification of HY specific T cells as before. Briefly, singlet cells were identified on a FSC-A vs FSC-H plot and small lymphocytes gated on the basis of standard light scatter properties. A “dump” excluded dead cells, monocytes and B-cells. T lymphocytes were then selected by CD3 expression. Finally, dextramer “positive” cells were visualised against CD8 expression. The phenotype of the HY specific T cells was then examined in detail. The memory phenotype is described as naïve (CD45RA+CCR7+), central memory (CM, CD45RA-CCR7+), effector memory (EM, CD45RA-CCR7-) and effector memory RA revertant (EMRA, CD45RA+CCR7-) (a). The effector memory phenotype is further subdivided into effector memory subsets on the basis of CD28 and CD27 expression into EM1 (CD27+CD28+), EM2 (CD27+CD28-), EM3 (CD27-CD28-), EM4 (CD27-CD28+) (b). HY specific T cells are shown in black as a density plot, superimposed on the overall CD8 T cell phenotype as a grey contour plot. The percentages in each gate for the HY specific T cells is shown in bold, and the overall CD8 T cell phenotype is shown in parenthesis for the memory phenotype on the basis of CCR7 and CD45RA (c) and the effector memory (CD45RA-CCR7-) cells further phenotyped on the basis of CD27 and CD28 expression (d).

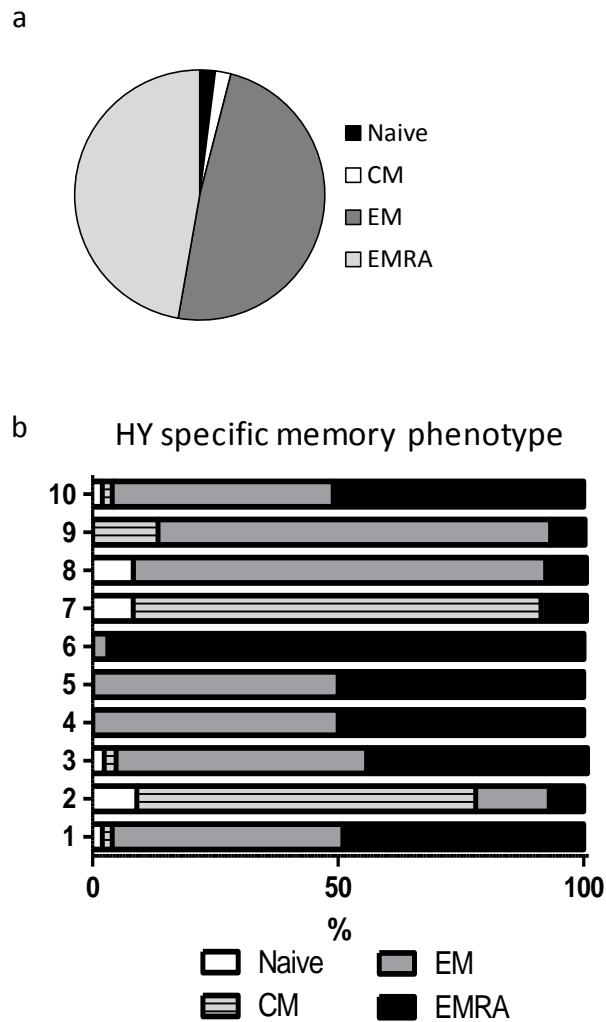
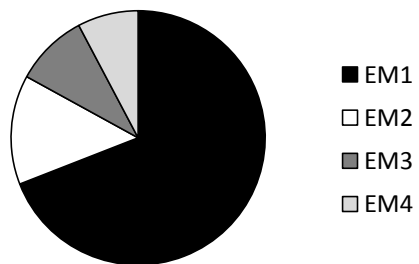


Figure 4.5.2 Memory phenotype of fetal specific T cells.

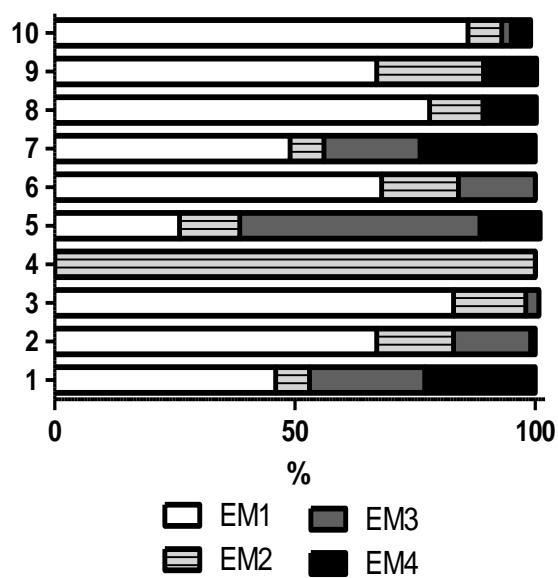
The memory phenotype is described as naïve (CD45RA+CCR7+), central memory (CM, CD45RA-CCR7+), effector memory (EM, CD45RA-CCR7-) and effector memory RA revertant (EMRA, CD45RA+CCR7-). The median phenotype (a) and individual results from 10 donors is shown (b).

a

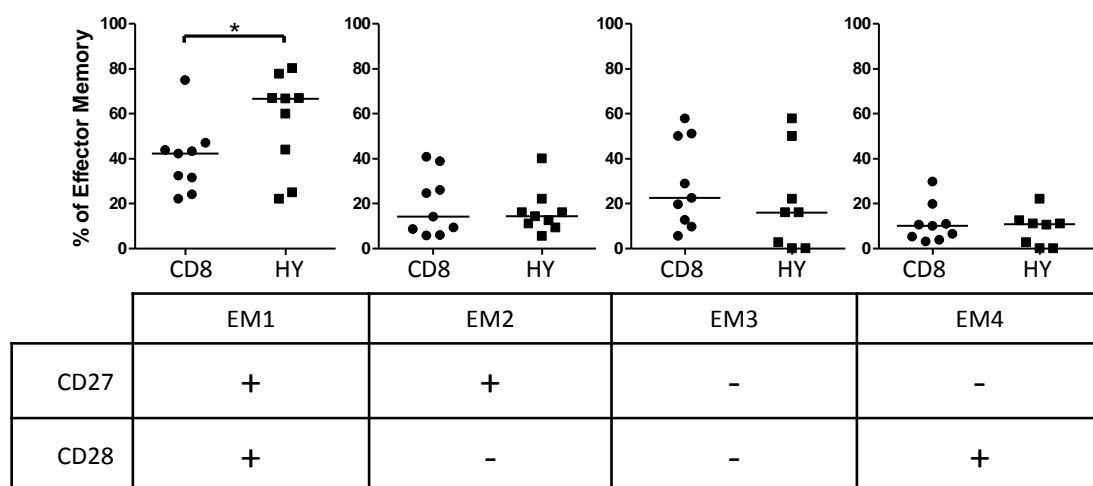


b

HY specific effector memory subsets



c



*Figure 4.5.3 The effector memory phenotype of fetal specific T cells.*

*The effector memory phenotype has been further subdivided into effector memory subsets on the basis of CD28 and CD27 expression into EM1 (CD27+CD28+), EM2 (CD27+CD28-), EM3 (CD27-CD28-), EM4 (CD27-CD28+). The median phenotype (a) and individual results from 10 donors is shown (b). The effector memory subset phenotype is compared between HY specific T cells and the overall CD8 T cell phenotype for each donor (c) and the EM phenotype then compared between the HY specific T cells and overall T cell subsets.*

## 4.6 Fetal antigen load

Quantitative real time PCR was conducted to detect male free fetal DNA load in maternal plasma during pregnancy.

A sensitive “Taqman” real time PCR assay was established to detect the multi-copy DSY14 gene on the Y chromosome. Use of this gene has been found to be more sensitive at low copy numbers than the single copy SRY gene (Zimmermann, El-Sheikhah et al. 2005). Standard curves were constructed with male DNA of known genome equivalents. The assay was found to be sensitive at less than 10 genome equivalents (GE) and the standard curve was linear over a large dynamic range up to  $10^5$  GE. A control gene beta-globin was also used to establish the total quantity of plasma DNA present. The beta-globin assay was primer limited, reducing its sensitivity, to enable multiplex assays to be conducted if required. Examples of the amplification curves from which the Ct values were determined are shown (Fig. 4.6.1a.). Example standard curves constructed from these are also shown (Fig. 4.6.1b). Standard curves for DYS14 and beta-globin were run in duplicate for every assay.

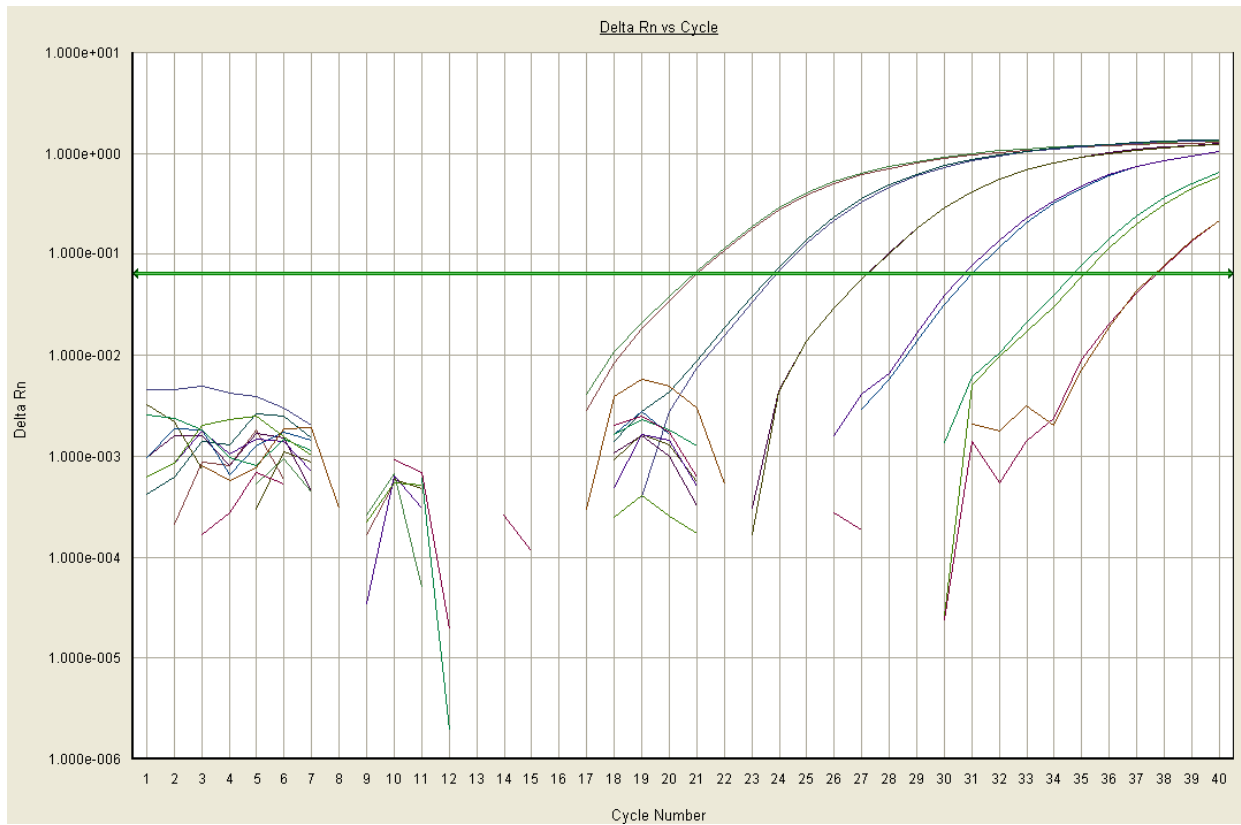
We found that the absolute quantity of male free fetal DNA was significantly elevated in the 3<sup>rd</sup> trimester of pregnancy (median 17 GE in the 1<sup>st</sup> trimester and 35 GE in the 2<sup>nd</sup> trimester to 184 GE in the 3<sup>rd</sup> trimester, a greater than 10 fold increase across gestation). After delivery the fetal plasma DNA was rapidly cleared from the maternal circulation and it could no longer be detected. (Fig. 4.6.2). We also examined the ratio of Dys14 to beta-globin to determine if the proportion of maternal plasma free fetal DNA which was fetal in origin changed across gestation. We found that as with the total fetal DNA that the proportion of

fetal DNA compared to the total plasma DNA increased in the 3<sup>rd</sup> trimester, from a median of 0.03 in the 1<sup>st</sup> trimester to 0.05 in the 2<sup>nd</sup> trimester and 0.33 in the 3<sup>rd</sup> trimester (Fig. 4.6.2b).

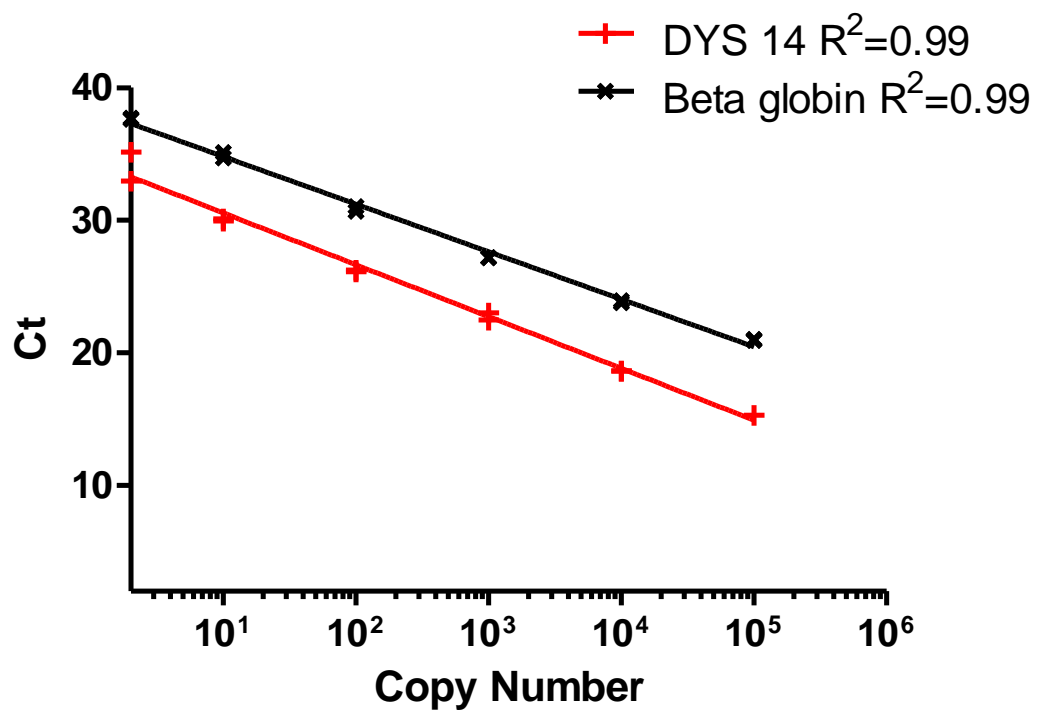
Changes in the maternal cellular immune response to fetal antigen have been examined alongside the quantification of free fetal DNA in maternal plasma. It can be observed in these 3 donors that the pattern for both the cellular response and the fetal DNA in maternal plasma is to accumulate during gestation. The major difference occurs after delivery when free fetal DNA is rapidly cleared, whereas the cellular immune response is not attenuated and in these cases actually increase further after delivery (Fig. 4.6.3).

We also attempted to detect intact fetal cells by extracting DNA from thoroughly washed PBMC's obtained from the maternal circulation during pregnancy. However, we could not consistently detect fetal cells within the PBMC fraction obtained during or after pregnancy.

a



b





*Figure 4.6.1 Standard curves and amplification plots for DYS14 quantitative real time PCR assay*

*Serial dilutions of male DNA consisting of  $10^5$ ,  $10^4$ ,  $10^3$ ,  $10^2$ , 10 and 2 genome equivalents of DNA were amplified using a primer/probe combination specific for DYS14 in singleplex reactions using an ABI Prism 7700, shown here in duplicate. The change in FAM fluorescent intensity ( $\Delta R_n$ ) plotted against cycle number is shown (a). Calibration curves are shown for both DYS14 and the control gene  $\beta$ globin.  $\beta$ globin was primer limited. These calibration curves were generated by plotting the  $C_t$  value for each sample, defined as the fractional cycle number at which the amplification plot crosses the threshold (solid horizontal line), against the initial number of genome equivalents. The Y intercept corresponds to the number of cycles required to detect a single gene and was consistently less than 40 cycles.*

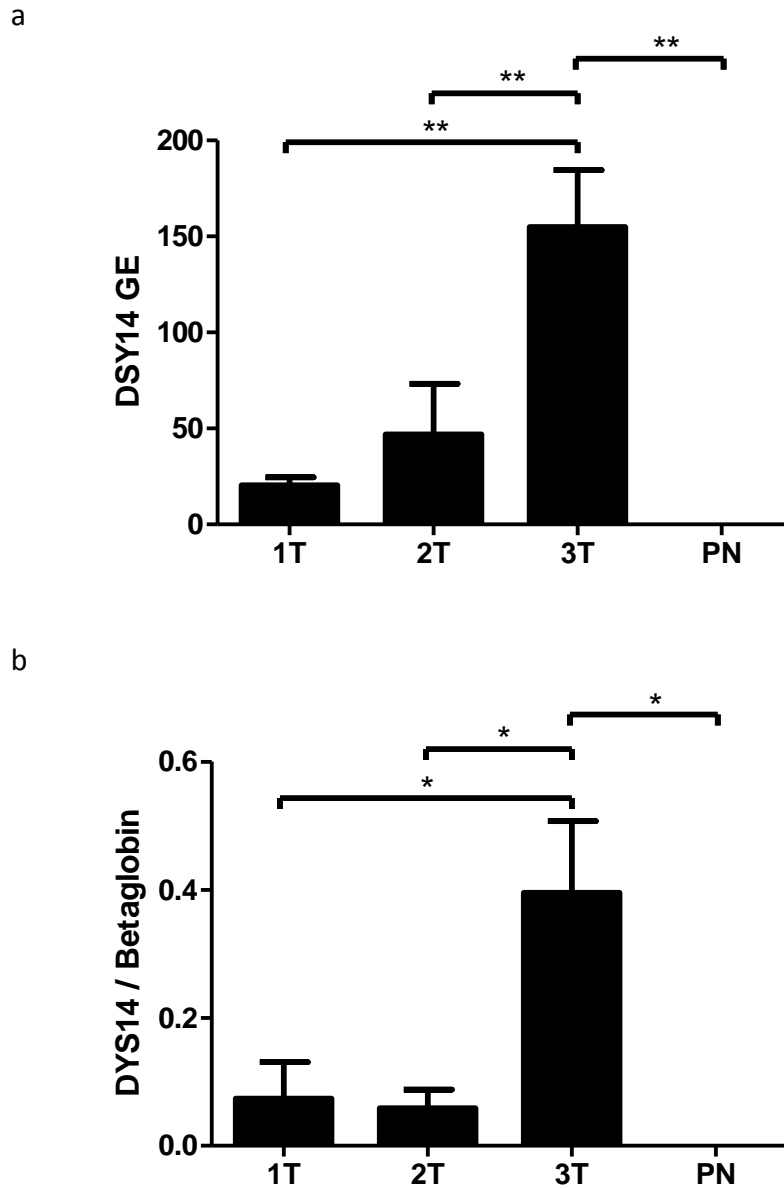


Figure 4.6.2 Male free fetal DNA across gestation.

Male free fetal DNA (DYS14 gene) in maternal plasma (a) and  $DYS14/\beta$ globin ratio in which the male antigen load has been normalised for total maternal plasma DNA (b) was measured by quantitative real time PCR in each trimester and the postnatal (PN) period ( $n=12$ , error bars indicate interquartile range, \* indicated  $p<0.05$ , \*\* indicates  $p<0.01$  by ANOVA with post test).

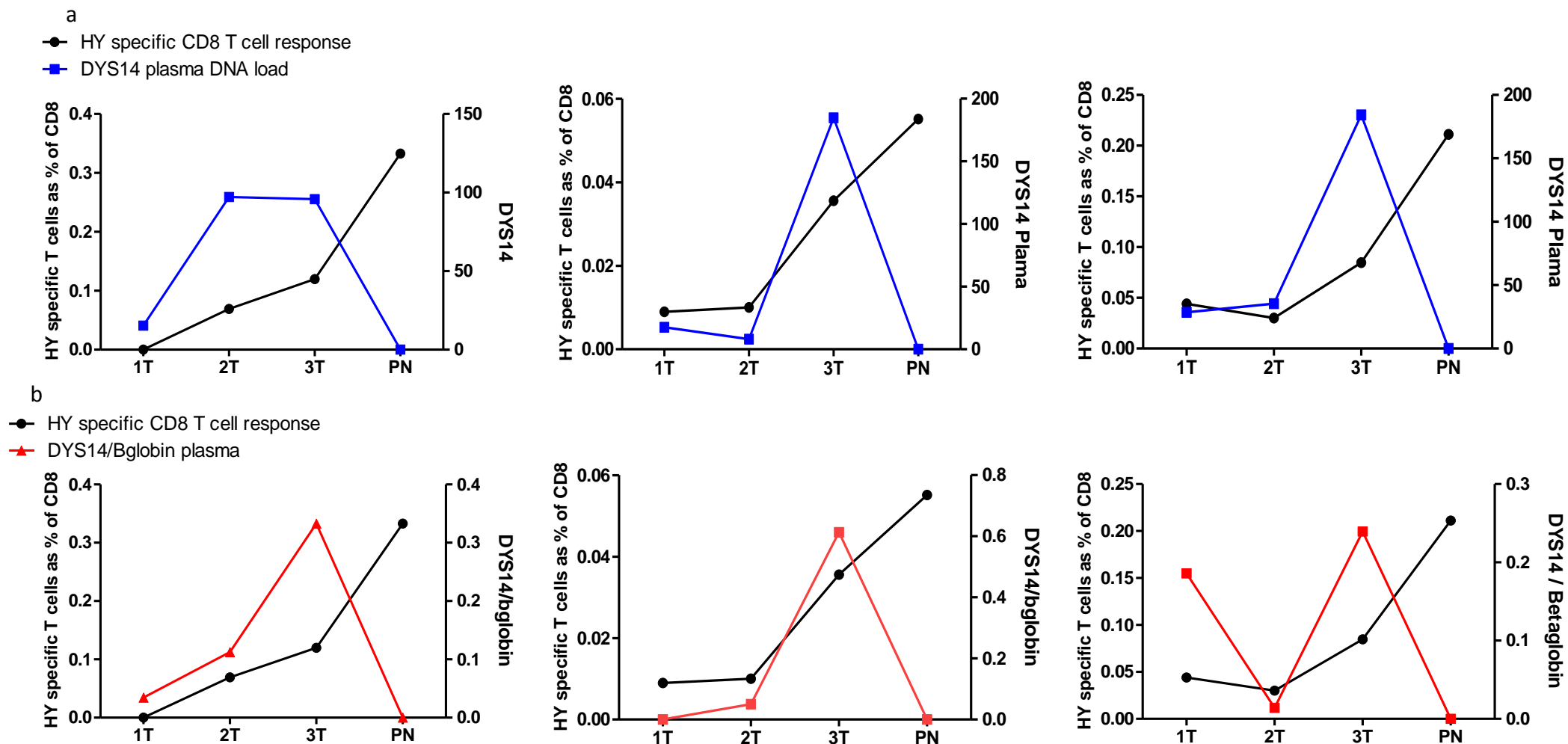


Figure 4.6.3. Correlation of HY specific T cell response with total DYS14 plasma DNA load and DYS14/ $\beta$  globin in maternal plasma in 3 donors

Quantitative real time PCR was used to measure the maternal plasma free fetal DNA load, using the male specific gene DYS14. This is correlated with the HY specific T cell response in 3 representative donors in the 1<sup>st</sup> trimester (1T), 2<sup>nd</sup> trimester (2T), 3<sup>rd</sup> trimester (3T) and postnatal period (PN). (a) The DYS14/ $\beta$ globin ratio demonstrate changes in fetal DNA are not simply a reflection of differences in the absolute quantities of free DNA in maternal plasma across gestation (b).

## 4.7 Discussion

We utilised MHC-peptide multimers to identify and characterise fetal minor histocompatibility antigen specific CD8 T cells within maternal peripheral blood during and after human pregnancy.

### ***Dextramers were superior to tetramers for the identification of HY specific T cells***

We found that using peptide-MHC dextramers resulted in antigen specific T cell staining which was brighter than dextramer staining, exhibited lower levels of background and enabled effective magnetic selection of fetal antigen specific cells. These features were important as we were seeking to identify a rare and potentially low affinity population of antigen specific cells.

### ***Meticulous flow cytometric gating to exclude aberrant binding events improved the assay specificity***

Antigen specific responses to HY antigen and tumour related antigens are known to be low frequency (Piper, McLarnon et al. 2007). It was therefore necessary to adopt a meticulous flow cytometric strategy, based on approaches optimised for the detection of rare antigen specific T cells (Chattopadhyay, Melenhorst et al. 2008) to minimise false positive events. This involved the use of multiple markers (CD14, CD19, viability dye, doublet

exclusion) to exclude cells which could aberrantly bind MHC-peptide multimers and a combination of CD3 and CD8 to identify CD8 T cells. This strategy proved beneficial compared to using a conventional gating strategy.

### ***Fetal antigen specific T cells can be detected during human pregnancy***

Cellular immune responses to fetal alloantigen have been reported in women with a history of pregnancy (Verdijk, Kloosterman et al. 2004; Piper, McLarnon et al. 2007; van Halteren, Jankowska-Gan et al. 2009). We conducted the first investigation to determine whether CD8 T cell immune responses to fetal minor histocompatibility antigens develop during pregnancy or only after parturition. Our results demonstrate that fetal specific CD8 T cell responses could be detected during pregnancy, at the same frequency as following delivery. Indeed, 28% of women develop T cell immune responses to fetal antigen from the first trimester of pregnancy. These results contrast with some murine models, which though also showing maternal cellular 'awareness' of fetal antigen during pregnancy have tended to suggest fetal specific CD8 T cell deletion during pregnancy (Tafari, Alferink et al. 1995; Jiang and Vacchio 1998; Vacchio and Hodes 2003; Vacchio and Hodes 2005).

An HY specific T cell response was detected more commonly in women who had more than 1 male pregnancy, though this was not statistically significant. This finding is in agreement with previous reports (Piper, McLarnon et al. 2007). It was very recently reported (Gammill, Guthrie et al. 2010) that contrary to expectations the level of fetal microchimerism does not appear to increase with an increasing number of pregnancies, and that the frequency of microchimeric cells remains constant with increasing pregnancies but that cells from subsequent pregnancies can take over and fill the same niche replacing the

microchimeric cell population. It is interesting to consider if this could be related to fetal antigen and thus microchimeric cell specific T cells which are controlling the expansion of the microchimeric cell numbers in subsequent pregnancies. This remains speculation as there have been no studies which have studied the degree of microchimerism and the cellular immune response in a way that would enable this question to be addressed.

In order to maximise the sensitivity of detection of HY-specific T cells we elected to use short term *in vitro* culture following peptide exposure. This technique has been widely employed to study a variety of antigen-specific responses including those associated with alloreactivity (James, Chai et al. 2003; Piper, McLarnon et al. 2007), viral infection (Reignat, Webster et al. 2002) and cancer (Coulie, Karanikas et al. 2001). We adopted a conservative approach and limited our assay to only a single episode of antigen exposure with a short 10 day culture period. This technique is unable to generate primary immune responses (Lalvani, Dong et al. 1997) and no responses were found in women without a history of miscarriage or male pregnancy. The magnitude of the HY-specific CD8<sup>+</sup> T cell response detected following this *in vitro* expansion correlated closely with the direct *ex-vivo* frequency within peripheral blood, although expansion in culture allowed greater sensitivity of detection.

#### ***Fetal specific T cells accumulate as pregnancy progresses, without evidence of deletion***

The dynamics of this response were further investigated in both a cross-sectional and longitudinal cohort of women. Both these approaches demonstrated that as pregnancy progressed there was an accumulation, rather than depletion, of fetal specific T cells. These then persisted into the postnatal period. The prospective, longitudinal assessment of this in an additional cohort of women enabled changes in the frequency of the response to be

observed over time in each individual. This was particularly informative given the heterogeneity of the response between different women. This confirmed the previous findings that there was an increase in HY specific T cells in women with a male pregnancy from the 1<sup>st</sup> trimester to the 3<sup>rd</sup> trimester and post natal time points.

There are many potential reasons why the effect observed in humans may be different from the previously reported murine studies. In particular there are important anatomical and immunological differences between murine and human pregnancy. In murine pregnancy the expression of MHC molecules at the decidual interface increases as pregnancy progresses (Rossant and Cross 2001). Whereas in human pregnancy HLA\*0201 which presents the FIDS epitope studied is not thought to be expressed by trophoblast at any point during normal pregnancy. It is interesting to speculate that some of the FAS/FAS-L dependent deletion seen in murine models may correspond with the presentation of HY antigens on the placenta later in pregnancy and that the lack of deletion in human pregnancy could be related to this antigen not being expressed by the trophoblast. However, even in murine models the findings of fetal specific T cell deletion has not been consistently reported. It has, for example, been demonstrated in an HY based model that even when the antigen was expressed on trophoblast and when there was evidence of homing of HY specific T cells to the decidua that deletion was not detected (Norton, Fortner et al. 2010). To investigate this further in a human context would be possible with a comparative study of HY antigens presented in the context of HLA-C, which is expressed on extravillous trophoblast in humans. However, no HY antigens expressed on HLA C have yet been described.

A further possible explanation for the differences between our findings and those of the previous murine models may be the nature of the model systems previously used to study this in murine pregnancy. In a transgenic system there are benefits in the ease with which antigen specific T cells can be tracked and phenotyped but as all T cells present are specific for a single antigen this wholly artificial situation alters the natural T cell repertoire and can alter the nature of the immune response observed , with a high antigen specific T cell frequency linked to developing a “tolerogenic” T cell response (Kurts, Kosaka et al. 1997). The antigen load is also important, with high and sustained antigen loads associated with CD8 down-regulation and deletion (Rocha, Grandien et al. 1995). The use of MHC-peptide multimers in our approach means that the physiological situation of a natural HY antigen, with physiological levels of antigen exposure and the low frequency of HY specific T cells, present within the natural repertoire *in vivo*, can be investigated directly.

### ***Relationship between fetal specific T cells and fetal antigen load***

There are several potential reasons why the accumulation of fetal specific T cells during gestation may occur. The most likely is increasing antigen availability leading to the proliferation of these cells, as is seen in response to viral antigens during primary viral infection during pregnancy (Marchant, Appay et al. 2003). We measured free fetal DNA in maternal plasma as a surrogate for the shedding of fetal material into the maternal circulation during pregnancy. As has been previously reported (Lo, Corbetta et al. 1997; Lo, Lau et al. 2000), we found increasing levels of fetal antigen shed into the maternal circulation in the 3<sup>rd</sup> trimester. Increasing antigen exposure could account for the expansion in fetal specific T cells. A similar phenomenon is seen if the frequency of tumour minor



histocompatibility antigen specific CD8 T cells is tracked in patients with myeloma. In these patients an increased frequency of response is seen at times of tumour relapse when antigen load increases (Goodyear, Piper et al. 2005).

There was no difference in the proportion of women in whom fetal specific T cells could be detected, or in the frequency of these cells, during and after pregnancy with a male fetus. This is intriguing in relation to the potential requirement for antigen during maintenance of the HY-specific immune response. Levels of fetal antigen in the maternal circulation are known to fall markedly following delivery (Lo, Zhang et al. 1999). Our measurement of free fetal DNA in maternal plasma, used as a surrogate for shed maternal antigen, confirmed this rapid clearance of free fetal DNA from the circulation. However, this does not necessarily mean that fetal antigen is being cleared in a similar fashion from maternal tissues such as lymph nodes. Indeed, it is recognised that after viral infection has resolved that antigen reservoirs can persist in lymph nodes and tissues and sustain memory T cell responses (Kim, Hufford et al. 2010). Furthermore, persistence of low levels of fetal antigen, in the form of fetal microchimerism following pregnancy is now well recognised and may be important in this (Bianchi, Zickwolf et al. 1996; Bianchi, Williams et al. 1997; Gammill, Guthrie et al. 2010). We did attempt to measure fetal cellular DNA from maternal blood. However, with the limited numbers of PBMCs available after cellular assays had been conducted, and the rarity of these cells, we were not able to consistently detect fetal cells in the maternal circulation. A recent study (Adams Waldorf, Gammill et al. 2010) specifically examined the prevalence of fetal cells in maternal lymphocytes enriched by magnetic selection from healthy pregnancy and concluded “the probability of detecting one fetal cell equivalent increased 6.2-fold each trimester....though fetal cells in PBMC were not detected for the majority of time points (only 20% of donors).” Intact fetal cells are clearly very rare in

the maternal circulation. Thus, while these fetal cells include cells capable of presenting antigen (Loubiere, Lambert et al. 2006) their rarity suggests that antigen is most likely indirectly presented by maternal antigen presenting cells (Fig. 1.5.1). Thus the development of a maternal cellular immune response would not rely on intact fetal cells in the maternal circulation as shed fetal antigen would suffice, nor would it be dependent on the fetal antigen presenting cells being an appropriate HLA type to present antigen to maternal T cells.

A limitation of measuring cells only in peripheral blood is that it is not possible to be sure if changes in their frequency relate to differences in tissue homing or mobilisation of antigen specific T cells into peripheral blood or true changes in their frequency. To further investigate this would require the investigation of fetal antigen specific T cells in maternal decidua and ideally secondary lymphoid tissue, alongside peripheral blood.

***Fetal specific CD8 T cells had a memory phenotype, and are not highly differentiated***

HY-specific CD8 T cells studied directly within maternal blood had an effector memory phenotype although the relative proportion of central memory, effector memory and revertant CD45RA<sup>+</sup> memory cells was variable and there was no clear correlation between memory subset distribution and clinical characteristics. One interesting feature was that HY-specific effector memory cells retained high levels of expression of CD27 and CD28. CD27<sup>+</sup>CD28<sup>+</sup> effector memory (EM1) cells share features with central memory cells and have longer telomeres than effector memory cells which have lost CD27 and CD28 (Romero, Zippelius et al. 2007). This relatively early-differentiated phenotype is likely to reflect limited antigen exposure *in vivo* and may indicate that, after priming, placental anatomy limits

exposure of HY-specific cells to fetal antigen. Alternatively, FOXP3<sup>+</sup> regulatory T cells, suggested to be important in feto-maternal tolerance during pregnancy, may potentially operate to limit T cell differentiation (McNally, Hill et al.). However, our finding that in the periphery pregnancy related changes in T regs are not significant across gestation suggests that for fetal antigen specific T cells in the periphery this T reg effect is less likely to be of importance.

***Influence of antigen presentation and expression on immune response has not been determined***

An important consideration regarding our study is that is that the FIDSYICQV peptide from HY has a ubiquitous pattern of tissue expression which is likely to play an important role in determining the nature of the HY-specific immune response. This was used as a model antigen because responses to this epitope are well characterised and had been detected in women after pregnancy. However, fetal-specific epitopes with more selective cellular expression, for example HA-1 on haematopoietic cells or antigens expressed only in certain stages of embryonic development, may vary the dynamics and phenotype of the response observed. The systematic assessment of responses to these other antigen types and the identification of further epitopes required to answer these questions will be an important area for future study.

***Conclusion***

Fetal minor histocompatibility antigen specific CD8 T cells can be commonly detected in normal pregnancy. The frequency of the fetal specific T cells increases during gestation, with no evidence of deletion. These cells are of a memory phenotype and are not highly differentiated.

# **Chapter 5**

## **Fetal specific T cell function**

## 5.1 Introduction

In chapter 4 we described the identification of HY minor histocompatibility antigen specific memory CD8 T cells in human pregnancy and the accumulation, rather than deletion, of these cells as pregnancy progresses. This chapter will address the function of these fetal specific T cells through fetal specific T cell clones generated during and after pregnancy.

It has been demonstrated that fetal specific T cell clones generated from women after pregnancy, even many years after delivery, retain their potential for cytotoxicity and IFN  $\gamma$  production (Verdijk, Kloosterman et al. 2004; Piper, McLarnon et al. 2007). However, the function of fetal specific T cells in women during pregnancy has not been investigated.

Many of the murine models of fetal specific T cells in pregnancy suggest that those cells which are not deleted are rendered hyporesponsive (Jiang and Vacchio 1998; Vacchio and Hodes 2003; Vacchio and Hodes 2005; Erlebacher, Vencato et al. 2007; Norton, Fortner et al. 2010). Vacchio et al. reported that the remaining fetal specific T cells were unable to proliferate in vitro, even if given IL-2, suggesting that this hypo-responsiveness is more profound than a conventional anergy state (Schwartz 2003; Redmond and Sherman 2005). But other models have shown no reduction in function (Norton, Fortner et al. 2010).

Due to the rarity of fetal specific T cells in the maternal circulation determining their function directly ex-vivo was not possible. The experimental approach utilised was the development of HY specific T cell clones. By generating T cell clones, with a pure T cell population and a known antigen specificity, these functional questions could be best addressed.

## 5.2 Fetal specific T cells isolated during pregnancy proliferate in culture

To assess the proliferation of fetal specific T cell in culture a subset of HLA\*0201 pregnant women with a male fetus in whom responses were seen following peptide lines expansion, and where there were sufficient PBMCs available (n=16) were further studied. It was demonstrated that HY specific T cells during pregnancy readily proliferated in culture increasing in frequency from a median frequency of 0.0077% of CD8 T cells (null values excluded) ex-vivo to 0.043% of CD8 T cells following peptide line expansion,  $p < 0.0001$  (Fig. 5.2 a). Furthermore, it was found that the frequency of fetal specific T cells found ex-vivo correlated well with the frequency observed following short term culture ( $p = 0.0043$ ) (Fig. 5.2b). There were 6 women (38%) in whom fetal specific T cells could not be detected ex-vivo despite their presence following short term culture, including an individual from whom HY specific T cell clones was obtained. This demonstrates the increased sensitivity of detection following short term culture. The process of short term culture was found to alter the T cell phenotype with the loss of CD45RA on memory cells previously re-expressing it. This is illustrated in Figure 5.2c. The memory phenotype of the HY specific T cells after expansion in a peptide line can be contrasted with the overall T cell phenotype following short term culture. HY specific T cells, due to their stimulation in the peptide line by antigen can be seen to have lost CD45RA. 85% are therefore now of an effector memory phenotype. This can be contrasted with the ex-vivo phenotype described in Figure 4.5.2. Thus HY specific T cell phenotype information was obtained only from ex-vivo experiments.

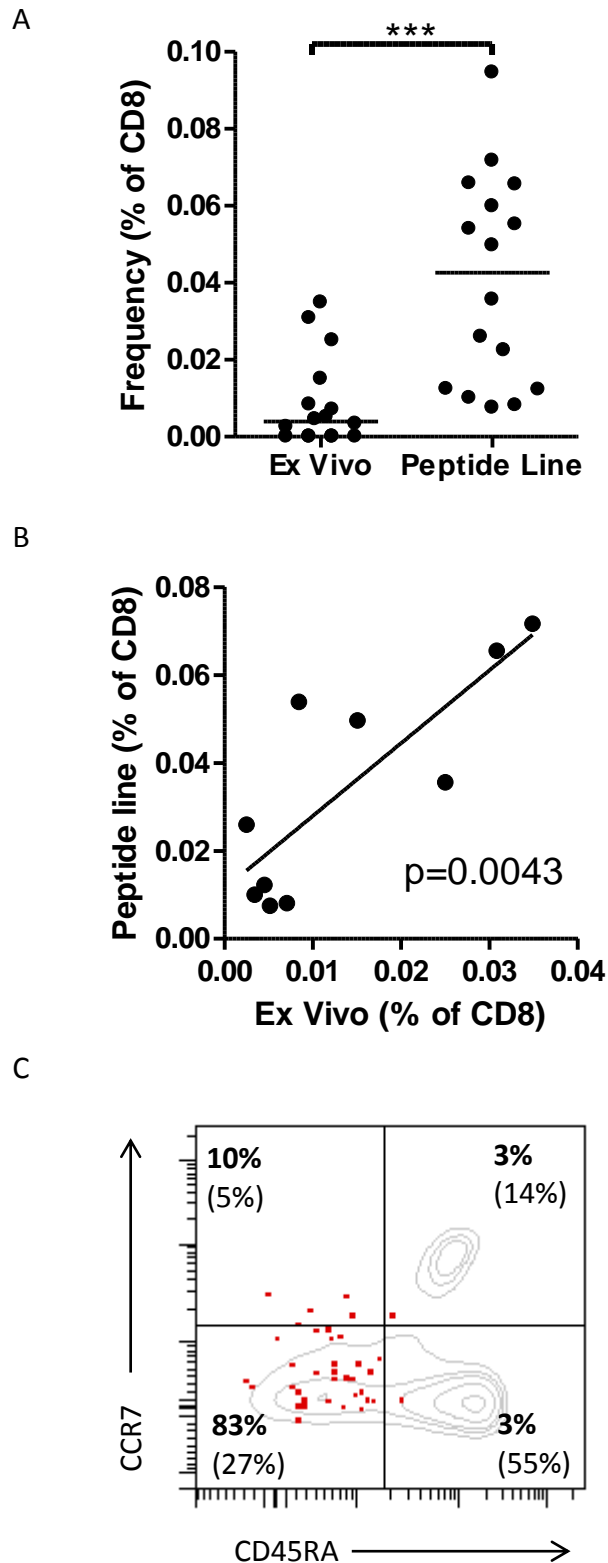


Figure 5.2 Proliferation of fetal specific T cells in culture.

The frequency of fetal specific CD8 T cells from women during pregnancy ex-vivo and following peptide line expansion is compared in paired samples ( $n=16$ ) (a). The frequency of fetal specific CD8 T cells is correlated between peptide line and ex vivo conditions (b) This flow cytometry plot demonstrates the memory phenotype



*of HY specific T cells following short term culture in a peptide line, on the basis of CCR7 and CD45RA. HY specific T cells are shown in red as a density plot, superimposed on the overall CD8 T cell phenotype as a grey contour plot. The percentages in each gate for the HY specific T cells are shown in bold, and the overall CD8 T cell phenotype is shown in parenthesis.*

### **5.3 Generation of fetal specific CD8 T cell clones**

Maternal PBMCs obtained during and after pregnancy were used to isolate fetal specific T cells and generate fetal antigen specific T cell clones. This was done to enable antigen specific T cell function to be examined. The generation of these clones involved exposing maternal PBMCs to a single pulse of antigen stimulation followed by 10 days culture with IL-2, IL-7 and IL-15. HY dextramer (FIDS) was then used to isolate the fetal specific CD8 T cells. Anti-PE beads and magnetic selection enabled enrichment of these cells. Cloning was then carried out by a limiting dilution technique. Following cloning the developing colonies were identified visually and picked prior to being screened by dextramer.

Through this technique multiple fetal specific T cell clones were generated (n=33 during pregnancy, n=77 post natal). This was, to our knowledge, the first isolation of fetal specific T cell clones from women during pregnancy.

Examples of HY specific T cell clones isolated during (Fig. 5.3.2) and after (Fig. 5.3.3) pregnancy are shown with histograms representing their staining by MHC peptide dextramers

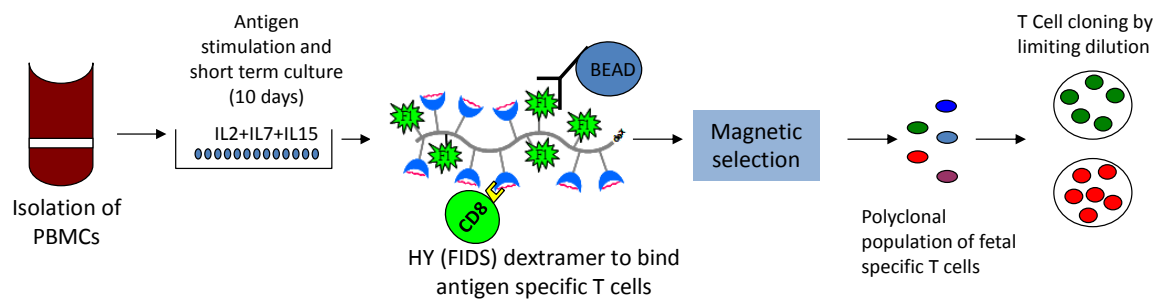
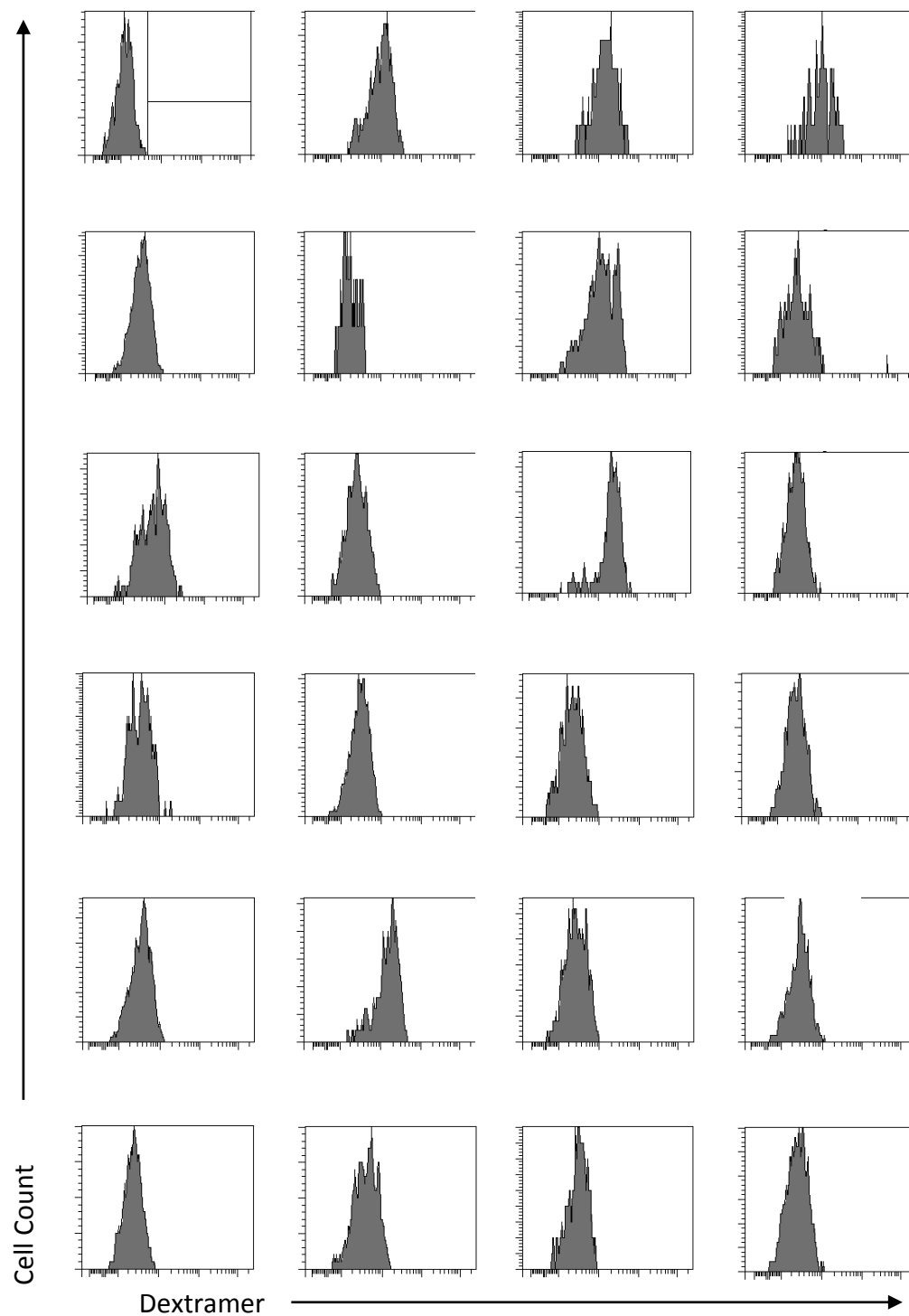


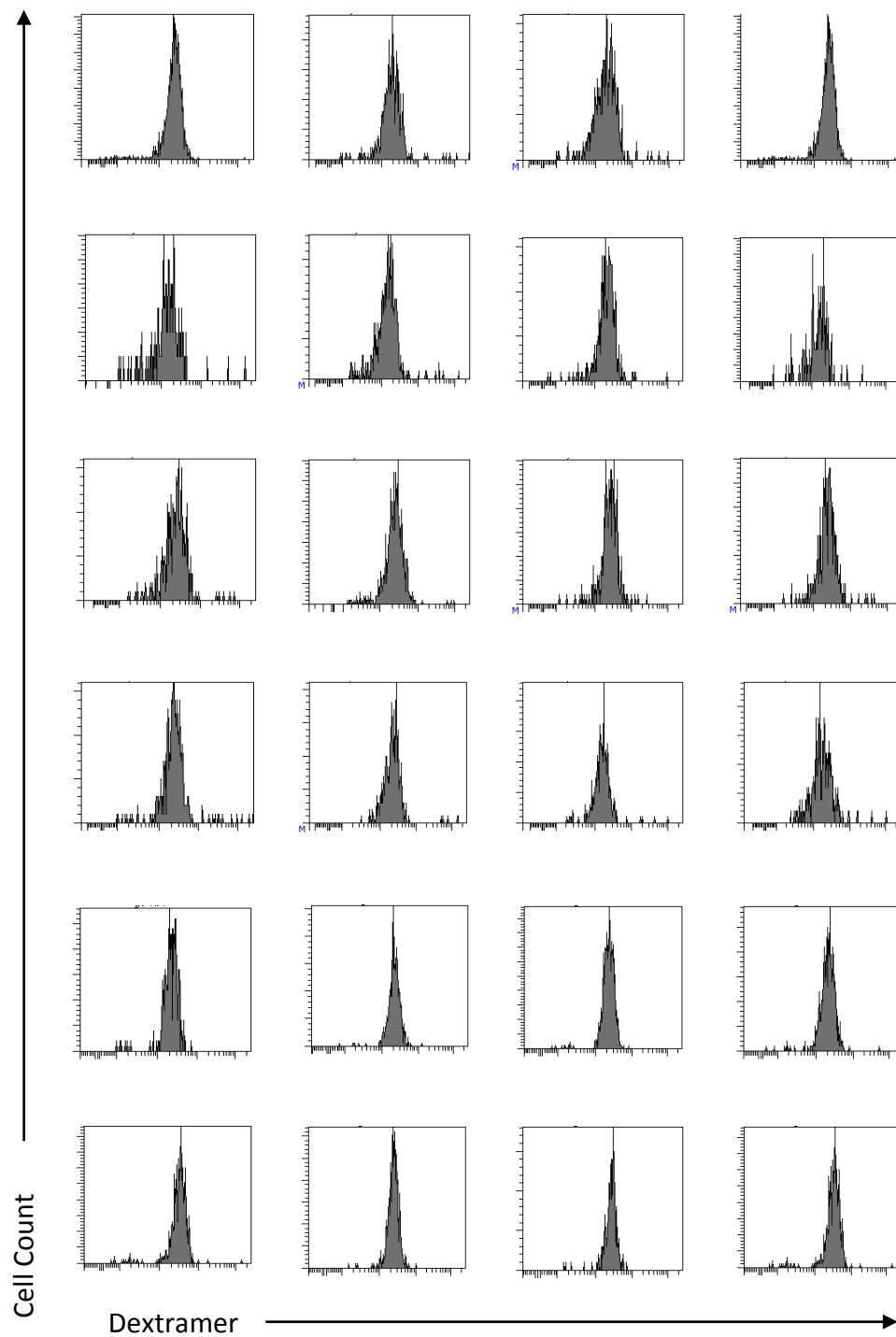
Figure 5.3.1 Schema for the generation of fetal antigen (HY) specific T cell clones.

Initially PBMCs were isolated by density gradient centrifugation. These were then stimulated for 1 hour with the HY peptide FIDS prior to short term (10 day) culture with the addition of IL-2, IL-7 and IL-15. HLA-peptide dextramer was used to isolate HY specific T cells. The dextramer incorporates the PE fluorochrome and anti-PE beads and magnetic selection could therefore be used to select these HY specific T cells. The selected cells after being checked for purity by flow cytometry were cloned through a limiting dilution method. Irradiated allogenic PBMCs stimulated with PHA from 3 healthy donors and irradiated lymphoblastoid cell lines were used as “feeder cells” to support the replication of the antigen specific T cell clones. Developing clones were identified by visual inspection of the developing colonies and these were then screened by dextramer prior to further functional analysis.



*Figure 5.3.2 Representative examples of fetal specific T cell clones.*

*Clones were obtained from women during normal pregnancy. These flow cytometry histograms show staining with the HY-A2 dextramer (FIDS) on the X axis and cell count on the y axis. The top left panel contains a negative control clone.*



*Figure 5.3.3 Representative examples of fetal specific T cell clones obtained from women after normal pregnancy.*

*These flow cytometry histograms show staining with the HY-A2 dextramer (FIDS) on the X axis and cell count on the y axis.*

## 5.4 Dextramer binding of fetal specific T cell clones

Interestingly, the intensity of staining of individual clones showed marked variation. Moreover, the intensity of staining remained consistent for individual clones during cell culture indicating that differential avidity for dextramer was an intrinsic cellular property. In particular a population of low intensity dextramer staining clones was isolated during pregnancy. The median of the MFI of clones isolated during pregnancy was 360, compared to 1644 for clones isolated after pregnancy ( $p < 0.0001$ ) (Fig. 5.4.1).

We used imaging flow cytometry (Amnis Imagestream) to examine the pattern of distribution of TCR and CD8 at the cell membrane in order to determine their co-localisation in lipid rafts (Drake and Braciale 2001) which has been revealed as an important determinant of function and staining by HLA-peptide multimers (Spencer and Braciale 2000). Dextramer-staining of the HY-specific T cell clones could be clearly visualised through this novel technique and demonstrated a punctuate pattern of membrane staining which co-localized with CD8 in all cases (Fig. 5.4.2).

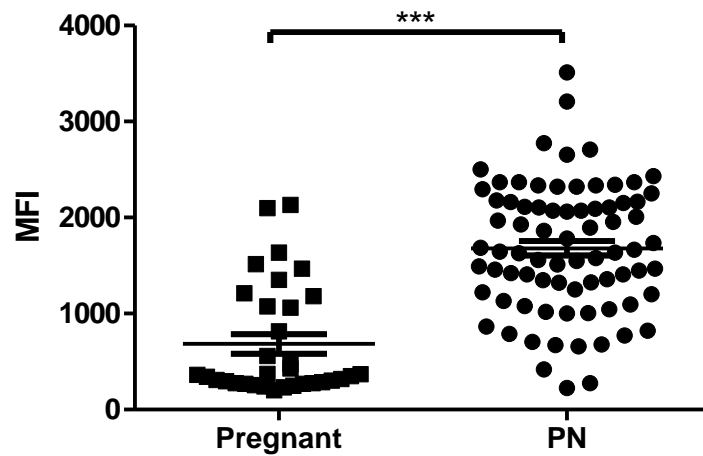


Figure 5.4.1 MHC peptide dextramer staining of T cell clones isolated during or after pregnancy.

The intensity of MHC-peptide dextramer staining is compared between T cell clones isolated during ( $n=33$ ) or after ( $n=77$ ) pregnancy, with a large number of low intensity T cell clones isolated during pregnancy (\*\*\*) indicates  $p<0.001$ ).

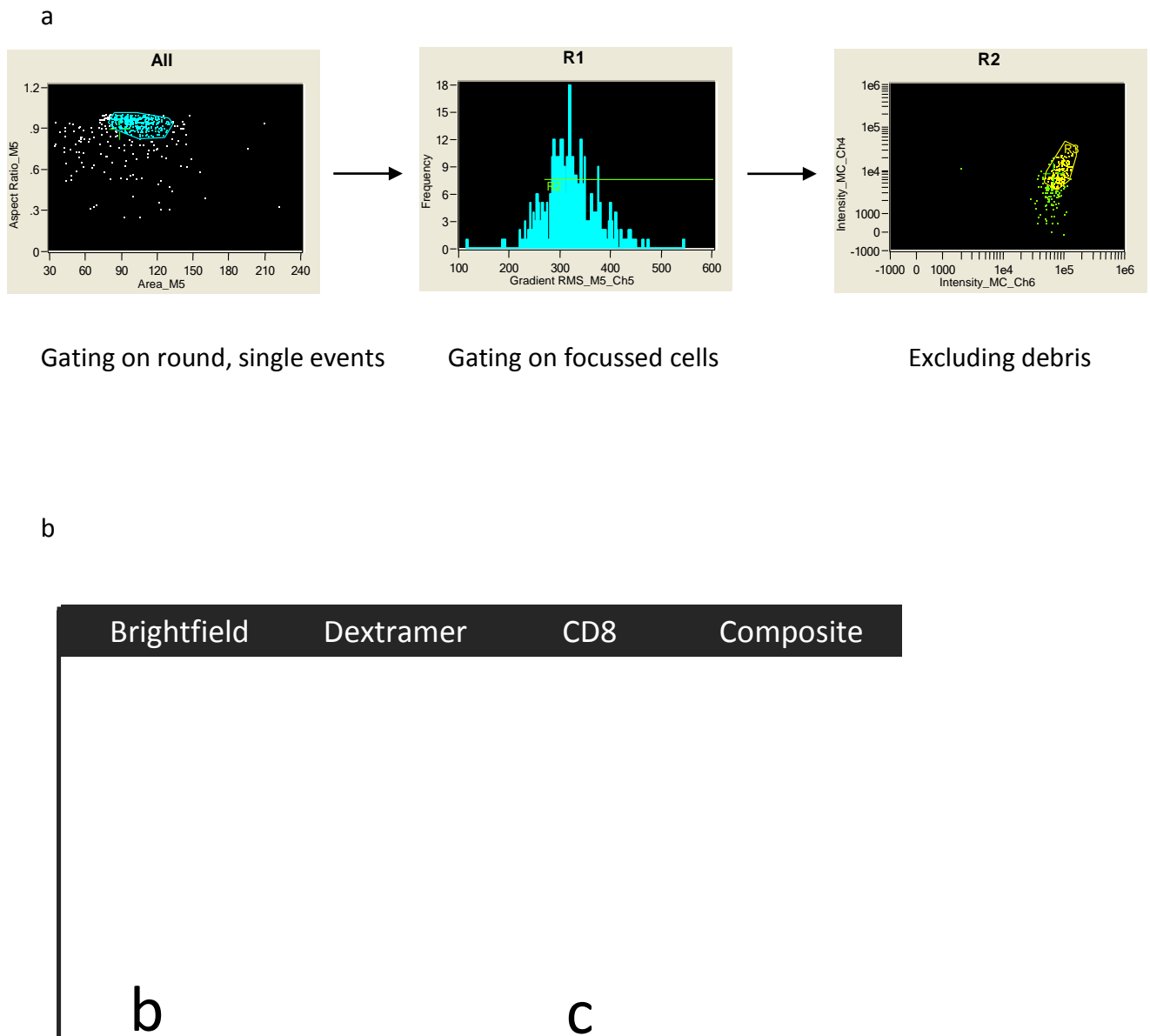


Figure 5.4.2 Imaging flow cytometry of fetal specific T cell clones.

The dextramer staining of fetal specific T cells is visualised by imaging flow cytometry using the AMNIS imagestream system. The gating strategy involved the exclusion of doublets, exclusion of cells which are out of focus and exclusion of debris (a). The images obtained for each cell include a brightfield image, dextramer staining (green), CD8 staining (red) and a composite image (co-localised staining yellow). The first 3 rows illustrate the HY HLA\*0201 FIDS dextramer and the final row negative control cells (b).

## 5.5 Function of fetal specific T cell clones

In view of the finding that HY-specific T cells are present within the blood of women who carry a concurrent male pregnancy it was important to examine the functional capacity of these T cells following stimulation *in vitro*. Clones were assessed for their ability to release IFN- $\gamma$  (n=17) and mediate cytotoxicity (n=18) following recognition of FIDSYICQV-pulsed target cells or primary male cells (Fig. 5.5.1). All of the clones demonstrated potent cytotoxic potential with the ability to lyse target cells at low effector : target ratios. Moreover, we could demonstrate that clones isolated during and after pregnancy were able to recognise natural male target cells revealing their capacity to recognise endogenously processed antigen. The HLA and peptide specificity was confirmed with HLA and peptide mismatched negative controls. There were no clones assessed in which dextramer staining was seen without antigen specificity also being demonstrated by the functional assays.

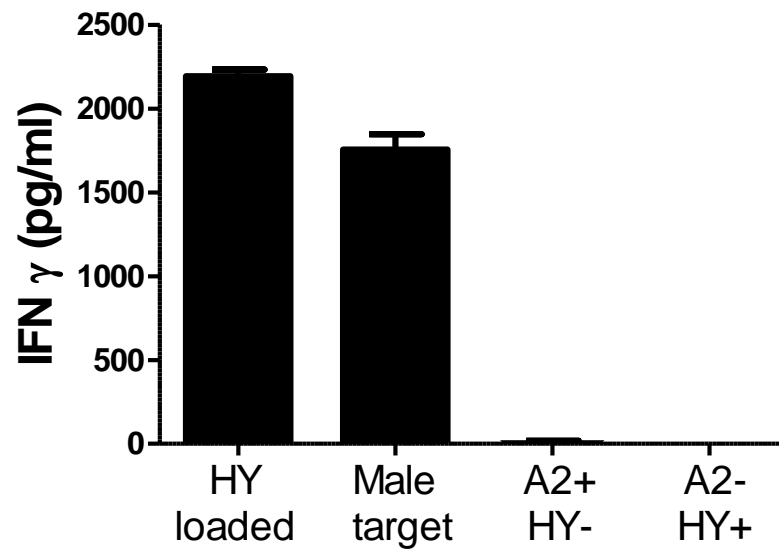
The assessment of multiple clones obtained both during and after pregnancy demonstrated there was no significant difference in production of IFN  $\gamma$  (Fig. 5.5.2a) or cytotoxicity (Fig. 5.5.2b) when clones generated during or after pregnancy were compared.

Interestingly, the cytotoxic capacity and cytokine release of individual HY-specific T cell clones was highly correlated when the cytotoxicity was determined at a 1:1 ratio of effector cell to target (Fig. 5.5.3). When the effector to target ratio was increased to a 5:1 ratio then the killing efficiency was so high for all clones examined that a correlation was no longer observed with cytokine production. Peptide titration experiments were carried out to determine the avidity of the T cell clones and contrast those clones isolated during and after pregnancy. The clones assessed in this assay were all identified as dextramer bright by flow cytometry. These clones had high avidity for peptide with 50% maximal function at peptide



concentration of  $10^{-6}$  or  $10^{-7}$ M. This is comparable with tumour related antigens previously described (Goodyear, Piper et al. 2005). It is noteworthy that when tetramer bright clones obtained during and after pregnancy were compared there were no differences seen in the relative binding affinity of these two populations (Fig. 5.5.4).

a



b

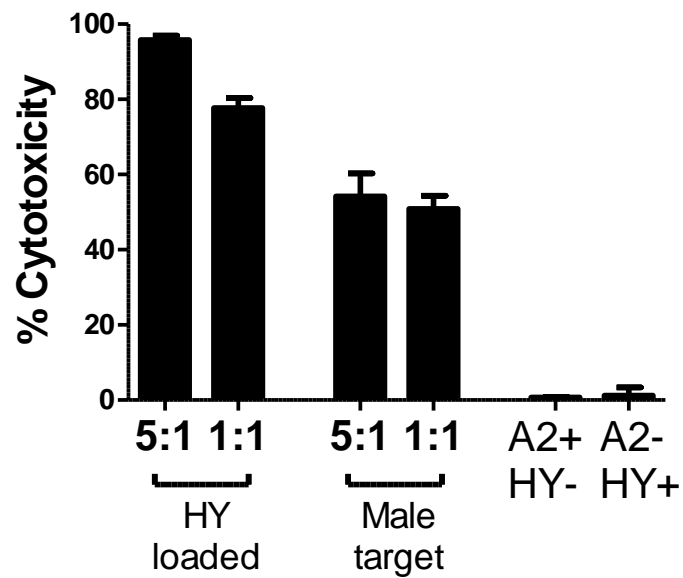


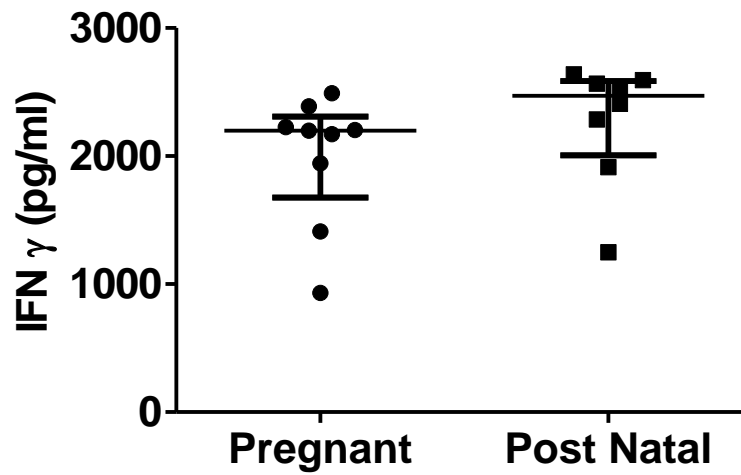
Figure 5.5.1 Representative examples of the assessment of T cell clone function and specificity.

IFN  $\gamma$  release was measured by ELISA of supernatant (a) and cytotoxicity by standard chromium release assay (b). Specificity was determined by screening the T-cell clones against not only HY loaded HLA matched targets and endogenously processed HY antigen (male cells), but also negative controls of HY loaded HLA mismatched

targets, HLA matched target with an irrelevant peptide and also T cells and targets alone (not shown).

Experiments were conducted in triplicate.

a



b

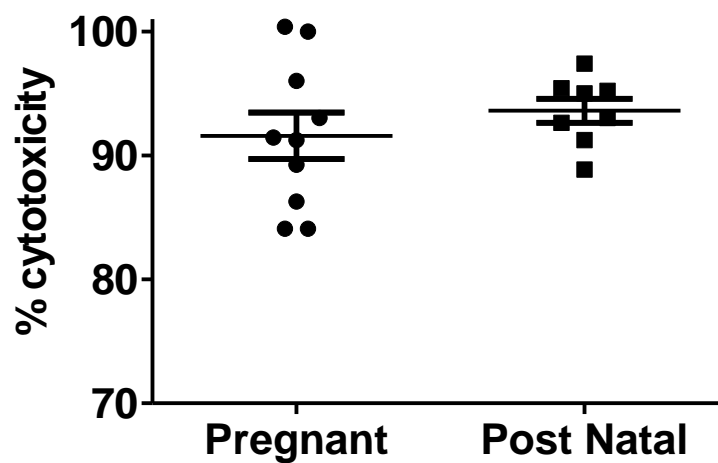


Figure 5.5.2 T cell clone function.

Summary data for the production of IFN  $\gamma$  ( $n=17$ ) (a) and cytotoxicity (shown at a 5:1 ratio) (b) ( $n=18$ ) are compared between T cell clones isolated during or after pregnancy. Bars indicate median.

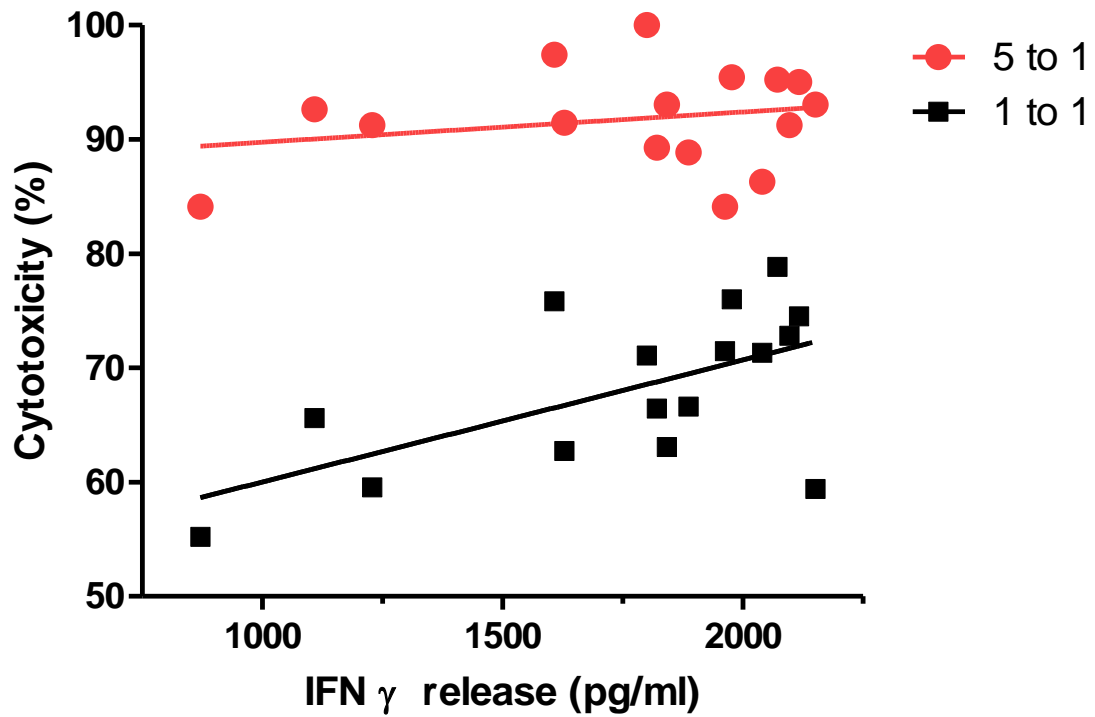


Figure 5.5.3 Correlation of cytotoxicity and IFN  $\gamma$  release by fetal specific T cell clones.

The cytotoxicity (measured as % killing) and IFN  $\gamma$  production (measured in pg/ml in the supernatant) of HY specific T cell clones in response to HY antigen loaded target cells at both a 5:1 and a 1:1 ratio is shown. The lines of best fit were calculated by linear regression. At a 1:1 ratio there is a significant correlation between cytotoxicity and IFN  $\gamma$  release ( $p < 0.05$ ).

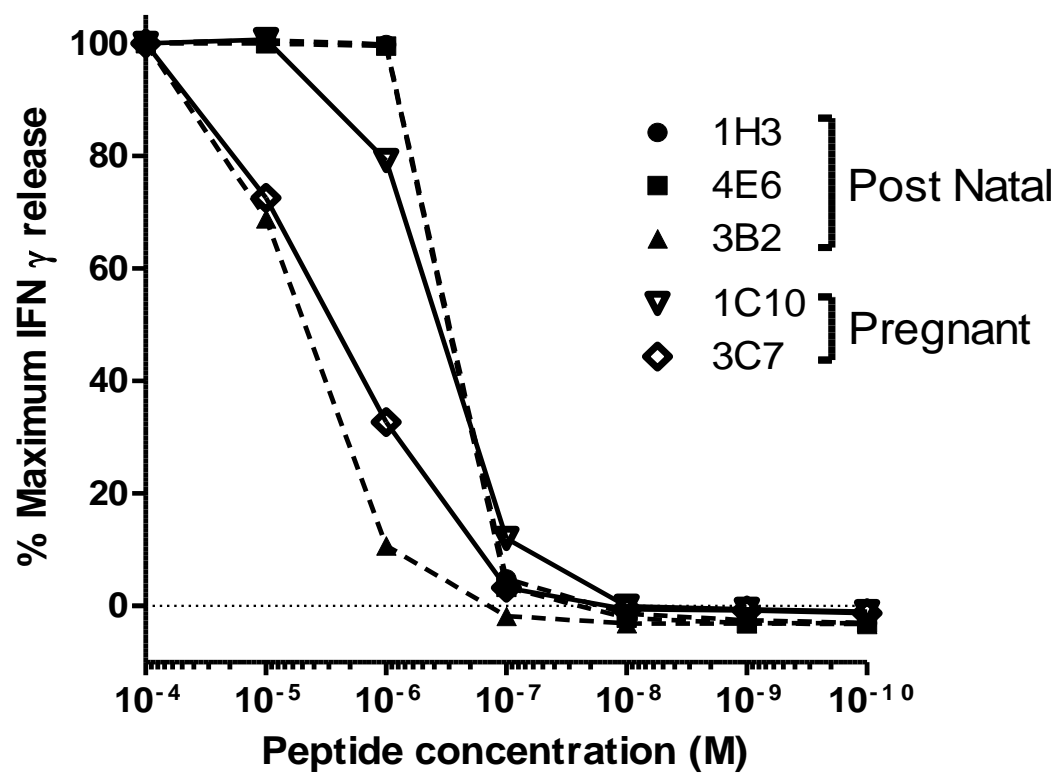


Figure 5.5.4 . Peptide binding affinity of fetal specific T cell clones.

The peptide binding affinity of T cell clones isolated both during and after pregnancy was assessed by peptide titration (n=5) These clones were all dextramer bright staining when assessed by flow cytometry.

## **5.6 Comparison of cellular function and T cell receptor sequence of dextramer bright and dim T cell clones**

The identification of antigen specific, dextramer dim staining clones during pregnancy (Fig. 5.4.3) led to the question of if these dim staining clones were functionally distinct. Bright and dim clones obtained from the same timepoint in the 2<sup>nd</sup> trimester, in the same individual, were therefore contrasted to establish if differences in cytokine secretion or cytotoxicity could be observed.

It was found that rather than the dim staining clones having impaired function they were potently cytotoxic, killing a median of 71% of peptide loaded targets at a 1 to 1 ratio, which was significantly more than the bright staining clones (median 61%,  $p=0.02$ ). This difference was not observed at a higher effector to target ratio, with very high killing efficiencies in both groups. IFN  $\gamma$  production was also significantly higher amongst the dim staining clones (2388 pg/ml v 2034 pg/ml,  $p=0.04$ ).

To further investigate this phenomenon 4 dextramer dim staining clones and 3 dextramer bright staining clones again from the same timepoint, in the same individual had their T cell receptor sequence determined. The TCR receptor sequencing was kindly performed by Mr Hayden Pierce. The histograms demonstrating the dextramer staining of these clones is shown, and they have been divided into those which are dim (Fig. 5.6.2 a) and those which are bright (5.6.2 b) The sequencing of their T cell receptors demonstrated that all the dim clones from this individual shared a TCR, for which the rearranged gene segments were  $\alpha$  V19, J39 and  $\beta$  V9, D1, J1-6 (5.6.2 c). The bright staining clones similarly

shared a TCR which was distinct from that on the dim clones, for which the gene segments were  $\alpha$  V24, J36 and  $\beta$  V6-5, D1, J1-1 (5.6.2 d).

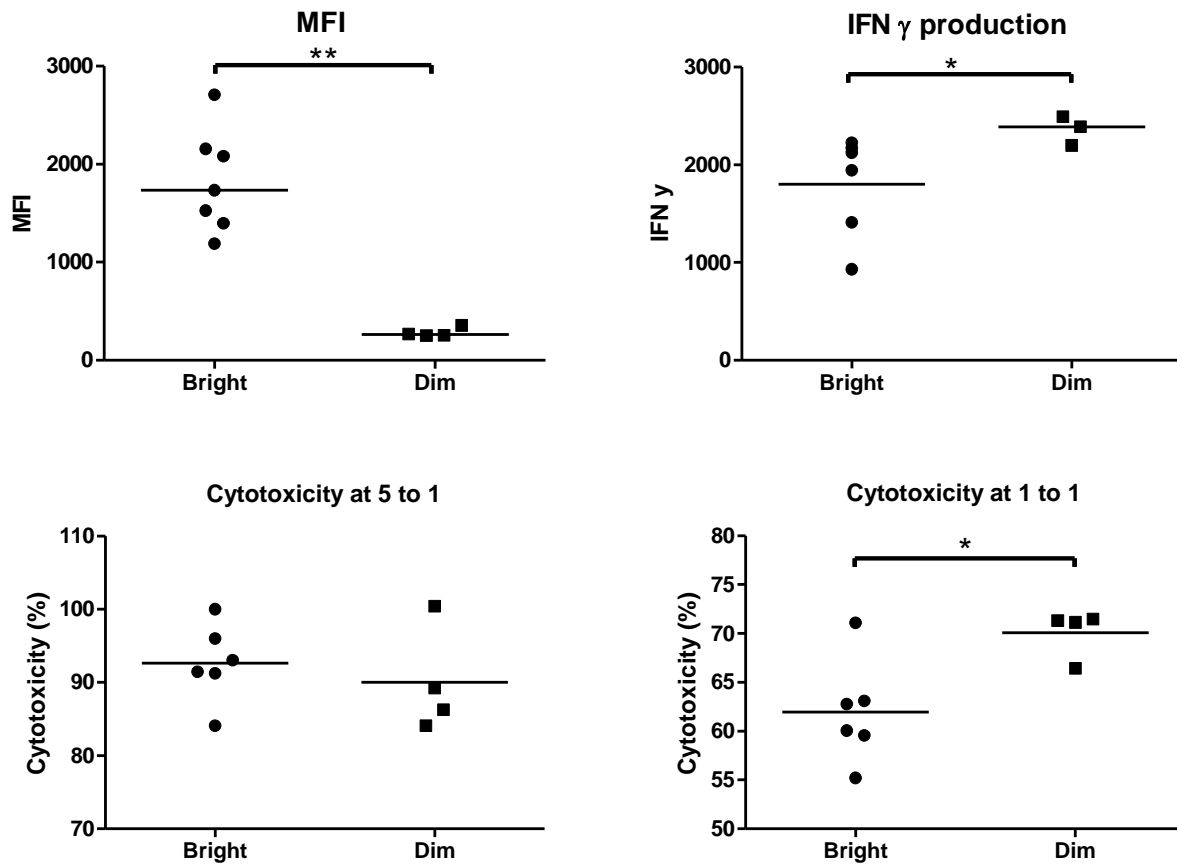
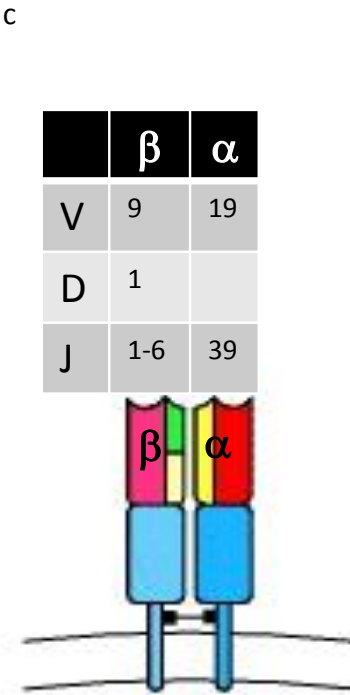
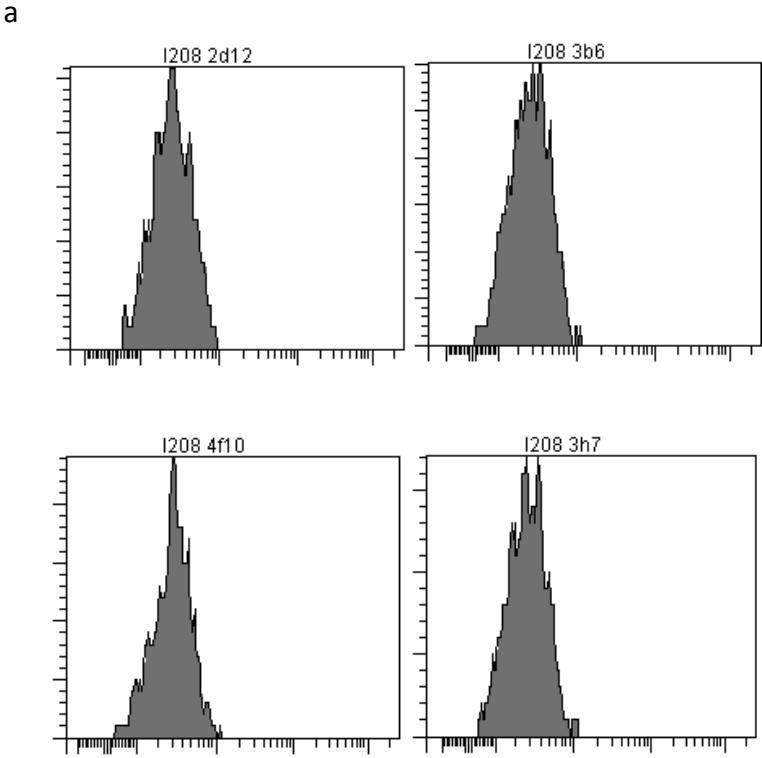


Figure 5.6.1 Functional assessment of tetramer bright and dim staining clones.

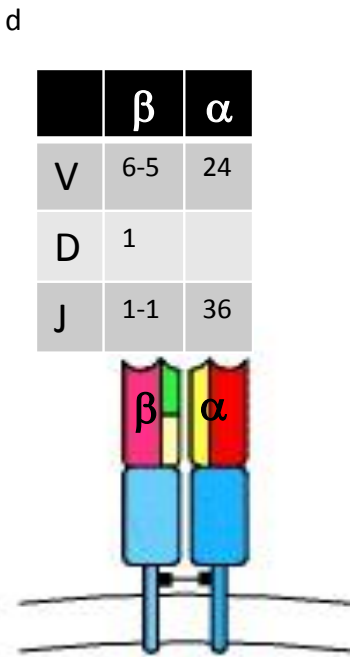
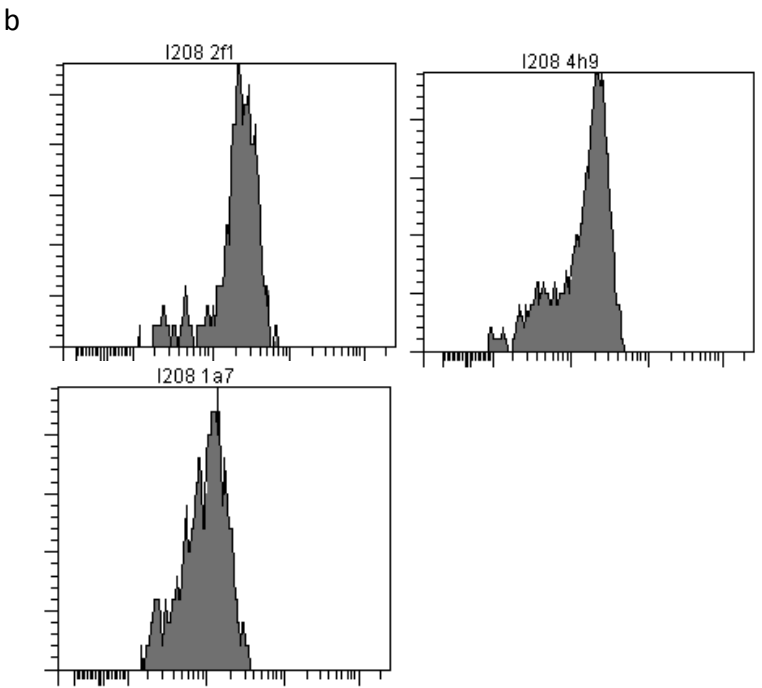
Comparison of tetramer bright and tetramer dim HY specific T cell clone function from clones isolated from the 2<sup>nd</sup> trimester, in the same donor during pregnancy. (\*= $p < 0.05$ )



Dextramer Dim



Dextramer Bright



*Figure 5.6.2. TCR receptor sequencing of dextramer bright and dim staining clones*

*4 dextramer dim staining clones and 3 dextramer bright staining clones were obtained from the same timepoint, in the same individual. Histograms demonstrating the dextramer staining of the dextramer dim (a) and dextramer bright (b) clones are illustrated. The sequencing of their T cell receptors was performed by Mr Hayden Pierce. All the dim clones from this individual shared a TCR, for which the rearranged gene segments were  $\alpha$  V19, J39 and  $\beta$  V9, D1, J1-6 (c). The bright staining clones similarly shared a TCR for which the gene segments were  $\alpha$  V24, J36 and  $\beta$  V6-5, D1, J1-1 (d).*

## **5.7 Investigation of overall CD8 T cell function during pregnancy**

To assess if pregnancy was associated with a global modulation of CD8 T cell function, which could account for the presence of functional fetal specific T cell clones in the absence of overt pregnancy pathology, we assessed if there was any global attenuation of CD8 T cell cytokine production after stimulation during pregnancy. IFN- $\gamma$  and IL-17 production by CD8 T cells was measured by intracellular cytokine staining following PMA/ionomycin stimulation in a cohort of 71 women. The gating strategy is shown (Fig 7.7.1 a). The cells were initially identified as small lymphocytes on the basis of their forward and side scatter properties and dead cells, monocytes and B cells excluded by a “dump” channel as previously (not shown). The left panel shows the subsequent gating of CD8 T cells as CD3+CD8+. The centre panel illustrates a representative example of IFN  $\gamma$  production and IL-17 production following stimulation with PMA-ionomycin. The right hand panel demonstrates a similarly treated isotype control. (Fig 5.7.1 a). IFN- $\gamma$  production was not seen to differ between pregnant women and those in the post natal period (Fig 5.7.1 b). To determine if there were more subtle changes that may have been missed by this type of

cross sectional analysis a prospective, longitudinal analysis of 17 women was conducted in which samples were obtained in each trimester and the post natal period and then cryopreserved. All timepoints from each individual were then simultaneously assessed. This similarly showed that IFN- $\gamma$  release by CD8 T cells did not vary during in each trimester or in the post natal period (Fig 5.7.1 c). IL-17 was produced by less than 1% of CD8 T cells and no significant differences were seen with gestation (data not shown).

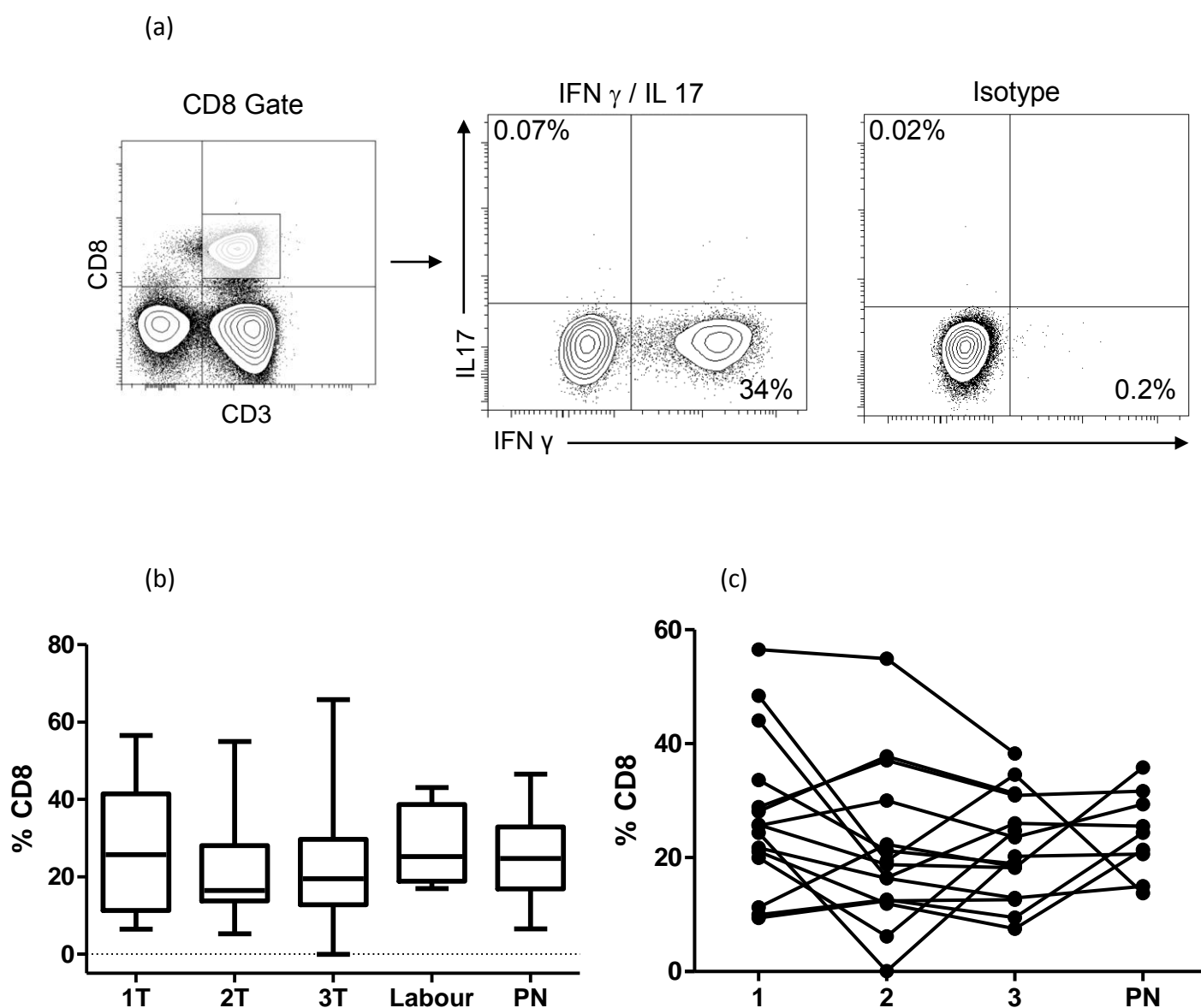


Figure 5.7.1. Global IFN  $\gamma$  production by CD8 T cells during pregnancy.

A representative flow cytometry plot illustrates the gating of CD3+CD8+ T cells and subsequent intracellular staining for IFN  $\gamma$  and IL-17, with appropriate isotype controls. The percentage of CD8 T cells which produce IFN  $\gamma$  upon stimulation with PMA/ionomycin is illustrated in a cross-sectional cohort ( $n=71$ ) of women bled during the 1<sup>st</sup> trimester (1T), 2<sup>nd</sup> trimester (2T), 3<sup>rd</sup> trimester (3T), in labour and in the post-natal period (PN) (b). In a subset of women ( $n=17$ ) responses were followed longitudinally with the same individual examined each trimester and in the postnatal period (c).

## 5.8 Discussion

Fetal specific T cells retained their ability to proliferate, demonstrated by their expansion when cultured in vitro. They also exhibited a broad and potent functional potential, with the ability to secrete IFN  $\gamma$  and kill target cells. Importantly, there was no reduction in function in clones isolated during pregnancy. We did however identify a population of fetal specific T cell clones isolated during pregnancy which had the unusual characteristics of only staining at a low intensity with MHC-peptide dextramer, suggesting that these are cells with a low affinity TCR/peptide-MHC interaction (Burlingham and Goulmy 2008). These dim staining clones appeared to secrete more IFN  $\gamma$  and be more cytotoxic than the bright staining clones and they had a distinct T cell receptor. The presence of functional fetal specific T cells in the absence of overt “rejection” could not be explained by a general suppression of T cell function during pregnancy, as IFN  $\gamma$  release in response to stimulation was found to be maintained by CD8 T cells throughout pregnancy and the postnatal period.

### ***Fetal specific T cells retain their proliferative potential***

The proliferative potential of HY-specific CD8<sup>+</sup> T cells when they were cultured with peptide in vitro is important because it has been previously reported in a murine transgenic system that HY-specific CD8<sup>+</sup> T cells which escaped deletion in pregnant female mice did not proliferate in response to antigen, even with the presence of IL-2 in the culture system (Jiang and Vacchio 1998). This hyporesponsiveness they observed may reflect a feature of the

transgenic TCR system they used with supraphysiological levels of antigen specific T cells. It could also reflect the fundamental biological and immunological differences between human and murine pregnancy (Moldenhauer, Hayball et al. 2010).

***Fetal specific T cell clones were generated from women during and after pregnancy***

The isolation of large numbers of fetal specific T cell clones and their detailed functional characterisation, which confirmed their specificity for HY antigen, has validated the generation of fetal specific CD8 T cells during pregnancy and the use of dextramer based techniques for detecting and isolating them.

***Fetal specific T cell clones generated during pregnancy retain their cytotoxicity and ability to produce IFN  $\gamma$***

The function of fetal specific T cell clones isolated during or after pregnancy was not found to differ significantly, with respect to cytotoxicity or IFN  $\gamma$  production. This is important as otherwise it could be suggested that the expansion of fetal specific T cells during pregnancy, in the absence of overt pathology, implies a functional deficiency in these cells (Chaouat, Petitbarat et al. 2010). Due to the rarity of these cells it was not possible to measure their function directly ex-vivo. However, we have demonstrated that with antigen exposure and the correct environment these cells certainly retain their functional potential. Thus, *in vivo*, given the appropriate environment we anticipate they could kill male cells and produce inflammatory cytokines. Whether this causes deleterious effects in normal pregnancy or causes pathology only in specific conditions remains to be determined.

Particularly immunogenic situations may be associated with increased antigen exposure or an inflammatory cytokine environment such as during placental disease or infection (Redline 2011). Further work to examine women with pathological pregnancies and to address the potential homing of these cells to the decidua would help address these questions.

The circulation of significant numbers of functional, fetal-specific CD8 T cells in healthy pregnancy is in agreement with some murine data which demonstrated that the presence of such cells did not affect litter size, even when the CD8 T cells were found at the maternal-fetal interface (Norton, Fortner et al. 2010). Our findings therefore suggest that in human pregnancy mechanisms other than deletion must be regulating their action *in vivo* enabling the fetus to escape from recognition by HY-specific T cells.

A potential mechanism that may be important may be the down regulation of HLA expression on trophoblast and syncytiotrophoblast. Though expression of HY antigen on these tissues did not affect pregnancy viability in a murine model (Norton, Fortner et al. 2010).

Alternative possible mechanisms of tolerance include the role of regulatory CD4+ T cells (Aluvihare, Kallikourdis et al. 2004). There is a substantial literature examining animal models in which donor cell chimerism is associated with T cell unresponsiveness and graft acceptance (Lubaroff and Silvers 1973; Ehl, Aichele et al. 1998; Cosimi and Sachs 2004), though whether the chimerism directly causes T cell unresponsiveness or is a consequence of it (Monaco 2002; Claas 2004) is less clear. The effect of antigen exposure during pregnancy on subsequent immunological tolerance has also been specifically investigated. Mouse models have demonstrated that exposure to male antigen through pregnancy makes these mice subsequently tolerant of syngenic male skin grafts and unable to generate

cytotoxic responses to male antigen (Simpson, Benjamin et al. 1981). The critical determinant of this effect in murine models seems to be the induction of regulatory T cells during antigen exposure during pregnancy. These regulatory T cells have been shown to have a potent effect on preventing rejection of the fetal allograft and subsequently inducing tolerance to fetal antigens (Aluvihare, Kallikourdis et al. 2004). An increase in T regulatory cells during human pregnancy had been reported, though as previously discussed this early work was not able to define these cells in detail due to the available markers of human regulatory T cells at that time (Somerset, Zheng et al. 2004). Work presented in chapter 3 of this thesis has demonstrated with the use of rigorous methods to define T regulatory cells, that in the periphery it is not possible to demonstrate pregnancy induced changes in their frequency, making this less likely to be a critical mechanism modulating the action of fetal specific T cells in human pregnancy. The study of fetal specific CD4<sup>+</sup> regulatory cells would be the best way to directly address these questions. However, the opportunity to conduct an antigen specific study of CD4 T cells is limited by poor definition of HLA class II HY epitopes and technical challenges associated with class II peptide-MHC multimers . With the rapid advances in class II tetramer technology this may soon become feasible (Massilamany, Upadhyaya et al. 2011).

CD8 T cells have also been suggested to have a potential regulatory role in tolerance during pregnancy, though the phenotype of these suggested regulatory cells is not well defined and their identification involved a murine foot pad in vivo transfer assay to assess their regulatory function. This assay is currently only used by one laboratory and the regulatory function was not demonstrated by more conventional assays (van Halteren, Jankowska-Gan et al. 2009) (Burlingham and Goulmy 2008). In our work the use of dextramers would have potentially identified all cells with a TCR specific for the fetal HY



antigen, irrespective of their function. The fact that we did not identify CD8 T cells with a fetal specific TCR that were not cytotoxic therefore adds support to the supposition that fetal specific regulatory CD8 T cells are not common in pregnancy or do not have a stable regulatory phenotype.

***A population of low avidity “dextramer dim” fetal specific T cells were observed during pregnancy, which are highly functional and have a distinct T cell receptor***

When HY-specific T cell clones were identified from women during pregnancy an interesting feature was observed. Individual clones were noted to stain with different levels of intensity following incubation with the HY dextramer. This feature was an intrinsic property of the T cell clones and was retained throughout the culture period. The intensity of HLA-peptide multimer staining has been shown to be a reliable indicator of avidity for peptide-MHC. Dim MHC-peptide multimer staining cells have been suggested by some to have regulatory properties (Cai, Lee et al. 2004). It was therefore of note that we observed a quite different situation, with the dextramer dim staining cells exhibiting higher levels of IFN  $\gamma$  release and cytotoxicity in response to antigen. It is possible that these highly cytotoxic cells have downregulated their TCR to reduce their activation and avoid deleterious effects. Fetal antigen specific T cells studied in murine TCR-transgenic models have been observed to show TCR downregulation (Tafari, Alferink et al. 1995) and it has been speculated that this is a mechanism to reduce functional capacity during exposure to male tissues (Burlingham and Goulmy 2008). Whether or not fixed modulation of the expression level of TCR is a particular feature of immune responses during pregnancy and the implications for this *in vivo* will need further study.

Identifying the TCR sequence of both tetramer bright and dim clones from the same individual presents exciting opportunities to further explore this. There is the potential to use these sequences to determine the structure of these distinct TCRs and examine their interaction with peptide-MHC and explore the implications of this on dextramer binding, TCR signalling and cell function.

***The identification of broadly functional fetal specific T cells during pregnancy has potentially important clinical consequences and possible therapeutic applications***

There may be important physiological and pathological roles of the cellular alloreactive T cell immune response that develops during pregnancy. Alloreactive responses in this context are not necessarily detrimental, such as the recognised role of uterine NK cells in promoting uterine vascular remodelling. Indeed, allogeneic pregnancy has been suggested to offer a survival advantage compared to syngeneic pregnancy (Menu, Chaouat et al. 1995), with a relative lack of CD8<sup>+</sup> T cell infiltration into decidua observed in pre-eclampsia (Rieger, Segerer et al. 2009).

However, there is also the potential that this phenomenon may have detrimental consequences. In 'villitis of unknown aetiology' (VUE) maternal CD8 T cell infiltration of the villous tree of the placenta is associated with fetal growth restriction, pregnancy loss and stillbirth (Redline 2007). These infiltrating lymphocytes may well be fetal antigen specific and thus homing to this site, but the nature of the antigen or why there is this apparent escape from the normal mechanisms of tolerance in these individuals remains to be determined.

Furthermore, the potential impact on fetal physiology was recently illustrated by the demonstration that maternal T cells trafficking into the fetal circulation are the main barrier to engraftment following in utero hematopoietic cell transplantation (Nijagal, Węgorzewska et al.). A detailed understanding of the biology of maternal fetal specific T cells, and developing means of modulating their action thus seems important in realising the potential of in utero haemopoietic cell transplantation.

The study of minor histocompatibility antigen specific T cells in the context of pregnancy offers important lessons for the use of minor histocompatibility antigen specific T cells in the broad and rapidly developing field of T cell based immunotherapy. Understanding the mechanisms operating during pregnancy which have evolved to abrogate a detrimental response towards fetal tissue may mimic strategies utilised by tumours to evade T cell based immunotherapy. Vaccination strategies designed to elicit minor histocompatibility antigen specific T cell responses directed against tumour related antigens (Riddell, Murata et al. 2002; Spaapen and Mutis 2008) can also be informed by this natural model in which the mother is primed by exposure to fetal antigens during pregnancy. The detection of T cell responses in normal pregnancy, without obvious pathology, also offers an important illustration of the care required when devising strategies to monitor the potential of these vaccination methods, as a simple measurement of the T cell response induced may not correlate with in vivo activity.

The generation of large numbers of functional clones during our work suggests that pregnancy, or the post natal period, may present an opportunity to utilise the natural priming of the maternal cellular immune system to generate a library of antigen specific T cell clones for potential therapeutic uses. Examples may include allogenic stem cell therapy

towards haemopoietic cell restricted antigens such as HA-1 to enhance the graft versus leukaemia effect post transplantation (Mutis, Ghoreschi et al. 2002; Marijt, Heemskerk et al. 2003; van Loenen, de Boer et al. 2011) or “tumour specific” antigens which are also found in placental or fetal tissue such as PLAC1 (Silva, Gnjatic et al. 2007; Dong, Peng et al. 2008; Fant, Farina et al. 2010).

## ***Conclusion***

Fetal specific T cells obtained during pregnancy proliferated in culture and T cell clones could be generated from women during and after pregnancy. The clones were cytotoxic and able to produce IFN  $\gamma$ . There was no functional difference between clones generated during or after pregnancy. A population of clones which stained dextramer with a low intensity during pregnancy were isolated but these still retained their cytotoxic and cytokine secreting functions.

The demonstration of the functional potential of fetal specific CD8 T cells during pregnancy has important implications for our understanding of maternal-fetal immunological interactions during pregnancy and how tolerance is maintained. It also has implications for the broader field of transplant immunology and cellular immunotherapy.

# **Chapter 6**

## **Maternal cellular immunity in recurrent miscarriage**

## 6.1 Introduction

Recurrent miscarriage is commonly defined as 3 or more consecutive pregnancy losses and affects 1% of couples (Rai and Regan 2006). In a majority of cases no known cause is found and an immune aetiology has been proposed (Section 1.4.1).

A range of immunological abnormalities have been suggested in women with recurrent miscarriage (Table 1.4.2.1), and this has led to the introduction of a range of immunomodulatory therapies (Table 1.4.2.2). However, there is no high quality evidence that these therapies are effective and their use is therefore not recommended until we better understand the pathogenesis of this condition, the potential mechanisms of action of these therapies and they have been subject to further trials (Section 1.4.2).

Immunity to HY antigens is of particular interest in women with recurrent miscarriage. In women with secondary recurrent miscarriage (women with a live child who have subsequent recurrent miscarriages) a firstborn boy is thought to be a poor prognostic factor (Section 1.4.3). This may be due to an immune response to HY antigens from the initial pregnancy, though this cellular response has not been demonstrated.

Progesterone is a steroid hormone essential for establishment and maintenance of pregnancy and proposed as a treatment for recurrent miscarriage (Table 1.4.2.2). The rationale for this is its immunomodulatory properties (Section 1.2.6).

We have characterised the T cell effector subsets of women with recurrent miscarriage and age matched, parous controls. We also identify a fetal (HY) specific immune response in these women. Finally, using HY specific T cell clones we investigated the immunomodulatory effect of progesterone on fetal specific T cells.

## 6.2 T cell effector subsets in recurrent miscarriage patients

T cell effector subsets were assessed in women with a history of unexplained recurrent miscarriage and in age matched, parous female controls. These subsets were measured in peripheral blood through a combination of intracellular staining for the cytokines IFN  $\gamma$  and IL-17 and assessment of the surface chemokine receptors CXCR3 and CCR4.

Intracellular staining for the cytokines IFN  $\gamma$  and IL-17 was conducted on CD4 T cells, following stimulation with PMA-Ionomycin, to define the T cell effector subsets TH1 and TH-17 respectively. Simultaneously the intracellular expression of IFN  $\gamma$  and IL-17 on CD8 T cells was determined. As previously due to the downregulation of CD4 upon stimulation by PMA-Ionomycin CD4 T cells were defined by CD3+CD8<sup>-</sup> expression, whilst CD8 T cells were defined as CD3+CD8<sup>+</sup> (Fig 3.1.1 b). The gating strategy and use of isotype controls to determine gating thresholds was conducted as before (Fig. 3.1.1).

Women with unexplained recurrent miscarriage (n=24) were recruited for these experiments from the recurrent miscarriage clinic at Birmingham Women's Hospital (Section 2.2.3).

The proportion of Th17 cells (defined as CD4<sup>+</sup> T cells demonstrated to be producing IL-17 by intracellular cytokine staining) was significantly higher in women with unexplained recurrent miscarriage (p=0.0009, Mann Whitney test). There was a 3 fold increase in the mean number of Th17 cells in women with recurrent miscarriage compared to controls (0.314 v 0.097).

The Th1 subset was defined as CD4<sup>+</sup> T cells which produced IFN- $\gamma$  by intracellular cytokine staining. Women with recurrent miscarriage had a significantly higher proportion of Th1 cells ( $p=0.0002$ ) with the mean number of T cells more than 2 fold higher in women with recurrent miscarriage (12.4 v 5.3). Interestingly, when CD8 T cells were examined there was no difference in the proportion of cells which produced IFN  $\gamma$  when women with recurrent miscarriage were compared to controls (26.1 v 30.0,  $p=0.36$ ).

In women with recurrent miscarriage there was a correlation between the frequency of TH1 and Th-17 cells within individuals ( $p=0.0025$ ,  $R^2$  0.36, shown in black). A similar trend was seen within the controls but this was not statistically significant ( $p=0.0553$ , shown in grey). Thus, those individuals with higher proportions of the inflammatory Th1 subset, similarly had higher levels of the TH17 subset. The difference between the women with recurrent miscarriage (black squares) and controls (red circles) is clearly visualised when production of these cytokines in both cohorts is graphically superimposed.

The Th1 and Th2 subsets were further defined by the surface chemokine receptors CXCR3 (Th1) and CCR4 (Th2) (see section 1.1.5). Gating to identify CD4 T cells was carried out as before (Fig. 3.1.1 a,b), but an unstimulated sample was used, as stimulation was found to lead to the downregulation of surface chemokine receptors. CXCR3 and CCR4 expression was then visualised and gating thresholds determined by FMO controls (Fig. 3.1.4a). As previously described the expression of these chemokine receptors was analysed by 2 methods, the percentage of cells expressing these markers and also the level of expression, by measuring the MFI.

CXCR3 expression on CD4 T cells was used as a marker of Th1 cells (Sallusto, Lenig et al. 1998; Appay, van Lier et al. 2008). We found that women with recurrent miscarriage had



a higher proportion of CD4 T cells expressing CXCR3 (4.1 v 11.2,  $p=0.0002$ ). If levels of CXCR3 expression were assessed by measuring the MFI this similarly was significantly increased in women with recurrent miscarriage (79.7 v 96.3,  $p=0.05$ ).

The chemokine receptor CCR4 was also examined. CCR4 was used as a marker of Th2 cells (Sallusto, Lenig et al. 1998; Appay, van Lier et al. 2008). We found that when the proportion of cells expressing CCR4 was compared between women with recurrent miscarriage and controls there was a reduction in women with recurrent miscarriage but this was not statistically significant (6.0 v 5.1,  $p=0.12$ ). However, when the levels of expression were examined by measuring the MFI of CCR4 on CD4 T cells this was significantly reduced in women with recurrent miscarriage, with the mean MFI reduced from 33.2 in the controls to -88.1 in women with recurrent miscarriage, this was highly significant  $p=0.0013$ .

A Th1/Th2 ratio was calculated from a ratio of the proportion of cells expressing CXCR3 divided by the proportion of cells expressing CCR4. There was a significant increase in the Th1/Th2 ratio in women with recurrent miscarriage (0.83 v 8.12  $p=0.0003$ ).

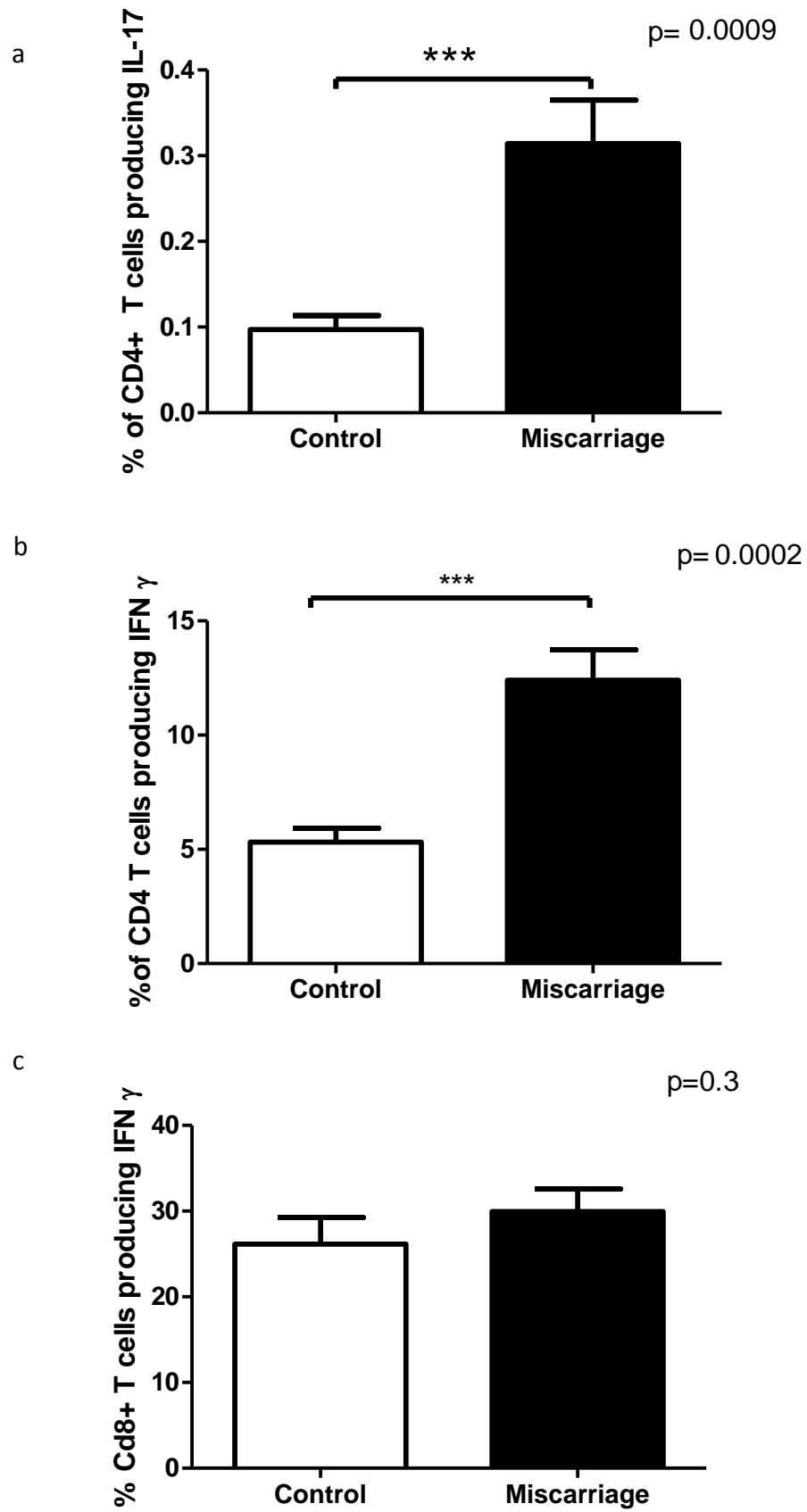


Figure 6.2.1 A comparison of cytokine production by T cells from women with recurrent miscarriage and controls.

*The production of IL-17 (a) and IFN  $\gamma$  (b) by CD4 T cells and IFN  $\gamma$  production by CD8 T cells is shown following PMA-Ionomycin stimulation. This was measured by intracellular cytokine staining and is expressed as a percentage of either total CD4 or total CD8 T cells. (Bars demonstrate mean with whiskers showing SEM, statistical analysis by Mann-Whitney test. Control n=15 and miscarriage n=24)*

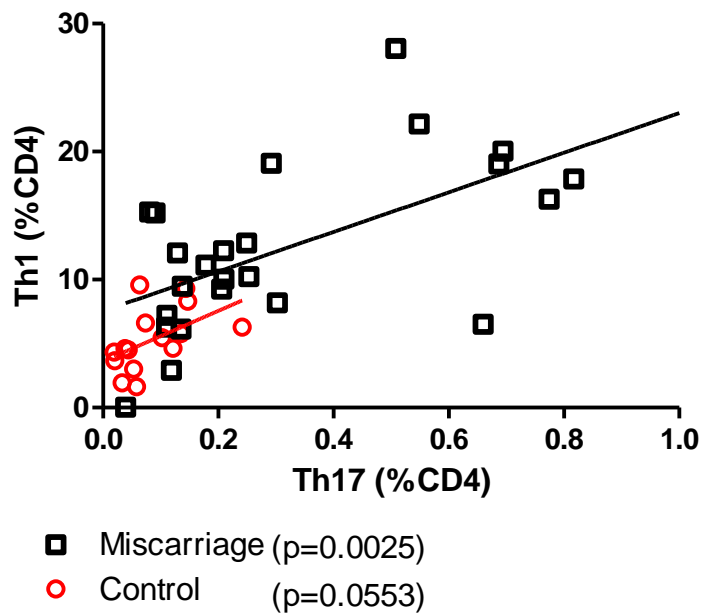


Figure 6.2.2 The proportion of Th1 and Th17 cells in women with recurrent miscarriage and controls

The proportion of Th1 and Th17 cells is correlated and contrasted between women with recurrent miscarriage (black squares) and controls (red circles). Th1 has been calculated as the percent of CD4 T cells secreting IFN  $\gamma$  following stimulation by PMA-Ionomycin and Th17 by the percent of CD4 T cells secreting IL-17 after the same stimulation. Linear regression lines are also calculated for both these groups.

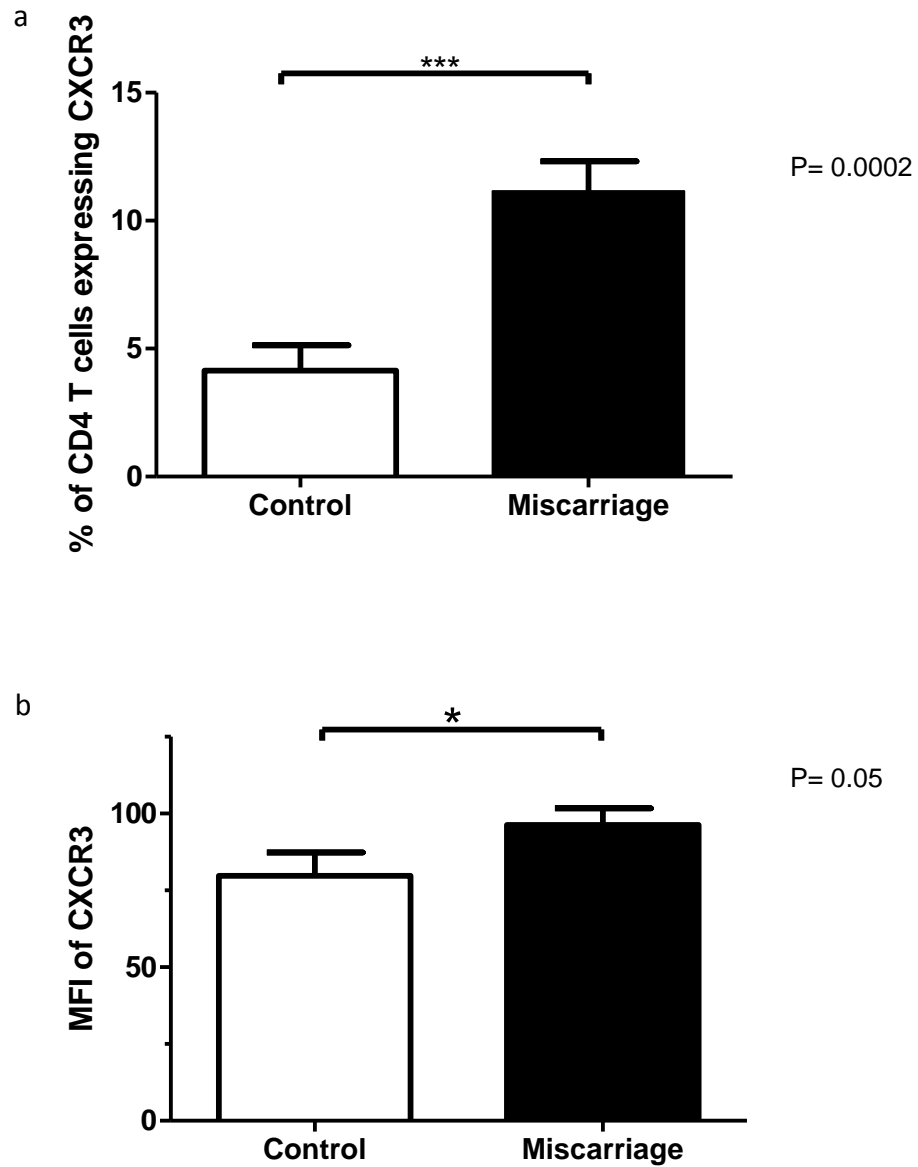
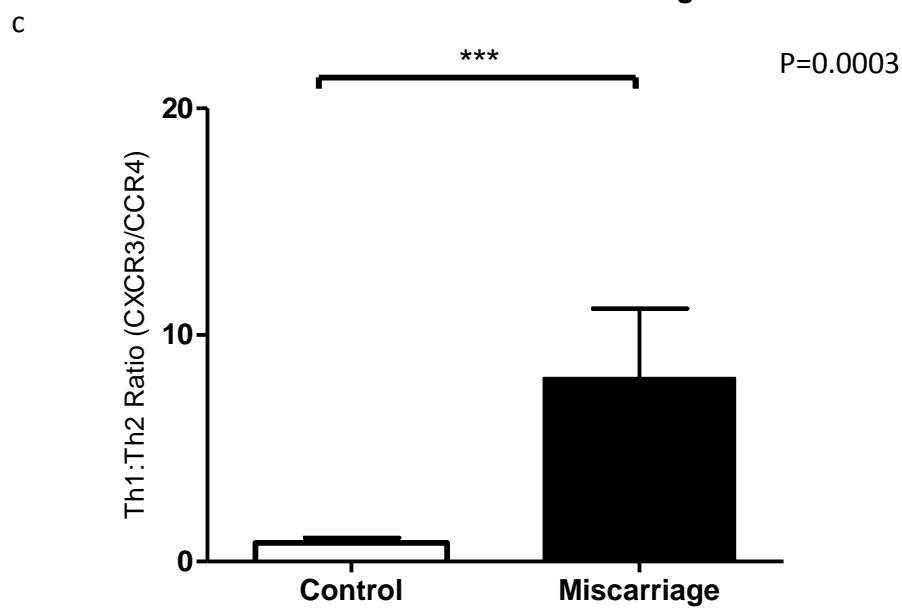
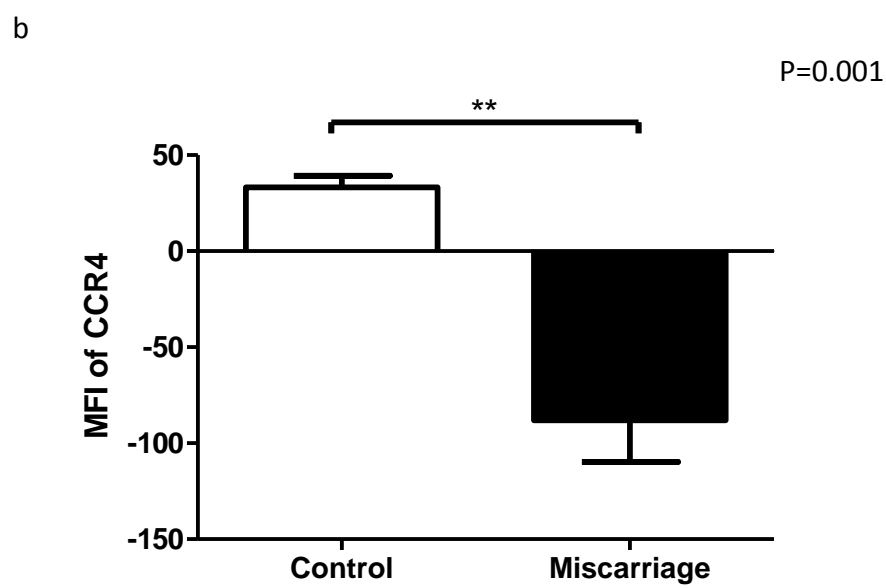
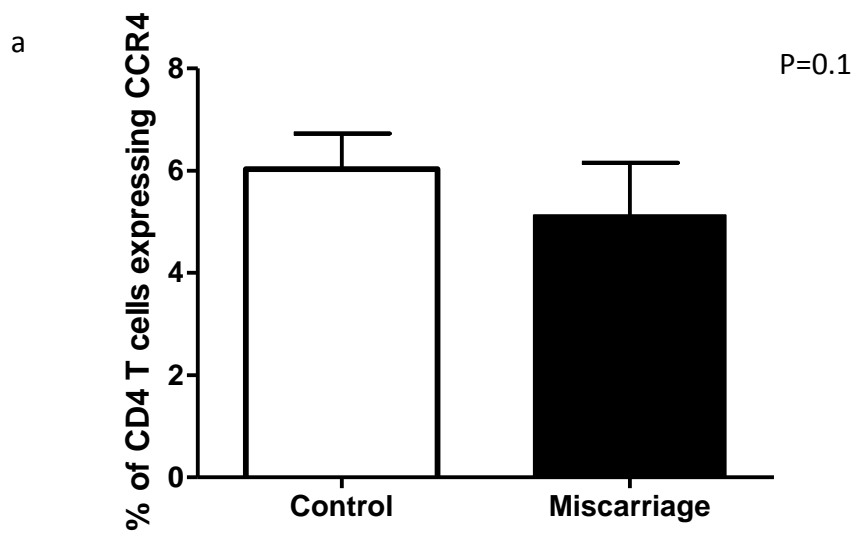


Figure 6.2.3 A comparison of the chemokine receptor CXCR3 expression on T cells of women with recurrent miscarriage and controls.

The production of CD4 T cells expressing CXCR3 is measured as a percent of all CD4 T cells (a) and measured by the median fluorescent intensity of CXCR3 (b) on cells without stimulation. (Bars demonstrate mean with whiskers showing SEM, statistical analysis by Mann-Whitney test. Control  $n=15$  and miscarriage  $n=24$ )



*Figure 6.2.4 A comparison of the chemokine receptor CCR4 expression on T cells of women with recurrent miscarriage and controls*

*The production of CD4 T cells expressing CXCR4 is measured as a percent of all CD4 T cells (a) and measured by the median fluorescent intensity of CCR4 (b) on cells without stimulation. The Th1:Th2 ratio is also contrasted between these groups. The ratio has been calculated using CXCR3 as a surrogate marker for Th1 and CCR4 as a marker for Th2 as follows; (CXCR3 expression (%CD4))/CCR4 expression (%CD4). (Bars demonstrate mean with whiskers showing SEM, statistical analysis by Mann-Whitney test. Control n=15 and miscarriage n=24)*

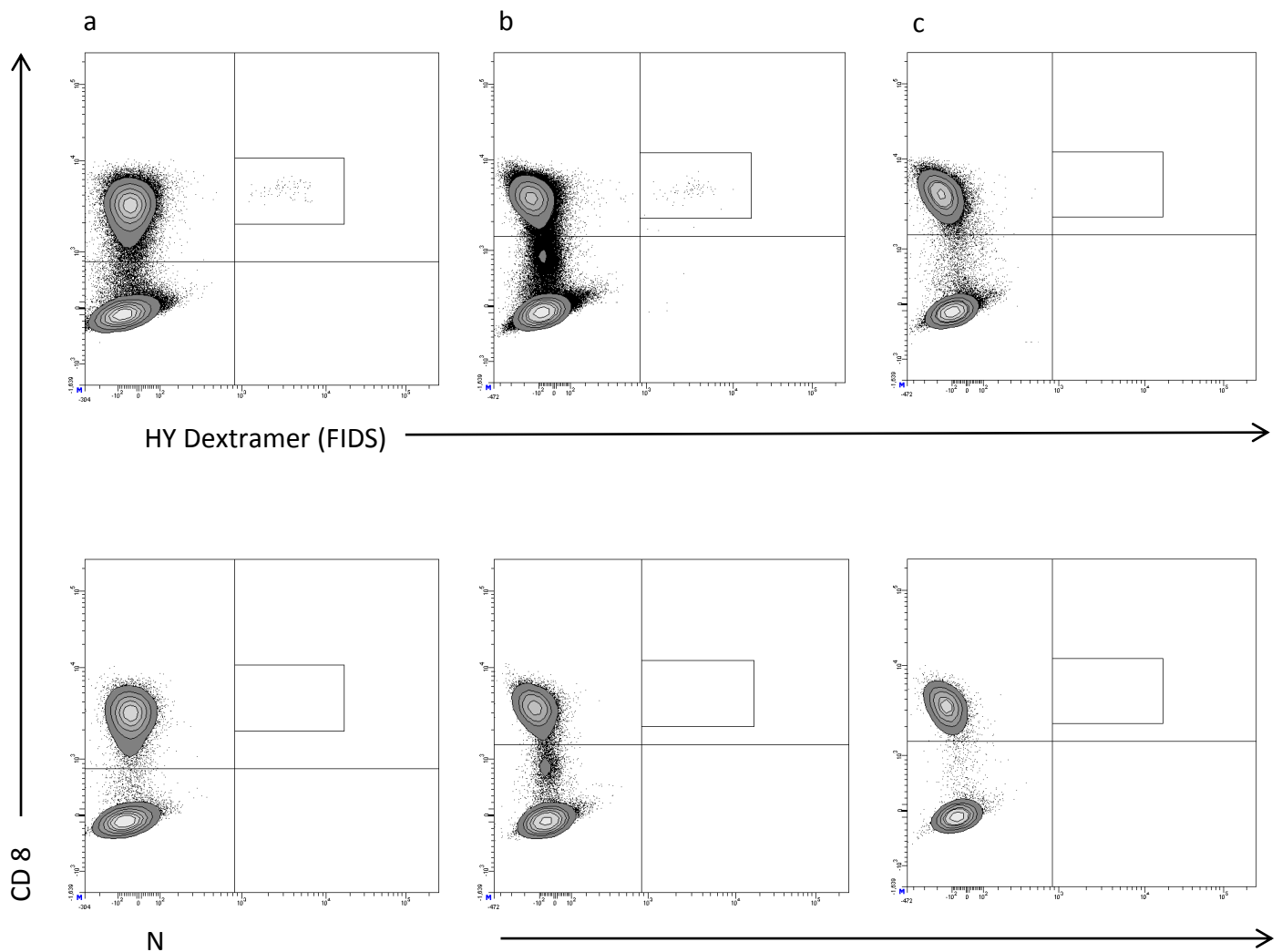
### **6.3 CD8 T cell responses to fetal antigens in recurrent miscarriage**

20 women with unexplained recurrent miscarriage, with the HA type HLA\*0201 were identified. HLA typing was performed as previously described with a PCR based method (section 2.10.3).

Frozen PBMCs from these women were thawed and cultured in sort term peptide lines, as previously described. Fetal specific T cells were then identified using dextramers containing the HY specific epitope (FIDS) as before (section 4.2).

A fetal specific T cell response could be detected in a proportion of women with recurrent miscarriage, including those who had only miscarriages and no live pregnancies. Examples from 2 donors in which a fetal specific T cell response (Fig 6.3 a,b) and a donor in which no response was seen are shown (Fig. 6.3 c). A fetal specific CD8 T cell response was seen in 8/20 women (40%). The mean response was 0.023% of CD8 T cells, with a range of 0.0017-0.14% of CD8 T cells. This detection rate of HY specific T cells (40% v 45%) and their frequency (0.023 v 0.029%) is not significantly different to that observed in parous non-pregnant females.





**Figure 6.3 Identification of fetal specific CD8 T cells in women with recurrent miscarriage.**

Fetal specific CD8 T cells are demonstrated in 3 representative women with recurrent miscarriages. In 2 examples (a) and (b) fetal specific T cells were identified and in (c) no fetal specific T cells were seen. The upper panels represent the use of the HY specific dextramer (FIDS) and the bottom panels a negative control with a HLA-A2 negative control dextramer. The gating strategy was as demonstrated previously, briefly, singlet cells were identified on a FSC-A vs FSC-H plot and small lymphocytes gated on the basis of standard light scatter properties. A “dump” channel was used to exclude dead cells, monocytes and B-cells. T lymphocytes were then selected by CD3 expression. Finally, the plots shown were produced which illustrate dextramer “positive” cells visualised against CD8 expression.

## 6.4 Modulation of fetal specific T cell function by progesterone

Fetal specific T cell clones can be generated from women during and after pregnancy (section 5.3). These were used as an *in vitro* model to study the immunomodulatory effect of progesterone on fetal specific CD8 T cells. Natural progesterone (P4) was added at a range of concentrations to T cell clones, incubated for 16 hours, with target cells presenting HY antigen. Negative controls included HLA mismatched or antigen mismatched targets. Changes in T cell function was measured by the assessment of IFN  $\gamma$  production in the culture supernatant by ELISA (see Fig. 6.4.1).

We found that progesterone (P4) at a 20 $\mu$ molar concentration caused a significant reduction in IFN  $\gamma$  release by HY specific T cells (Median 36% reduction,  $p < 0.001$ ). This is a concentration similar to that found in human term decidua (Fig. 6.4.2a). The reduction was even more profound (15.8% reduction,  $p < 0.0001$ ) at a higher concentration of progesterone. Interestingly, EBV specific CD8 (Fig. 6.4.2b) and CD4 T cell clones (Fig. 6.4.2c) did not show this modulation by P4 at physiological concentrations, with a reduction in IFN  $\gamma$  release only seen at the supraphysiological (pharmacological) concentration of 100 $\mu$ molar.

It was important to determine if this effect was due to modulating antigen presentation. We found that there was no difference in the effects of progesterone on IFN  $\gamma$  release by HY specific T cell clones whether antigen was processed endogenously by the target cell or loaded onto the cell surface (Fig. 6.4.3).

The effect of progesterone on cell viability was assessed to ensure that this reduction in cytokine production was not simply a viability effect. This was done by flow cytometric

(using propidium iodide) and microscopy based (using trypan blue) viability assessment. No change in cell viability were observed at up to 100  $\mu$ M with this incubation period (data not shown).

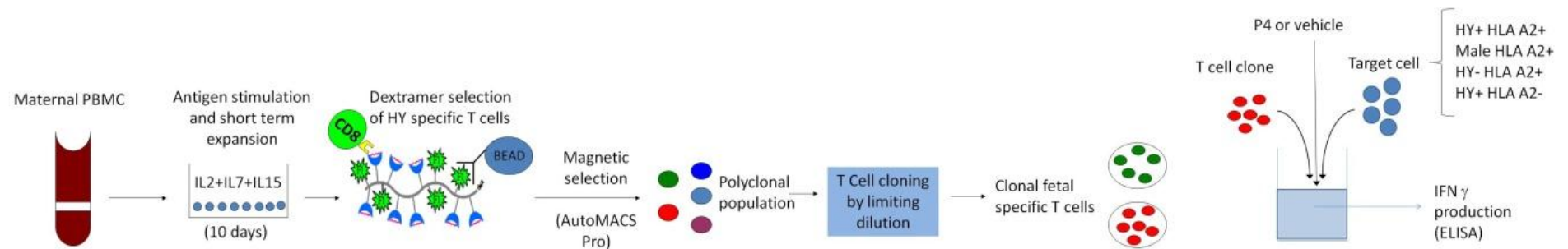
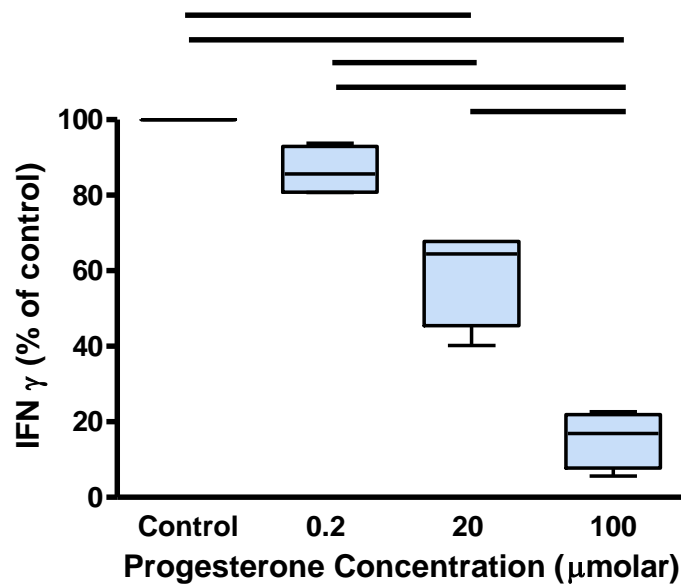


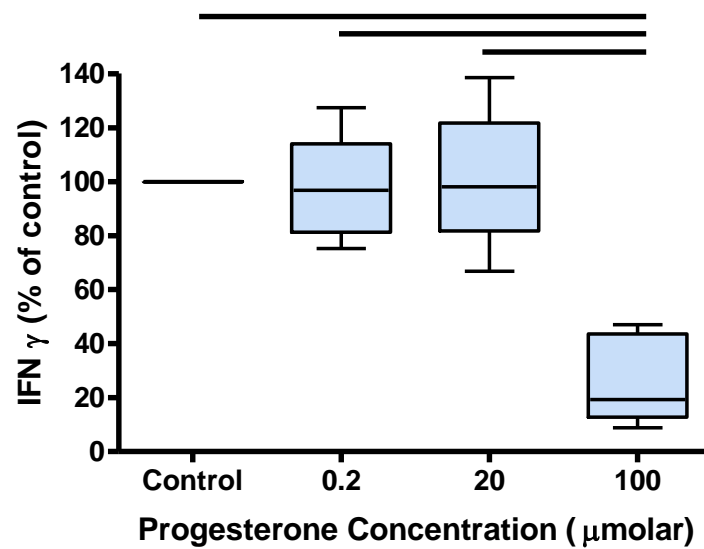
Figure 6.4.1 Schema for assessment of the functional effect of progesterone on fetal antigen (HY) specific T cell clones.

Initially PBMCs were isolated by density gradient centrifugation. These were then stimulated for 1 hour with the HY peptide FIDS prior to short term (10 day) culture with the addition of IL-2, IL-7 and IL-15. HLA-peptide dextramer was used to isolate HY specific T cells. The dextramer incorporates the PE fluorochrome and anti-PE beads and magnetic selection could therefore be used to select these HY specific T cells. The selected cells after being checked for purity by flow cytometry were cloned through a limiting dilution method. Irradiated allogenic PBMCs stimulated with PHA from 3 healthy donors and irradiated lymphoblastoid cell lines were used as “feeder cells” to support the replication of the antigen specific T cell clones. Clones were then screened by dextramer. Function was then measured by IFN  $\gamma$  production in supernatant after co-culture of the T cell clones with HLA matched target cells loaded with FIDS peptide, or negative controls which included irrelevant peptide and HLA mismatched target. The effect of progesterone was measured by the addition of progesterone at varying concentration or vehicle alone (DMSO) to the culture media. IFN  $\gamma$  production was measured by ELISA of culture supernatant at 16 hours.

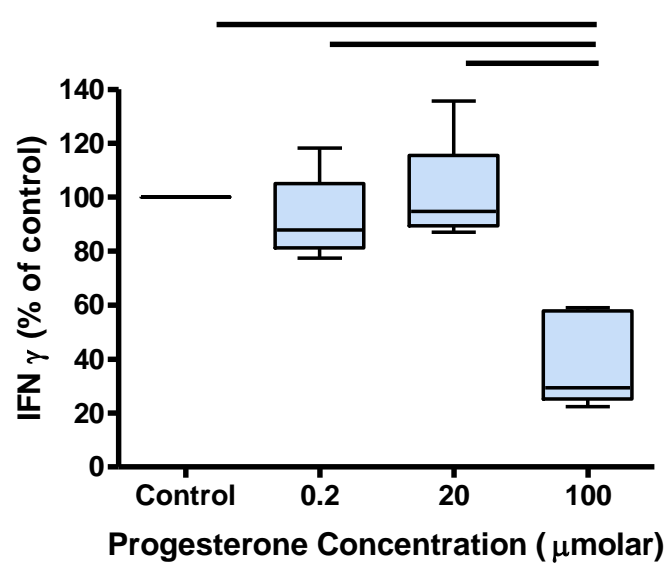
a) HY specific CD 8



b) EBV specific CD 8



c) EBV specific CD 4



*Figure 6.4.2 Modulation of IFN  $\gamma$  release by progesterone*

*HY specific T cell clones (n=5) (a), EBV specific CD8 (n=5) (b) and EBV specific CD4 (n=5) (c) T cell clones were assayed at a range of progesterone concentrations and the production of IFN  $\gamma$  measured after 16 hours measured in the culture supernatant. Each sample was tested in triplicate. HLA mismatched and peptide mismatched negative controls were used in every experiment. (Boxes represents median and interquartile range and whiskers range. Statistical analysis by Kruskal-Wallis with post test, horizontal bars indicate statistical significance, >0.05)*

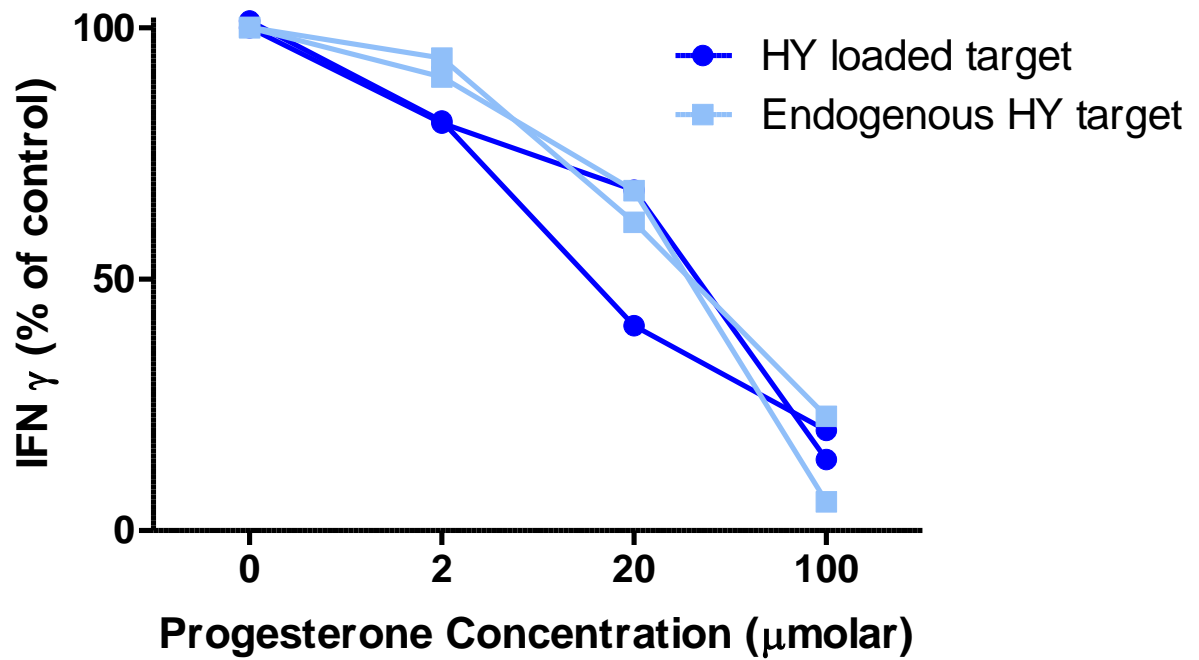


Figure 6.4.3 Effect of antigen presentation on the progesterone induced modulation of IFN  $\gamma$  release by HY specific T cell clones .

IFN  $\gamma$  release by HY specific T cell clones (n=4) are compared at a range of progesterone concentrations when the target was either a female HLA-A2 LCL loaded with peptide or a male LCL which was therefore processing and presenting endogenous HY peptide. Assays were conducted in triplicate and the results plotted here are the mean of these replicates.

## 6.5 Discussion

### ***Recurrent miscarriage was associated with an increased frequency of Th1 and Th17 cells***

Women with a history of recurrent miscarriage were found to have an altered cellular immune response. In particular, in response to activation with PMA and ionomycin, CD4 T cells demonstrated increased IFN  $\gamma$  release and IL17 production. Chemokine receptors associated with the Th1 and Th2 subsets were also examined on un-stimulated cells and these showed an increase in surface expression of CXCR3 in recurrent miscarriage, with reduced levels of CCR4 in women with recurrent miscarriage. This also suggests a skewing towards a Th1 type response with an increased Th1/Th2 ratio in women with recurrent miscarriage.

The association of a Th1 type response with adverse pregnancy outcomes has been proposed since early murine models (Wegmann, Lin et al. 1993). Enthusiasm for this concept stems from the transplantation setting in which Th1 dependent effector mechanisms are implicated in allograft rejection (Erdmann, Jung et al. 2004; Burns, Wang et al. 2005) and Th2 type cytokines in allograft tolerance (Nickerson, Steurer et al. 1994; Strom, Roy-Chaudhury et al. 1996; Li, Zand et al. 1998). A translation of this from transplantation to pregnancy led to the suggestion that Th1 cytokines would be deleterious to pregnancy and Th2 cytokines advantageous. Indeed, injection of INF  $\gamma$ , TNF  $\alpha$  and IL-2 in abortion prone murine matings increased abortion rates and this was reduced by IL-10 (Chaouat, Assal Meliani et al. 1995). However, this is likely to be too simplistic an interpretation as even knocking out multiple Th2 type cytokines does not affect pregnancy rates (Fallon, Jolin et al. 2002). Previous studies have examined the Th1/Th2 profile in women with recurrent miscarriage but have



not been consistent in their findings when peripheral blood PBMCs have been examined. Piccinni et al. reported no change in IFN  $\gamma$ , Th2 type cytokines, LIF or M-CSF (Piccinni, Beloni et al. 1998), whilst others have reported a Th1 skewed response of peripheral blood mononuclear cells of women with recurrent miscarriage in response to trophoblast (Hill, Polgar et al. 1995) and mitogens (Raghupathy, Makhseed et al. 2000). However, it may not be as simple as Th1 type cytokines such as IFN  $\gamma$  necessarily being detrimental to pregnancy. Indeed, IFN  $\gamma$  has been demonstrated to be essential for the remodelling of the spiral arteries which is critical for normal placentation (Ashkar, Di Santo et al. 2000). Thus it may be that the timing, quantity and regulation of cytokines during pregnancy is important with no clear dichotomy of those which are “good” and “bad”.

The importance of the Th1/Th2 balance was extended to normal pregnancy with the suggestion that normal pregnancy is also associated with a shift from a Th1 to a Th2 profile as part of maternal immunological tolerance of the fetus. However, in our studies of normal pregnancy this effect was not seen (section 3.1). Our findings therefore suggest that changes in T cell cytokine production measured in the periphery are of greater importance in pathological than normal pregnancy.

In addition to the Th1 and Th2 subsets there has now been recognised a 3<sup>rd</sup> functionally distinct effector subset termed Th17, which is defined by the secretion of IL-17 (Bettelli, Carrier et al. 2006). As this subset was only recognised recently there is still little known about its role in reproduction. It is though being increasingly recognised that Th17 cells are important effectors in a wide range of human diseases (Korn, Bettelli et al. 2009). Our findings confirm the early report by Liu et al. that these cells are more frequent in women with recurrent miscarriage (Liu, Wu et al. 2011). We also examined Th17 cells using

longitudinal studies of normal pregnancy which demonstrated that Th17 cells are also more frequent in the 1<sup>st</sup> trimester of normal pregnancy (section 3.1). We must therefore be cautious in ascribing these cells to having a wholly deleterious function. It may be that Th17 cells have a more complex role with both physiological roles in normal pregnancy but dysfunction in pathological pregnancy. A greater insight into the role of Th17 cells in reproductive biology *per se* will be required to understand their role within recurrent miscarriage.

This study was conducted on a small heterogeneous cohort of women with recurrent miscarriage, in whom no cause was found by conventional investigations. It lacked the numbers to identify clinical factors associated with alterations in the immunological response. It would be interesting in future work to assess a larger cohort with complete clinical information including where possible fetal karyotype information to determine if there are particular clinical factors associated with an abnormal cellular immune response which may make particular subgroups a more suitable target for immunological therapies.

The presence of a difference in the cellular immune function of women with recurrent miscarriage compared to healthy controls is a clinically important observation. It suggests that immunological therapies which address this dysfunction may have a role in treating these women. From our data it can also be observed that there are within the cohort of women with recurrent miscarriage a number of women who appeared to have no increase in IFN  $\gamma$  and IL-17 release, whilst a subgroup had substantial increases. It is fascinating to speculate whether those women with the greatest abnormality in cellular immune function may be those most likely to benefit from immunological therapies. Future clinical trials of immunomodulatory agents such as progesterone, immunoglobulin or

biological therapies may benefit from utilising these immunological parameters as a basis for patient selection.

From our data it cannot be determined if recurrent miscarriage is caused by this immunological dysfunction or if this is acquired as a result of being exposed to multiple miscarriages, with miscarriage itself having a role in altering the maternal cellular immune function. Prospective studies would be required to answer this question conclusively.

### ***Miscarriage induces a fetal specific T cell response***

Utilising MHC-peptide multimer technology we examined the maternal CD8 T cell response mounted against fetal HY antigens. In 40% of women a fetal specific immune response was demonstrated. This included women who had only previously had recurrent early miscarriages without any pregnancies reaching the 2<sup>nd</sup> trimester.

Thus, our data enables us to determine that a fetal specific immune response is also common in women with recurrent miscarriage and is induced by miscarriage alone without the requirement for pregnancies to progress past the 2<sup>nd</sup> trimester. This has not been determined previously in an antigen specific manner. However, previous epidemiological studies had suggested that a fetal specific HY response was likely to be important in recurrent miscarriage. These studies had identified that women with secondary recurrent miscarriage with a previous live born male child had a poorer prognosis and that furthermore these women have an excess of female fetuses and the birth weights of their infant was also lower (Christiansen, Pedersen et al. 2004; Nielsen, Andersen et al. 2008; Nielsen, Steffensen et al. 2009). Interestingly, previous studies have similarly found an

association between male gender of the fetus and obstetric complications such as placental abruption, pre-eclampsia, stillbirth and neonatal morbidity (Bracero, Cassidy et al. 1996; Kramer, Usher et al. 1997; Basso and Olsen 2001; Haddad, Mercer et al. 2001). This provides an indication that anti-HY immunity may negatively effect outcome and placental or fetal growth.

However, this epidemiological evidence was, in the context of miscarriage, restricted to women with secondary recurrent miscarriage as the sex of the live born fetus could be determined. Our demonstration that an HY response is also initiated in women with primary recurrent miscarriage means that the HY effect may also be important in these women. Whether the measurement of an HY specific immune response is in itself an important prognostic factor or would influence the gender of future children is an important question. Now that a methodology has been determined to enable the determination of this response from frozen samples the use of a historical cohort with full prognostic information should enable this question to be answered. It would however be restricted in the case of this epitope to women who are HLA-A2 and would therefore not be applicable to the whole population.

It would be fascinating to determine in particular how the HY response measured correlates with the gender of the previous miscarriages as this information was only available for live-born children in this cohort. However, it is not routine practice in the UK to karyotype the abortus and therefore information was not available from these women on the number of male pregnancies they have had. Similarly, it is therefore not possible to match the recurrent miscarriage cohort with a control group which have had the same

number of pregnancies to determine if women with recurrent miscarriage have an exaggerated fetal specific response.

The finding of the HY response being of a similar prevalence and magnitude in these women to the response detected in parous women without recurrent miscarriage means that differences in the function of these HY responsive cells or in their regulation may be important additional factors in the pathology. This also warrants further investigation and could be done initially through fetal specific T cell clones developed from these women being functionally assessed. The identification of fetal specific T regulatory cells would be particularly important but would be technically challenging due to the small number of known class II HY epitopes and technical challenges with class II HLA-peptide multimers.

#### ***Progesterone modulates IFN $\gamma$ production by fetal specific T cell clones***

Fetal (HY) specific T cells can be isolated from women during pregnancy and HY specific T cell clones generated. These cells retain their cytotoxicity and ability to produce IFN  $\gamma$ . How the function of these cells is modulated so that they do not exert deleterious effects in normal pregnancy is unclear. We hypothesised that progesterone may be exerting an important immunomodulatory effect and used fetal specific T cell clones as an *in vitro* model to examine the effect of progesterone on the suppression of the maternal cellular immune responses to fetal antigen.

The production of IFN  $\gamma$  by fetal specific T cell clones was significantly reduced by progesterone at concentrations similar to those found at the decidual interface. This was not

due to changes in cell viability or due to an effect on antigen processing. This same effect was not observed with EBV specific CD8 or CD4 T cell clones.

Progesterone is a steroid hormone crucial for the establishment and maintenance of human pregnancy. It has been long suggested that the high concentrations of progesterone found at the maternal-fetal interface may inhibit the maternal immune response against the fetal allograft (Siiteri, Febres et al. 1977). It has also been suggested that progesterone can alter the function of CD4 T cell clones, causing them to shift from a TH1 to a Th2 phenotype (Piccinni, Giudizi et al. 1995; Piccinni 2006), though no effect was seen on CD8 T cells. To the best of our knowledge this is the first demonstration of progesterone having important effects on the modulation of CD8 T cells and fetal specific T cells in particular.

This demonstration of the immunomodulatory effect of progesterone on fetal specific clones is important as it suggests that progesterone may provide a potential pharmacological method of manipulating fetal specific CD8 T cell responses. There is much interest in the potential use of progesterone supplementation as a therapy to prevent miscarriage. Existing evidence suggests that progesterone therapy may be of benefit in women with a history of recurrent miscarriage and is not associated with an increase in adverse events (Haas and Ramsey 2008). However, the evidence is not yet sufficiently robust for it to be recommended in clinical guidelines (Regan, Backos et al. 2011). This question is therefore currently being comprehensively tested in an international multicentre trial (Coomarasamy, Truchanowicz et al. 2011). If an important mechanism of its action is via the modulation of maternal T cell responses such as a reduction in IFN  $\gamma$  production then immunological assays identifying women with high levels of T cell IFN  $\gamma$  production may be useful in selecting patients who would benefit from therapy. Similarly, it may be that

functional assays measuring the alteration in the T cell response with progesterone supplementation *in vitro* may indicate those most likely to respond to progesterone therapy clinically.

The mechanism of progesterone's action in this context is still unclear. Potential mechanisms may be through the classical nuclear progesterone receptor, though the current consensus is that these receptors are not found in T cells. This makes it more likely that progesterone is acting via the more recently recognised membrane progesterone receptors of which mPR $\alpha$  and mPR $\beta$  have been identified in T cells (Dosiou, Hamilton et al. 2008). Alternative mechanisms may be through the non-genomic inhibition of Na(+)/H(+)-exchange 1 (NHE1) (Chien, Chang et al. 2006; Chien, Liao et al. 2007) or direct inhibition of K<sup>+</sup> channels (Ehring, Kerschbaum et al. 1998). Our work is ongoing to establish which progesterone receptors are present in different T cell subsets including fetal specific T cells. We aim by selectively blocking these putative pathways to determine how progesterone is acting in this context. Further work will also establish how a wider range of cytokines are affected, and not only examine secreted cytokines but through intracellular cytokine staining determine if progesterone is acting to alter cytokine production or secretion.

We are currently extending this work to a clinical cohort of women with recurrent miscarriage who are participating in a randomised trial of progesterone therapy versus placebo. We are examining the cellular immune response and serum cytokine levels of these women to establish if P4 supplementation taken therapeutically has the same effects on T cell function as we have observed in our *in vitro* system. We also aim to determine if the pre pregnancy cellular immune status or the *in vitro* response to progesterone predicts the clinical response and outcome.

## ***Conclusion***

Women with a history of recurrent miscarriage demonstrated a higher proportion of Th1 and Th17 cells in their peripheral blood than controls. This difference in cellular immunity could be associated with an increased alloimmune response and thus be detrimental to pregnancy. If this cellular immune dysfunction characterises a subgroup of women who would benefit from immunomodulatory therapies requires further investigation. Furthermore, a fetal specific T cell response was demonstrated in women with recurrent miscarriage. This finding strengthens the assertion that abnormal cellular immunity may be part of the aetiology of recurrent miscarriage and further work linking fetal specific responses with prognosis will be important. Finally, it was shown that progesterone could attenuate the function of fetal specific T cells at physiological concentrations. Understanding how progesterone acts in this context may enable the better selection of women with recurrent miscarriage for progesterone therapy and even enable progesterone therapy to be used to modulate CD8 T cell responses in other scenarios outside of reproductive immunology such as in graft versus host disease post transplantation.



# **Chapter 7.**

## **General Discussion**

Viviparous pregnancy, in which the fetus develops within the maternal body, represents a significant challenge for the maternal immune system, as it requires a state of functional immunological tolerance of the semi-allogenic fetus. This immunological tolerance is not, as originally suggested, solely dependent on a strict anatomical separation of the maternal and fetal blood supply. Indeed, human haemochorial placentation leads to a close juxtaposition between placental villi and the uteroplacental circulation with intimate cellular contact between maternal and fetal tissue (Moffett and Loke 2006). However, the unique microenvironment at the decidual interface modulates potential fetal maternal interactions through restricted MHC expression by human trophoblast (Rouas-Freiss, Goncalves et al. 1997), tryptophan depletion (Mellor, Sivakumar et al. 2001), regulation of complement (Xu, Mao et al. 2000), FasL expression by trophoblast and corticotrophin releasing hormone (Makrigiannakis, Zoumakis et al. 2001), regulatory T cells (Aluvihare, Kallikourdis et al. 2004) and leukaemia inhibitory factor (Stewart, Kaspar et al. 1992).

Moreover, it is now recognized that transfer of fetal cells and non-cellular material into the maternal circulation occurs from early in the prenatal period (Lo, Lo et al. 1996) and that fetal cells may then persist lifelong in the maternal circulation (Gammill, Guthrie et al. 2010) and tissues (O'Donoghue, Chan et al. 2004), a phenomenon termed “fetal microchimerism”. This transplacental exchange therefore permits immunological interactions between fetal and maternal tissue to occur during pregnancy at a peripheral interface, where modulation by the microenvironment of the decidua will not occur, and raises the important question as to how these are initiated and regulated (Taglauer, Adams Waldorf et al. 2010).

T cells constitute a highly diverse and important component of the cellular immune system. Rapid advances in our understanding of T cell diversity and complexity means that despite considerable investigation of the role of T cells in reproductive immunology, there are still many important areas requiring investigation and clarification. We initially conducted an examination of the effector and regulatory subsets of T cells in maternal peripheral blood throughout gestation and the postnatal period. Longitudinal analysis revealed an elevation in Th17 cells in the 1<sup>st</sup> trimester of normal pregnancy. This requires further investigation to determine if Th17 cells have a physiological role in early pregnancy rather than the inflammatory, detrimental role normally hypothesised based on their function in autoimmune disease. The often cited shift from a Th1 to Th2 phenotype was not in our cohort observed through the direct study of the peripheral T cell phenotype or plasma cytokines. A strength of our study was that longitudinal analysis of changes within individuals showed clearly that a Th1 to Th2 shift was not required for successful pregnancy. Similarly we detected no global increase in regulatory T cells in maternal peripheral blood during normal pregnancy, again suggesting that this cannot be considered an essential mechanism supporting maternal tolerance of the fetus during normal human pregnancy.

The T cell repertoire is determined by an individual's previous infection history. The impact that this has on reproductive immunology is little understood, there have however been suggestions that CMV serostatus is linked to an increased risk of poor placentation and the development of pre-eclampsia. Also, primary CMV infection and, less commonly, secondary infection during pregnancy can cause fetal disease and morbidity. The balance between maternal immune competence and viral pathogenicity is thus delicately poised and the understanding of this clinically important. CMV is known to be a very immunodominant antigen and infection, in the elderly in particular, can lead to the oligoclonal expansion of

differentiated T cells, skewing the T cell repertoire. We found that the effect of previous CMV infection was also dramatic within a pregnant cohort. A more differentiated CD8 T cell memory phenotype was found in women with previous CMV infection. CMV status must therefore be considered whenever investigating CD8 T cell memory subsets and differentiation between different pregnant cohorts. How these profound immunological changes during pregnancy that are induced by previous CMV infection alter maternal health or pregnancy related outcomes now requires further investigation.

Further to this investigation of the global changes in the maternal cellular immune response we utilised MHC-peptide multimers to establish changes in fetal antigen specific T cells during pregnancy (findings are summarised in Figure 7.1). Previous murine models have utilised transgenic systems and though the findings have not been consistent they have suggested that fetal antigen specific CD8 T cells may be deleted during pregnancy as a mechanism of fetal-maternal tolerance. However, we determined that fetal minor histocompatibility antigen specific CD8 T cells can be commonly detected during normal pregnancy. Indeed, the frequency of the fetal specific T cells increases during gestation, with no evidence of deletion. These cells are of a memory phenotype but are not highly differentiated.

Fetal specific T cells obtained during pregnancy proliferated in culture and T cell clones could be generated from women during and after pregnancy. The clones were cytotoxic and able to produce IFN  $\gamma$ . There was no functional difference between clones generated during or after pregnancy. A population of clones which stained dextramer with a low intensity during pregnancy were isolated but these still retained their cytotoxic and cytokine secreting functions.

The demonstration of the functional potential of fetal specific CD8 T cells during pregnancy has important implications for our understanding of maternal-fetal immunological interactions during pregnancy and tolerance to this antigen at least is maintained by mechanisms other than deletion. The generation of fetal antigen specific T cells during normal pregnancy also has implications for the broader field of transplant immunology and cellular immunotherapy.

Women with a history of recurrent miscarriage demonstrated a higher proportion of Th1 and Th17 cells in their peripheral blood than controls. This difference in cellular immunity could be associated with an increased alloimmune response and thus be detrimental to pregnancy. If this cellular immune dysfunction characterises a subgroup of women who would benefit from immunomodulatory therapies requires further investigation. Furthermore, a fetal specific T cell response was demonstrated in women with recurrent miscarriage. This finding strengthens the assertion that abnormal cellular immunity may be part of the aetiology of recurrent miscarriage and further work linking fetal specific responses with prognosis will be important. Finally, it was shown that progesterone could attenuate the function of fetal specific T cells at physiological concentrations. Understanding how progesterone acts in this context may enable the better selection of women with recurrent miscarriage for progesterone therapy and even enable progesterone therapy to be used to modulate CD8 T cell responses in other scenarios outside of reproductive immunology.

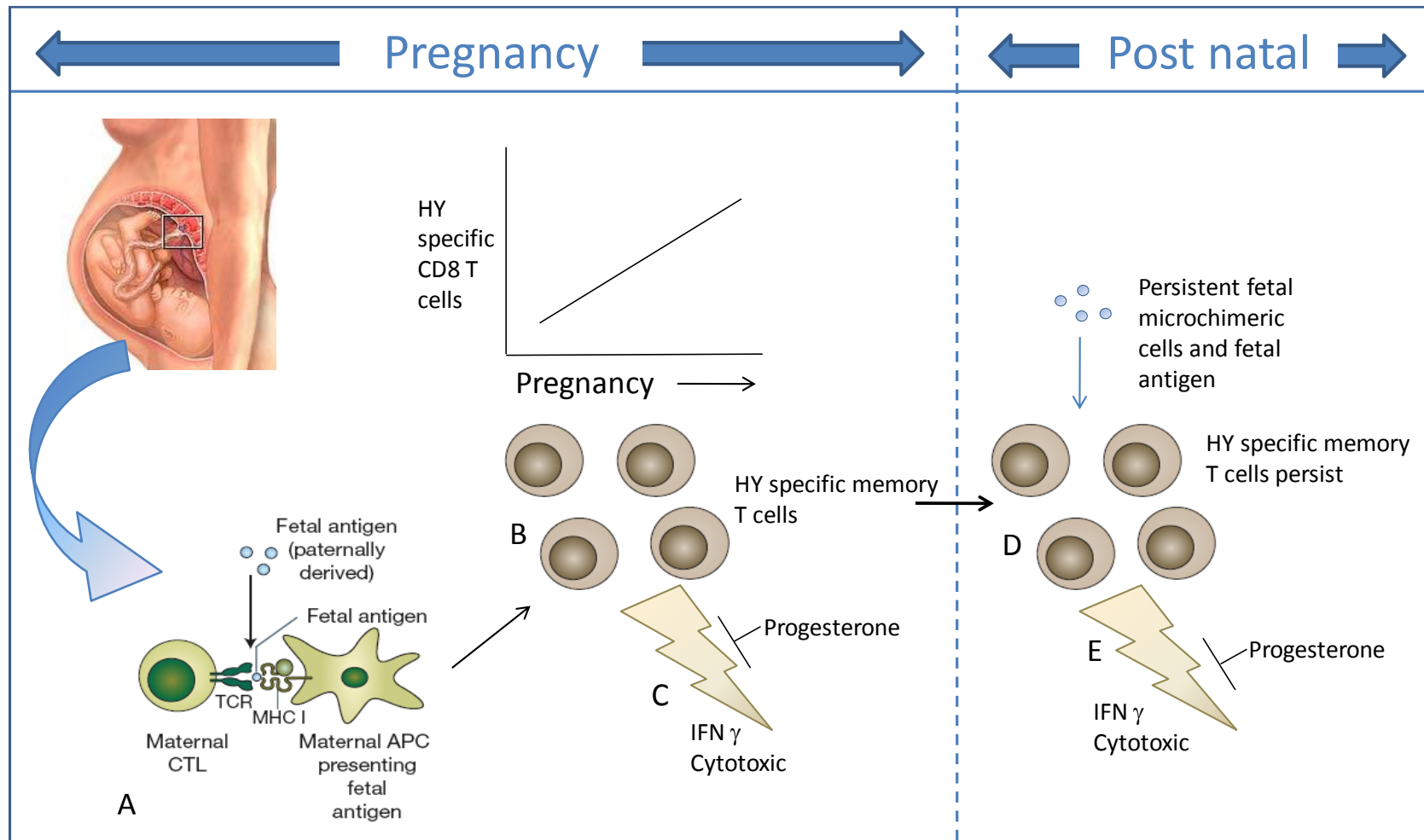


Figure 7.1 Summary of the fetal specific CD8 T cell response during human pregnancy

During pregnancy fetal antigens are transferred into the maternal circulation. This occurs from the 1<sup>st</sup> trimester. Priming of maternal CD8 T cells against fetal antigens leads to the expansion of fetal specific CD8 T cells which can be detected in the periphery from the 1<sup>st</sup> trimester. (A) As pregnancy progresses rather than these fetal specific cells being deleted they appear to increase in frequency within the maternal circulation. (B) They were found to be of a memory phenotype and were not highly differentiated. Fetal specific T cell clones generated from women during pregnancy retained their function and were able to kill male target cells and produce IFN  $\gamma$ . (C) After pregnancy these fetal specific memory T cells persisted at a similar frequency, and could even be found many years after pregnancy. Their may be a role of persistent fetal antigen in maintaining this response. Clones produced from these cells were similarly functional and their cytokine production could be attenuated by progesterone. (D) (APC=Antigen Presenting Cell, IFN  $\gamma$ =Interferon  $\gamma$ )

# **Chapter 8.**

## **References**

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