THE DEVELOPMENT AND FUNCTION OF THYMIC MICROENVIRONMENTS

by

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ABSTRACT

The thymus is organised into distinct microenvironments, and trafficking through these regions enables thymocytes to receive essential signals for the generation of a diverse and self-tolerant T-cell repertoire. Thymic epithelial cells (TEC) represent a key stromal cell type during defined stages in T cell development, yet the mechanisms regulating their development are only partly understood.

An ontogenetic approach was employed to study stages of cortical thymic epithelial cell (cTEC) development. This study identifies a previously unreported population of cTEC progenitors expressing CD205 and β5T and has defined distinct checkpoints in the development of the cTEC lineage. Furthermore, the importance of thymic crosstalk during specific stages of cTEC development and also the requirement for RANK-RANKL signalling for the development of various medullary thymic epithelial cell (mTEC) subsets has also been defined. Additionally, the importance of chemokine-mediated signalling for the establishment and compartmentalisation of the thymus has been highlighted by employing laser capture microdissection and studying thymus microenvironments in mice deficient for particular chemokine related signalling pathways.

Overall this study has provided novel insight into the development of the thymic cortex and will help to understand how these cells become specialised in their ability to support positive selection of developing T cells.

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CONTENTS

ABBREVIATIONS

-/-	deficient
2-dGuo	2-deoxyguanosine
AIRE	autoimmune regulator
AP-1	activator protein 1
APC	antigen presenting cell
APECED	polyendocrinopathy-candidiasis-ectodermal dystrophy
Bcl-2	B cell lymphoma 2
BCR	B cell receptor
BrdU	bromodeoxyuridine
bTEC	bipotent thymic epithelial cell
C	constant
CCL	CC chemokine ligand
CCR	CC chemokine receptor
CD	cluster of differentiation
CDM	CED-5, DOCK180, Myoblasticity
cDNA	complementary DNA
CDR	complementarity-determining region
Cld	claudin
CLIP	class II associated invariant chain peptide
cMES	cortical mesenchyme
CMJ	corticomedullary junction
cTEC	cortical thymic epithelial cell
CTL	cytotoxic T lymphocyte
CTLA-4	cytotoxic T lymphocyte antigen 4
CXCL	CXC chemokine ligand
CXCR	CXC chemokine receptor
DAPI	4,6-diamidino-2-phenylindole
DC	dendritic cell
DL	delta like
DMEM	Dulbecco's Modified Eagle's Medium
DN	double negative
DNA	deoxyribonucleic acid
DOCK	dedicator of cytokinesis
DP	double positive
ECM	extracellular matrix molecule
EpCAM-1	epithelial cell adhesion molecule 1
ER	endoplasmic reticulum
ES	embryonic stem
FACS	fluorescence activated cell sorting
FCS	fetal calf serum
FGF	fibroblast growth factor
FGFR	fibroblast growth factor receptor
FoxN1	forkhead box N1
FoxP3	forkhead box P3

FTOC	fetal thymus organ culture
Gcm2	glial cell missing homolog 2
GFP	green fluorescent protein
Gp38	glycoprotein 38
GTP	guanosine triphosphate
HLA	human leukocyte antigen
Hoxa3	homeobox A3
HSA	heat stable antigen
HSC	haematopoietic stem cell
ICAM-1	intercellular adhesion molecule-1
IFN	interferon
Ii	invariant chain
IL	interleukin
Int	intermediate
IS	immunological synapse
ISP	immature single positive
ITAM	immunoreceptor tyrosine based activation motif
J	junctional
K	keratin
LAT	linker for activation of T cells
LCM	laser capture microdissection
LEF-1	lymphoid enhancer-binding factor 1
LFA-1	lymphocyte function associated antigen 1
Lin	lineage
LPS	lipopolysaccharide
LT	lymphotoxin
LTβR	lymphotoxin β receptor
LTi	lymphoid tissue inducer cell
MHC	major histocompatibility complex
mRNA	messenger RNA
mTEC	medullary epithelial cell
NC	neural crest
NF-κB	nuclear factor kappa B
NFAT	nuclear factor of activated T cells
NK	natural killer
NS	not significant
PAMP	pathogen associated molecular pattern
Pax1	paired box 1
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PD-1	programmed death 1
PDGFRα	platelet derived growth factor receptor α
PD-L1	programmed death ligand 1
PD-L2	programmed death ligand 2
PI3K	phosphoinositide-3-kinase
Plt	paucity of lymph node T cells
pre-Tα	pre-TCR alpha chain
pre-TCR	pre-TCR complex

PRR	pattern recognition receptor
PSGL-1	P-selectin glycoprotein ligand 1
RAG	recombinase activating gene
RANK	receptor activator NF-κB
RANKL	RANK ligand
RF10-H	RPMI-1640 Hepes
RNA	ribonucleic acid
RORγ	retinoic acid receptor-related orphan receptor γ
RT	reverse transcription
RTOC	reaggregate thymus organ culture
Sca-1	stem cell antigen 1
SCF	stem cell factor
SCZ	subcapsular zone
SLP-76	SH2 domain containing leukocyte protein of 76kDa
SP	single positive
Tbx1	T box 1
TCF-1	transcription factor 1
TCR	T cell receptor
TdT	terminal deoxynucleotidyl transferase
TEC	thymic epithelial cell
TECK	thymus expressed chemokine
Th1	T helper 1
Th2	T helper 2
TNF	tumour necrosis factor
TNFRSF	tumour necrosis factor receptor superfamily
TRA	tissue restricted antigen
TRAF6	TNF receptor associated factor 6
Treg	T regulatory
TSSP	thymus-specific serine protease
UEA-1	Ulex Europaeus Agglutinin 1
V	variable
VCAM	vascular cell adhesion molecule 1
WT	wildtype
YFP	yellow fluorescent protein
ZAP-70	zeta chain associated protein kinase 70

CHAPTER 1

GENERAL INTRODUCTION

1.1 Overview of the Immune System.

1.1.1 The Role of the Immune System.

The immune system has evolved to protect organisms from disease by recognising and destroying foreign pathogens and tumour cells. The immune system is made up of a complex and organised network of cells and molecules that are specialised in their role of defending the body against infection whilst maintaining tolerance to self-tissues (Delves and Roitt, 2000). There are two systems that function together to eliminate pathogens and provide an effective defence system. The evolutionary ancient innate or 'natural' immune system initiates a rapid, non-specific response to foreign pathogens and lacks immunological memory. By contrast, the adaptive or 'acquired' immune system is characterised by the clonal expansion of T and B lymphocytes, which express antigen specific receptors and confers lifelong immunity to re-infection with the same antigen.

The innate immune system includes mechanical, chemical and cellular elements, which act in concert as the first line of defence (Basset et al., 2003). The mechanical element comprises the physical barrier of the epithelia preventing access to tissues and organs of the body. Epithelial cells are held together by tight junctions and comprise the skin and line the body's respiratory, gastrointestinal and urogenital tracts. These epithelial cells form an effective barrier against the external environment by secreting anti-microbial enzymes (such as lysozyme and pepsin) and mucus that can inhibit attachment of the microbe. Should these microbes evade the outer defences, other innate immune mechanisms are in place to detect and eliminate the invaders.

The chemical element of innate immunity is in the form of hydrolytic proteins or peptides and inflammatory cytokines such as TNF-α and IL-6, which induce fever and

enhance epithelial responses to pathogens (Basset et al., 2003). The key cellular components of innate immunity include the macrophages, dendritic cells (DCs), mast cells, neutrophils and the natural killer (NK) cells, which can become activated during an inflammatory response against an infection (Janeway and Medzhitov, 2002). The basis of this activation is the recognition of pathogen associated molecular patterns (PAMPs) such as LPS, by pattern recognition receptors (PRRs) (Basset et al., 2003; Medzhitov and Janeway, 1997). Additionally, cells of the innate immune system such as the macrophages have receptors for antibodies and complement that enhance the process of phagocytosis when such cells encounter microbes coated in antibody or components of the complement pathway. Although the innate immune response can be effective in eliminating or preventing the spread of an infection, in some circumstances these defences can be breached and hence an adaptive immune response will follow.

1.1.2 The Adaptive Immune Response.

The adaptive immune response is distinguishable from innate immunity based on the specific recognition of foreign antigens by specialised classes of lymphocytes and the differentiation of pathogen specific effector cells (Janeway and Medzhitov, 2002). Following elimination of antigen during the primary immune response, a small number of effector cells persist and these are known as memory cells. This defines the secondary immune response and ensures a more rapid and effective response on subsequent exposure, thereby providing lasting immunological memory.

Adaptive immunity is comprised of a variety of cell types including B cells, NK T cells, γδ T cells, αβ CD4 helper T cells and αβ CD8 cytotoxic T cells. All these cells derive

from pluripotent haematopoietic stem cells (HSCs) in the bone marrow. Adaptive immunity has evolved to generate a large repertoire of T and B lymphocytes, which in contrast to the cells of the innate immune system have the capability of targeting a multitude of pathogens via the diverse expression of unique antigen specific receptors (Turner et al., 2006).

Adaptive immune responses are initiated by DCs, which develop in the bone marrow and lie at the interface between the innate and adaptive immune system (Basset et al., 2003). Immature DCs reside in the peripheral tissues where they are continuously sampling their environment by a process of endocytosis until they receive a danger signal. Such signals are either endogenous, consisting of molecules such as heat shock proteins produced from cells undergoing stress or abnormal cell death, or exogenous such as LPS on foreign microbes (Gallucci and Matzinger, 2001). Upon receiving a danger signal, the immature DCs undergo a programme of maturation, which transforms them into highly efficient antigen presenting cells (APCs) and potent activators of naïve T cells (Guermonprez et al., 2002). The maturation of DCs is typified by the increase in surface expression of co-stimulatory markers such as CD40, CD80 and CD86, greater antigen processing efficiency and increased MHC molecule expression (Banchereau et al., 2000). The maturation of DCs coincides with their migration to the secondary lymphoid organs (such as the spleen and lymph nodes) where they prime naïve antigen specific T cells and consequently activate the adaptive immune response. The organisation of these secondary lymphoid organs provide specialised microenvironments integral in providing optimal cellular interactions between B and T lymphocytes and DCs (Fu and Chaplin, 1999).

Another important cell that plays a vital role in the adaptive immune response is the B lymphocyte. B cells develop in the bone marrow and express a B cell receptor (BCR) that is the membrane bound form of the antibody that is secreted following B cell activation and

differentiation into a plasma cell. Antibodies or immunoglobulins bind specifically to pathogens with the function to neutralise them or prepare them for uptake by phagocytes. The activation of B cells and their differentiation into antibody secreting plasma cells and thereafter memory cells is triggered by antigens and usually requires assistance from T lymphocytes (MacLennan et al., 2003).

1.1.3 T Lymphocytes.

T lymphocytes play a central role in adaptive immunity by orchestrating the immune response to infection. T cells derive from precursors in the bone marrow and in contrast to B cells continue their development in the thymus (see section 1.2). T cells express a T cell receptor (TCR) and unlike B cells that can recognise antigen in its native form, T cells can only recognise processed antigen peptides presented by APCs in the context of Major Histocompatibility Complex (MHC) molecules. There are two major classes of T cells defined by the surface expression of the co-receptors CD4 and CD8. Following antigen recognition and the initiation of T cell signalling (see section 1.1.6) naïve $CD4^+$ and $CD8^+$ T cells differentiate into effector T cells, which have differing effector functions.

1.1.3.1 $CD8^+$ cytotoxic T cells.

$CD8^+$ cytotoxic T cells are selected in the thymus to recognise and respond to foreign peptides in the context of MHC class I (Bevan, 2004). Naïve $CD8^+$ T cells exit the thymus and differentiate into potent cytotoxic $CD8^+$ T lymphocytes (CTLs) following the recognition of antigen. CTLs kill virally infected or abnormal cells by inserting perforins into the cell membrane of target cells, producing pores that provide a route for proteolytic enzymes that

induce apoptosis in the target cell. Additionally, CTL also activate apoptosis via the engagement of their Fas ligand with the Fas molecule on target cells. The destruction of virally infected cells prevents viral replication and the release of cytokines (such as IFN-γ) induces an antiviral state in neighbouring cells that renders them resistant to infection (Delves and Roitt, 2000; Yewdell and Haeryfar, 2005).

1.1.3.2 $CD4^+$ helper T cells.

$CD4^+$ T cells are selected in the thymus to recognise and respond to foreign peptides in the context of MHC class II. $CD4^+$ T cells play an integral role in the immune response through their ability to stimulate antibody production in B cells and to recruit neutrophils, eosinophils and basophils to sites of infection and inflammation (Zhu and Paul, 2008). Following activation by antigen, naïve $CD4^+$ T cells differentiate into either T helper 1 (Th1) or T helper 2 (Th2) effector cells (Mosmann and Coffman, 1989). Th1 cells secrete cytokines including IFN-γ and IL-2 and are involved in targeting intracellular pathogens and are thought to be responsible for the induction of autoimmune disease (Zhu and Paul, 2008). Th2 cells target extracellular pathogens including helminths and secrete cytokines including IL-4, IL-5 and IL-10 and help B cells make antibody (Zhu and Paul, 2008). $CD4^+$ T cells can also differentiate into Th17 cells and regulatory T cells (Tregs). Th17 cells secrete cytokines including IL-21 and IL-17 and play a role in the induction of autoimmune tissue injury. (Bettelli et al., 2006). Tregs express CD4, CD25 and the transcription factor FoxP3. Tregs are made in the thymus and are essential for maintaining peripheral tolerance and preventing autoimmune disease by suppressing T cell responses (Vignali et al., 2008).

1.1.4 Antigen Processing and Presentation to T cells.

The recognition of antigens in the peptide-binding groove of surface expressed MHC molecules by the TCR is at the forefront of the T cell immune response. To activate T cells, MHC proteins must first be loaded with and present peptide antigens. There are two subtypes of MHC involved in TCR recognition; class I and class II (Vyas et al., 2008). A third subtype also exists and is important for the complement activation pathway of the humoral immune response (Yung Yu et al., 2000).

MHC class I molecules are expressed on the surface of almost all nucleated cells and initiate an immune response to virally infected cells, intracellular bacterial infections or malignant cells by presenting endogenously derived peptides to $CD8^+$ cytotoxic T cells (Flutter and Gao, 2004). Endogenous proteins to be loaded onto MHC class I molecules are degraded by proteasomes in the cytosol for MHC class I presentation (Trombetta and Mellman, 2005). Peptide transporters transfer the resulting peptides to the endoplasmic reticulum (ER) and are loaded onto new MHC class I molecules. Following association with peptide, MHC class I molecules are rapidly transferred via the Golgi apparatus to the cell surface membrane (Guermonprez et al., 2002).

In contrast to MHC class I molecules, expression of MHC class II molecules are mostly restricted to professional APCs such as DCs, B cells, macrophages and also epithelial cells in the thymus. MHC class II molecules associate with peptides derived from exogenous antigens, which have been internalised into vesicles via the endocytic pathway (Guermonprez et al., 2002). Assembly of the MHC II molecule occurs in the ER where the presence of the invariant chain (Ii) in the peptide binding groove, targets the complex to the endosomal pathway (Watts, 2004). Within the endosomes or lysosomes the Ii chain is degraded by cysteine proteases of the cathepsin family. The accessory molecule HLA-DM (or H-2M in

mice) catalyses the dissociation of the CLIP (class II associated invariant chain peptide) from the peptide binding groove of the MHC class II molecule for antigenic peptide loading (Bryant and Ploegh, 2004). Following CLIP removal and peptide loading, peptide-MHC class II complexes are delivered to the plasma membrane for presentation to $CD4^+$ helper T cells.

The process of antigen presentation in the context of MHC class I and class II molecules is also imperative for the intra-thymic positive selection of T cells expressing diverse, self-MHC restricted T cell receptors capable of recognising a multitude of peptides (see section 1.2.5).

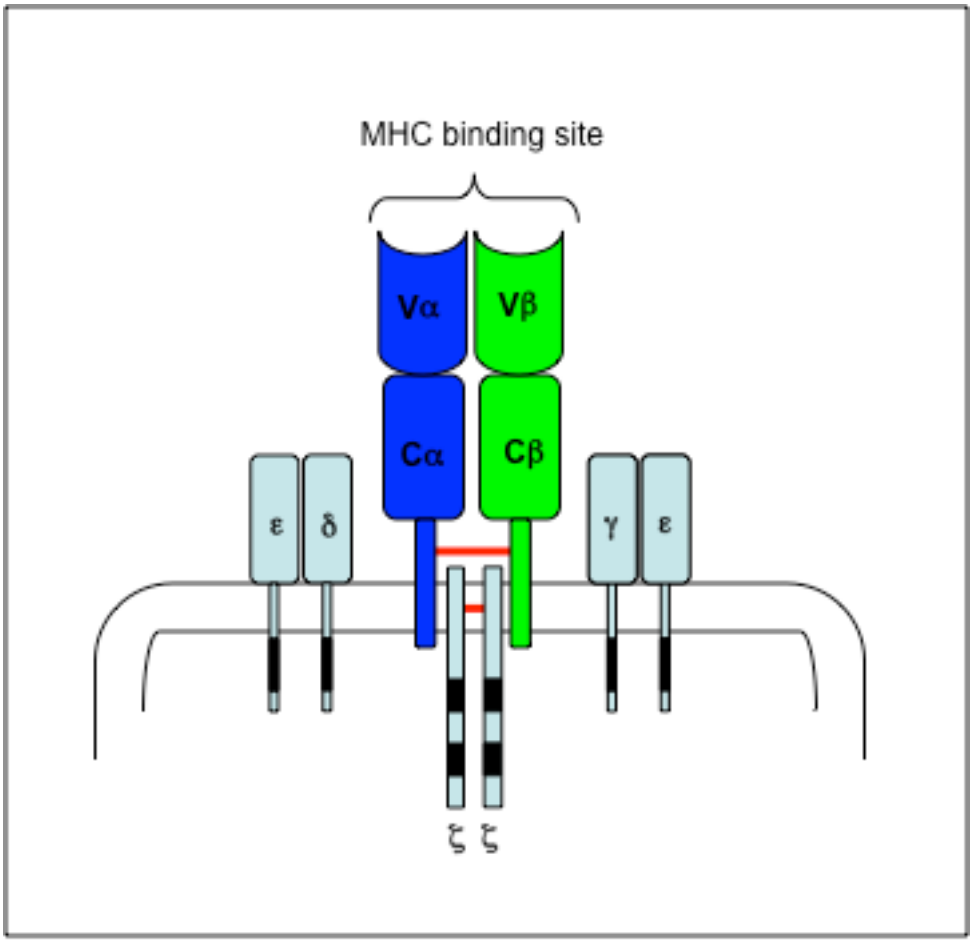
1.1.5 Structure of the T Cell Receptor.

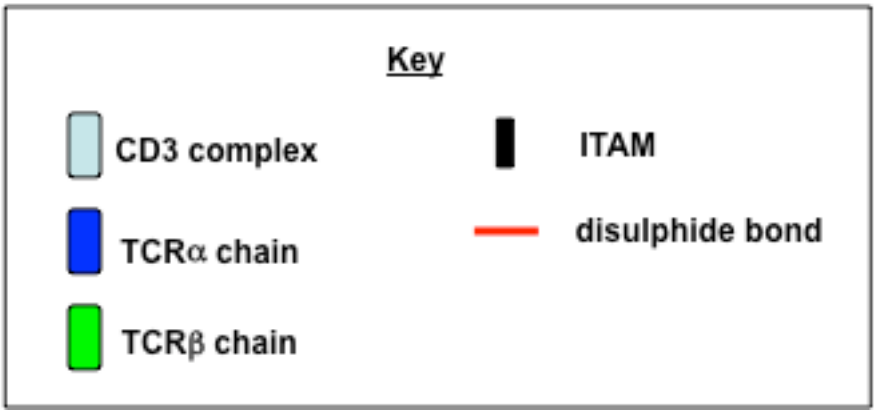
One of the most important interactions during an immune response is that of the TCR with peptide-MHC complexes. In addition to delivering essential signals at distinct stages of T cell development in the thymus, the TCR is vital for the activation and differentiation of mature T cells into effector and memory cells in the periphery (Call and Wucherpfennig, 2005). The TCR is associated with the CD3 complex as described in section 1.1.6 and illustrated in figure 1.1. The majority of T cells express the αβTCR, which engages with processed antigen peptides in the context of MHC molecules expressed on APCs (van der Merwe and Davis, 2003). There are T cells that also express the γδTCR and these cells constitute only 1-5% of the circulating peripheral lymphocytes (Carding and Egan, 2002). The γδ T cells are mostly found within epithelial tissues such as the skin and unlike αβ T cells they recognise unprocessed antigen in a non- MHC restricted manner (Hayday, 2000; Hayday and Pennington, 2007). Data and discussion of T cells in this study will refer to αβ-T cells unless otherwise stated.

Figure 1.1 Structure of the T cell Receptor and CD3 Complex.

The T cell receptor is composed of an α- and β-chain joined together by a disulphide bond. Each chain is comprised of a variable (V) region and a constant (C) region. The Vα and Vβ domains encode regions of hypervariability known as complementarity-determining regions (CDRs) and these form the peptide-MHC binding site. The TCR is associated with the CD3 complex, which is comprised of CD3γ, CD3δ, two molecules of CD3ε and the additional CD3ζ subunit, which exists primarily as a disulphide-linked homodimer. Each CD3 subunit contains cytoplasmic domains with conserved immunoreceptor tyrosine based activation motifs (ITAMs).

Image adapted from Janeway et al (2005).


MHC binding site
Vα
Vβ
Cα
Cβ
ε
δ
γ
ε
ζ ζ


Key
CD3 complex
TCRα chain
TCRβ chain
ITAM
disulphide bond

The αβTCR is composed of an α-chain and a β-chain, linked together by disulphide bonds to form a heterodimer. Each chain is comprised of a variable (V) domain and a constant (C) domain (Saito et al., 1984), which are spliced together during T cell development in the thymus. The V domain of the β-chain is encoded by variable (V), diversity (D) and junctional (J) gene segments, whereas only the V and J gene segments encode the V region of the α-chain (Turner et al., 2006). The Vα and Vβ domains encode regions of hypervariability known as complementarity-determining regions (CDRs) and this forms the peptide-MHC binding site (Chothia et al., 1988; Davis et al., 1998).

The immune system has the remarkable capacity to generate a vastly diverse repertoire of antigen receptors specific for the multitude of pathogens we may encounter in a lifetime. Such diversity is achieved through a complex and highly regulated site-specific genomic process called V(D)J recombination (Alt et al., 1992). A combinatorial rearrangement of the V, D and J segments generates diversity in the variable region of each TCR chain and further receptor structure diversity is achieved by the addition or removal of nucleotides at joining sites by the enzyme terminal deoxynucleotidyl transferase (TdT) (Fugmann et al., 2000; Komori et al., 1993). V(D)J recombination in both T and B cells is regulated by the proteins encoded by the recombination activating genes RAG-1 and RAG-2, which are expressed in developing lymphocytes undergoing antigen receptor assembly. Defects in either of these genes result in severe immunodeficiency due to a defect in the production of functional lymphocytes bearing antigen receptors (Mombaerts et al., 1992; Schwarz et al., 1996).

Engagement of the TCR with APCs presenting antigen peptides in the context of MHC class I or class II is imperative for T cell activation and requires association with a multi-component signalling complex.

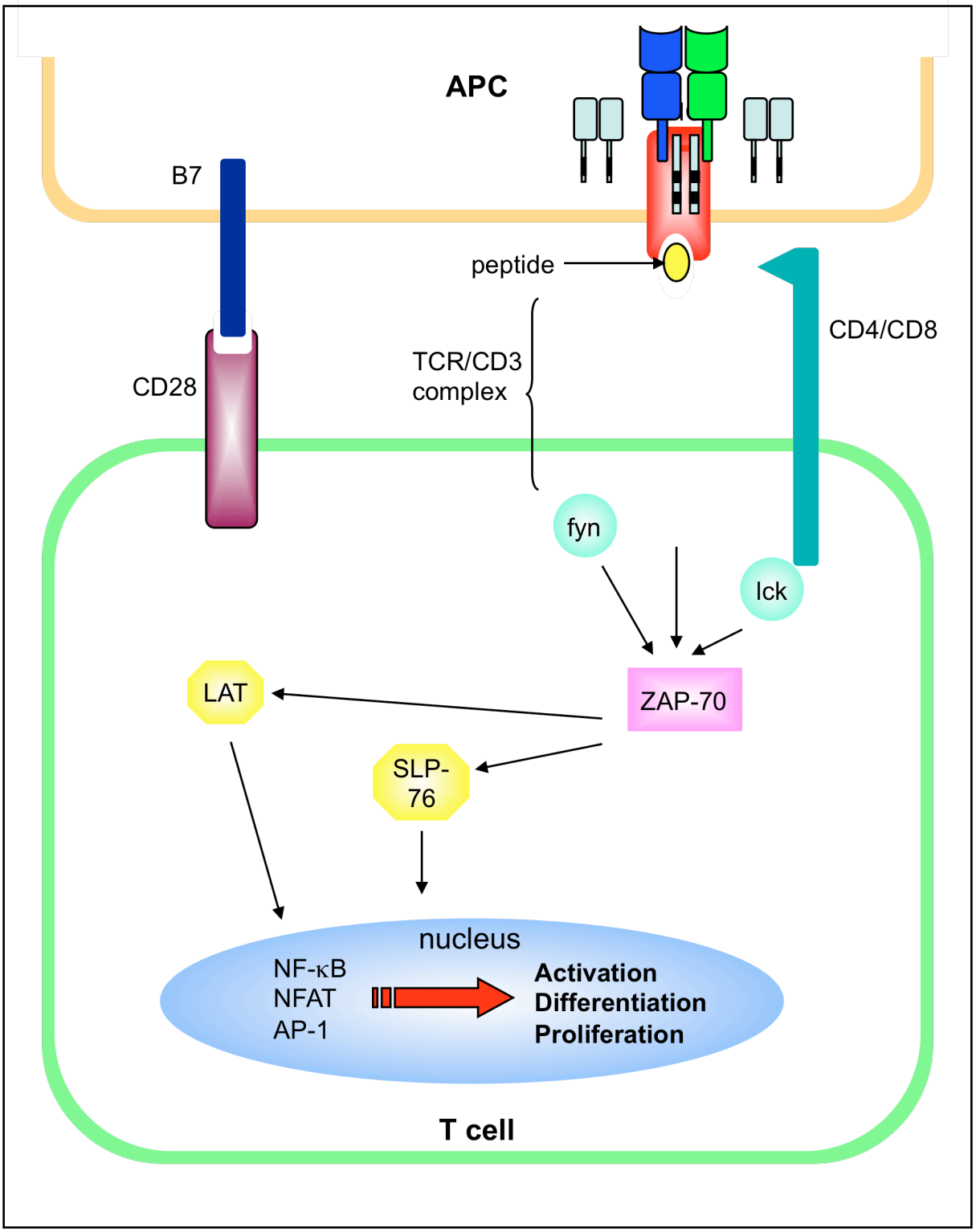
1.1.6 TCR and CD3 Signalling.

The sustained engagement of TCR with peptide-MHC on the cell surface of APCs leads to the cross-linking of the TCR, which initiates a complex intracellular signalling cascade culminating in the activation of T cell effector functions (Ehrlich et al., 2002) (figure 1.2). The specialised contact area between T cells and APCs has been termed the 'immunological synapse' and is defined by the 'bull's eye' distribution of the TCR with other cell surface signalling and adhesion molecules associated with the propagation of a signal from the T cell surface to the nucleus (Grakoui et al., 1999; Kane et al., 2000). The centre of the bull's eye is enriched for the TCR and peptide-MHC complex, whereas the ring contains the integrin LFA-1 and its major counter receptor ICAM-1 (Bromley et al., 2001a).

Due to a short cytoplasmic domain, the αβTCR is incapable of signalling to the cells alone and hence associates with a group of invariant molecules that are non-covalently associated with the αβ heterodimer and are collectively termed the CD3 complex. This complex, which is also important for surface expression of the αβTCR, consists of CD3γ, CD3δ and two molecules of CD3ε, which are encoded by genes on mouse chromosome 9 and are structurally related to the immunoglobulin family (Call and Wucherpfennig, 2005; Chan et al., 1994). The additional subunit CD3ζ has a large cytoplasmic domain and exists primarily as a disulphide-linked homodimer with a small percentage of TCRs containing heterodimers of ζ and η (a splice variant of ζ) (Call and Wucherpfennig, 2005; Clayton et al., 1992). Each CD3 subunit contains cytoplasmic domains with conserved immunoreceptor tyrosine based activation motifs (ITAMs), which are important in mediating intracellular signal transduction following TCR recognition of antigen (Clevers et al., 1988; Kane et al., 2000). Upon TCR engagement by antigen presented by MHC molecules, T cell

Figure 1.2 Overview of the Immunological Synapse.

Overview showing the key signalling pathways initiated following TCR engagement with peptide loaded MHC molecules on antigen presenting cells (APCs). Signalling pathways culminate in the activation of transcription factors involved in T cell effector functions.


APC
B7
peptide
CD4/CD8
TCR/CD3
complex
CD28
fyn
lck
LAT
ZAP-70
SLP-
76
nucleus
NF-κB
NFAT
AP-1
Activation
Differentiation
Proliferation
T cell

signalling is initiated following binding of the co-receptors CD4 (on helper T cells) or CD8 (on cytotoxic T cells) to the constant regions of MHC class II and MHC class I molecules respectively.

The recruitment and activation of co-receptor associated $p56^{lck}$ (tyrosine kinase belonging to the Src family) and receptor associated $p59^{fyn}$ (also belonging to the Src family) initiates Lck and Fyn mediated phosphorylation of ITAMs in the cytoplasmic tails of the CD3 complex (Lin and Weiss, 2001; Straus and Weiss, 1992; Turner et al., 1990). The consequence of phosphorylated ITAMs is the recruitment and activation of another tyrosine kinase belonging to the Syk family called ZAP-70. ZAP-70 associates with the CD3ζ chain and is important for the propagation of the signal by acting as a scaffold for the binding of additional adapter proteins such as LAT and SLP-76 (Deindl et al., 2007; Samelson, 2002). Events occurring downstream of LAT and SLP-76 phosphorylation include calcium mobilisation via the generation of inositol phospholipids and cytoskeletal reorganisation via Vav-dependent activation of Rac, which is integral for the formation of the immunological synapse (Ardouin et al., 2003; Turner and Billadeau, 2002). The culmination of these signalling pathways is the activation of transcription factors such as nuclear factor κB (NF-κB), nuclear factor of activated T cells (NFAT) and activator protein 1 (AP-1), which initiate transcription of genes involved in differentiation, proliferation and effector actions of T cells.

Signalling by the TCR/CD3 complex only, in the absence of co-stimulatory signals fails to activate T cells and instead induces apoptosis. Signals delivered to the TCR by co-stimulatory cell surface molecules on APCs are important for optimal activation of T cells and the induction of tolerance by the provision of inhibitory signals (Sharpe and Freeman, 2002). One of the most understood co-stimulatory pathways is B7-CD28. B7 members include B7.1 (CD80) and B7.2 (CD86) expressed on APCs, which augment and sustain T cell responses by

interacting with CD28 on T cells (Bromley et al., 2001b). CTLA-4 is expressed on activated T cells and binds to B7.1 and B7.2 to deliver an inhibitory signal to the T cell (Linsley et al., 1991). Other members of the B7 family include PD-L1 (CD274) and PD-L2 (CD273), which bind to PD-1 on T cells and this pathway is thought to be involved in the induction of T cell tolerance. (Liang et al., 2003).

The generation of T lymphocytes that are specific for a wide spectrum of antigens occurs in the thymus. The establishment of this self-tolerant and diverse T cell repertoire is dependent on the unique structure of the thymus and this is of considerable interest. Further discussion and data within this thesis relates to this subject.

1.2 T Cell Development.

1.2.1 Structure and Function of the Thymus.

The thymus is an encapsulated primary lymphoid organ situated in the upper anterior thorax above the heart and whose emergence during evolution occurred in parallel with the appearance of V(D)J recombination (Boehm, 2008). The thymus plays a fundamental role in establishing a fully functional immune system by supporting the development of T cells that are highly specialised in their ability to mount a reactive immune response against specific antigens. The importance of the thymus in T cell development was first demonstrated in 1961 when thymectomised neonatal mice developed severe immunodeficiency (Miller, 2002). After birth, the thymus starts to undergo a gradual process of involution or atrophy, where the thymus shrinks in size and is replaced by fat and the production of new T cells is decreased.

The thymus consists of numerous lobules separated by mesenchymal septae, with each lobe comprising a unique three-dimensional complex network of non-lymphoid stromal cells,

which provides a supporting framework to mediate T cell development. (Anderson and Jenkinson, 2001). The two main regions of the stroma comprise of the peripheral cortex and the central medulla, each containing several distinct types of thymic epithelial cells (TECs) (Boyd et al., 1993). These distinct regions generate highly specialised intra-thymic microenvironments, which direct the maturation of immature T cell precursors into a diverse repertoire of self-tolerant and antigen-specific T cells. To achieve this, T cells must undergo a series of checkpoints including, commitment to the T cell lineage, TCR gene rearrangements, selection of the αβ T cell repertoire, CD4 and CD8 lineage commitment and post-selection maturation. This highly orchestrated development of thymocytes is defined by their migration through the different microenvironments of the thymus allowing them to receive essential signals from the thymic stroma. However, prior to intra-thymic T cell development, haematopoietic precursors must first colonise the thymus.

1.2.3 Colonisation of the Thymus by Thymocyte Progenitors.

T cells develop from migrant progenitors that originate in different haematopoietic sites depending on the stage of development (Anderson et al., 2006). The liver and yolk sac are the primary sites of haematopoiesis in the fetus, whereas progenitors colonising the thymus in the adult are derived from the bone marrow. The very early thymic progenitors or HSCs have self-renewal capacity and can give rise to all lymphocyte populations and other blood cell types. In mice, multipotent HSCs lack lineage markers (Lin^-) indicative of differentiated cells but do express stem cell antigen 1 (Sca-1) and CD117 (c-Kit) and are termed LSK cells (Ikuta and Weissman, 1992). LSK cells represent the most immature HSCs in the bone marrow.

T cell development is dependent on the colonisation of the thymus by HSC-derived T cell progenitors. Progenitors in the thymus have an extremely limited capacity to self-renew and so continuous replenishment of the thymic pool is necessary. This seeding of the thymus from progenitors migrating through the bloodstream requires a continuous input of progenitors to maintain T cell development and this is thought to occur in periodic waves (Foss et al., 2001). Before vascularisation is established, precursors colonise the fetal thymus at day 12 of gestation (E12) by exiting nearby blood vessels and entering the perithymic mesenchyme surrounding the thymus before migrating across the epithelial basement membrane (Itoi et al., 2001; Suniara et al., 2000). However, in the postnatal thymus circulating progenitors enter the thymus via blood vessels in a region of the thymus called the cortico-medullary junction (CMJ) (Lind et al., 2001).

There is still considerable debate as to the precise phenotypic identity of precursors seeding the thymus and if these cells represent cells with multiple lymphoid lineage potential or cells committed to the T cell lineage (Jenkinson et al., 2006). Studies have implicated the role of Notch signalling in T cell commitment. Notch genes encode a family of transmembrane receptors, which have a role in cellular differentiation and cell fate decisions (Maillard et al., 2005). Four mammalian Notch homologues exist (Notch 1-4) and their ligands include Jagged 1, Jagged 2, Delta-like 1 (DL1) and Delta-like 4 (DL4), which are expressed on thymic epithelial cells. The essential role of Notch signalling in T cell development is evidenced by the absence of T cells and the presence instead of B cells in the thymus of mice with perturbed Notch signalling (Wilson et al., 2001). Initially it was argued that intrathymic Notch signalling determines lineage choice of progenitors entering the thymus, which have T cell and B cell potential (Pui et al., 1999). However, a study has shown that T and B lineage choice occurs prior to precursor recruitment to the thymus and so

argues against a role for intrathymic Notch signalling in determining T/B lineage choice during initial fetal thymus colonisation (Harman et al., 2005). These studies may suggest that intrathymic Notch signalling may in fact be required for continued T cell differentiation and for revealing T cell fate in already committed progenitors entering the thymus. Notch signalling pathway is also thought to have a role in subsequent T cell development including, regulation of TCRβ rearrangements, αβ versus γδ and CD4 versus CD8 lineage commitment (Anderson et al., 2006).

The precise mechanisms that mediate recruitment of thymic progenitors from the bone marrow are yet to be fully defined. However, such recruitment of progenitors is known to differ in the fetal and post-natal thymus. Thymic colonisation in the fetal thymus is regulated by a chemotactic mechanism. In the fetal thymus it has been shown that CCL21/CCR7 and CCL25/CCR9 mediated chemokine signals are essential for early thymus colonisation prior to vascularisation but are not required post-vascularisation (Liu et al., 2006). A study analysing chemokine expression on the first wave of progenitors entering the E12 thymus, identified heterogeneity within these early thymic migrants as defined by the expression of multiple chemokine receptors including, CCR7, CCR9 and CXCR4 (Jenkinson et al., 2007a). A population of cells lacking this expression pattern was also observed suggesting that other chemokine independent mechanisms are important or other as yet unidentified chemokines play a role in progenitor colonisation.

In the post-natal thymus, recruitment of thymic progenitors in the adult is regulated by a family of carbohydrate binding proteins called selectins, which have an important role during inflammatory responses by recruiting effector cells to sites of infection (Rossi et al., 2005). P-selectin was found to be expressed extensively throughout the thymus vasculature and is a key mediator of the homing and recruitment of circulating cells. The cognate ligand

for P-selectin is P-selectin glycoprotein ligand 1 (PSGL-1). PSGL-1 is expressed on LSK cells in the bone marrow and early progenitors in the thymus and hence implicates the role of selectins in facilitating progenitor recruitment to the thymus. A recent study has shown that the periodic thymic expression of P-selectin and the CCR9 ligand CCL25, which is also important for thymic progenitor immigration, is likely to contribute to thymic gating (Gossens et al., 2009). This study also demonstrated a role for sphingosine-1-phosphate (S1P) in the expression of P-selectin.

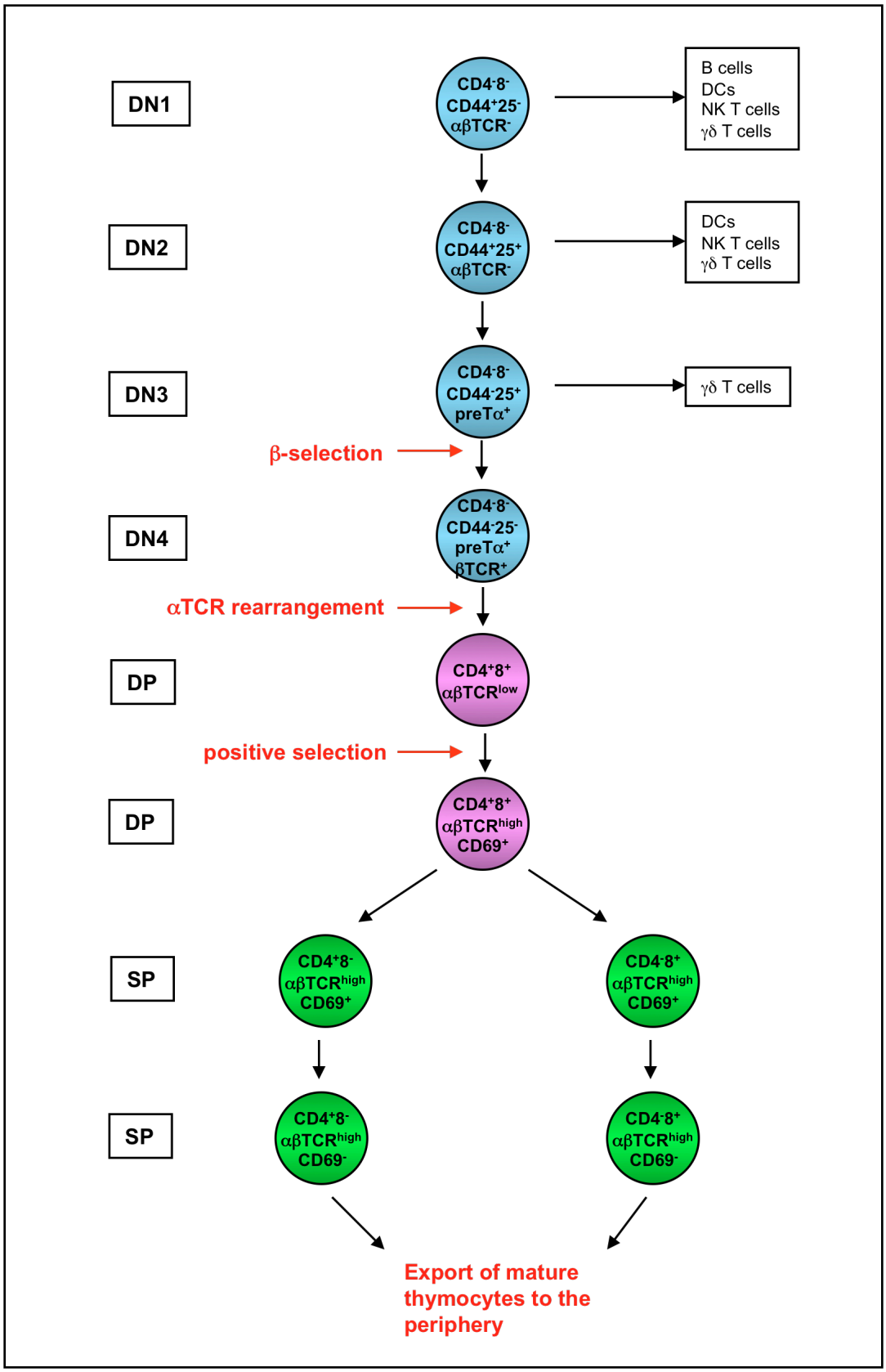
Once T cell progenitors enter the thymus they subsequently undergo a tightly regulated process of T cell development.

1.2.4 Early Intra-thymic T Cell Development.

Following thymic colonisation, thymocytes differentiate through various phenotypically distinct stages as defined by expression of cell surface markers including the co-receptors CD4 and CD8 (Figure 1.3). Thymic progenitors interact closely with thymic stromal cells throughout the various stages of T cell development allowing them to receive important signals for their differentiation. This development correlates with an orchestrated migration of thymocytes through different thymic microenvironments (see section 1.2.7). Following progenitor colonisation in the fetal thymus, thymocyte development proceeds in an organised manner with sequential developmental stages occurring at different stages of ontogeny. However, T cell development in the adult represents a dynamic equilibrium between colonising progenitors and newly arising mature T cells with all stages of development present (Zuniga-Pflucker and Lenardo, 1996).

Figure 1.3 Stages of Intrathymic T Cell Development.

Stages of T cell development in the thymus as defined by phenotypic changes in the expression of cell surface markers including the co-receptors CD4 and CD8. Thymocytes differentiate from double negative (DN) to double positive (DP) and finally to single positive (SP) cells. Alternative lineage potentials are indicted in the boxes and red arrows show the essential checkpoints during T cell development.


DN1
CD4-8-
CD44+25-
αβTCR-
B cells
DCs
NK T cells
γδ T cells
DN2
CD4-8-
CD44+25+
αβTCR-
DCs
NK T cells
γδ T cells
DN3
CD4-8-
CD44-25+
preTα+
γδ T cells
β-selection
DN4
CD4-8-
CD44-25-
preTα+
βTCR+
αTCR rearrangement
DP
CD4+8+
αβTCRlow
positive selection
DP
CD4+8+
αβTCRhigh
CD69+
SP
CD4+8-
αβTCRhigh
CD69+
CD4-8+
αβTCRhigh
CD69+
SP
CD4+8-
αβTCRhigh
CD69-
CD4-8+
αβTCRhigh
CD69-
Export of mature
thymocytes to the
periphery

The earliest described progenitors that first enter the thymus at the CMJ are described as lineage double negative (DN) cells because they lack expression of CD4 and CD8. During the DN stage each precursor entering the thymus gives rise to approximately 1 million daughter cells and hence is the most proliferative stage of T cell development (Lind et al., 2001). The DN stage is further sub-divided into four separate stages based on the differential expression of CD44 (phagocytic glycoprotein 1), CD25 (IL-2 receptor α), CD117 and CD127 (IL-7 receptor α chain) (Godfrey et al., 1993; Zuniga-Pflucker and Lenardo, 1996). As these early thymocytes differentiate through the DN substages, they undergo a progressive loss in multi-lineage potential and start to acquire a phenotype characteristic of T cells. Cells at the double negative 1 stage (DN1) are defined as $CD25^{-}$ $CD44^{hi}$ $CD117^{+}$ $CD127^{-}$. This subset is the most immature cell type in the thymus and is thought to contain subsets of common lymphoid progenitors with multi-lineage potential, including T and B cells, DCs and NK T cells but not myeloid cells. However, this definition of DN1 cells is very much simplified and heterogeneity has been identified within this stage as defined by the expression of other haemato-lymphoid lineage markers such as CD24 (Porritt et al., 2004) and Flt3 (Sambandam et al., 2005). These various DN1 progenitor subsets all home to the thymus but have different proliferative capacities and lineage potentials.

DN1 cells acquire CD25 and CD127 and transition to the double negative 2 (DN2) stage of development (defined as $CD25^{+}$ $CD44^{hi}$ $CD117^{+}$ $CD127^{+}$) and these cells are found predominantly in the mid-cortical region of the thymus. DN2 cells are thought to have lost B cell potential and have limited NK T cell potential but can still give rise to αβ and γδ T cells and DCs (Schmitt et al., 2004). At this stage of development, expression of the RAG genes are upregulated (Wilson et al., 1994) and rearrangement of the TCRγ and δ loci occurs, which precede rearrangement at the TCRβ locus (Livak et al., 1999). TCRβ rearrangement is

completed upon transition to the double negative 3 (DN3) stage of development, which is defined as $CD25^{+}$ $CD44^{lo}$ $CD117^{lo}$ $CD127^{lo}$. IL-7 signalling has been shown to be important at this early stage of T cell development. IL-7 is produced by thymic epithelial cells and binds to IL-7Rα, (CD127), which is complexed with the common cytokine receptor γ chain (γc) (Zamisch et al., 2005). A study using OP9-DL1 cells, a stromal cell line known to support T cell development *in vitro* (Schmitt and Zuniga-Pflucker, 2002), demonstrated that proliferation and differentiation of DN1 and DN2 cells required the presence of IL-7, whilst differentiation beyond the DN3 stage was IL-7 independent (Balciunaite et al., 2005).

DN3 cells are localised in a region of the thymus adjacent to the capsule, called the subcapsular zone (SCZ). Cells at this stage of development have absolutely committed to the T cell lineage and can give rise to αβ and γδ T cells. The complete loss of non-T lineage potential, coinciding with the large-scale gene rearrangement of TCR chains, marks the irreversible commitment to the T cell lineage. The exact branch point of αβ and γδ lineages and the molecular mechanisms determining this lineage decision have not yet been fully characterised. However, a study has reported a differential role for Notch signalling during αβ and γδ T lineage diversification (Ciofani et al., 2006). In the context of αβ T cell development, rearranged TCRβ chain pairs with an invariant pre-Tα and CD3 components on the cell surface forming the pre-TCR complex initiating further differentiation of the thymocyte (Baldwin et al., 2005). The pre-TCR complex consists of the TCRβ chain disulphide linked to the pre-TCRα, which is homologous to the TCRα chain but contains a single extracellular immunoglobulin-like structural domain (Groettrup et al., 1993; von Boehmer et al., 1999; Zuniga-Pflucker and Lenardo, 1996). Only cells with a functional pre-TCR are selected for expansion and further maturation and this process is called β-selection and defines the first checkpoint of T cell development. The failure to express a functional pre-

TCR prevents further thymocyte differentiation and leads to apoptosis. β-selection ensures that only thymocytes that have successfully generated functional in frame TCRβ progress to the next stage of T cell development. This occurs through pre-TCR signalling, which requires association with the CD3 complex and activation of the protein tyrosine kinases lck and fyn (as described in section 1.1.5) (von Boehmer et al., 1999).

The pre-TCR/CD3 complex prevents further DNA recombination at the TCRβ locus by a process of allelic exclusion, which ensures that developing T cells express only one TCRβ chain (Aifantis et al., 1997; Petrie et al., 1993). Signalling through the pre-TCR complex terminates β-selection and drives the proliferation and differentiation of DN cells to the next stage of T cell development (Ardouin et al., 1998).

Thymocytes emerging from β-selection at the DN3 stage undergo a dramatic increase in proliferation and down-regulate CD25 and CD44 to make the transition to what is known as the double negative 4 stage (DN4) of T cell development. However, these cells do not represent true DN cells as they are in the process of up-regulating CD4 and CD8 expression to become double positive (DP) thymocytes (Petrie and Zuniga-Pflucker, 2007). DP cells constitute the entire region of the cortex. This phase of T cell development is marked by the rearrangement of the TCRα gene locus, which displaces the pre-Tα and pairs with the already expressed β chain. This is then followed by surface expression of low levels of αβTCR assembled with the CD3 complex. Following upregulation of the co-receptors, a large population of DP cells are generated and these constitute approximately 90% of thymus cellularity (Germain, 2002). This transition of DN cells to DP cells occurs via a subset of thymocytes called immature single positive (ISP) cells, which can be defined as either $CD4^+$ $CD8^-$ $CD3^-$ or $CD4^-$ $CD8^+$ $CD3^-$ (Hugo et al., 1990; Tatsumi et al., 1990). Such $CD4^+$ or $CD8^+$

ISP cells represent a rapidly cycling intermediate precursor for $CD4^+$ $CD8^+$ cells. Notch signalling has been implicated in this transition of DN cells to DP cells (Huang et al., 2003).

The successful rearrangement of the TCRα and β chains prepares the DP thymocyte for T cell repertoire selection, which defines the next checkpoint of T cell development (Zuniga-Pflucker and Lenardo, 1996).

1.2.5 Thymic Selection of the αβTCR Repertoire.

Immature $CD4^+$ $CD8^+$ $CD3^{lo}$ double positive (DP) thymocytes expressing low levels of αβTCR are subjected to stringent positive and negative selection events in the cortical region of the thymus. Interactions between developing thymocytes and self-MHC molecules expressed on the thymic stroma define this stage of development and represent a vital checkpoint in the development of T cells.

DP cells have a relatively short life span of approximately 3-5 days (Egerton et al., 1990) during which the RAG genes continue to be expressed and there is further rearrangement at the TCRα locus. Approximately 90% of DP cells are thought to die by neglect unless there is an appropriate interaction between the TCR with self-peptide/MHC complexes, presented by cortical epithelial cells, ensuring their survival and differentiation into single positive (SP) cells. Survival of pre-selection DP cells has been reported to be regulated by the expression of the anti-apoptotic factor Bcl-2 as well as initiation of the Wnt signalling pathway via the transcription factors TCF-1 and LEF-1 (Ioannidis et al., 2001; Pongracz et al., 2003). Wnt proteins belong to a family of secreted glycoproteins and are expressed by the thymic epithelium. Wnts bind to frizzled receptors on developing T cells, which initiates distinct signalling pathways resulting in the stabilisation of β-catenin

(Anderson et al., 2006). Stabilised β-catenin translocates to the nucleus where it binds to TCF-1 and LEF-1 resulting in expression of Wnt target genes.

Successful engagement of the TCR on DP cells with self-peptide/MHC on the stroma prevents further TCRα rearrangement and leads to various developmental fates depending on the strength and the nature of the interaction between the TCR and peptide-MHC (Ober et al., 2000). DP cells that can bind to self-peptide presented on MHC I with low affinity will differentiate into $CD4^-$ $CD8^+$ (SP8) T cells, whereas DP cells that bind to self-peptides presented on MHC II will differentiate into $CD4^+$ $CD8^-$ (SP4) T cells. This process ensures that only thymocytes expressing receptors with the potential to recognise foreign peptides in the context of self-MHC are selected to mature into single positive T cells (Anderson and Jenkinson, 2001). DP cells that interact with self-MHC with too high an affinity are potentially autoreactive and will die by apoptosis in a process called negative selection (Anderson et al., 2006). Only a small fraction of thymocytes (approximately 3-5%) will survive this checkpoint in T cell development (Goldrath and Bevan, 1999).

Early studies have shown that radioresistant stromal cells are responsible for imposing MHC-restricted antigen recognition by developing T cells (Bevan, 1977; Zinkernagel et al., 1978). It become increasingly clear that the cortical thymic epithelial cells (cTECs) have a unique ability to mediate and support positive selection of thymocytes from the DP to SP stage (see section 1.3.6). Experiments using reaggregate thymic organ cultures (RTOCs), where thymocytes were cultured with thymic stroma expressing MHC II, have established the importance of MHC II^+ cTECs in supporting positive selection of DP thymocytes (Anderson et al., 1994a; Anderson et al., 1994b). Sustained interactions of DP cells with MHC are important for further maturation into functional SP4 and SP8 cells and this is evidenced by the upregulation of the early activation marker CD69. Experiments involving the

reaggregation of $CD4^+$ $CD8^+$ $CD69^-$ (preselection) thymocytes with MHC II deficient stroma failed to generate SP4 thymocytes with no evidence of CD69 expression, thereby further confirming the importance of MHC interactions to initiate positive selection (Hare et al., 1999). However, in the same study $CD4^+$ $CD8^+$ $CD69^+$ thymocytes did not require MHC expression on stroma for differentiation to the SP stage of development. Therefore, CD69 expression discriminates between MHC-dependent and MHC-independent stages of thymocyte development, where the initial stages of positive selection require sustained TCR-MHC interactions. However, further functional and phenotypic differentiation to SP4 and SP8 cells does not require continuous signalling through the TCR, although still requires interaction with the thymic epithelium. Other phenotypic changes at this stage include, the upregulation of another activation/selection marker CD5 and down-regulation of RAG 1 and 2 (Merkenschlager et al., 1997). Further maturation into SP thymocytes is also dependent on the upregulation of particular cytokine receptors, most notably the IL-7 receptor α chain (CD127) and the common γ chain (CD132), which is an important component of IL-2, IL-4, IL-7, IL-9 and IL-15 cytokine receptors (Hare et al., 2000).

In addition to these changes, the levels of CD4 and CD8 expression decreases during selection giving rise to a $CD4^{low}$ $CD8^{low}$ TCR^{int} population (Lucas and Germain, 1996). These cells are thought to differentiate into either SP4 or SP8 thymocytes via a transitional population of cells defined as $CD4^+$ $CD8^{low}$ (Singer et al., 2008). The precise cellular and molecular mechanisms that underlie CD4/CD8 lineage decisions are still subject to debate. However, a study showed that constitutively activated Notch1 promotes the development of SP8 cells over SP4 cells, thereby implicating a role for Notch signalling in CD4 and CD8 lineage fate (Robey et al., 1996).

Following TCR mediated repertoire selection positively selected thymocytes migrate to the medulla where they are subject to post-selection maturation.

1.2.6 Post-Selection Maturation and Export to the Periphery.

Positively selected thymocytes induced to differentiate into SP4 and SP8 cells expressing high levels of TCR are rapidly relocated to the medulla where they are thought to spend approximately 14 days awaiting export to the periphery (Egerton et al., 1990). During this stay in the medulla, thymocytes undergo a process of receptor tuning and maturation, which is accompanied by further deletion of potentially auto-reactive thymocytes that have escaped negative selection in the cortex by exposure to tissue restricted antigens (TRAs) (Takahama, 2006). This process of negative selection is supported by the medullary thymic epithelial cells (mTEC) and in particular a subset expressing the autoimmune regulator AIRE is vital for regulating central tolerance (see section 1.3.7.1).

In the medulla SP4 and SP8 cells undergo waves of expansion with at least 6 rounds of cell division and this is developmentally regulated as post-selection expansion was greater in neonatal thymocytes compared to adult thymocytes (Hare et al., 1998). Although this expansive phase is independent of TCR/MHC interactions, it is dependent on continuous signals from thymic epithelial cells. Notably IL-7 signalling, which plays an important role in the early stages of T cell development, mediates intra-thymic expansion of positively selected thymocytes and supports the establishment of a peripheral T cell pool in the neonate (Hare et al., 2000). IL-7 produced by MHC II^+ thymic epithelium signals to SP cells following their upregulation of IL-7Rα and γ chains.

Recently selected SP thymocytes residing in the medulla undergo further sequential changes in the expression of particular cell surface markers to become recent thymic emigrants (RTEs). Such phenotypic changes include the down-regulation of CD69 and heat stable antigen (HSA) and the upregulation of CD62L, Qa-2 and CD45RB and this differentiation occurs simultaneously with the acquisition of functional maturity (Gabor et al., 1997a; Jin et al., 2008).

Following this differentiation process, mature thymocytes are thought to migrate towards the post-capillary venules at the CMJ and are exported at a rate of 1-2% of total thymocytes per day (Gill et al., 2003). Export of cells from the thymus occurs synchronously and is a heterogeneous mix of cells including αβ $CD4^+$ T cells, αβ $CD8^+$ T cells, γδ T cells, NK T cells and Tregs (McCaughtry et al., 2007). The export of T cells is not regulated by the size of the peripheral T cell pool but rather intrinsically regulated within the thymus (Gabor et al., 1997b).

Thymic emigration occurs in a poorly defined chemokine dependant mechanism via blood vessels at the CMJ and in the medulla, where naïve T cells enter the blood and lymphatic system to take up residence in the secondary lymphoid organs, including the spleen and lymph nodes, where they await stimulation by antigen.

1.2.7 Directed Migration of Thymocytes and the Role of Chemokines.

During their development, thymocytes migrate through the various regions of the thymus continuously interacting with the thymic stroma (figure 1.4). The stratified organisation of thymocytes forms specialised microenvironments in the post-natal thymus providing important signals for each stage of T cell development (Anderson et al., 2006). The

signals that mediate thymocyte trafficking are yet to be fully elucidated. However, there is increasing evidence to suggest an important role for chemokines and their receptors during this process (Takahama, 2006).

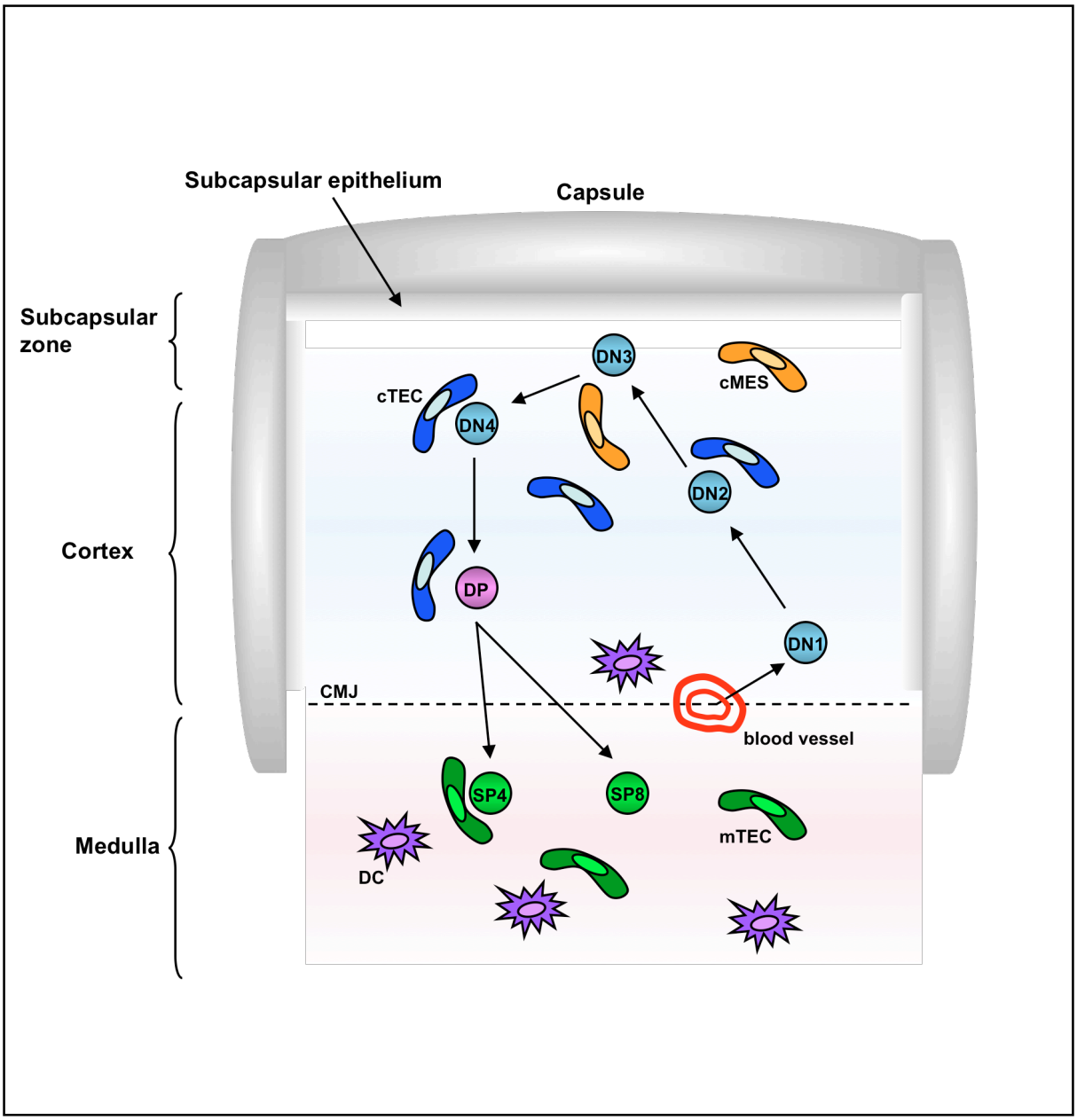
Chemokines are a family of chemotactic cytokines that were thought to primarily regulate leukocyte traffic and recruitment of leukocytes during infection and inflammation. However, they are also known to play a role in lymphoid trafficking in the absence of inflammation and also the development of lymphoid organs. Chemokines stimulate cell migration via G-protein-coupled seven-transmembrane receptors (Rossi and Zlotnik, 2000). Many studies have identified the production of chemokines by distinct thymic stromal cells and the expression of their corresponding receptors on different thymocyte subsets. This differential expression pattern suggests the essential role that chemokines play in regulating the complex trafficking of thymocytes within the post-natal thymus (Ladi et al., 2006). It is important to note that the requirement for chemokine receptor mediated migration of thymocytes is likely to differ in the fetal thymus, which is neither vascularised or structured during progenitor colonisation. Studies in mice with mutations in genes encoding for chemokines or their receptors have identified several groups important in post-natal thymocyte migration.

Progenitors enter the thymus via large postcapillary venules at the CMJ and their differentiation through the DN1 to DN4 substages corresponds with their directed migration through the cortex towards the subcapsular zone (SCZ). DN1 progenitors, which are largely absent from the outer cortex, are abundant at the CMJ and typically spend approximately 9-10 days confined to this area (Porritt et al., 2003). Migration out of this area into the mid-

Figure 1.4 Organisation of the Thymic Microenvironment.

During T cell development, thymocytes undergo directed migration through the different thymic microenvironments where they receive essential signals from specialised stromal cells.

Arrows indicate direction of migration. DN, double negative, DP, double positive, SP4, single positive CD4 T cell, SP8, single positive CD8 T cell, CMJ, corticomedullary junction, cMES, cortical mesenchyme, cTEC, cortical thymic epithelial cell, mTEC, medullary epithelial cell.



Key

Cortical thymic epithelial cell (cTEC)

Medullary thymic epithelial cell (mTEC)

Cortical mesenchyme cell (cMES)

Dendritic cell (DC)

cortex corresponds with the differentiation to the DN2 stage of development, which lasts approximately 2 days. Commitment to the T cell lineage and β-selection defines differentiation to the DN3 stage and correlates with polarised migration towards the SCZ where DN3 cells begin to accumulate (Lind et al., 2001; Porritt et al., 2003). The lifespan of DN3 cells is approximately 4 days. The SCZ provides a microenvironment that supports the final stages of DN proliferation and transition to the DP stage. The acquisition of CD4 and CD8 during the transition from DN to DP correlates with a reversion of the migration polarity guiding the DP thymocytes back inwards across the cortex towards the medulla (Misslitz et al., 2004)

The polarised migration of DN cells towards the SCZ, against the flow of DP cells moving inwards, implicates delivery of a chemotactic signal from thymic stromal cells in the outer cortex and/or SCZ. Signalling through CXCL12 (stromal derived factor 1, SDF-1) and its receptor CXCR4 has been identified as an absolute requirement for the proper localisation of DN progenitors in the cortex (Plotkin et al., 2003). CXCR4 is expressed by all DN subsets with the highest expression in DN2 and DN3 cells. The ligand, CXCL12 is expressed on cortical epithelial cells but not in the medulla. Cells lacking CXCR4 fail to differentiate past the DN1 stage and this clearly indicates the importance of CMJ to cortex migration for normal T cell development (Plotkin et al., 2003). Another chemokine important for the polarised migration of DN cells towards the SCZ is CCL25 (thymus expressed chemokine, TECK), which is found on epithelial cells in the cortex and medulla. Its receptor CCR9, although expressed at low levels on DN2 and DN3 cells, is predominantly on DP cells and is down-regulated following transition to the SP stage. Premature over expression of CCR9 leads to an accumulation of DN3 cells in the cortex (Uehara et al., 2006), whereas CCR9 deficiency results in the failure of DN3 cells to localise to the SCZ suggesting an important

role for CCR9 in SCZ homing (Benz et al., 2004). Surprisingly, thymocytes failing to migrate to the SCZ in $CCR9^{-/-}$ mice are still able to undergo normal T cell development, thereby suggesting that homing to this area is not an absolute requirement for T cell development (Benz et al., 2004). Signalling through the chemokine receptor CCR7 was also found to be important for the normal differentiation of DN cells (Misslitz et al., 2004). A transitional population of DN cells called DN1-2 ($CD25^{int}$ $CD44^{high}$) express CCR7 before its expression on SP4 and SP8 cells. $CCR7^{-/-}$ mice have an accumulation of DN cells at the DN1 and DN1-2 stage, whereas the proportion of DN2, DN3 and DN4 cells are severely reduced suggesting a partial block or delay in the transition of DN1 cells to the DN2 stage. The DN1-2 cells are aberrantly located at the CMJ suggesting that signalling through CCR7 is essential for the efficient migration of DN1-2 cells to the mid-cortex allowing for their differentiation to the next stage of development. Together the studies by Plotkin et al (2003), Benz et al (2004) and Misslitz et al (2004) demonstrate the vital role of CXCR4, CCR9 and CCR7 signalling for successful migration of early progenitors in the postnatal thymus. Arrest of thymocytes at different stages of development correlates with abnormal thymic architecture indicating the importance of cross-talk between developing thymocytes and thymic epithelial cells.

Differentiation of DP thymocytes to mature SP4 and SP8 cells correlates with their relocation to the medulla. It has been identified that CCR7 signalling is essential for cortex to medulla migration of developing thymocytes (Ueno et al., 2004). TCR engagement of immature cortical DP cells upregulates CCR7 expression. The ligands for CCR7, CCL19 and CCL21 are predominantly produced in the medulla. In a normal steady state thymus, DP thymocytes produced in the cortex undergoing positive selection following TCR/peptide-MHC interactions, undergo a series of maturation stages (as described in section 1.2.5)

including upregulation of CCR7. This is subsequently followed by migration to the medulla in response to CCL19 and CCL21 production where they become mature SP4 and SP8 cells. CCL19 but not CCL21 is known to be important for attracting newly generated T cells out of the thymic medulla to the circulation in newborn mice (Ueno et al., 2002). In mice deficient for CCR7 or CCR7 ligands (*plt/plt* mice) positively selected thymocytes that are differentiating into SP cells are arrested in the cortex and fail to localise to the medulla and these cells showed no defects in maturation and selection (Ueno et al., 2004). These mice also exhibited normal export of T cells. This suggests that the medulla may not be required for the maturation and export of T cells. However, T cells produced in the absence of CCR7 mediated cortex-medulla migration were incapable of acquiring tolerance to organ-specific antigens, thereby suggesting that cortex to medulla migration is essential for the establishment of central tolerance rather than for supporting maturation or export of T cells (Kurobe et al., 2006).

As summarised in this section, the development of a diverse and self-tolerant T cell repertoire is a tightly regulated process and is clearly dependent on the thymic epithelial cells, which constitute the major stromal cell type of the different thymic microenvironments. However, developing thymocytes are also important in regulating the establishment of organised cortical and medullary compartments through a process of crosstalk. Thus, research aimed at elucidating the developmental origins of thymic epithelial cells will reveal how such cells become specialised in their ability to support T cell development.

1.3 The Thymic Stroma.

1.3.1 Composition of Thymic Stroma.

The composition and cellular compartmentalisation of the thymic stroma provides optimal microenvironments to support the different stages of T cell development as described above. The stroma, defined as the non-haematopoietic component of the thymus, is extremely heterogeneous and is comprised of many cell types with specialised functions. The stroma can be characterised by the expression of keratin (K) with keratin^{+} cells representing thymic epithelial cells and keratin^{-} cells representing a mixture of mesenchymal cells, fibroblasts and endothelial cells. Keratin^{+} epithelial cells are further subdivided into cortical epithelial cells (cTECs) and medullary epithelial cells (mTECs), which display further heterogeneity.

1.3.1.1 Cortical epithelium.

The cortex is characterised by a meshwork of reticular, MHC I and II positive cortical epithelial cells (cTECs) that can be identified by the expression of specific cell markers including MTS44, keratin 8 and Ly51 (Gray et al., 2006). By histology cTECs are typically identified as K8^{+} K5^{-}, whereas by flow cytometry, cTECs are identified as epithelial cell adhesion molecule 1^{+} (EpCAM-1^{+}) Ly51^{+}. Although not a commonly used marker, CD205 (also known as DEC205) is known to be specifically expressed on cTECs in the adult thymus and is also expressed on DCs (Jiang et al., 1995). The non-epithelial component of the cortex includes EpCAM-1^{-} Ly51^{+} cortical mesenchyme cells (cMES) (Muller et al., 2005). However, there are very few markers specific for the thymic cortex that enables their isolation and study.

1.3.1.2 Medullary epithelium.

The cellular composition of the medulla is extremely heterogeneous and consists of bone marrow derived DCs and macrophages as well as epithelial cells, which display further heterogeneity. mTECs have a globular morphology and express an array of co-stimulatory markers such as CD40 (Dunn et al., 1997) and CD80 (B7.1) (Nelson et al., 1993), which are implicated in T cell activation. By histology mTECs are typically identified on the basis of a $K5^{+}$ $K8^{-}$ phenotype and by flow cytometry on the basis of an EpCAM-1^{+} $Ly51^{-}$ phenotype (Anderson et al., 2007). Cells of this lineage also bind the lectin Ulex Europaeus Agglutinin 1 (UEA-1) (Gray et al., 2002). Subsets of mTECs also express the autoimmune regulator AIRE. By comparison to cTECs, mTECs also express MHC I and II molecules. However, in contrast to cTECs, mTECs express varying levels of MHC II with the presence of an MHC II^{low} and ^{high} population. The non-epithelial components of the medulla include extracellular matrix proteins (such as ER-TR7 and perlecan), podoplanin and DCs and macrophages, which also form an integral element of the stroma.

1.3.2 Thymus Organogenesis.

Thymus organogenesis occurs in several consecutive stages and is dependent on interactions between cells of all three embryonic germ layer origins including, endoderm-derived epithelium, ectoderm-derived neural crest mesenchyme and mesoderm-derived haematopoietic cells and endothelial cells (Manley, 2000).

Development of the thymus is initiated at around day 9 of embryonic gestation (E9) in the mouse, with the first morphological signs of the thymus becoming apparent at approximately E10.5 (Manley, 2000). The early stages of thymus organogenesis is closely tied to that of the parathyroid gland with both organs developing from bilateral organ

primordia arising from an embryonic region called the 3rd pharyngeal pouch. Pharyngeal pouches, which initially constitutes a double-layered membrane composed of an ectodermal and endodermal cell sheet, represent specialised pockets of the foregut endodermal tube and contain primordia for organs and tissues that are later found in the chest, neck and head regions including the thymus and the parathyroid gland (Rodewald, 2007). The common primordium begins to develop at E11.5 via budding and outgrowth from the 3rd pharyngeal pouch endoderm with each endodermal primordium containing precursors for one thymus lobe and one parathyroid gland. However, as early as day 10 of gestation, parathyroid and thymus specific domains can already be identified before separation into distinct organs by the complementary expression of the transcription factors Gcm2 and FoxN1, respectively (Gordon et al., 2001). Gcm2 is first expressed in a specific region of the 3rd pharyngeal pouch at E9. By contrast, FoxN1 expression does not appear until after the formation of the common primordium (E11.5).

A mesenchymal capsule of neural crest (NC) origin surrounds each primordium and is thought to be important in supporting the growth and development of the primordium as well as its importance in regulating the differentiation of thymic epithelial cells (TECs). Interactions between epithelial and mesenchymal cells prior to thymocyte colonisation define these early events in thymus organogenesis. The NC mesenchyme surrounds the thymic primordium until eventually invaginating the epithelial primordium to contribute to the septae and a network of fibroblast cells.

At around E12 (when the thymic rudiment is undergoing the first wave of progenitor colonisation) the common primordium separates from the pharynx and begins to migrate towards the anterior chest cavity so that by E13.5 the thymus and parathyroid have separated into distinct organs (Blackburn and Manley, 2004). During this stage of organogenesis the

thymus reaches its normal anatomical position at the midline above the heart to form a bilobed organ, with the parathyroid located adjacent to the thyroid gland in the neck. Studies have also reported the presence of a cervical thymus, which develops after birth and has a normal cortex and medulla organisation with the ability to support T cell development (Dooley et al., 2006; Terszowski et al., 2006).

At this stage of organogenesis, which occurs in the absence of thymocytes, TECs are homogenous as they lack cortical and medullary compartmentalisation and are yet to reach functional maturity. The patterning and differentiation of the thymic rudiment, which is required to establish normal organisation and function of cortical and medullary regions, defines the later stages of thymus development (approximately E15.5-birth) (Manley, 2000; Rodewald, 2007). The rapid accumulation of thymocytes leads to the formation of a functional thymic microenvironment capable of supporting full T cell development. This patterning and differentiation of the thymus requires stage specific interactions between TECs and thymocytes via a process of thymic crosstalk.

1.3.3 Molecular Regulation of Thymus Organogenesis: A Role for FoxN1.

Many genes have been implicated in early development of the thymic anlage including Hoxa3, Pax1 and Tbx1 and mutations in these genes can lead to thymic aplasia, hypoplasia, or failure of the lobes to migrate towards the chest (Hollander et al., 2006; Rodewald, 2007). However, in the context of this study the transcription factor FoxN1 (formerly known as whn) is the most important. FoxN1, which is encoded by the gene mutated in the nude mouse, belongs to a family of forkhead box transcription factors, which also includes Foxp3 important for determining the development and function of Tregs (Coffer and Burgering, 2004). FoxN1 is expressed in hair follicles and all TEC subsets. The FoxN1 protein contains

a winged-helix DNA binding domain and a transcriptional activation domain (Schuddekopf et al., 1996). Mice and humans with a mutation in the Foxn1 gene, resulting in an absence of the Foxn1 protein display the nude phenotype characterised by loss of hair and congenital athymia.

The expression of Foxn1 is first detectable at E11.5 following the budding and outgrowth of the thymic anlagen from the 3rd pharyngeal pouch (Gordon et al., 2001; Nehls et al., 1996). Hence, FoxN1 is not required for the initiation of thymus organogenesis, as nude mice display normal formation of the thymus primordium and normal migration of the anlage to its correct anatomical location (Hollander et al., 2006). Aberrant thymus development in the nude mouse is first detected at approximately E12, where the thymic anlage does not further develop and there is a failure of the epithelial cells to form the characteristic 3D meshwork that is typical of a normal thymus. Hence FoxN1 is thought to be essential for cell autonomous regulation of TEC differentiation, where it acts to regulate the growth and differentiation of the epithelial cells (Blackburn et al., 1996). Moreover, the thymic epithelial rudiment in the nude mouse fails to induce haematopoietic cell colonisation and persists in the adult as a rudimentary thymus with a cystic epithelial structure. Hence, nude mice have severe immunodeficiency due to the failure of the thymus to support T cell development. The precise role of FoxN1 in the development of TECs is unclear. However, it has been reported that epithelial cells in the nude anlagen have a respiratory like phenotype with regard to their organisation and appearance, suggesting that FoxN1 may be important in directing the epithelial cells towards a thymic fate (Dooley et al., 2005).

The molecular mechanisms that regulate FoxN1 expression and function are not completely understood. A study has demonstrated a role for the canonical Wnt pathway,

where overexpression of certain Wnt proteins (detected in 3rd pharyngeal pouch at E10.5) increased FoxN1 transcription in TECs (Balciunaite et al., 2002).

It is clear that there are many key issues relating to thymus organogenesis that are of importance. However, in the context of this study the identification of progenitors or stem cells with TEC forming potential and the molecular regulators of their development through ontogeny are of particular interest.

1.3.4 Identification of Bipotent Thymic Epithelial Progenitors.

The embryonic origins of the cortical and medullary regions is of great interest and previously it had been controversial as to whether the thymic epithelium is of a dual germ layer origin with contribution from the endoderm and ectoderm. The 'dual germ layer origin' model originally suggested that the cortex develops from the ectodermal derived epithelium and the medulla develops from endodermal derived epithelium (Cordier and Heremans, 1975). Such a model would imply that for TEC differentiation to precede then contributions from both endoderm and ectoderm are required. However, the prevailing view from chick-quail studies supports the 'single origin' model where the thymic epithelium is solely derived from progenitors or stem cells of endoderm origin, which are committed to the TEC lineage (Le Douarin and Jotereau, 1975). A further study in mice also argued against the 'dual origin' model and provided evidence to support the 'single origin' model (Gordon et al., 2004). Ectopic transplantation of isolated pharyngeal endoderm generated a functional thymus with organised cortex and medullary areas, indicating that contribution from the ectoderm is not required. However, despite this confirmation of a single germ layer origin, it was still unclear

whether cTECs and mTECs arise from a distinct progenitor pool or a common progenitor population.

The existence of bipotent progenitors that can develop into both cortical and medullary lineages were first demonstrated in studies identifying TEC populations in the early thymus expressing both cTEC and mTEC markers, which are typically rare in an adult thymus (Ropke et al., 1995). Such 'double positive' TECs are thought to be predominant in patients with thymic epithelial tumours (e.g. myasthenia gravis) (Ritter and Boyd, 1993). Early organogenesis of the thymus is defined by a lack of anatomical compartmentalisation and TECs are homogenous lacking phenotypic markers indicative of a mature cortical or medullary phenotype, such as expression of MHC II (Jenkinson et al., 1981). Heterogeneity in adult cTECs and mTECs are can be distinguished by their keratin expression profiles, which are $K5^{-}K8^{+}$ and $K5^{+}K8^{-}$ respectively (Rodewald, 2007). However, all immature TECs in the embryo co-express K5 and K8 and these cells are infrequently scattered throughout the cortex and predominate mostly at the CMJ in the adult (Klug et al., 1998). Such cells are present at a higher frequency in both CD3εtg26 and $RAG1^{-/-}$ mice displaying blocks in thymocyte development, highlighting the possible role of lymphostromal interactions in TEC development. This co-expression of keratins precedes the appearance of mature $K5^{-}K8^{+}$ cTECs and $K5^{+}K8^{-}$ mTECs, thereby suggesting that these 'double positive' TECs may arise from a common progenitor pool (Anderson et al., 2007). However, these studies do not show direct evidence that the 'double positive' TECs represent bipotent progenitors.

In 2006 two seminal studies provided definitive proof for the existence of a bipotent TEC (bTEC) progenitor. In a clonal assay by Rossi et al (2006), a single TEC was isolated from an E12 yellow fluorescent protein (eYFP) transgenic mouse. This single cell (identified on the basis of expression of a pan-epithelial marker EpCAM-1) was microinjected into a

non-fluorescent host thymus and then grafted under the kidney capsule of a recipient mouse. Immunohistological analysis of the transplanted thymus revealed that the single YFP^{+} cell gave rise to cells in the cortex and medulla. Contributions to a single TEC lineage were strikingly absent. Not only did this study demonstrate that all TEC progenitor activity is already present at E12, this study elegantly demonstrated that a bipotent pool of progenitors are present in the early thymus and that they can develop into cells of the cTEC and mTEC lineage.

A genetic approach by Bleul et al (2006) provided further evidence for the existence of bTECs. Using *in vivo* cell lineage analysis, eYFP expression was randomly switched on in TECs by Cre recombinase under the control of the human Keratin 14 promoter ($K14^{Cre}$) (the K14 promoter is active in epithelial progenitor cells). In newborn mice, labelled TECs gave rise to either mTEC clusters only, cTEC clusters only or both mTEC and cTEC progeny. In this experiment $K14^{Cre}$ randomly marked common and committed TEC progenitors and their progeny and so provided evidence for the presence of bi and unipotent progenitors for the cortex and medulla (Bleul et al., 2006). To address whether single TEC progenitors can form functional thymus units, Bleul et al (2006) created a conditional mutant allele of FoxN1, which was reverted to wild-type function in single TECs *in vivo*. Neonatal mice showed organised cortex and medulla, with expression of AIRE, and elevated numbers of T cells. This study clearly showed that the block in TEC development in the nude mouse does not result in a loss of TEC progenitors but rather suggests that bTEC progenitors may lie dormant in the nude mouse and can be induced to differentiate into functional TECs following a genetic reversion to wild-type (Rodewald, 2007).

Despite the significant breakthroughs in the identification of bipotent progenitors that can development to TECs of both cortical and medullary lineage, such common progenitors

have yet to be phenotypically characterised and isolated. Initial studies identified MTS24$^+$ TECs in the embryo as a putative TEC progenitor (Bennett et al., 2002; Gill et al., 2002). Heterogeneity in MTS24 expression was observed as early as E12, a stage in which bipotent epithelial cells predominate, with equal proportions of MTS24$^-$ and MTS24$^+$ populations. It was found that MTS24$^+$ TECs were able to give rise to functional thymic tissue containing both cortex and medulla when ectopically transferred, whereas MTS24$^-$ TECs were unable to establish a thymus. However, reassessment of TEC progenitor potential using MTS24, found that MTS24 expression does not discriminate between progenitor and non-progenitor populations as both MTS24$^-$ and MTS24$^+$ cells could give rise to a functional thymus (Rossi et al., 2007a). Furthermore, the potential of MTS24$^+$ TECs to give rise to a functional thymus diminished by E18 suggesting that at this stage of development MTS24$^+$ TECs are likely to represent quiescent, mature cells rather than thymus forming progenitor cells.

Studies have also yet to resolve the issue of self-renewal capacity of TEC progenitors. It is yet to be addressed whether there is self-renewing pool of bipotent progenitors remaining throughout life or if a fixed number of bipotent progenitors exists with self-renewal capacity restricted to downstream lineage committed progenitors (Anderson et al., 2007). Another important unresolved issue is whether bipotent progenitors persist in the adult. Indeed studies suggest their existence as K5$^+$ K8$^+$ cells expand significantly during thymic atrophy, prior to thymic regeneration (Popa et al., 2007), with another study showing reversal of thymic atrophy with restored thymic architecture and thymic output (Gray et al., 2006). These studies demonstrate plasticity in TEC microenvironments and provide evidence that TEC progenitors persist and can be induced to proliferate and differentiate in the post-natal thymus (Anderson et al., 2007).

To summarise, despite the identification at a functional level that cTECs and mTECs arise from a common progenitor pool, the lack of specific markers means that the quest to identify and characterise the thymic epithelial stem cell remains elusive. (Figure 1.5 shows a simplified overall scheme of TEC development).

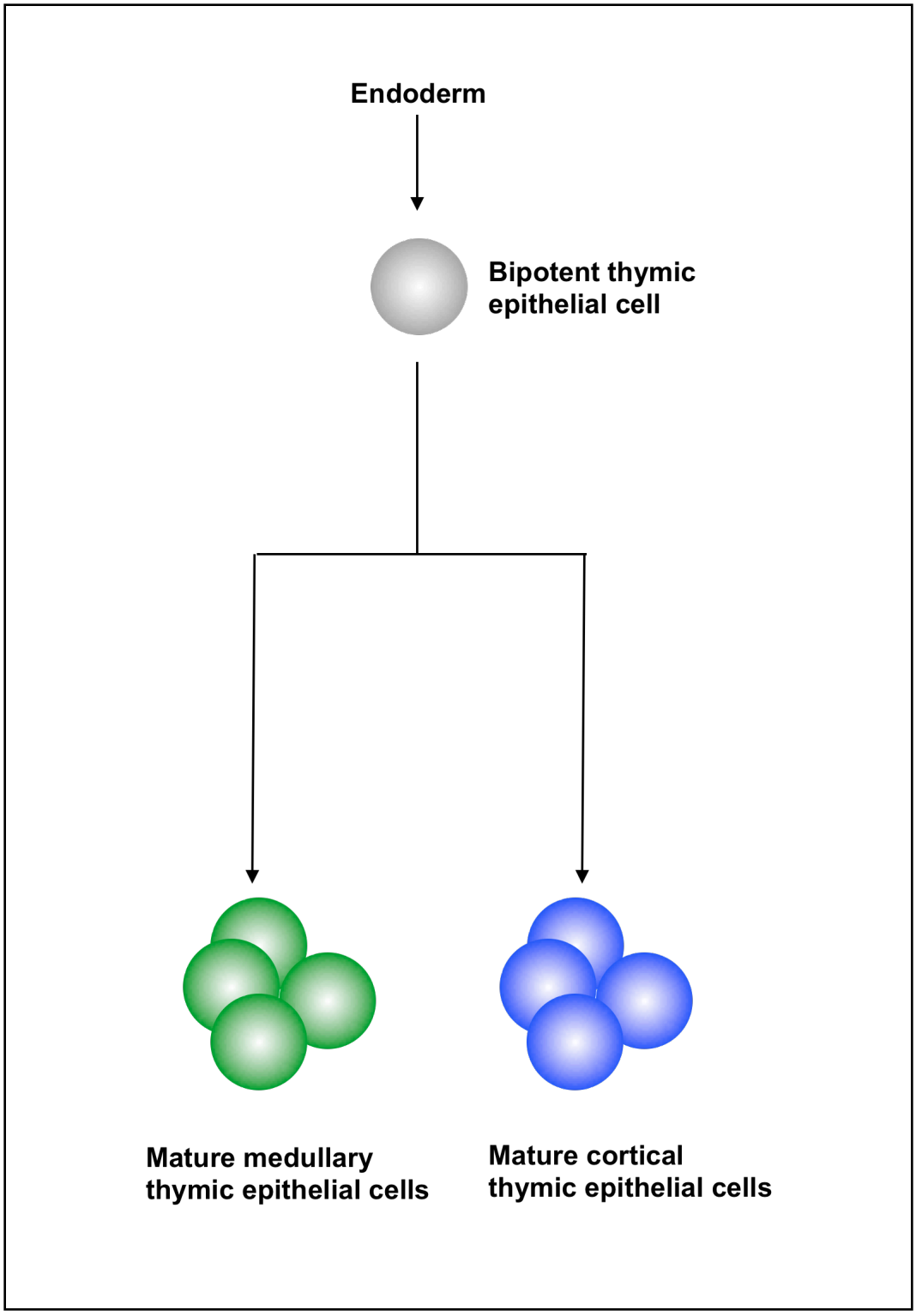
1.3.5 Regulation of Bipotent TEC Development: The Role of the Mesenchyme.

Although thymocytes are required for later stages of TEC development, the development and differentiation of bipotent progenitors into cTEC and mTEC lineages occurs independently of thymic crosstalk. This suggests that signals for TEC differentiation may be cell autonomous or derived from additional cells. Studies have demonstrated a role for the mesenchyme in regulating TEC development.

As described in section 1.3.1, epithelial-mesenchymal interactions between the 3rd pharyngeal pouch endoderm and the surrounding neural crest-derived mesenchyme during embryogenesis are vital for the formation and development of thymic primordium. The mesenchyme is comprised of a heterogeneous population of cells that changes throughout thymus development. In the fetal mouse, neural crest derived mesenchyme cells contribute to thymic capsule, interlobular septae and the stromal cell network, such as fibroblasts (Yamazaki et al., 2005). Mesenchymal cells in the adult make up the thymic capsule and septae and also constitute a small proportion of cortical stromal cells defined as EpCAM^{-} Ly51^{+} and these have been assigned as cortical mesenchymal cells (cMES) (Muller et al., 2005). A population of mesenchymal fibroblasts expressing MTS15 have also been identified and these cells were found to express a number of chemokines and growth factors important for both thymocyte and epithelial cell development (Gray et al., 2007b).

Figure 1.5 Development of Thymic Epithelial Cells.

Development of mature cortical and medullary thymic epithelial cells from an endoderm derived bipotent thymic epithelial cell.


Endoderm
Bipotent thymic epithelial cell
Mature medullary thymic epithelial cells
Mature cortical thymic epithelial cells

The mesenchyme is thought to have a role in T cell development by acting directly on DN2 cells through the provision of extracellular matrix (ECM) molecules and they are located in the cortex in association with non-epithelial stromal cells (Anderson et al., 1997; Anderson et al., 1993). The mesenchyme also has a role in the regulation of thymic epithelial cell proliferation. At E12, an age that comprises mostly of TEC progenitors at the bipotent stage, there is high proliferation of TECs, which declines throughout thymus ontogeny. The removal of mesenchyme from E12 fetal thymic lobes demonstrated its importance in the expansion of epithelial cells (Jenkinson et al., 2003). Mesenchymal cells regulate this expansion through the provision of fibroblast growth factors (FGFs), namely FGF7 and 10. The receptor for FGF7 and 10 is FGFR2-IIIb and the deletion of this receptor was found to be required for the proliferation of TECs after E12, further demonstrating the importance of the mesenchyme (Revest et al., 2001). However, TEC differentiation beyond E12 is independent of ongoing mesenchyme derived signals (Jenkinson et al., 2003). A majority of the mesenchyme expresses platelet derived growth factor receptor α (PDGFRα) and these cells were found to be important for the expansion of epithelial cells to provide sufficient intrathymic niches to support normal numbers of developing thymocytes. (Jenkinson et al., 2007b). It has also recently been demonstrated that the epithelial progenitor pool in the thymus has a limited capacity for proliferation and a restriction of the size of this pool affects the number of mature TECs (Jenkinson et al., 2008).

Events occurring downstream of bipotent progenitors and the mechanisms that regulate commitment and differentiation leading to the development of cortex and medulla are not clear (Anderson et al., 2007). However, some advances have already been made regarding development and function of TEC lineages and in particular mTEC development.

1.3.6 Development of cTECs and their Specialised Role During Positive Selection.

Due to a paucity of cTEC markers, very little is known about cTEC development and regulation and how distinct lineages may reflect functional specialisation (Anderson et al., 2007). In contrast to our understanding of mTEC development (discussed in section 1.3.7), cTEC progenitors downstream of bTECs have yet to be identified. Furthermore, whilst many abnormalities in mTEC development as a result of genetic deficiencies have been reported, reports of abnormalities in cTEC development have been scarce.

cTECs express both MHC I and II molecules have been identified as efficient and unique mediators of positive selection (Anderson et al., 1994b). However, whilst some have argued against a role for cTECs in central tolerance, there is speculation that cTECs can induce central tolerance by supporting negative selection of potentially autoreactive thymocytes (Goldman et al., 2005). A recent study (McCaughtry et al., 2008) demonstrated unequivocally that the clonal deletion of thymocytes can occur in the cortex and requires no involvement of the medulla and can occur in the absence of cortex-medulla migration, as previously thought (Kurobe et al., 2006). The elimination of these autoreactive T cells by apoptosis is induced by rare $CD11c^{+}$ cortical DCs (McCaughtry et al., 2008) and this tolerising mechanism helps to eliminate TCRs reactive to ubiquitous self antigens.

The unique ability of cTECs to support positive selection is based on poorly defined antigen-processing and antigen-presenting capabilities. Cortical epithelium is likely to express unique cell surface molecules providing signals that promote positive selection rather than negative selection. These molecules are likely to be distinct from mTECs and DCs and are unlikely to be the same as those required for activation of mature T cells (Anderson and Jenkinson, 2001).

In the context of $CD8^+$ T cell selection, the generation of peptides for MHC I presentation requires proteasomes. A catalytic proteasome subunit called β5T was recently identified as being exclusively expressed by cTECs and is essential for generating MHC I-restricted $CD8^+$ T cells during thymic selection (Murata et al., 2007). β5T deficiency in mice was associated with a significant reduction in SP8 thymocytes, with no apparent affect on numbers of SP4 thymocytes. It is thought that in contrast to constitutive proteasomes, which produce high affinity ligands, thymoproteasomes such as β5T produce low affinity MHC I ligands in cTECs, thereby supporting positive selection (Murata et al., 2007).

MHC II-restricted selection of $CD4^+$ T cells by cTECs involves lysosomal endoproteinases called cathepsins. More specifically cathepsin L was identified as essential for degradation of class II invariant chain peptides (CLIP) in cTECs but not in bone marrow derived APCs, which require cathepsin S (Beers et al., 2005; Nakagawa et al., 1998). A recent study has also highlighted the role of thymus-specific serine protease (TSSP), which is expressed in the endosomal compartment of cTECs only, in the positive selection of a subset of $CD4^+$ T cells (Gommeaux et al., 2009). TSSP is thought to be involved in the processing of self-peptides for MHC II presentation.

The studies of Murata et al (2007) and Gommeaux et al (2009) highlight the adaptive phenotype of cTECs in regulating the functional specialisation of cTECs for positive selection (Boehm, 2009).

1.3.7 Specialisation of the Medulla.

1.3.7.1 The role of mTECs in central tolerance.

Central tolerance is established via a combination of distinct mechanisms that include clonal deletion of thymocytes that bear TCRs with high affinity for peptide/self-MHC complexes and the positive selection of $FoxP3^+$ $CD4^+$ $CD25^+$ regulatory T cells (Tregs) (Anderson et al., 2007).

mTECs express a wide spectrum of tissue restricted antigens (TRAs) previously thought to be expressed only by specialised tissues in the periphery and this has been termed promiscuous gene expression (Derbinski et al., 2001). The self-antigens presented by mTECs represent all the organ-specific antigens, mirroring the peripheral self and thereby allowing the pre-emptive encounter of potentially destructive T cells with peripheral self-antigens (Kyewski and Klein, 2006). The regulation of this unusual gene expression pattern is poorly understood. However, a subset of mTECs expressing AIRE is known to be involved. AIRE encodes a 545 amino acid protein that is localised in the nucleus and acts as transcription factor for promiscuous gene expression of TRAs (Anderson et al., 2007). AIRE is defective in human autoimmune condition autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED), which is characterised by the presence of autoimmune antibodies specific for multiple self-antigens. Mice with defects in AIRE also show similar signs of autoimmunity with multi-organ lymphocytic infiltration and presence of autoantibodies (Peterson et al., 2008). Interestingly, a recent study claims that whilst AIRE expression in the perinatal period is sufficient to induce self-tolerance, AIRE mediated tolerance in the adult is largely dispensable because a previously generated tolerised T cell pool can control newly emerging autoreactive T cells (Guerau-de-Arellano et al., 2009).

AIRE clearly has a role in regulating central tolerance through TRA expression. However, a recent study has shown that $AIRE^+$ mTECs may also have a role in the selection of $FoxP3^+$ Tregs (Aschenbrenner et al., 2007). The absence of MHC II^+ mTECs was found to be associated with a decrease in Tregs and that mTECs specifically generated antigen specific Tregs suggesting that $AIRE^+$ mTECs shapes the Treg repertoire and so provides a microenvironment for their selection.

1.3.7.2 Identification and development of medullary epithelial progenitors.

In contrast to cTEC development, many advances in the development of the mTEC lineage has been made and most significantly, mTEC specific progenitors that develop downstream of bipotent progenitors have been identified.

A study by Rodewald et al (2001) showed that the medulla develops from clonal islets arising from single progenitors. Using chimaeric mice that were generated by injecting embryonic stem (ES) cells into MHC mismatched blastocysts, they found that individual medullary islets arose from either ES cell or blastocyst origin but not both. Furthermore this study also demonstrated the formation of medullary islets from disaggregated cell suspensions of TECs obtained from E16 thymus. Reaggregate thymus organ cultures (RTOC) were made from TEC suspensions from MHC mismatched donors. RTOCs were grafted into MHC II knockout mice and the resulting graft showed evidence of clonal islets of IA^b or IA^d origin but not both. This study suggests that the medulla develops as isolated medullary islets from single progenitors as opposed to developing from an epithelial cell layer and such individual islets are likely to coalesce during thymus ontogeny to form confluent regions of medulla. Most significantly this study identifies the existence of an mTEC progenitor cell, and such a

cell is still present at day 16 of gestation. This study also demonstrates the remarkable capacity of isolated epithelial cells to self-reorganise into an organised and functionally competent microenvironment supporting T cell development *in vivo* (Rodewald et al., 2001).

Following the identification of the existence of mTEC progenitors many studies have sought to further phenotypically characterise the development of such progenitors. The medullary epithelium is extremely heterogeneous. Two distinct mTEC subsets include the $CD80^-$ mTECs expressing a limited array of TRAs and the $CD80^+$ mTECs, which express AIRE and a wide array of TRAs (Derbinski et al., 2005; Derbinski et al., 2008). Until recently it was uncertain if this heterogeneity reflects functionally distinct lineages or different developmental stages in a single mTEC lineage. A recent study identified a precursor-product relationship where a subset of RANK expressing $Ly51^-$ $EpCAM^+$ $CD80^-$ $AIRE^-$ mTECs were precursors for a mature subset of $Ly51^-$ $EpCAM^+$ $CD80^+AIRE^+$ mTECs (Rossi et al., 2007b). This study also found that RANKL signals from thymic $CD4^+$ $CD3^-$ lymphoid tissue inducer cells (LTi) promote the maturation of these immature mTECs into mature mTECs via RANK-RANKL signalling. Another study provided evidence that $AIRE^+$ mTECs develop from mTEC progenitors expressing the tight junction proteins claudin 3 and claudin 4 and UEA-1 (Cld3, 4^+ UEA-1^+) and that these cells were present as early as E13.5 (Hamazaki et al., 2007). They also found that grafting these Cld3, 4^+ TECs into nude mice exclusively generated mTEC clusters.

Furthermore, mTEC proliferation has been identified in the adult thymus suggesting a developmental pathway where differentiation and replacement of mTECs arises from an mTEC progenitor pool undergoing cellular turnover (Gray et al., 2006). A further study identified a large pool of non-cycling cells within the immature mTEC subset suggesting that these cells provide a 'reservoir' of mTEC committed progenitors that maintain the continuous

renewal of mTECs in the adult thymus (Gabler et al., 2007). Such studies support a terminal differentiation model where $AIRE^+$ mTECs represent end-stage cells expressing the differentiation marker involucrin, which are subsequently replaced by a pool of immature mTEC subsets (Yano et al., 2008) and TRAs become more diverse as the mTECs mature.

The identification of precursor-product relationships has been helpful in elucidating some of the stages of mTEC development and has aided the identification of various molecular regulators of these stages (figure 1.6).

1.3.7.3 Regulation of mTEC development.

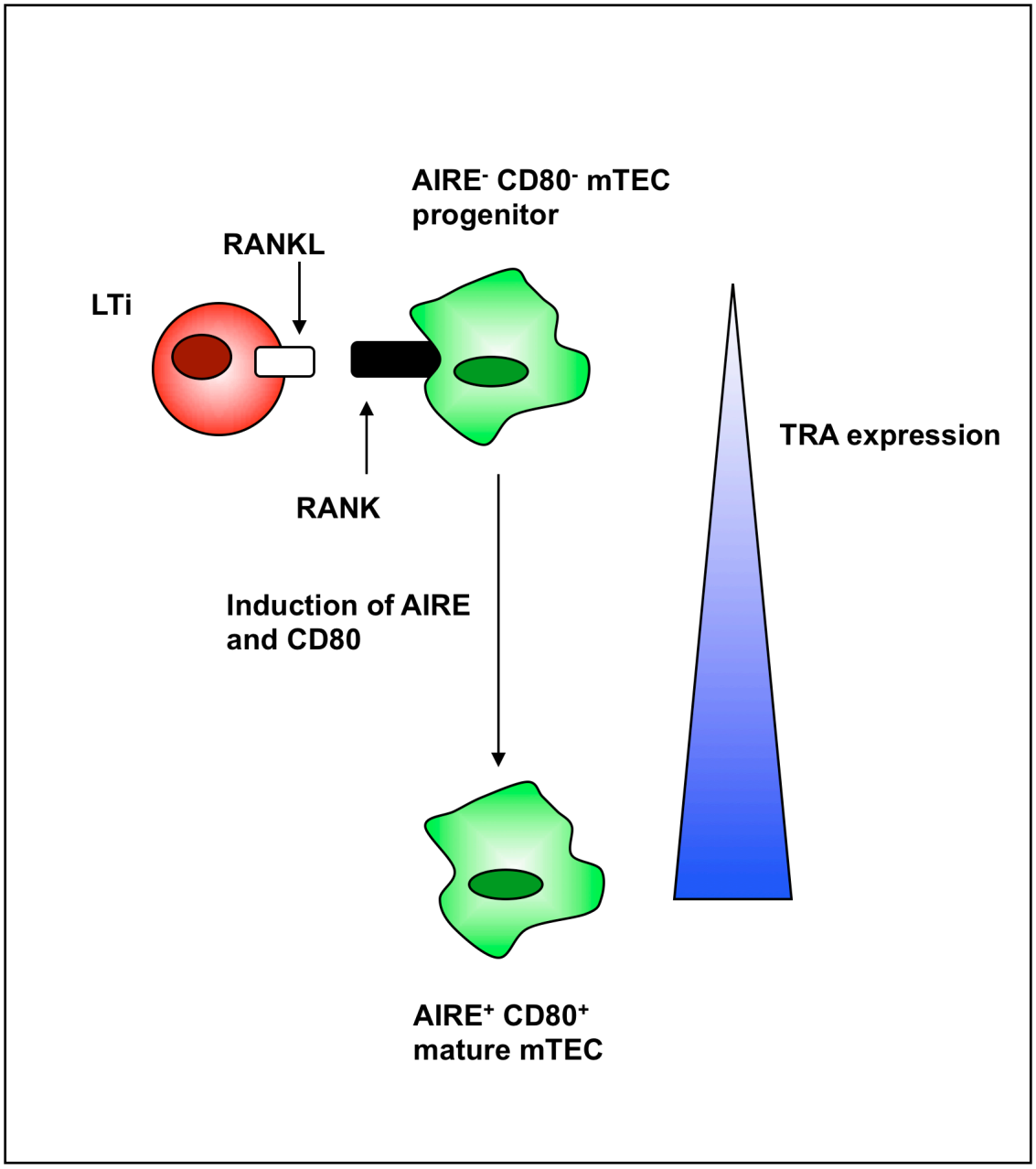
Mechanisms and signalling pathways that underlie mTEC development are poorly understood. However, many studies have implicated a number of tumour necrosis factor receptor superfamily (TNFRSF) members such as lymphotoxin β receptor (LTβR), receptor activator of NF-κB (RANK) and CD40.

Lymphotoxin is expressed by lymphocytes and signals through LTβR, which is expressed on stromal cells and is vital for inducing cytokines, chemokines and other molecules that organise and maintain the specialised microenvironments of lymphoid tissues. The precise role of LTβR in the formation of the medulla is still a matter of dispute with initial studies suggesting a role in the induction of $AIRE^+$ mTECs and control of autoimmunity (Boehm et al., 2003; Chin et al., 2003), whereas subsequent studies find that LTβR is not essential for expression of AIRE (Martins et al., 2008; Rossi et al., 2007b; Venanzi et al., 2007).

Figure 1.6 Development of AIRE$^+$ Medullary Thymic Epithelial Cells.

The terminal differentiation model of medullary thymic epithelial cell (mTEC) development states that immature AIRE$^-$ CD80$^-$ mTEC progenitors expressing RANK interact with lymphoid tissue inducer (LTi) cells to induce AIRE and CD80 expression to become mature AIRE$^+$ CD80$^+$ mTECs. This developmental step is accompanied by an increase in expression of tissue-restricted antigens (TRAs).

Image adapted from Anderson et al (2007).


AIRE- CD80- mTEC
progenitor
RANKL
LTi
RANK
Induction of AIRE
and CD80
TRA expression
AIRE+ CD80+
mature mTEC

The most well understood mediator of mTEC development is RANK-RANKL signalling. As described in the previous section, RANK signals from LTi cells are important in regulating the development of $CD80^+$ mTECs, which are known to also express AIRE (Rossi et al., 2007b). RANKL is expressed on both thymic and splenic LTi cells, which were previously known to be important for secondary lymphoid organ development and organisation. $RANK^{-/-}$ mice were found to have marked absence of $AIRE^+$ mTECs by histology and nude mice receiving $RANK^{-/-}$ thymus lobes via kidney capsule transfer were found to develop severe symptoms of autoimmunity (Rossi et al., 2007b). This study highlighted the importance of LTi derived RANKL signals in the development of $AIRE^+$ mTECs and as a key regulator of central tolerance.

Subsequent studies have shown a possible role for CD40-CD40L signalling in mTEC development (Akiyama et al., 2008; Hikosaka et al., 2008). CD40 is expressed on all TECs whereas the ligand is expressed on $CD4^+$ thymocytes. $CD40^{-/-}$ has been associated with a reduction of $AIRE^+$ mTECs but not a complete ablation (White et al., 2008). These studies suggest that whilst RANKL provide initial signals for $AIRE^+$ mTEC differentiation, $CD4^+$ thymocytes provide additional signals for maintenance. The identification of these molecular mediators has highlighted the importance of thymic crosstalk where lymphocyte derived signals play a vital role in the development of TECs.

1.4 General Aims of Study.

As discussed in this chapter, the steps of T cell development have been well described. However, stages and mechanisms regulating the development of thymic stromal cells and

establishment of various thymic microenvironments are yet to be fully elucidated. Hence, the aims and objectives of this thesis are as follows;

- To investigate TEC development downstream of bTEC progenitors.
    - Characterise stages of cTEC development.
    - Phenotypically assess heterogeneity within the mTEC lineage and elucidate signals involved in their development.
- To study the establishment of thymic microenvironments with particular regard to the role of chemokine-induced signals for the establishment of the SCZ.

CHAPTER 2

GENERAL MATERIALS AND METHODS

2.1 Mice.

The use of inbred mouse models in the context of thymus development is vital to fully understand the sequence of events occurring during organogenesis and so provides a source of accurately timed embryonic, neonatal and adult material. Wildtype mice were used to characterise the normal development of thymocytes and thymic stromal cells. Knockout and transgenic mice were used to attribute particular genes and mechanisms to key stages in thymus development.

2.1.1 Mouse Husbandry and Breeding.

All mice used in this study were housed and maintained in the Biomedical Sciences Unit (BMSU), University of Birmingham, in accordance to home office regulations. Mice were obtained from either commercial suppliers, other research groups or from the BMSU in-house stock. Timed matings were obtained by placing a male mouse into a cage with two or three female mice overnight. Successful matings were identified by the presence of a vaginal plug (VP), the day of which was designated as day zero of gestation (E0). The gestation of mice can take between 18-21 days, with the day of birth designated postnatal day 0 (P0). This makes it possible to obtain fetal and neonatal material of specified developmental stages. Details of all wildtype and genetically modified mouse strains used in this study are outlined in Table 2.1.

Table 2.1 WildType and Genetically Modified Mouse Strains Used In This Study.

Mouse Strain	MHC haplotype	CD45 Isotype	Phenotype	Source
Balb/c	H-2^d	CD45.2	Wild type	BMSU
C57/BL6	H-2^b	CD45.2	Wild type	BMSU
Balb/c nude (nu/nu)	H-2^d	CD45.2	Deficiency in the FoxN1 gene. Abnormal thymic epithelial cell development. Thymus rudiment is not populated by lymphoid precursors and consequently nude mice do not have T cells.	BMSU
CD3 epsilon transgenic (CD3ε26Tg)	H-2^b	CD45.2	Over expression of the human CD3 ε transgene. Lack of T cells due to a block at the earliest stage of T cell development ($CD4^- 8^- 25^- 44^+$, double negative 1 stage) (Wang *et al.*, 1995).	Jackson Lab
$Rag1^{-/-}$ (C57/BL6)	H-2^b	CD45.2	RAG1 deficiency leads to a defect in the production of functional lymphocytes bearing antigen receptors. Block at the $CD4^- 8^- 25^+ 44^-$ (double negative 3) stage of T cell development (Mombaerts *et al.*, 1992).	Jackson Lab
$DOCK2^{-/-}$ (C57/BL6)	H-2^b	CD45.2	Deficiency in DOCK2 (a downstream mediator of chemokine signalling) leads to disruption of chemokine induced lymphocyte migration. Failure to respond to CXCL12, CCL19 and CCL21.	Y. Fukui, Kyushu University, Japan
$CCR9^{-/-}$ (C57/BL6)	H-2^b	CD45.2	Deficiency in CCR9 leads to failure of T cells to respond to CCL25 signalling.	W. Agace, University of Lund, Sweden

2.2 Medium and Tissue Culture Reagents.

2.2.1 Medium.

All media and additives were obtained from Sigma, Poole, UK, unless otherwise stated. Isolation of tissues and general handling of cells was carried out in RPMI-1640 Hepes (RF10-H) supplemented with 10% heat inactivated fetal calf serum (FCS) (Table 2.2). The long term culture of cells and tissues was carried out in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FCS and other additives as indicated in Table 2.3 and 2.4. Aliquots of media were prepared in advance and stored at 4°C.

2.2.2 Additives.

To eliminate all lymphoid and dendritic cells from fetal thymic organ cultures, 2-deoxyguanosine (2-dGuo) (Sigma) was added to culture media (Jenkinson et al., 1982). Stock solutions of 9mM 2-dGuo were stored at -20°C prior to addition to culture medium to give a final concentration of 1.35mM. To prepare stromal cell suspensions, 2.5% stock of trypsin in Hank's balanced salt solution (HBSS, Sigma) was diluted 10-fold in 0.02% EDTA in Ca^{++} Mg^{++} free phosphate buffered saline (PBS), to give a working concentration of 0.25% trypsin.

Table 2.2 Preparation of RPMI-1640 Hepes (RF10-H).

Medium and additives	Volume	Final concentration
RPMI-1640 + 20mM Hepes, with L-glutamine, without bicarbonate	10ml	-
200nM L-glutamine	100µl	2mM
5000 IU/ml Penicillin and Streptomycin	200µl	100 IU/ml
Heat-inactivated fetal calf serum (FCS)	1ml	10%

Table 2.3 Preparation of Dulbecco's Modified Eagle's Medium (DMEM).

Medium and additives	Volume	Final concentration
Dulbecco's Medium with 3.7g/I bicarbonate, without glutamine	10ml	-
100x non-essential amino acids	100µl	-
1M Hepes – final conc.	100µl	10mM
$5x10^3$ M2-Mercaptoethanol	100µl	-
200mM L-glutamine – final conc.	200µl	4mM
5000 IU/ml penicillin and streptomycin	200µl	100IU/ml
Heat-inactivated FCS 10%	1ml	10%

Table 2.4 Constituents of 100x Non-Essential Amino Acids.

Constituents	Concentration (mg/ml)
L-alanine	8.9
L-asparagine	15.0
L-aspartic Acid	13.0
Glycine	7.5
L-glutamic Acid	14.2
L-proline	11.5
L-serine	10.5

2.3 Isolation of Mouse Tissue.

All handling of murine adult, embryonic and neonatal tissue was performed in a laminar flow hood to provide a sterile environment, using sterile surgical instruments and sterile solutions.

All adult mice used in this study were culled by cervical dislocation of the neck and the thymus was dissected out and placed in RF10-H. For embryonic work, pregnant mice were culled and the uterus was removed whole. Embryos in their amniotic sacs were removed from the uterus and washed in PBS. Placentas and sacs were then removed and the embryos were washed in 1:1 PBS:RF10. Thymic lobes were dissected out from the thoracic tree (encompassing the heart, lungs, thymus and trachea) and transferred into RF10-H before either explantation into organ culture or processing for FACs analysis or immunohistology.

2.4 Fetal Thymus Organ Culture (FTOC).

Fetal thymus organ culture (FTOC) is a well established system that supports a complete programme of T cell development *in vitro*, whilst maintaining the integrity of the 3D architecture of the thymic stromal cells (Jenkinson and Anderson, 1994). This system promotes efficient interactions between developing T cells and stromal cells, thus emulating the specialised microenvironment that is typical of a mature thymus *in vivo* (figure 2.1 illustrates this technique).

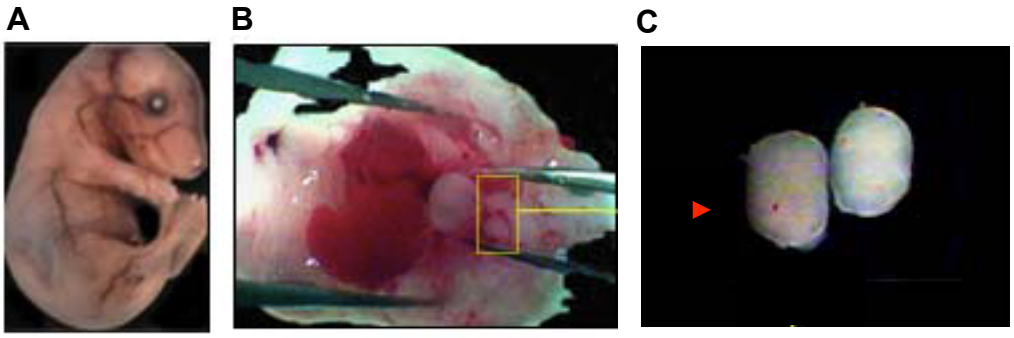
Thymic lobes were removed from mouse embryos at the required stage of gestation (usually E15 for FTOC). Using a finely drawn mouth-controlled glass pipette, lobes were placed on 0.8μm sterile nucleopore filters (Millipore) on sterile artiwrap sponges (approximately 1cm^2 in size) (Medipost Ltd) in DMEM. The nucleopore filters maintain organ cultures at the liquid-air interface, for optimal exchange with both the medium and gas phase vital for optimal growth (Jenkinson et al., 1982).

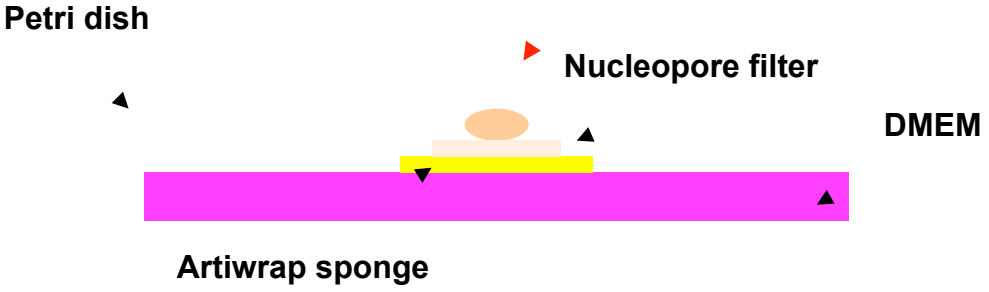
For normal organ culture, lobes were set up in 90mm, single vent sterile Petri dishes (Sterilin) containing 4mls DMEM. When required, 2-dGuo was added to the DMEM to give a final concentration of 1.35mM. A maximum of six lobes were cultured on a single sponge and filter, with two sponges and filters per dish. Petri dishes were then placed in humidified chambers. Cultures were gassed with 10% CO_2 to get the desired pH (7.2-7.4) for approximately 10 minutes before being sealed and transferred to a 37°C incubator for a minimum of 7 days. For optimal lymphoid and dendritic cell depletions by 2-dGuo, fetal lobes were cultured for 5-7 days.

Figure 2.1 Fetal Thymus Organ Culture.

Mouse embryos at day 15 of gestation were used as a source of thymus lobes (A). Thoracic cavity was exposed and thymus lobes can be visualised lying above the heart (red box) (B). Lobes were isolated (C) and explanted in organ culture (D).

Images A-C courtesy of F. Kinsella, University of Birmingham.


A
B
C


Petri dish
Nucleopore filter
DMEM
Artiwrap sponge

2.5 Isolation of Cell Types.

To study development and phenotype of thymic stromal cells, methods for enrichment of specific thymic epithelial cells was employed. Two approaches for this are highlighted below and include high-speed cell sorting and immunomagnetic bead separation.

2.5.1 Isolation of Thymocytes.

To obtain cell suspensions of thymocytes for flow cytometry adult thymic lobes or embryonic lobes were gently teased apart in RF10-H medium with fine forceps to release the cells. Cells were counted in a haemocytometer and centrifuged.

2.5.2 Isolation of Thymic Stromal Cells.

In order to obtain single cell suspensions of thymic stromal cells, freshly dissected embryonic thymic lobes or embryonic lobes that have been in organ culture for 7 days were washed three times in Ca^{++} Mg^{++} free PBS (Sigma) to remove all traces of serum that can inactivate trypsin. Washed lobes were then suspended in 600µl 0.25% trypsin (Sigma) diluted in 0.02% EDTA (Sigma) and then incubated at 37°C for approximately 15-20 minutes (precise timing depends on the stage of thymus development and days in organ culture). After this, dissaggregation of the lobes was aided by gentle pipetting, until a stromal cell suspension had formed. The activity of the trypsin was blocked by adding an equal volume of RF10-H. Cells were centrifuged for 10 minutes at 1000rpm and the supernatant removed. The cell pellet was resuspended in 1ml RF10-H and the cells were counted in a haemocytometer.

2.5.3 Bead Coating and Immunomagnetic Separation Techniques.

For further enrichment and selection of thymic epithelial cells, an immunomagnetic separation technique was employed using Dynabeads (Dynal, Wirral, UK). Beads were prepared the day before use by washing in RF10 three times to remove sodium azide and then incubated at 4°C overnight in the presence of the appropriate antibody, which was diluted to the required concentration in PBS. Details of antibodies used to coat Dynabeads are outlined in table 2.5. Tissue culture derived CD45 supernatant was coated onto anti-rat IgG Dynabeads, whilst anti-CD205 and anti-CD40 biotin were coated onto streptavidin Dynabeads. Following incubation, beads were washed three times in RF10 to remove all excess antibody and resuspended in 100µl of RF10. Beads were then added to cell suspensions in 200µl of RF10 at an approximate ratio of 10:1. Cells and beads were centrifuged for 10 minutes at 1000 rpm, at 4°C in round bottomed sterile cryogenic vials (Cryovial, Quebec, Canada) to ensure a large surface area for optimal cell to bead interactions. This was repeated a second time after gentle pippetting of the cells and beads. Such interactions result in the formation of 'rosettes', clusters of cells bound to beads, which were visualised by light microscopy throughout the process. Once positive binding was detected, cells rosetted to beads were isolated from unbound cells using an Eppendorf (1.5ml) Dynal Magnetic Particle Concentrator (Dynal). Supernatant containing the unbound populations was removed and rosettes were washed gently to eliminate any none specifically binding cells.

To obtain cells of defined epithelial populations for RT-PCR, cell suspensions depleted of CD45 were treated with biotin conjugated CD205 and CD40 streptavidin beads as described above. Rather than discarding, the CD205 and CD40 rosettes were snap frozen for RT-PCR.

Table 2.5 Details of Antibodies Used to Coat Dynabeads for Immunomagnetic Separation.

Bead type	Coating antibody and dilution	Use
Anti-rat IgG	Anti-mouse CD45 tissue culture supernatant used neat (ATCC)	Depletion of $CD45^+$ haematopoietic cells from freshly dissected embryonic or organ cultured thymus lobes.
Streptavidin	Anti-mouse CD205 biotin used 1:20	Selection of $CD205^+$ thymic epithelial cells from freshly dissected embryonic thymus lobes.
Streptavidin	Anti-mouse CD40 biotin used 1:50	Selection of $CD40^+$ thymic epithelial cells from freshly dissected embryonic thymus lobes.

2.5.4 Preparation of Cells for High Speed Sorting.

Suspensions of thymocytes or stromal cells were immunolabelled for flow cytometric high speed sorting in a similar manner to cell suspensions prepared for flow cytometry, which is described in section 2.6.

Following completion of immunolabelling, cells were washed and resuspended. Single colour controls, of approximately 100,000 cells, were resuspended in 200μl RF10-H. Experimental samples of no less than $1x10^6$ were resuspended in 1ml RF10-H. Suspensions were filtered to remove any adherent cell clumps through a 30μm mesh-membrane filter unit (Miltenyi Biotech) and then transferred to polypropylene FACs tubes (Becton Dickinson) before sorting. Sorting was performed on a MoFlo high speed sorter (Dako Cytomations). Sorted cells were collected into FACs tubes containing 500μl RF10-H and then centrifuged

and counted. A small sample was kept to reanalyse to determine purity of the sorted population of cells. Sorted cells were snap-frozen for cDNA extraction (as described in section 2.8).

2.6 Immunolabelling and Flow Cytometry.

2.6.1 Antibodies and Immunoconjugates.

Table 2.6 outlines all primary antibodies used for flow cytometry. Secondary antibodies, used when the primary was not conjugated to a fluorochrome, are listed in table 2.7. All antibodies were titrated to optimise working concentrations prior to use.

2.6.2 Immunolabelling of Cell Suspensions for Flow Cytometric Analysis.

Cell suspensions of thymocytes or stromal cells were prepared as described in section 2.5. Cells were counted and samples of less than 1 x 10^6 were aliquoted into 1.5ml eppendorf tubes and centrifuged. All suspensions were made up in RF10-H.

After removal of the supernatants, cell pellets were resuspended in 50µl of the relevant primary and secondary antibody. Samples were then incubated for 30 minutes on ice. Appropriate single colour controls were also set up. Where primary antibodies were not directly conjugated to a fluorochrome, a control sample was needed to set the negative peak of florescence and measure non-specific binding of the antibody. To achieve this, the primary antibody was omitted from the protocol and replaced with 50µl PBS. Once the immunolabelling was complete, cells were washed in 1ml PBS and centrifuged. When required, the relevant secondary antibodies were added to cells and incubated for 30 minutes

Table 2.6 Details of Primary Antibodies for Flow Cytometric Immunolabelling.

Specificity (clone)	Species/Isotype	Source	Working dilution
Anti-mouse CD45.2 FITC (clone 104)	Mouse IgG2a	eBioscience	1:200
Anti-mouse CD205 (DEC205) biotin (clone NLDC145)	Rat IgG2a	AbCAM	1:50
Purified anti-mouse EpCAM-1 (clone G8.8) conjugated to Alexa 647 by A. White, University of Birmingham	Rat	Purified EpCAM-1 a gift from A. Farr, University of Washington, USA	1:800
Anti-mouse CD40 PE (clone 3/23)	Rat IgG2a	Serotec	1:20
Anti-mouse Ly51 (6C3) PE (clone BP-1)	Mouse IgG2a	eBioscience	1:500
Anti-mouse CD80 biotin (B7.1) (clone 16-10A1)	Armenian hamster IgG	eBioscience	1:400
Anti-mouse PD-L2 (B7.DC) biotin (clone TY25)	Rat IgG2a	eBioscience	1:100
Anti-mouse PD-L1 (B7-H1) biotin (clone MIH5)	Rat IgG2a	eBioscience	1:100
Anti-mouse CD4 PE (clone GK1.5)	Rat IgG2b	eBioscience	1:1200
Anti-mouse CD8α FITC (clone 53-6.7)	Rat IgG2a	eBioscience	1:200
Anti-mouse CD25 (IL-2Rα) APC (clone PC61.5)	Rat IgG1	eBioscience	1:200
Anti-mouse/human CD44 (clone IM7)	Rat IgG2b	eBioscience	1:400
Anti-mouse TCRβ biotin (clone H57-597)	Armenian hamster IgG2	BD	1:200
Anti-mouse IAd (MHC II) FITC (clone AMS-32.1)	Mouse IgG2b	BD	1:600

Continued.

Anti-mouse IAb (MHC II) FITC (clone AF6-120.1)	Mouse IgG2a	BD	1:600
Anti-BrdU FITC	-	BD	1:50
Anti-mouse lineage markers biotin (including CD11b (Mac-1), Ly-6G (erythroid cells), CD3 ε chain, CD45R/B220	Rat IgG2b	BD	1:200
Anti-mouse CD4 biotin (GK1.5)	Rat IgG2b	eBioscience	1:100
Anti-mouse CD8 biotin (clone CT-CD8b)	Rat IgG2b	eBioscience	1:100
Anti-mouse IAd PE (clone AMS-32.1)	Mouse IgG2b	BD	1:200
Anti-mouse Ly51 biotin (clone 6C3)	Rat IgG2a	eBioscience	1:200
Anti-mouse AIRE conjugated to Alexa 488 by A. White, University of Birmingham	Rat	Purified AIRE a gift from H. Scott, University of Melbourne, Australia	1:200
Anti-mouse PD-1 PE (clone J43)	Armenian hamster IgG1	eBioscience	1:100
Anti-mouse CD69 biotin (clone H1.2F3)	Armenian Hamster IgG1	BD	1:100
gp38 supernatant (clone 8.1.1)	Hamster	Grown from a hybridoma cell line, gift from A. Farr, University of Washington, USA	1:2
Anti-mouse VCAM-1 (clone 429)	Rat IgG2a	eBioscience	1:100
Anti-mouse ICAM-1 (clone 3E2)	Rat	BD	1:100
Anti-mouse PDGFRα (clone APA5)	Rat IgG2a	eBioscience	1:200

Table 2.7 Details of Secondary Antibodies for Flow Cytometric Immunolabelling.

Specificity (clone)	Species/Isotype	Source	Working dilution
Streptavidin PE Cy7	-	eBioscience	1:500
Streptavidin APC	-	BD	1:400
Streptavidin PE	-	eBioscience	1:1200

on ice. After washing, cells were then resuspended in 200µl of PBS. Samples were then transferred to 12.5ml polystyrene FACS tubes (Becton Dickinson) for FACS analysis.

2.6.3 Flow Cytometric Analysis.

Acquisition was performed using a Becton Dickinson LSR flow cytometer and subsequent analysis was carried out using FloJo software. Prior to acquisition of experimental samples, any relevant negative control samples were examined in order to set negative peaks for populations not expressing the tested antibodies. For experiments using more than two fluorochromes, single colour controls were used to set up the machine to set the positive peaks for populations expressing the tested antibodies, but also to adjust compensation levels required between the various fluorochrome detection channels.

Where possible at least 100,000 events were recorded per experimental sample, with forward and side scatter gates to exclude non-viable cells, while fluorochrome detection channel gates were set in accordance to negative and positive peaks generated from the control samples.

2.7 Immunohistology Techniques.

2.7.1 Sectioning and Fixation of Tissues.

To produce sections of embryonic and adult thymi, organs previously frozen in liquid nitrogen, were mounted onto a microtome in OCT compound (Tissue Tek) at a defined orientation. 5μM thick sections were cut and mounted onto 4 spot glass slides (Hendley-Essex) for immunohistology. All slides were dried at room temperature for an hour and then fixed for 20 minutes in acetone (Baker) at 4°C. Slides were allowed to air dry for 10 minutes before storage in polythene grip seal bags at -20°C until further use.

2.7.2 Immunolabelling of Frozen Tissue Sections.

Slides were allowed to dry at room temperature within grip seal bags for 10 minutes. In order to examine slides by confocal microscopy, sections were stained with fluorochrome-conjugated anti-mouse antibodies diluted in PBS containing 1% bovine serum albumin (BSA). Slides were pre-blocked with 10% normal goat serum and secondary and tertiary step antibodies were pre-absorbed with 10% normal mouse serum. For intracellular staining (such as AIRE), antibodies were made in PBS containing Tween (Sigma). Details of all primary antibodies used in this study are outlined in table 2.8 and details of secondary and tertiary antibodies are detailed in table 2.9. After staining the sections, slides were mounted in Vectashield with DAPI (VectorLabs). DAPI is nuclear stain and allows for visualisation of the whole tissue section.

Confocal images were acquired using a Zeiss LSM510 laser scanning confocal head with a Zeiss Axio Imager Z1 microscope (Zeiss, Welwyn Garden City, UK). Digital images were recorded in four separately scanned channels with no overlap in the detection of

emissions from the respective fluorochromes. FITC or Alexa 488 conjugated antibodies were excited with a 488nm argon laser, TRITC and Alexa 555 and 594 conjugated antibodies were excited with a 561nm helium laser, Alexa 647 conjugated antibodies were excited with the 633nm helium laser and finally DAPI was detected using the 405nm diode laser. Confocal micrographs were stored as digital arrays of 2048x2048 pixels with 8 bit sensitivity.

Table 2.8 Details of Primary Antibodies used for Immunohistology.

Specificity (clone)	Species/isotype	Source	Working dilution
Anti-mouse CD205 (DEC205) FITC (clone NLDC145)	Rat IgG2a	AbCAM	1:50
Anti-mouse CD40 biotin (clone 3/23)	Rat IgG2a	BD	1:50
Purified anti-mouse EpCAM-1 (G8.8) conjugated to Alexa 647 by A.White, University of Birmingham	Rat	Purified EpCAM-1 a gift from Andy Farr, University of Washington, USA	1:150
Anti-mouse CD25 FITC (clone 7D4)	Rat IgM	BD	1:150
Purified anti-mouse CD4 (clone GK1.5) conjugated to Alexa 647 by D.Withers, University of Birmingham.	Rat	Purified CD4 from eBioscience	1:100
Anti-mouse CD8β biotin (clone CT-CD8b)	Rat IgG2b	eBioscience	1:100
Anti-mouse keratin 8 (clone KS8.7)	Mouse IgG1	Progene	1:10
Anti-mouse pan-cytokeratin FITC (clone C-11)	Mouse IgG1	Sigma	1:600
Anti-mouse keratin 5 purified	Rabbit	Covance	1:1500

Continued.

Anti-mouse CD31 biotin (clone ER-MP12)	Rat IgG2a	BMA Biomedicals	1:100
Purified anti-mouse AIRE	Rat	A gift from H. Scott, University of Melbourne, Australia	1:200
gp38 supernatant (clone 8.1.1)	Hamster	Grown from a hybridoma cell line, gift from A. Farr.	1:2
Perlecan supernatant	Rat	A gift from Z. Lokmic, University of Muenster, Germany	1:2
ER-TR7 supernatant	Rat	Grown from a hybridoma cell line, gift from W. van Ewijk, Leiden University, Netherlands	1:2

Table 2.9 Details of Secondary and Tertiary Antibodies Used for Immunohistology.

Specificity (clone)	Species/isotype	Source	Working dilution
Anti-FITC	Rabbit	Biosource	1:200
Anti-rabbit FITC IgG (H+L)	Goat	Southern Biotech	1:100
Anti-biotin TRITC	Mouse	Stratech Scientific	1:400
Anti-hamster biotin IgG (H+L)	Goat	Vector Laboratories	
Streptavidin Alexa 555	Goat	Invitrogen	1:1000
Anti-rabbit Alexa 647 IgG (H+L)	Goat	Invitrogen	1:400
Anti-rat Alexa 595 IgG (H+L)	Goat	Invitrogen	1:400

2.8 Preparation of Samples for Gene Expression Analysis.

All gene expression analysis was carried in collaboration with S. Parnell, University of Birmingham.

2.8.1 Snap Freezing of Cell Populations.

Cell populations obtained following MoFlo sorting or immunomagnetic bead separations were transferred into 1.5ml RNase-free eppendorf tubes (Camlab) and centrifuged at 1000rpm for 10 minutes. Supernatants were removed to leave a dry pellet. Closed eppendorfs were immersed in liquid nitrogen before storage at -80°C until further use.

2.8.2 mRNA extraction.

mRNA was extracted from sections obtained by either laser capture microdissection (described in section 4.2), MoFlo sorted cells or cells obtained after immunomagnetic separation, using a thermoMACS^{TM} heating block and protocol (Miltenyi). The whole procedure was carried out in a laminar flow cabinet. Lysis/binding and wash buffers were allowed to equilibrate to room temperature prior to carrying out the protocol. Frozen tissue sections or cells obtained from MoFlo cell sorting were suspended in 900µl of lysis/binding buffer and vigorously vortexed for 3 minutes to ensure total cell lysis. For cells obtained from immunomagnetic bead separation, cells were separated from the beads by suspending rosettes in lysis/binding buffer and using a 21-gauge needle to lyse the cells. The resulting mix was then centrifuged briefly at 13,000 rpm to separate the cells from the beads. Beads were discarded.

Cell lysates were then centrifuged at 13,000 rpm for 3 minutes in LysateClear columns. 50µl of oligo microbeads were added to each lysate and gently mixed to allow the

polyA tails of the mRNA present to hybridise to the oligo microbeads. Lysates were then placed in μMACS columns previously primed with 100μl lysis/binding buffer. As the sample passed through the column, the magnetically labelled mRNA remained bound to the column. Columns were rinsed twice with 200μl lysis/binding buffer to remove proteins and DNA, followed by a rinse with 100μl of wash buffer 4 times to remove ribosomal RNA and DNA. In order to continue with cDNA synthesis directly, the bound mRNA was not eluted from the column. 100μl of equilibration/wash buffer was added to the column twice and allowed to run through. Meanwhile, the lyophilised enzyme mix was dissolved in 20μl of resuspension buffer and applied on top of the column followed by 1μl of sealing solution applied directly to the top of the column to prevent evaporation. The thermoMACS^{TM} was then turned on to 42°C and left for 1 hour and 15 minutes for reverse transcription to take place. Following the incubation period, the column was rinsed twice with 100μl equilibration/wash buffer. To release the cDNA, 20μl release solution was added to the column and then left for 30 minutes at 42°C. Following the incubation period, 50μl elution buffer was added to elute the cDNA, which was collected into an eppendorf. cDNA was placed at -20°C until ready for use. High yields and purity of cDNA was obtained with this procedure.

2.8.3 Quantitative RT-PCR.

Quantitative PCR was performed on the RotorGene RG-3000 (Corbett Research) using SYBR green with primers specific for various genes of interest. Details of all primers used in this study are outlined in Table 2.10. β-actin was used as the housekeeping gene for sample normalisation, prior to amplifying the target genes of interest. Primers were made by Sigma-Genosys and primer pairs were designed using the aid of PrimerBank software

(http://pga.mgh.harvard.edu/primerbank/index.html) to minimise the possibility of dimerisation that could lead to non-specific PCR amplification. PCR reactions were carried out in triplicates or duplicates in 15μl volumes in reaction buffer containing 1X QPCR SYBER Green Mix (Quantace) and 200nM-300nM forward and reverse primers. The amplification program comprised of an initial 'hot start' (95°C for 15 minutes), followed by cycling at 95°C for 15 seconds, 58 to 62°C (depending on primer pair) for 20 seconds and 72°C for 5 seconds (39 cycles). The fluorescent signal produced from the amplicon was acquired at the end of each polymerisation step and at the end amplification, a melt curve profile was obtained (72-99°C, hold 30 seconds on 1st step then 5 seconds on next steps) in order to check specificity of amplicon. Reaction amplification efficiency and the Ct values were obtained from the Rotor Gene 6.0 software (Corbett Research, Sydney Australia) using standard curves primarily obtained from cDNA from FTOCs. For calculation of the relative expression values for samples normalised to β-actin, the Pfaffl model that takes gene-dependent differences in the amplification efficiency into account was employed. Specific amplification of target genes was normally verified by post-amplification melt curve analysis and also by fractionation of PCR products on a 2.0% agarose gel, which were identified by their fragment size.

Table 2.10 Details of primers.

Primer	Forward 5' end primer sequence	Reverse 3' end primer sequence	Product size (base pairs)
β-actin (NM_007393.2)	CGTGAAAAGATGACCCAGATCA	TGGTACGACCAGAGGCATACAG	100
AIRE (NM_009646)	TGCATAGCATCCTGGACGGCT	CCTGGGCTGGAGACGCTCTTT	187
CCL19 (NM_011888.2)	GGGGTGCTAATGATGCGGAA	CCTTAGTGTGGTGAACACAAC	137
CCL21 (NM_011335.2)	GCTGCCTTAAGTACAGCCAGA	CTTCCTCAGGGTTTGCACAT	150
RANK (NM_009399.3)	GCTGGCTACCACTGGAACTC	GTGCAGTTGGTCCAAGGTTT	182
Claudin 3 (NM_009902.3)	ACCAACTGCGTACAAGACGAG	CAGAGCCGCCAACAGGAAA	78
Claudin 4 (NM_009903.2)	GTCCTGGGAATCTCCTTGGC	TCTGTGCCGTGACGATGTTG	112
LTβR (NM_010736.3)	GAGCAGAACCGGACACTAGC	GAAGGTAGGGATGAGCACC	256
Cathepsin L (NM_009984.3)	ATCAAACCTTTAGTGCAGAGTG	CTGTATTCCCCGTTGTGTAGC	136
Cathepsin S (NM_021281.2)	CCATTGGGATCTCTGGAAGAAAA	TCATGCCCACTTGGTAGGTAT	155
β5T (NM_175204.4)	ACTCCCGACACTCCCAGAC	CCGTGACGAAAGCGAAAAGC	149
DOCK2 (NM_033374.2)	TGTCACAGGGCATTTTCCCTA	TCGTACATCATGGACTGCACT	208
CCL25 NM_009138.2	CGGAAGTAGAATCTCACAGCA	TTACCAGCACAGGATCAAATG	105
CCR9 NM_001166625.1	AGGCCAAGAAGTCATCCAAGC	CCTTCGGAATCTCTCGCCAA	251
CXCL12 (NM_021704.2)	GCTCTGCATCAGTGACGGTA	TGTCTGTTGTTGTTCTTCAGC	162
CXCR4 NM_009911.3	GAAGTGGGGTCTGGAGACTAT	TTGCCGACTATGCCAGTCAAG	125
CCR7 NM_007719.2	TGTACGAGTCGGTGTGCTTC	GGTAGGTATCCGTCATGGTCTT	162
FGF-7 NM_008008.3	CTCTACAGGTCATGCTTCCACC	ACAGAACAGTCTTCTCACCCT	174
FGF-10 NM_008002.3	CAGCGGGACCAAGAATGAAG	TGACGGCAACAACTCCGATTT	77
FGFR2 NM_010207.2	TTCTCCTAGTTACCCCGACAC	AGGCAGACAGGGTTCATAAGG	94

CHAPTER 3

CHECKPOINTS IN THE DEVELOPMENT OF CORTICAL THYMIC EPITHELIAL CELLS

3.1 Introduction.

The thymus is comprised of a unique three-dimensional network of stromal cells, which provide specialised microenvironments for the development of a diverse and self-tolerant T cell repertoire. Thymic microenvironments are anatomically compartmentalised into cortical and medullary regions, which house the key stages of T cell development. Thymic epithelial cells (TECs) constitute the major cell type of the cortex and medulla and are phenotypically and functionally heterogeneous. Cortical thymic epithelial cells (cTECs) support the positive selection of thymocytes that recognise self-peptide/MHC II complexes with low affinity, whereas medullary thymic epithelial cells (mTECs) regulate self-tolerance via the deletion of potentially autoreactive T cells by a process of negative selection.

TECs provide a number of signals to developing thymocytes including; peptide/MHC ligands for TCR repertoire selection (Cosgrove et al., 1992), soluble growth factors such as Wnts (Pongracz et al., 2003), IL-7 and stem cell factor (SCF) for survival and expansion (Rodewald et al., 1997) and Notch ligands for differentiation and lineage commitment (Jenkinson et al., 2006). Indeed, the importance of TECs has been further demonstrated during thymic atrophy where a reduction in TEC frequency and function is associated with a reduction in T cell output (Chidgey et al., 2007). Furthermore, the autoimmune deficiencies that arise as a result of abnormal TEC development highlight the importance of TECs for maintaining self-tolerance (Naquet et al., 1999). Thus, characterising stages and mechanisms of cTEC and mTEC development and the interactions between thymocytes and stromal cells is central to our understanding of intrathymic T cell development.

The discovery of a bipotent TEC (bTEC) progenitor that can develop into both cortical and medullary lineages was a significant finding and provided novel insight into TEC development (Bleul et al., 2006; Rossi et al., 2006). In the context of mTEC development,

most notably the subset expressing AIRE, several molecular mediators have now been identified as important, including the cell surface receptors RANK, CD40 and LTβR (Akiyama et al., 2008; Boehm et al., 2003; Hikosaka et al., 2008; Irla et al., 2008; Rossi et al., 2007b; White et al., 2008). Furthermore, a precursor-product relationship has been identified within the mTEC lineage (Hamazaki et al., 2007; Rossi et al., 2007b) along with the finding that mTEC committed progenitors have self-renewal capacity (Gabler et al., 2007). In addition, defects in thymocyte development have been associated with mTEC abnormalities, highlighting the importance of thymic crosstalk in the development of the medulla. In contrast to our understanding of mTEC development, stages and mechanisms regulating cTEC development downstream of bTEC progenitors are poorly characterised and a cTEC progenitor has yet to be identified (figure 3.1). Clearly, further studies that attempt to isolate cTEC progenitors and define important mechanisms that regulate cTEC development are vital for understanding how cTECs become specialised in their ability to mediate positive selection of developing thymocytes.

In view of this, the objective of this chapter was to characterise cTEC development occurring downstream of bTEC progenitors using the endocytic receptor CD205 (also known as DEC205), which is expressed in the thymic cortex and DCs but not the medulla (Jiang et al., 1995) and the recently identified proteasome subunit β5T (Murata et al., 2007).

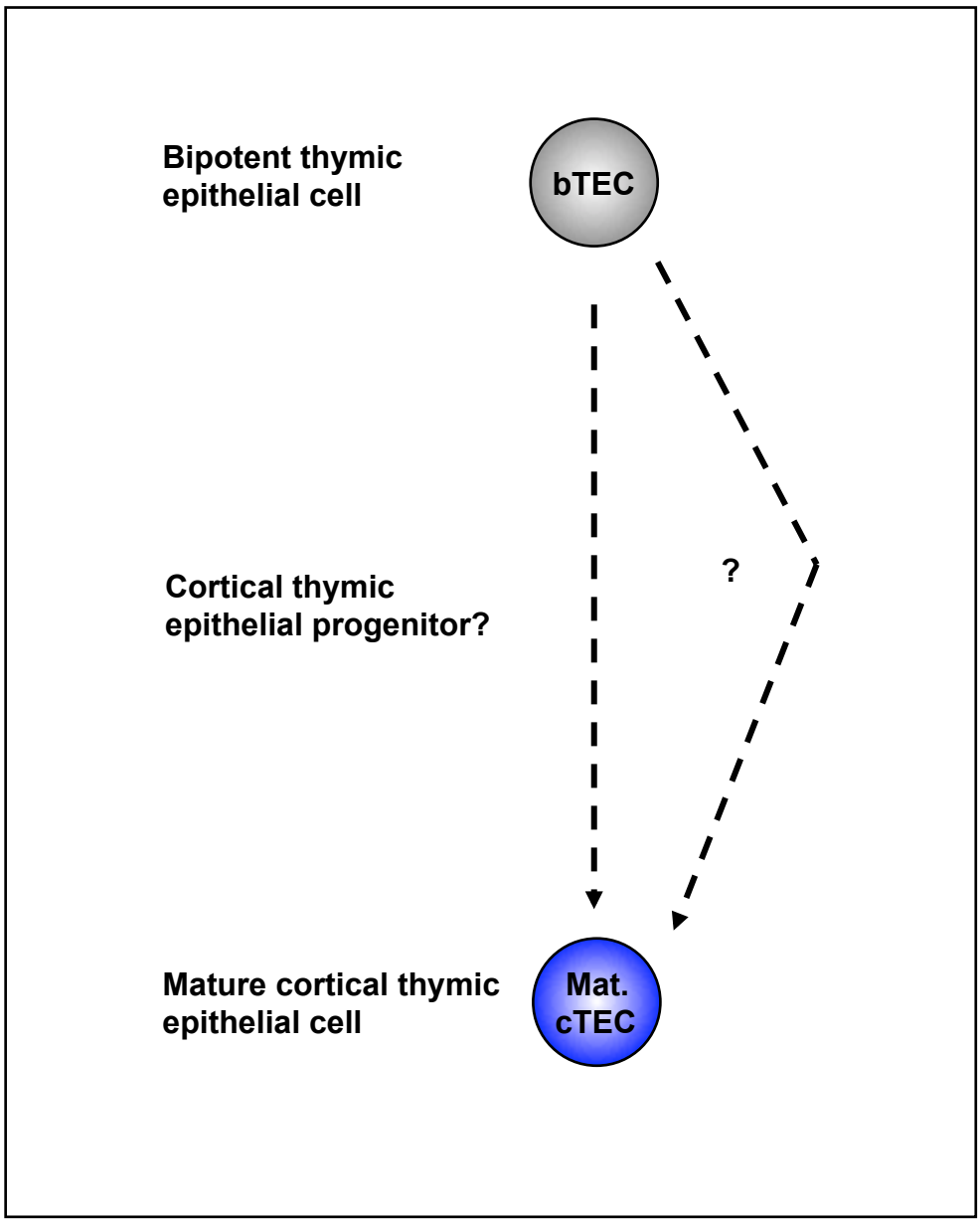
Thus, the aims and objectives of this chapter were to;

- Identify stages in cTEC development.
- Assess proliferative status of cTEC subsets.
- Identify cellular and molecular regulators of cTEC development.

Figure 3.1 A Previous Model of Cortical Thymic Epithelial Cell Development.

A common bipotent thymic epithelial cell (bTEC) progenitor develops into a mature cortical thymic epithelial cell (cTEC) via an unknown developmental pathway. A cTEC progenitor downstream of the bipotent TEC progenitor has not been identified and the precise molecular and cellular regulators of this pathway have not been elucidated.

bTEC, bipotent thymic epithelial cell, Mat, mature, cTEC, cortical thymic epithelial cell.


Bipotent thymic epithelial cell
bTEC
Cortical thymic epithelial progenitor?
?
Mature cortical thymic epithelial cell
Mat. cTEC

3.2 Specialised Materials and Methods.

3.2.1 BrdU Assay to Measure Cellular Proliferation.

To assess proliferation of stromal cell subsets, a bromodeoxyuridine (BrdU) flow cytometry protocol was employed (BD Pharmingen). BrdU (an analogue of the DNA precursor thymidine) is incorporated into newly synthesised DNA by cells entering and progressing through the S phase of the cell cycle. Thus, this assay allows for the identification of actively cycling cells. BrdU assays were carried out in collaboration with G. Desanti, University of Birmingham.

Freshly isolated thymus lobes or lobes placed in fetal thymus organ culture (FTOC) for 7 days (as described in section 2.4) were placed in DMEM containing BrdU at a final concentration of 10μM for an overnight incubation (note: for the negative controls, lobes were cultured without BrdU). Lobes were then removed from culture and enzymatically disaggregated to obtain suspensions of stromal cells. To enrich for stromal cells, cells were depleted of $CD45^+$ haematopoietic cells by an immunomagnetic bead separation technique (as described in section 2.5.3). Purity of the CD45 negative fraction was checked by flow cytometry. The purified stromal cell suspension was then surface stained for the relevant primary and secondary antibodies. Upon completion of the cell surface stain, the BrdU protocol was carried out on all samples, including the negative controls, according to the manufacturers instructions. Washed cells were incubated with cytofix/cytoperm buffer for 25 minutes on ice in order to fix and permeabilise cells. This was followed by washing the cells with perm/wash buffer and then incubating the cells for 10 minutes on ice with cytoperm plus buffer. Cells were then washed again with perm/wash buffer and re-fixated with cytofix/cytoperm buffer for 5 minutes on ice. After another wash with perm/wash buffer, cells were incubated with DNase for 1 hour at 37°C in order to expose incorporated BrdU.

Following the incubation period, cells were again washed with perm/wash buffer. Cells were then stained with a fluorescent anti-BrdU antibody made up in perm/wash buffer for 20 minutes on ice. Finally, cells were washed with perm/wash buffer and then resuspended in PBS before acquisition on an LSR flow cytometer (BD Biosciences).

3.3 Results

3.3.1 Characterisation of Stromal Cells in a Wildtype Thymus.

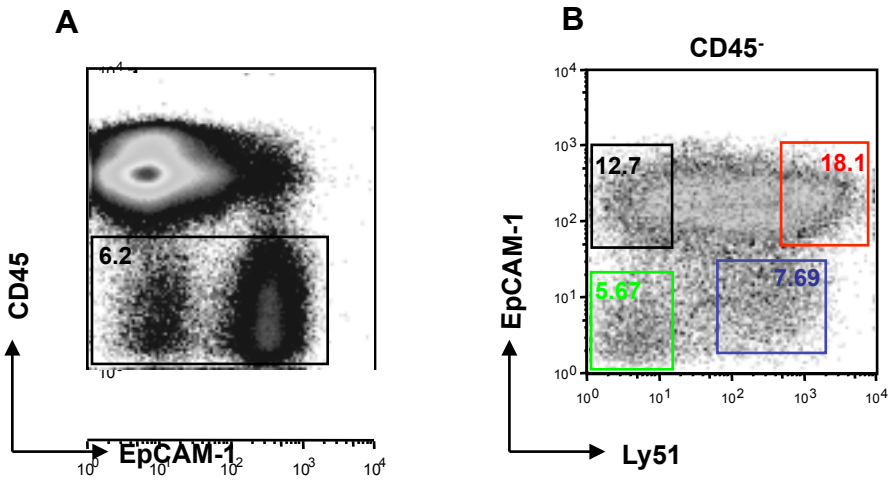
To determine the basic stromal cell composition of an adult thymus, embryonic thymus lobes were dissected from wildtype (WT) embryos at day 15 of gestation (E15) and placed in FTOC for 7 days. To avoid long phases of dissaggregation, this *in vitro* system was employed instead of using thymi from neonatal or adult mice. This well established system supports a complete programme of T cell development *in vitro* and the thymic stromal cells retain their 3D architecture allowing for efficient interactions between developing T cells and stromal cells (Jenkinson and Anderson, 1994). Thus, this system emulates the specialised thymic microenvironment that is typical of a mature thymus *in vivo* (equivalent in age to an approximately 3 or 4 day old neonate).

FTOC lobes removed from culture were enzymatically disaggregated to obtain cell suspensions of thymic stromal cells. Stromal cells were then stained for flow cytometry for CD45 (haematopoietic cell surface marker), EpCAM-1 (a cell surface marker expressed on both cTECs and mTECs) and Ly51 (a cTEC specific marker). The non-haematopoietic compartment of the thymus identified as $CD45^-$ (figure 2.2A), is comprised of a heterogeneous population of stromal cells (figure 2.2B).

Figure 3.2 Characterisation of Stromal Cells in a Wildtype Thymus.

Thymus lobes dissected at day 15 of gestation from WT mouse embryos were placed in fetal thymus organ culture (FTOC) for 7 days. Following culture, lobes were enzymatically digested to obtain suspensions of thymic stromal cells. Cells were stained for flow cytometry for CD45, EpCAM-1 and Ly51. A $CD45^-$ non-haematopoietic thymic stromal cell compartment is shown (A). Thymic stromal cells are further subdivided into different subsets based on their EpCAM-1 and Ly51 expression (B). Table summarises the different thymic stromal cell subsets and their phenotype (C).

Percentages of cells within gated areas are indicated. Data representative of three experiments.



C

Gate	Phenotype	Cell type
———	CD45^{-} EpCAM-1^{+} Ly51^{-}	Medullary thymic epithelial cell (mTEC)
———	CD45^{-} EpCAM-1^{+} Ly51$^{+/high}$	Cortical thymic epithelial cell (cTEC)
———	CD45^{-} EpCAM-1^{-} Ly51^{+}	Cortical mesenchymal cell (cMES)
———	CD45^{-} EpCAM-1^{-} Ly51^{-}	Non epithelial cell

A population of cTECs was identified on the basis of a $CD45^-$ $EpCAM1\text{-}1^+$ $Ly51^{+/high}$ phenotype. mTECs were identified as $CD45^-$ $EpCAM\text{-}1^+$ $Ly51^-$. A $CD45^-$ $EpCAM\text{-}1^-$ $Ly51^+$ phenotype distinguishes a non-epithelial stromal cell type previously described as the cortical mesenchyme (cMES) (Muller et al., 2005). A population of stromal cells identified as $CD45^-$ $EpCAM\text{-}1^-$ $Ly51^-$ was also observed and these cells are likely to represent a non-epithelial population of cells comprising of fibroblasts and endothelial cells. The table in figure 2.2 summarises the stromal cell populations in the thymus and their phenotype as described above.

3.3.2 An Adult Wildtype Thymus has a Defined Stromal Architecture.

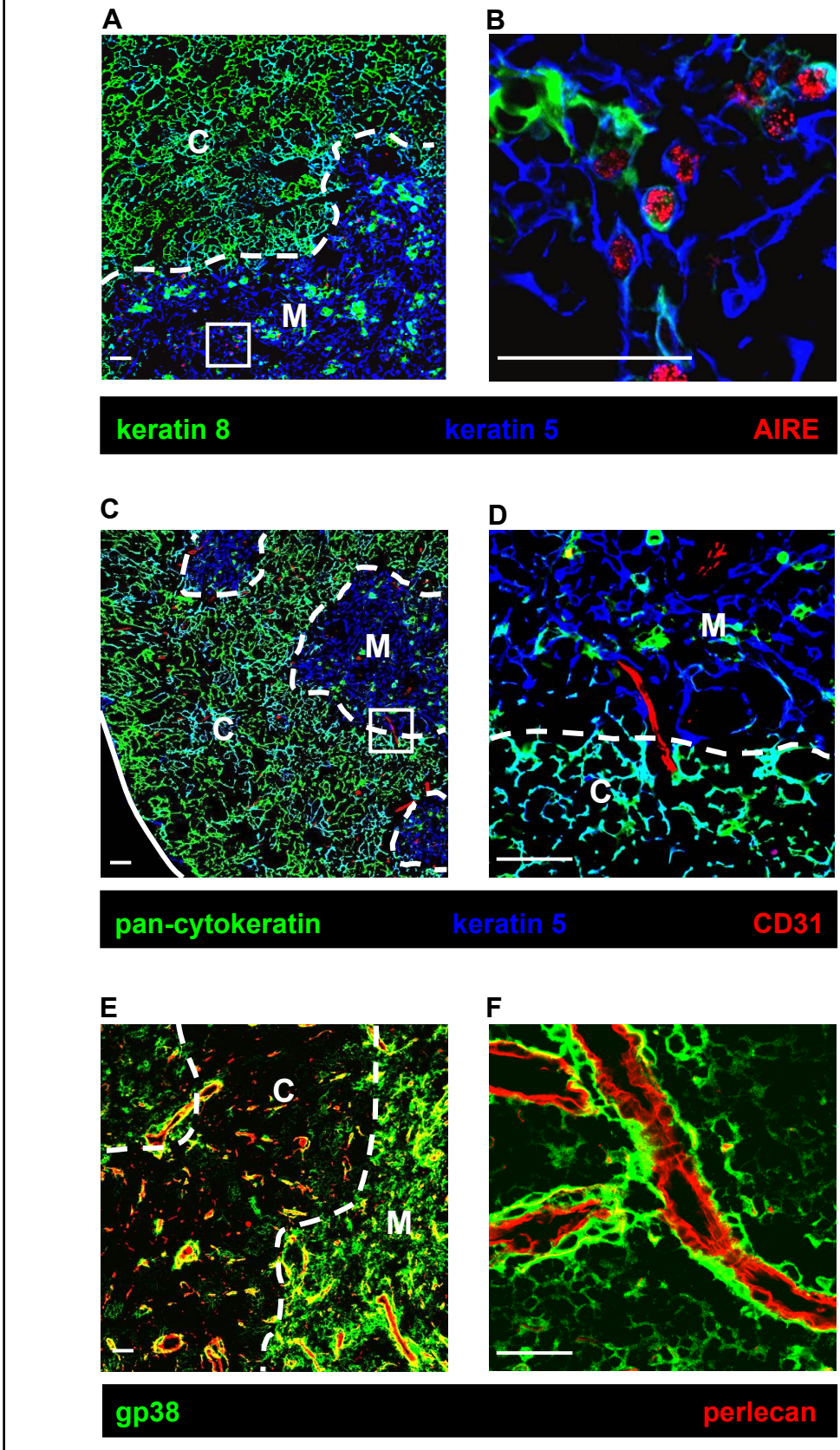
To identify the basic organisation of stroma in an adult WT thymus, frozen thymus tissue sections from a 4-6 week old WT mouse were stained for confocal microscopy analysis for a panel of stromal cell markers (figure 3.3A-F). Cortex and medulla are identified on the basis of the nuclear stain DAPI (not shown). Cortical and medullary epithelial cells were identified by the differential expression of keratins (figure 3.3A-D), where the cortex typically expresses keratin 8 (K8) and a pan-cytokeratin marker (typically follows the same expression pattern as K8), whereas the medulla expresses keratin 5 (K5). $K8^+$ $K5^+$ cells were observed at the corticomedullary junction (CMJ) and some cells were scattered through the cortex (figure 3.3A). The autoimmune regulator AIRE is expressed specifically by mTECs, which are predominantly $K5^+$ (figure 3.3A and B) and has a punctate appearance as illustrated in figure 3.3B. To identify endothelial vessels in the thymus, sections were also stained for the surface marker CD31 (figure 3.3C and D). Endothelial cells appear to predominate at the CMJ, which represents the site of entry for T cell progenitors. Another important stromal marker is

Figure 3.3 An Adult Wildtype Thymus has a Defined Stromal Architecture.

Frozen tissue sections of thymus dissected from 4-6 week old WT mice were stained for keratin 8, keratin 5 and AIRE (A and B), Pan-cytokeratin, keratin 5 and CD31 (C and D) and gp38 and the extracellular matrix protein perlecan (E and F). High power magnifications of boxed areas are shown in the right hand panels.

Cortex and medulla are identified on the basis of DAPI staining (not shown). The solid line denotes edge of tissue and dashed line denotes the corticomedullary junction. Bar indicates a scale of 50μm.

C, cortex, M, medulla.


A
B
C
M
keratin 8
keratin 5
AIRE
C
D
M
C
M
pan-cytokeratin
keratin 5
CD31
E
F
C
M
gp38
perlecan

transmembrane glycoprotein 38 (gp38, also called podoplanin), which is known to be expressed by stromal cells in T cell dependent areas of peripheral lymphoid tissue and has been implicated in the presentation of chemokines (Farr et al., 1992). Extracellular matrix proteins (ECM), such as perlecan are important components of basement membranes. Staining of adult thymus sections with gp38 and perlecan revealed that gp38 is predominantly expressed in the medulla as a large network with some $gp38^{+}$ cells scattered throughout the cortex (figure 3.3E and F). Perlecan was also observed in the medulla on vessel like structures in close association with gp38 (figure 3.3E and F).

3.3.3 CD205 Expression Defines Cortical Epithelial Cells but not Medullary Epithelial Cells.

Having established that a WT thymus is comprised of a heterogeneous population of stromal cells with defined cortical and medullary regions (sections 3.3.1 and 3.3.2), we then turned our focus on trying to characterise the development of these thymic microenvironments with a particular emphasis on the development of cTECs.

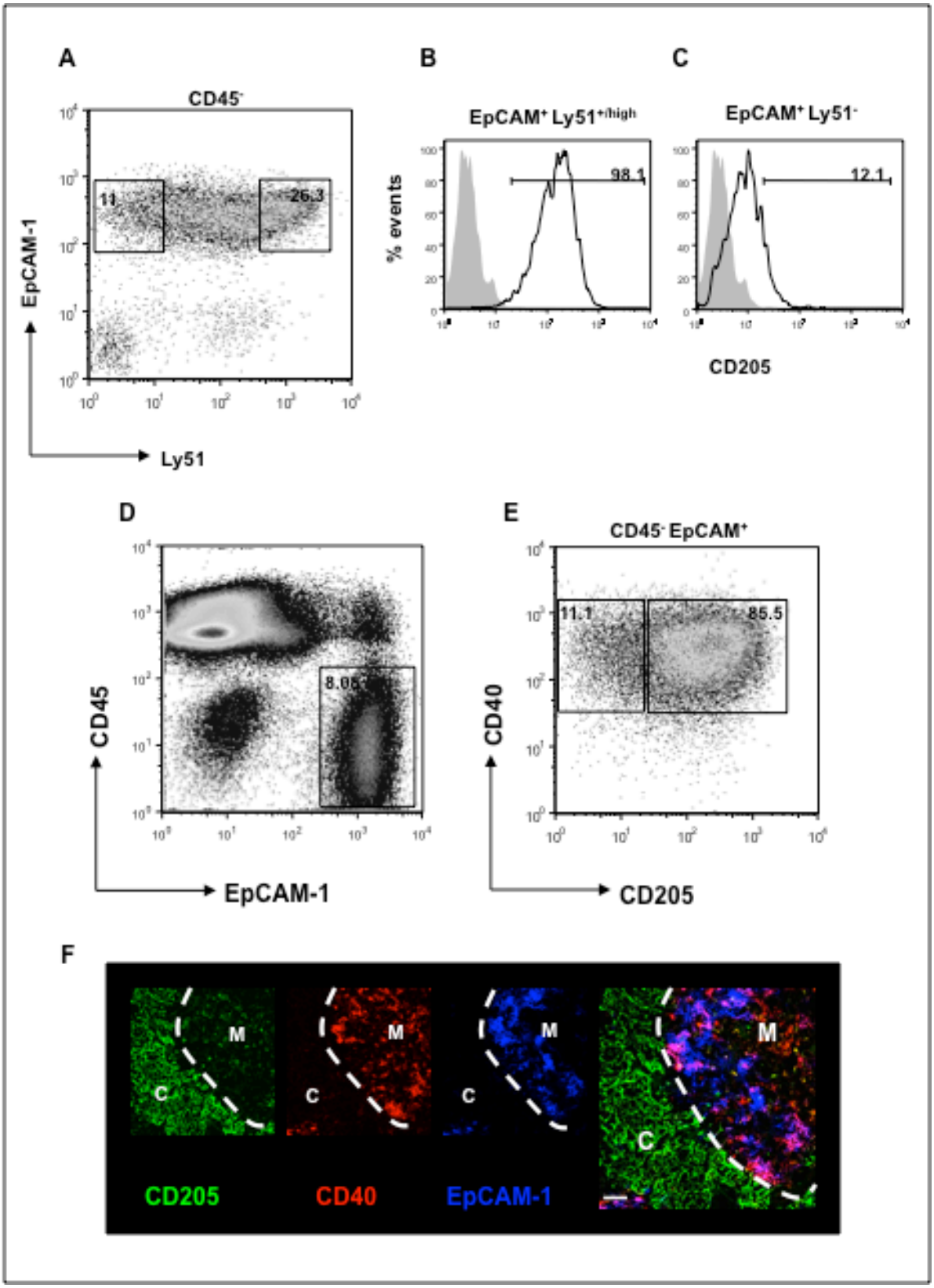
Due to paucity of cTEC associated markers, successful characterisation of cTEC development has previously been limited. CD205 was chosen in our study to characterise development of the cortex due to its previously known specificity as a cTEC marker in an adult thymus (Jiang et al., 1995; Nonaka et al., 2007). To confirm the specificity of CD205 in our study, a stromal cell suspension was obtained from 7 day FTOC lobes, which were stained for CD45, EpCAM-1 and Ly51 to determine CD205 expression in cTECs and mTECs (figure 3.4). CD205 expression was largely restricted to a cTEC population, defined as $CD45^{-}$ EpCAM-1^{+} $Ly51^{+/high}$ (figure 3.4B), whereas expression of CD205 on mTECs, defined as $CD45^{-}$ EpCAM-1^{+} $Ly51^{-}$, was minimal (figure 3.4C).

Figure 3.4 CD205 Expression Defines Cortical Epithelial Cells but not Medullary Epithelial Cells.

Thymus lobes dissected at day 15 of gestation from WT mouse embryos were placed in fetal thymus organ culture (FTOC) for 7 days. Following culture, lobes were enzymatically digested to obtain suspensions of thymic stromal cells. Cells were stained for flow cytometry for CD45, EpCAM-1, Ly51 and CD205. A typical EpCAM-1 and ly51 profile gated on $CD45^-$ cells is shown (A). Histograms show CD205 expression on gated cortical (defined as $EpCAM\text{-}1^+$ $Ly51^{+/high}$) and medullary cells (defined as $EpCAM\text{-}1^+$ $Ly51^-$) (B and C). Stromal cells were also stained for CD45, EpCAM-1 and CD205 with CD40. By gating on thymic epithelial cells (defined as $CD45^-$ $EpCAM\text{-}1^+$) (D), CD205 and CD40 expression was analysed (E). Percentages of cells within gated areas are indicated. Shaded grey areas on histograms represent the negative control. Data representative of three experiments.

Frozen tissues sections of a 4-6 week old thymus were stained for EpCAM-1, CD205 and CD40 (F). The dashed line denotes the corticomedullary junction. Bar indicates scale of 50μm.

C, cortex, M, medulla.


A
CD45-
EpCAM-1
Ly51
11
26.3
B
EpCAM+ Ly51+/high
% events
98.1
C
EpCAM+ Ly51-
12.1
CD205
D
CD45
EpCAM-1
8.05
E
CD45- EpCAM+
CD40
CD205
11.1
85.5
F
M
C
CD205
CD40
EpCAM-1

Having confirmed CD205 as a cTEC specific marker, CD205 expression was analysed in 7 day FTOCs together with the co-stimulatory marker CD40, which is expressed by all TECs in the adult and is important for the development of mTECs. Expression of CD205 and CD40 was analysed within a TEC specific population defined as $CD45^-$ $EpCAM\text{-}1^+$ (figure 3.4D). A large population of $CD45^-$ $EpCAM\text{-}1^+$ $CD40^+$ $CD205^+$ cTECs was observed with a smaller population of $CD45^-$ $EpCAM\text{-}1^+$ $CD40^+$ $CD205^-$ mTECs present (figure 3.4E).

Histological analysis of CD205, CD40 and EpCAM-1 in a 4-6 week old thymus further confirmed CD205 expression as cortical specific based on the expression pattern of the nuclear stain DAPI (DAPI staining not shown), which appears dense in the cortex (figure 3.4F). (Note: flow cytometry analysis indicates that all mature TECs are $EpCAM\text{-}1^+$ and $CD40^+$, whereas by histological analysis, EpCAM-1 and CD40 expression appears to be medullary restricted because expression levels in the cortex of these two markers are below detection point.)

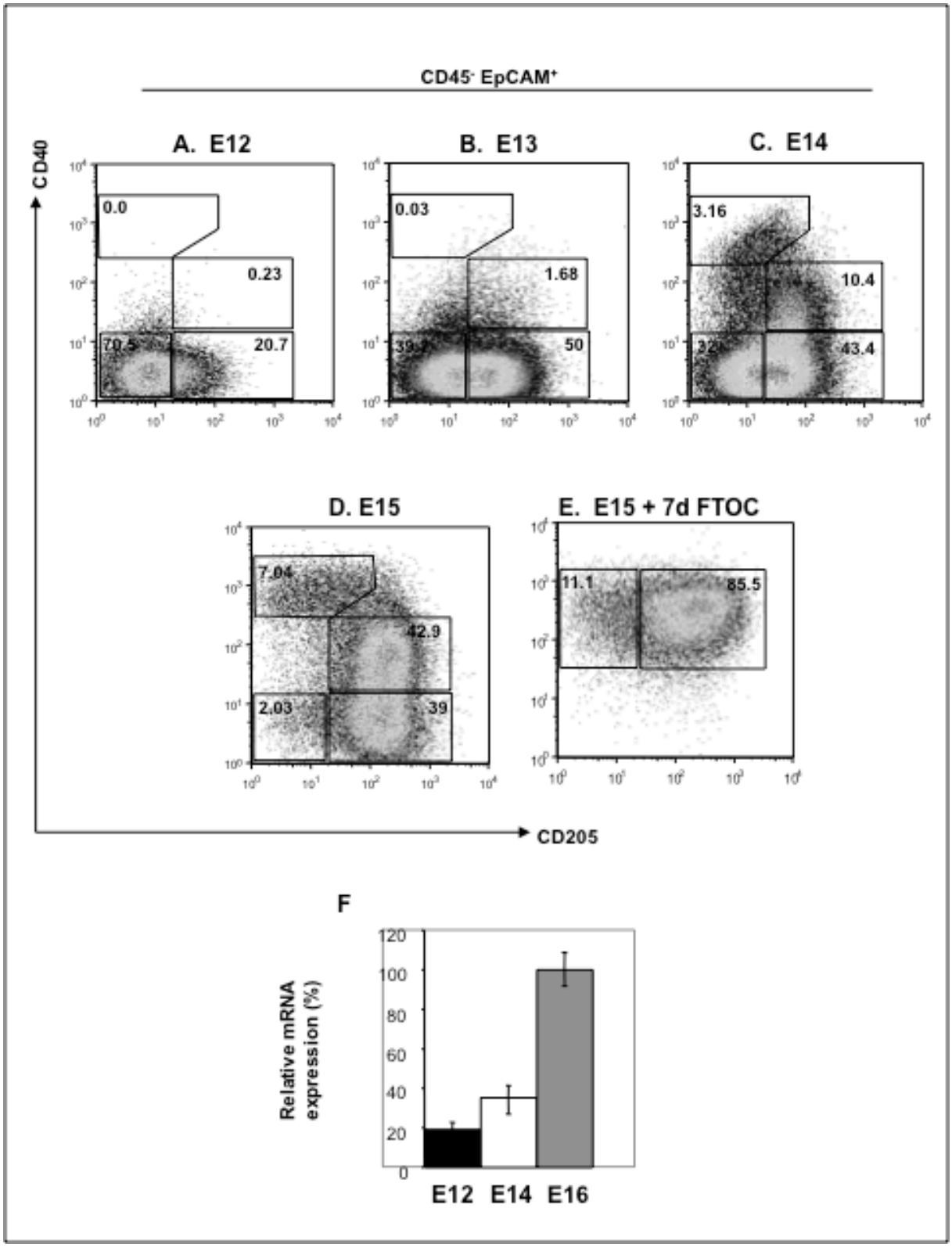
3.3.4 Ontogeny of Cortical Epithelial Cell Development in a Wildtype Thymus.

Having characterised CD205 as a cTEC marker (section 3.3.3), CD205 was then used to identify the appearance of a cortical lineage through ontogeny. Embryos at E12 were chosen as the starting point as it has previously been shown that the dominant population of cells in the E12 thymus are $EpCAM\text{-}1^+$ TECs capable of giving rise to both cTECs and mTECs (Rossi et al., 2006). To investigate the precise timing of cTEC development during ontogeny, thymic lobes were dissected from various ages of mouse embryos (E12-E15). To emulate a mature thymus E15 lobes were placed in FTOC for 7 days. Stromal cell suspensions obtained from freshly dissected and cultured lobes were stained for CD45, EpCAM-1, CD205 and CD40. By gating on $CD45^-$ $EpCAM\text{-}1^+$ TECs, CD205 and CD40

Figure 3.5 Ontogeny of Cortical Thymic Epithelial Cell Development.

Freshly isolated WT thymus lobes of the indicated ages and E15 lobes placed in FTOC for 7 days were enzymatically digested to obtain cell suspensions of thymic stromal cells. Cells were stained for flow cytometry for CD45, EpCAM-1, CD205 and CD45. CD205 and CD40 expression on $CD45^-$ $EpCAM\text{-}1^+$ thymic epithelial cells are shown for each stage of development (A-E). Percentages of cells within gated areas are indicated. Data representative of at least three experiments.

Quantitative RT-PCR of the cortical specific marker β5T, normalised to the housekeeping gene β-actin, was carried out on purified $EpCAM\text{-}1^+$ thymic epithelial cells isolated by MoFlo sorting from the indicated stages of gestation (F). PCRs were carried out in triplicate and error bars indicate the standard error of the mean. Data representative of at least two distinct sorting experiments.


CD45- EpCAM+
A. E12
B. E13
C. E14
D. E15
E. E15 + 7d FTOC
CD40
CD205
0.0
0.23
70.5
20.7
0.03
1.68
39.2
50
3.16
10.4
32
43.4
7.04
42.9
2.03
39
11.1
85.5
F
Relative mRNA expression (%)
120
100
80
60
40
20
0
E12 E14 E16

expression was analysed at all indicated stages of gestation (figure 3.5A-E). In contrast to the large population of $CD205^+$ cTECs observed in 7 day FTOCs (figure 3.4E and 3.5E) a small but detectable population of $CD205^+$ cTECs was observed at E12 (figure 3.5A). This population increased in frequency throughout ontogeny. In contrast to a mature thymus, TECs at both E12 and E13 initially emerged as $CD40^-$. However, at E14 and E15 an emergence of a small population of $CD205^-$ cells expressing high levels of CD40 was observed (figure 3.5C and D). Most striking is at both E14 and E15 distinct populations of $CD205^-$ $CD40^+$ and $CD205^+$ $CD40^-$ cells were present. At both these stages of ontogeny a gradual acquisition of CD40 expression by $CD205^+$ cTECs was also observed. By contrast, all mature TECs in 7 day FTOC lobes express CD40 (figure 3.5E). Interestingly, by using quantitative RT-PCR on total TEC populations we observed that the appearance of the small $CD205^+$ $CD40^-$ population at E12 was accompanied by expression of the cortical specific proteasome subunit β5T (figure 3.5F). Expression of the β5T gene in total TECs increased at later developmental stages (E14 and E16).

Together, the above data suggests the appearance of a cortical lineage of TECs expressing CD205 and β5T during early embryonic thymus development, with a population of medullary TECs expressing high levels of CD40 appearing later in development.

3.3.5 Requirement for FoxN1 During Cortical Thymic Epithelial Cell Development.

Nude mice have a deficiency in the FoxN1 gene, which is important for TEC development past the initial formation of the thymus. The nude phenotype results in an arrest at the immature TEC progenitor stage preventing the differentiation to the cortical and medullary lineages. Hence, nude mice lack T cells due to a non-functional thymus. To determine if FoxN1 deficiency has an effect on the development of $CD205^+$ cTECs during

ontogeny, thymi from nude mouse embryos were dissected at different stages of gestation (E12-E15) (Note: at embryonic ages 12-15 the nude thymus remains a small alymphoid rudiment) (figure 3.6). Stromal cell suspensions obtained from embryonic nude thymi were stained for CD45, EpCAM-1, CD205 and CD40. After gating on $CD45^-$ $EpCAM\text{-}1^+$ TECs, CD205 and CD40 expression was analysed and compared to age and background matched WT embryonic thymi (see figure 3.5A-E for representative examples of CD205 and CD40 expression on TECs obtained from different ages of embryonic WT mice). In contrast to a WT E12 thymus (figure 3.5A), CD205 expression was noticeably absent from E12 TECs deficient for FoxN1 (figure 3.6A) and these cells remained negative at later stages of development (figure 3.6B-D). In addition, FoxN1 deficient TECs also lacked CD40 expression. Therefore, acquisition of CD205 and CD40 during TEC development occurs in a FoxN1 dependent manner.

Furthermore, β5T expression was measured by quantitative RT-PCR in total TECs from E12 nude mice and compared to total TECs from E12 WT mice (figure 3.6E). Analysis showed the complete absence of β5T in the E12 nude thymus, further confirming the absence of a cTEC lineage in mice deficient for FoxN1.

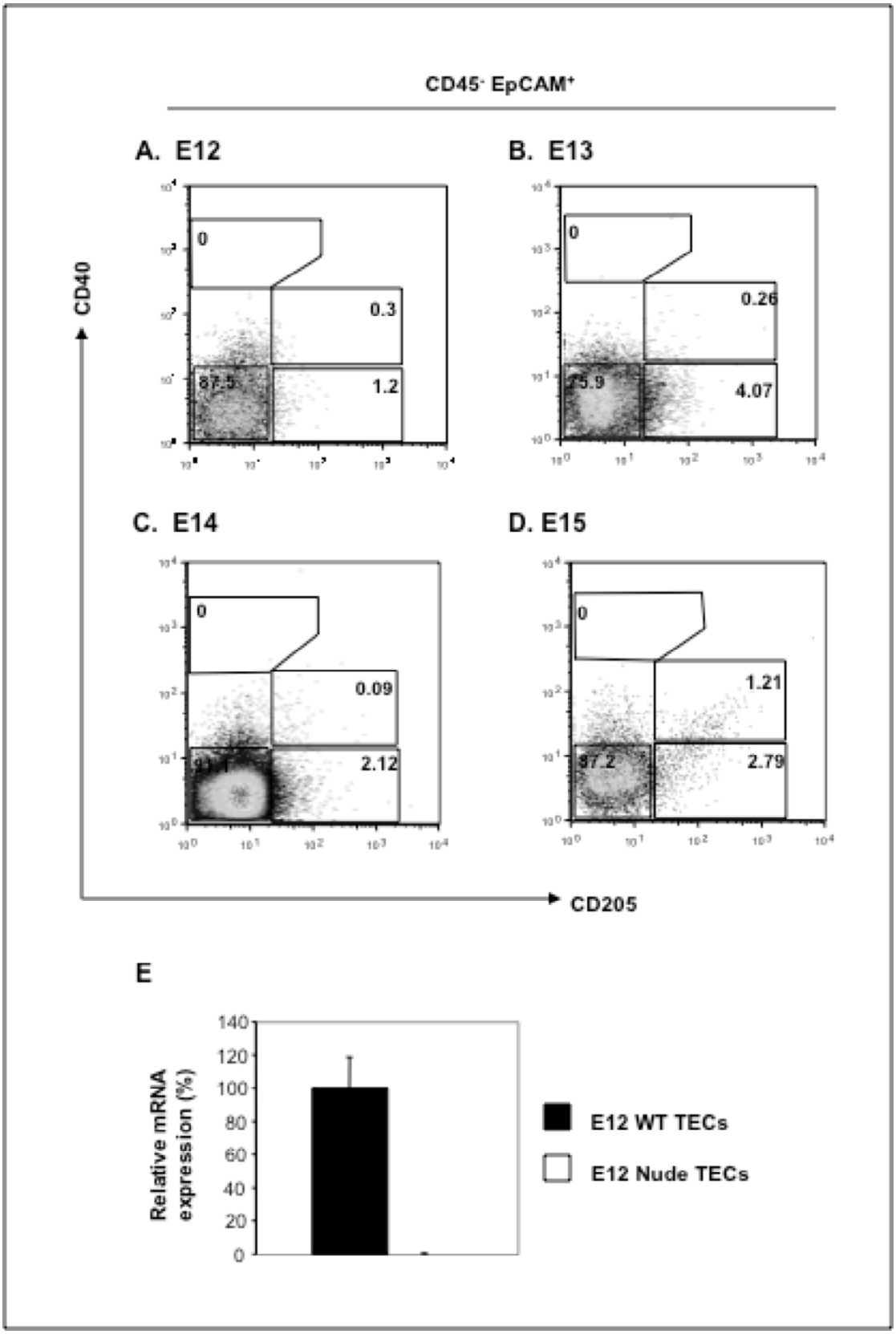
3.3.6 Genotypic Profile of Immature cTEC and mTEC Populations During Early Thymus Development.

Having identified distinct populations of $CD205^+$ $CD40^-$ cTECs and $CD205^-$ $CD40^+$ mTECs at E15, which are absent in nude mice where TEC development is perturbed, we then aimed to further characterise the genotype of these cells by analysing the expression of a panel of cTEC and mTEC associated genes by RT-PCR. Stromal cell suspensions were

Figure 3.6 Requirement for FoxN1 During Cortical Thymic Epithelial Cell Development.

Freshly isolated nude (FoxN1 deficient) thymus lobes of the indicated ages were enzymatically digested to obtain cell suspensions of thymic stromal cells. Cells were stained for flow cytometry for CD45, EpCAM-1, CD205 and CD45. CD205 and CD40 expression on $CD45^-$ $EpCAM\text{-}1^+$ thymic epithelial cells are shown for each stage of development (A-D). Percentages of cells within gated areas are indicated. Data representative of at least three experiments.

Quantitative RT-PCR of β5T, normalised to the housekeeping gene β-actin, was carried out on purified $EpCAM\text{-}1^+$ thymic epithelial cells isolated by MoFlo sorting from WT and nude mice at E12 of development (E). PCRs were carried out in triplicate and error bars indicate the standard error of the mean. Data representative of at least two distinct sorting experiments.


CD45- EpCAM+
A. E12
B. E13
C. E14
D. E15
CD40
CD205
0
0.3
87.5
1.2
0
0.26
75.9
4.07
0
0.09
2.12
0
1.21
97.2
2.79
E
Relative mRNA expression (%)
140
120
100
80
60
40
20
0
E12 WT TECs
E12 Nude TECs

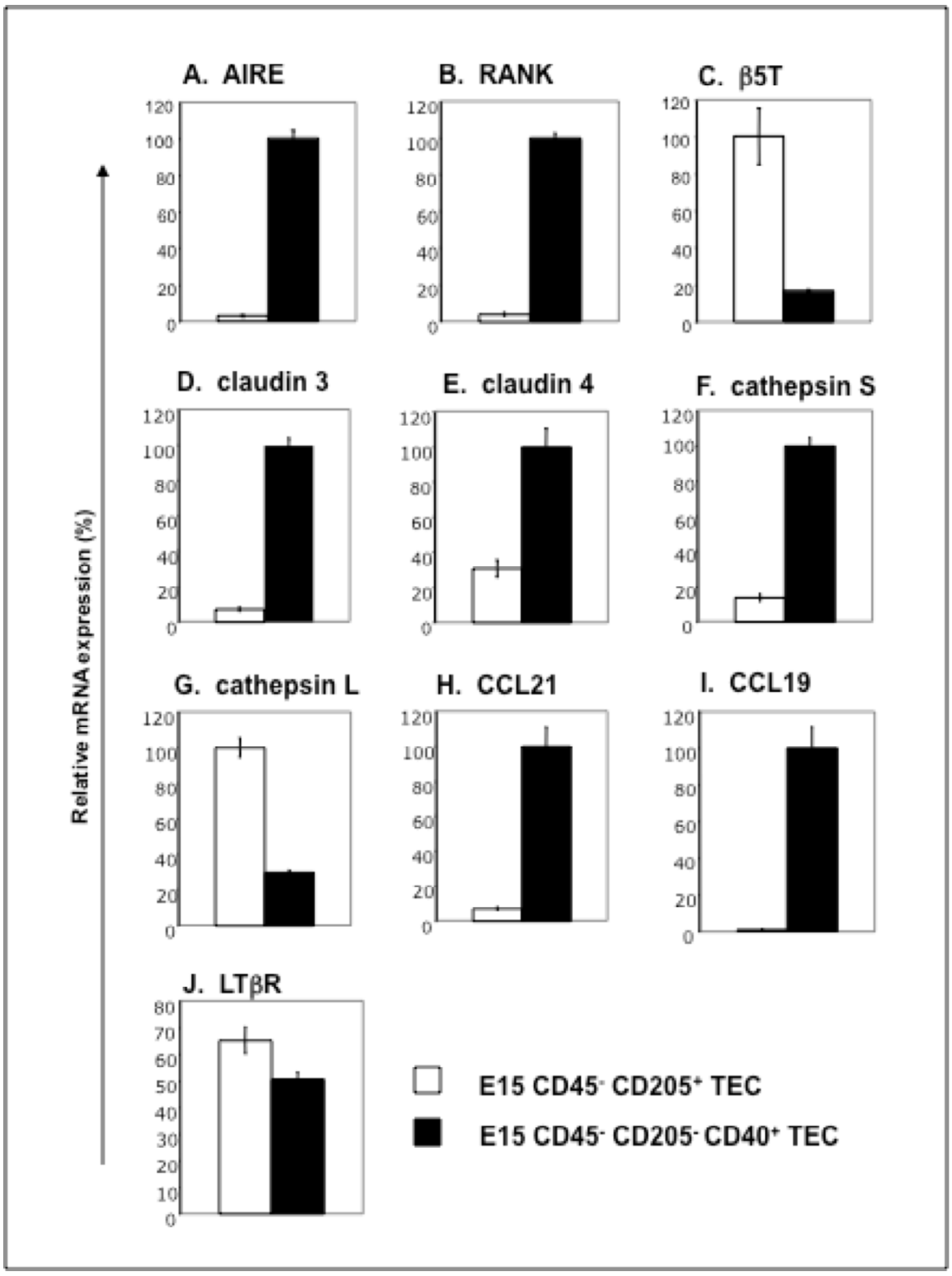
obtained from freshly dissected E15 thymus lobes. Using immunomagnetic Dynal beads, cell suspensions were depleted of $CD45^+$ haematopoietic cells to enrich for stromal cells. Also using Dynal beads, populations of $CD205^+$ and $CD40^+$ cells were positively selected for. These cells were then processed for mRNA and quantitative RT-PCR was carried out for a variety of cTEC and mTEC specific genes (figure 3.7A-J). Relative gene expression was calculated for samples normalised to the housekeeping gene β-actin.

The medullary specific autoimmune regulator gene AIRE was expressed only in the $CD40^+$ mTEC population with minimal expression detected in the $CD205^+$ cTEC population (figure 3.7A). RANK is expressed by stromal cells and its ligand RANKL is expressed by both thymic and splenic lymphoid tissue inducer cells (LTi) but not thymocytes. Signalling through RANK has been shown to be important for the development of $AIRE^+$ mTECs. In the present study RANK was highly expressed by $CD40^+$ mTECs with almost undetectable expression in the $CD205^+$ cTEC population (figure 3.7B). The cTEC associated proteasome subunit β5T was detected in the $CD205^+$ cTEC population but was markedly low in the $CD40^+$ mTEC population (figure 3.7C). Claudin 3 (Cld3) and 4 (Cld4) are tetraspannin adhesion molecules that are the main proteins that contribute to the architecture of tight junctions. A recent study identified that $AIRE^+$ mTECs are derived from a unique subset of progenitors that are $Cld3^+$ and $Cld4^+$ (Hamazaki et al., 2007). In this study we found that both genes are expressed at high levels by $CD40^+$ mTECs with minimal expression in the $CD205^+$ cTEC population (figure 3.7D and E). Cathepsins S and L are cysteine proteases that are differentially expressed on APCs and play key roles in MHC II presentation by aiding the degradation of invariant chains. Whilst cathepsin S expression is typically found on bone marrow derived APCs, cathepsin L expression in the thymus is restricted to cTECs. In this study we identified cathepsin S expression predominantly in the $CD40^+$ mTECs (figure 3.7F),

Figure 3.7 Genotypic Profile of Immature cTEC and mTEC Populations During Early Thymus Development.

Quantitative RT-PCR was used to compare relative mRNA expression of a panel of thymic epithelial cell expressed genes associated with the cortical and medullary lineages (A-J). The relative mRNA levels, normalized to the housekeeping gene β-actin, was assessed from E15 CD45 depleted TECs subdivided into $CD205^{+}$ cortical cells and $CD205^{-}$ $CD40^{+}$ medullary cells obtained following immunomagnetic bead separation.

PCRs were carried out in triplicate and error bars indicate the standard error of the mean. Data representative of at least two distinct sorting experiments.


A. AIRE
B. RANK
C. β5T
D. claudin 3
E. claudin 4
F. cathepsin S
G. cathepsin L
H. CCL21
I. CCL19
J. LTβR
Relative mRNA expression (%)
E15 CD45- CD205+ TEC
E15 CD45- CD205- CD40+ TEC

whereas cathepsin L was most highly expressed in the $CD205^+$ cTEC population (figure 3.7G). CCL19 and CCL21 are chemokines important in the later stages of T cell development and are chemoattractive for single positive thymocytes. In this study both chemokines were highly expressed in $CD40^+$ mTECs with almost undetectable levels in the $CD205^+$ cTEC population (3.7H and I).

Lymphotoxin β receptor (LTβR) is expressed by stromal cells and is involved in lymphotoxin signalling, which is important for activating cytokines and chemokines and other growth factors that are important in maintaining the various lymphoid microenvironments. Interestingly, in this study LTβR was most highly expressed by $CD205^+$ cTECs with high expression also detected in the $CD40^+$ mTECs (figure 3.7J).

To summarise, our genotypic data suggest that the $CD205^+$ population at E15 express cTEC associated genes, whereas the $CD40^+$ population express a panel of mTEC associated genes. Collectively, data discussed thus far identifies $CD205^+$ cells at E15 as putative cTEC progenitors. Further experiments were carried out in order to demonstrate this.

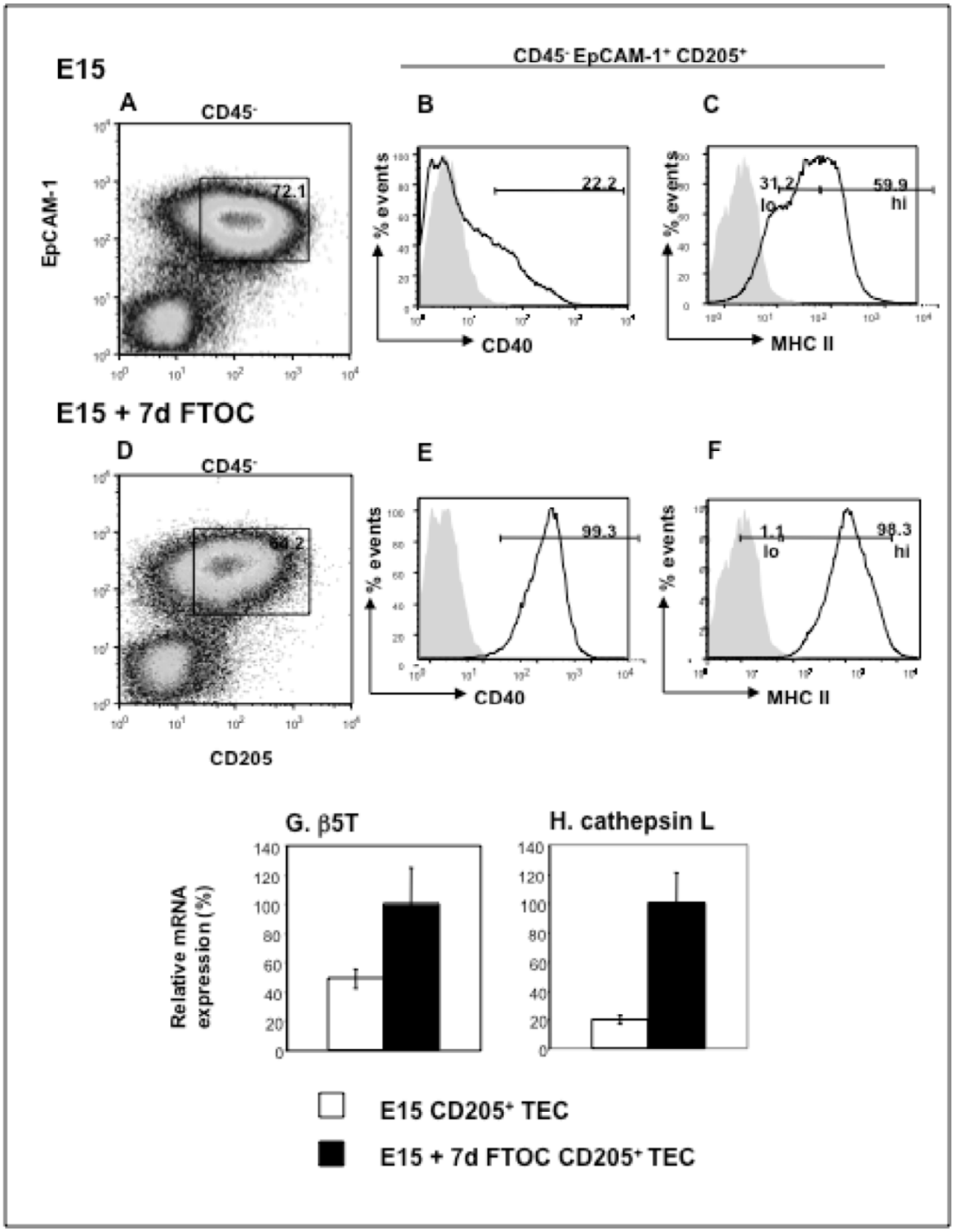
3.3.7 Identification of a Putative Cortical Thymic Epithelial Cell Progenitor.

We propose that $CD205^+$ cells in the E15 thymus represent immature cTECs that later differentiate into mature $CD205^+$ cTECs. Should this be correct, such immature and mature $CD205^+$ cells should show a precursor-product relationship, which has previously been demonstrated during mTEC development. Acquisition of CD40 expression by $CD205^+$ cTECs correlates with an increase in thymus maturity during ontogeny (section 3.3.4). In addition, MHC II expression can also be used as an indication of TEC maturity. Therefore, to identify if $CD205^+$ cTECs in an E15 thymus represent putative progenitors of more mature

Figure 3.8 Identification of a Putative Cortical Thymic Epithelial Cell Progenitor.

Freshly isolated E15 WT thymus lobes and E15 lobes placed in fetal thymus organ culture (FTOC) for 7 days were enzymatically digested to obtain cell suspensions of thymic stromal cells. Cells were stained for flow cytometry for CD45, EpCAM-1 and CD205. CD40 and MHC II expression on $CD45^-$ $EpCAM\text{-}1^+$ $CD205^+$ cells are shown for both immature cortical cells (A-C) and mature cortical cells (D-F). Percentages of cells within gated areas are indicated. Shaded grey areas on histograms represent the negative control. Data representative of at least three experiments. hi, high, lo, low.

By quantitative RT-PCR the relative mRNA levels of β5T and cathepsin L, normalised to the housekeeping gene β-actin was measured in E15 $CD45^-$ $CD205^+$ cortical cells and compared to $CD45^-$ $EpCAM\text{-}1^+$ $CD205^+$ cortical cells from 7d FTOC (G and H). PCRs were carried out in triplicate and error bars indicate the standard error of the mean. Data representative of at least two distinct sorting experiments.


E15
CD45⁻ EpCAM-1⁺ CD205⁺
A
CD45⁻
EpCAM-1
72.1
B
% events
22.2
CD40
C
% events
31.2
lo
59.9
hi
MHC II
E15 + 7d FTOC
D
CD45⁻
CD205
E
% events
99.3
CD40
F
% events
1.1
lo
98.3
hi
MHC II
G. β5T
H. cathepsin L
Relative mRNA expression (%)
140
120
100
80
60
40
20
0
E15 CD205⁺ TEC
E15 + 7d FTOC CD205⁺ TEC

$CD205^+$ cTECs, CD40 and MHC II expression was analysed in E15 $CD205^+$ cTECs and compared to mature $CD205^+$ cTECs from 7 day FTOCS (figure 3.8). Stromal cell suspensions from freshly dissected E15 lobes and organ-cultured lobes were stained for CD45, EpCAM-1, CD205 and either CD40 or MHC II. CD40 or MHC II expression was analysed in a cTEC population defined as $CD45^-$ $EpCAM\text{-}1^+$ $CD205^+$ in both E15 and FTOC lobes (3.8A and D). Whilst a small proportion of cTECs at E15 expressed CD40 (figure 3.8B), mature cTECs from 7 day FTOCs were all positive for CD40 (figure 3.8E). Similarly, cTECs from E15 showed a relatively heterogeneous expression of MHC II with the presence of cells expressing low levels of MHC II (figure 3.8C). However, cTECs from 7 day FTOCs were all expressing high levels of MHC II with a notable reduction in the proportion of cells expressing low levels of MHC II (figure 3.8F).

We subsequently compared expression of the cTEC associated genes β5T and cathepsin L in $CD205^+$ cells sorted from both E15 and 7 day FTOC lobes. Quantitative RT-PCR analysis showed that mature $CD205^+$ cTECs from 7 day FTOCs expressed higher levels of both genes compared to immature $CD205^+$ cTECs from E15 lobes (figure 3.8G and H).

Collectively, the data presented above confirms that $CD205^+$ cells at E15 are immature cTEC progenitors that increase in maturity to become mature $CD205^+$ cTECs expressing high levels of CD40, MHC II, β5T and cathepsin L.

3.3.8 Induction of MHC II Expression and Proliferative Status Define Distinct Stages During cTEC Development.

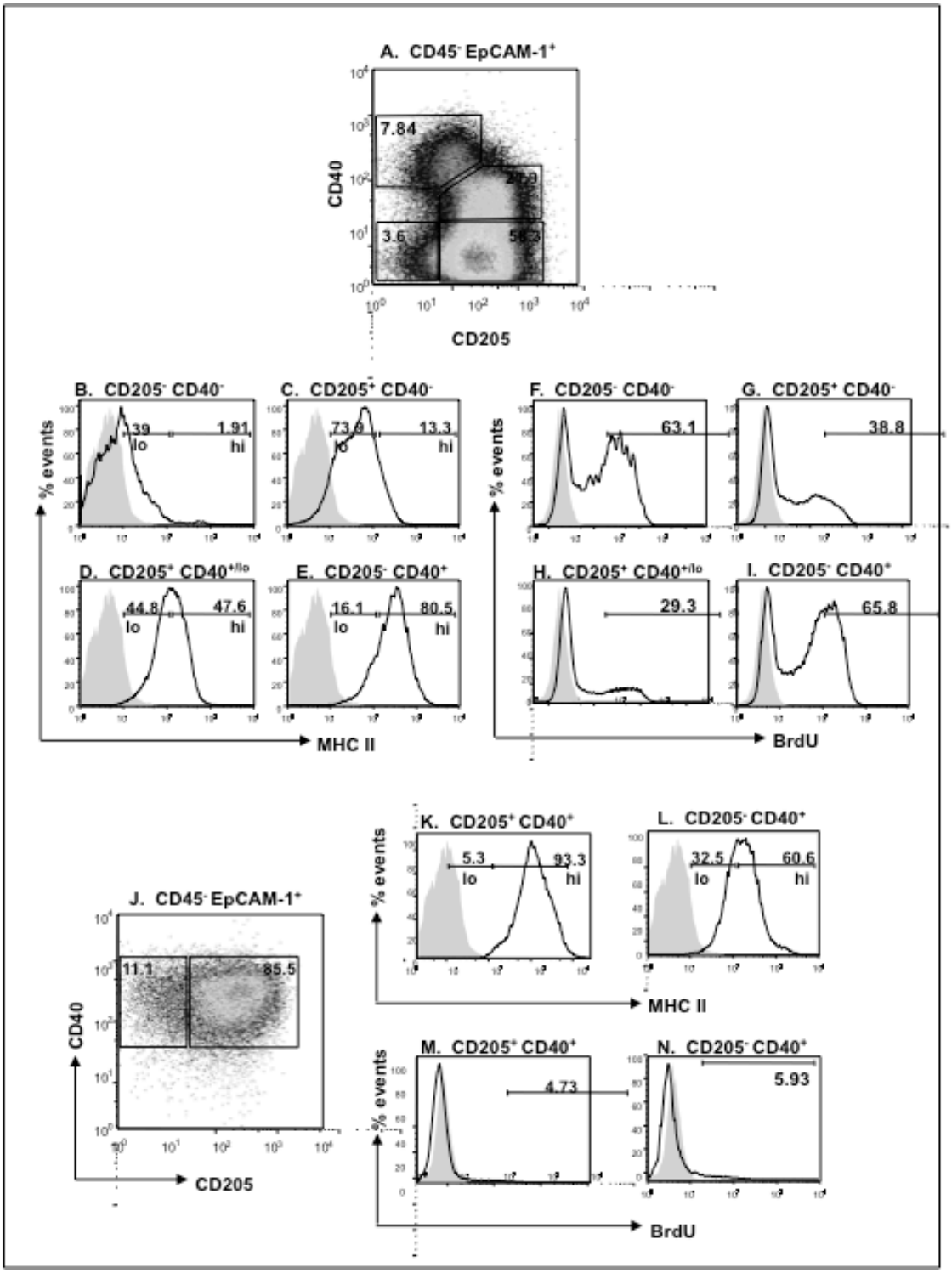
As described above, the acquisition of MHC molecules during TEC development is indicative of their maturity and is essential for their ability to mediate positive selection of the developing TCR repertoire. Hence, MHC II expression has previously been used to identify

precursor-product relationships within the mTEC lineage (Gray et al., 2006). However, in the context of cTEC development changes in MHC II expression are unclear. To investigate this, MHC II expression was measured in developing TEC populations at E15 (3.9B-E) and compared to mature TECs in FTOCs (figure 3.9K and L). In contrast to the uniform high levels of MHC II in mature $CD205^+$ cTECs in 7d FTOC (figure 3.9K), $CD205^-$ $CD40^-$ TECs at E15 are largely negative for MHC II (figure 3.9B). However, $CD205^+$ $CD40^-$ cTECs display heterogeneity with regard to their MHC II expression with the presence of an $MHC\ II^{-/low}$ subset still evident (figure 3.9C). Following the up-regulation of CD40 expression, the $CD205^+$ cTECs become predominantly $MHC\ II^+$ (figure 3.9D). Interestingly, this data also suggests that MHC II induction occurs prior to CD40 expression.

TECs are known to undergo phases of proliferation during their development to aid their expansion (Gray et al., 2006; Jenkinson et al., 2003) but how this relates to the development of the cTEC lineage is unknown. In order to investigate this, freshly isolated E15 thymus lobes and E15 thymus lobes placed in FTOC for 7 days were pulsed with BrdU overnight. Incorporation of BrdU in dividing cells was measured by flow cytometry in developing TEC populations at E15 (figure 3.9F-I) and compared to mature TECs in FTOCs (figure 3.9M and N). Analysis of the proliferative status of the different TEC subsets at E15 showed that the majority of $CD205^-$ $CD40^-$ TECs incorporate BrdU suggesting that these cells are highly proliferating (figure 3.9F). Acquisition of CD205 and then CD40 expression correlated with a decline in the proliferative status of these cells (figure 3.9G and H). In marked contrast, mature $CD205^+$ $CD40^+$ cells in 7 day FTOCs do not incorporate BrdU and so indicates that these cells are quiescent non-dividing cells (figure 3.9M).

Figure 3.9 MHC II Expression and Proliferative Status Defines Distinct Stages During Cortical Thymic Epithelial Cell Development.

Freshly isolated E15 WT thymus lobes and E15 lobes placed in FTOC for 7 days were enzymatically digested to obtain cell suspensions of thymic stromal cells. MHC II expression (B-E, K and L) and BrdU incorporation (F-I, M and N) was analysed in $CD45^-$ $EpCAM\text{-}1^+$ TECs subdivided on the basis of CD205 and CD40 expression in E15 (A) and 7d FTOC (J) lobes. Histograms shown are generated by gating on CD40 and CD205 subsets of $EpCAM\text{-}1^+$ TECs depleted of $CD45^+$ cells using magnetic beads prior to staining. Shaded grey areas represent the negative controls and numbers in histograms indicate percentage of cells within the gates. Data representative of at least three separate experiments.


A. CD45- EpCAM-1+
7.84
3.6
CD40
CD205
B. CD205- CD40-
1.39
lo
1.91
hi
C. CD205+ CD40-
73.9
lo
13.3
hi
F. CD205- CD40-
63.1
G. CD205+ CD40-
38.8
% events
D. CD205+ CD40+/lo
44.8
lo
47.6
hi
E. CD205- CD40+
16.1
lo
80.5
hi
H. CD205+ CD40+/lo
29.3
I. CD205- CD40+
65.8
MHC II
BrdU
J. CD45- EpCAM-1+
11.1
85.5
CD40
CD205
K. CD205+ CD40+
5.3
lo
93.3
hi
L. CD205- CD40+
32.5
lo
60.6
hi
% events
MHC II
M. CD205+ CD40+
4.73
N. CD205- CD40+
5.93
% events
BrdU

MHC II expression and BrdU incorporation was also measured in the CD205^{-} CD40^{+} immature and mature mTEC population at E15 and in 7d FTOC lobes respectively. Interestingly, whilst we show that immature CD205^{-} CD40^{+} mTECs are rapidly proliferating as illustrated by the high incorporation of BrdU (figure 3.9I), a majority of these cells are expressing high levels of MHC II (figure 3.9E). Whilst the more mature CD205^{-} CD40^{+} mTECs from cultured lobes have retained their high levels of MHC II (figure 3.9L), they have lost their proliferative capabilities as indicated by their lack of BrdU incorporation (figure 3.9N).

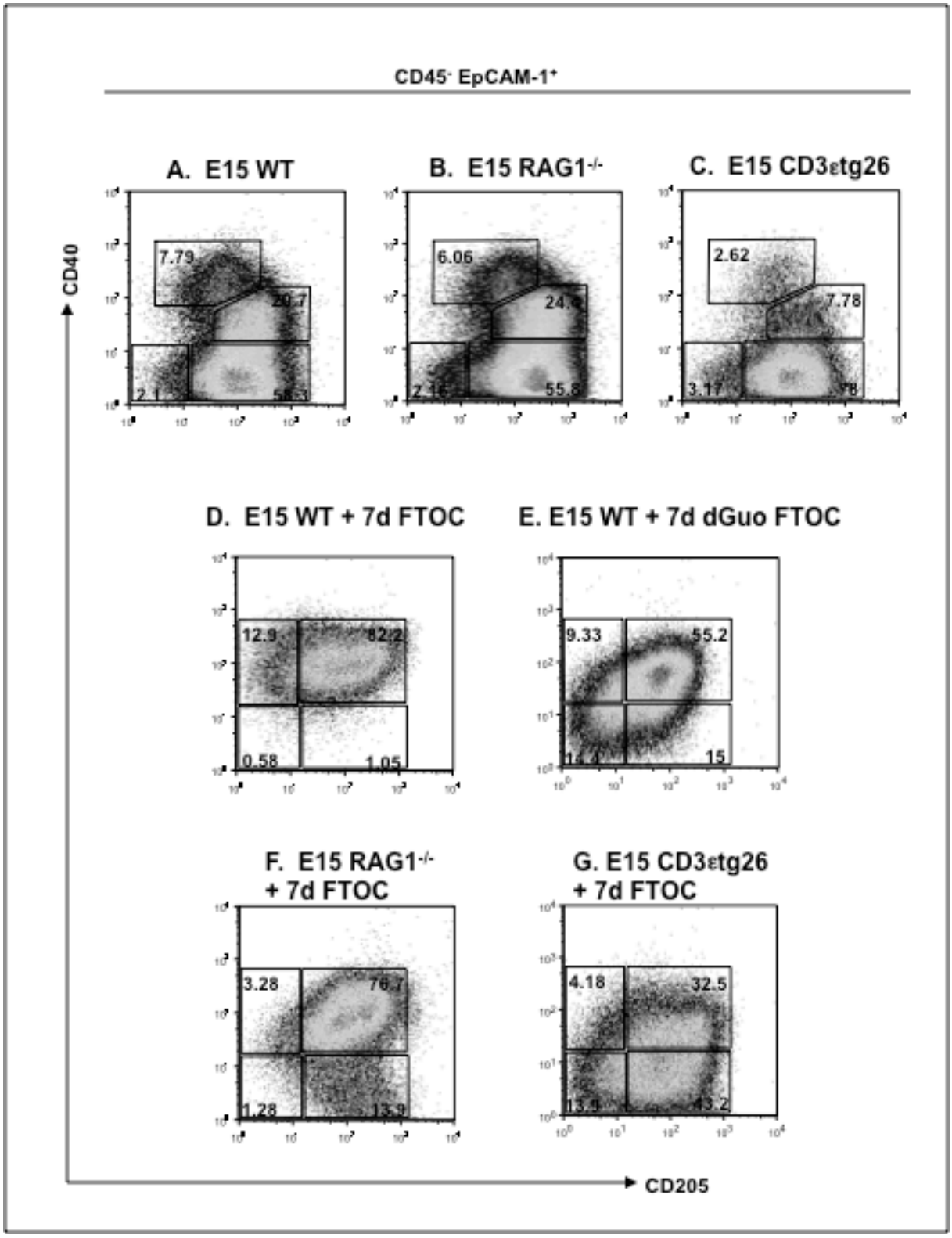
To summarise, the above data thus demonstrates that the sequential acquisition of CD205 and CD40 during cTEC development correlates with a decline in proliferation as indicated by BrdU incorporation and also an increase in maturity as indicated by MHC II expression.

3.3.9 Thymocytes Are Required For Induction of CD40 Expression On Thymic Epithelial Cells.

Lymphostromal interactions are important in the maintenance of thymic microenvironments via a process of thymic crosstalk. Therefore, we aimed to determine if the absence of thymocytes affects the expression of CD205 and CD40 on TECs by investigating the development of these cells in mice with blocks in thymocyte development at different stages. CD205 and CD40 expression on TECs was analysed in RAG1$^{-/-}$ mice where thymocyte development is blocked at the double negative 3 (DN3) stage due to an absence of TCR gene rearrangement. We also analysed TEC development in CD3εtg26 mice, which via an unknown mechanism, display a block at the very earliest stage of thymocyte development (DN1) (Wang et al., 1995).

Figure 3.10 Thymocytes Are Required For Induction of CD40 Expression On Thymic Epithelial Cells.

Freshly isolated E15 thymus lobes and E15 lobes placed in fetal thymus organ culture (FTOC) for 7 days from WT, $RAG1^{-/-}$ and CD3εtg26 mice were enzymatically digested to obtain cell suspensions of thymic stromal cells. 7d FTOCs supplemented with 2-deoxyguanisine (2-dGuo) to eliminate lymphoid cells were also analysed. CD205 and CD40 expression was analysed in $CD45^{-}$ $EpCAM\text{-}1^{+}$ TECs (A-G). Percentages of cells within gated areas are indicated. Data representative of at least three experiments.


CD45- EpCAM-1+
A. E15 WT
B. E15 RAG1-/-
C. E15 CD3εtg26
CD40
7.79
20.7
2.1
58.3
6.06
24.9
2.16
55.8
2.62
7.78
3.17
78
D. E15 WT + 7d FTOC
E. E15 WT + 7d dGuo FTOC
12.9
82.2
0.58
1.05
9.33
55.2
14.4
15
F. E15 RAG1-/-
+ 7d FTOC
G. E15 CD3εtg26
+ 7d FTOC
3.28
76.7
1.28
13.9
4.18
32.5
13.9
43.2
CD205

To assess the contribution of thymocytes to the development of mature cTEC and mTECs, 7d FTOC lobes were treated with 2-deoxyguanisine (2-dguo), which selectively eliminates all lymphoid cells (figure 3.10E). CD205 and CD40 expression on $CD45^-$ $EpCAM\text{-}1^+$ TECs was measured and data reveals that whilst CD205 levels are relatively normal compared to 7d FTOC cultured in the absence of 2-dguo (figure 3.10D), CD40 levels are low.

At E15, proportions of $CD205^+$ $CD40^-$ cTECs were normal in $RAG1^{-/-}$ (figure 3.10B) mice and higher in CD3εtg26 mice (figure 3.10C) compared to a WT (3.10A). However, whilst $RAG1^{-/-}$ mice show a relatively normal level of expression of CD40, there was a marked reduction of TECs expressing CD40 in CD3εtg26 mice. In contrast to WT 7 day FTOCS (figure 3.10D), E15 $RAG1^{-/-}$ 7 day FTOC lobes showed a slight decrease in CD40 expression on TECs with the presence of a small population of $CD40^-$ cells (figure 3.10F). E15 CD3εtg26 7 day FTOC lobes showed a much larger decrease in TECs expressing CD40 with the presence of a large population of $CD40^-$ cells (figure 3.10G).

3.3.10 Stage Specific Requirement for Thymocyte Crosstalk During Later Stages of cTEC Development.

As illustrated in section 3.3.9, thymocytes are important during TEC development. However, with regard to the development of the cortex, the precise thymocyte subsets and the stages of cTEC development they regulate are unclear. Therefore, we aimed to further analyse cTEC development in mutant mice in which thymocyte development is blocked at different stages (figure 3.11). Stromal cell suspensions were obtained from E15 background matched WT, $RAG1^{-/-}$ and CD3εtg26 mice and analysed for CD45, EpCAM, CD205 and either CD40 or MHC II expression and BrdU incorporation. Levels of CD40, MHC II and

BrdU incorporation were measured in the cTEC population defined as CD45$^-$ EpCAM-1$^+$ CD205$^+$. By comparison to E15 WT, EpCAM-1$^+$ CD205$^+$ cTECs are present in both E15 RAG1$^{-/-}$ and CD3εtg26 mice at similar proportions (figure 3.11A-C). However, whilst EpCAM-1$^+$ CD205$^+$ cTECs from E15 RAG1$^{-/-}$ mice show further evidence of maturation as demonstrated by the similar levels of CD40 and MHC II expression compared to WT (figure 3.11E and H), EpCAM1$^+$ CD205$^+$ cTECs from E15 CD3εtg26 mice show a dramatic absence of cells expressing both CD40 and MHC II (figure 3.11F and I). This data suggests that whilst the initial stages of cTEC development occur independently of thymocytes, the presence of DN1-DN3 cells are essential for further maturation of CD205$^+$ cTEC.

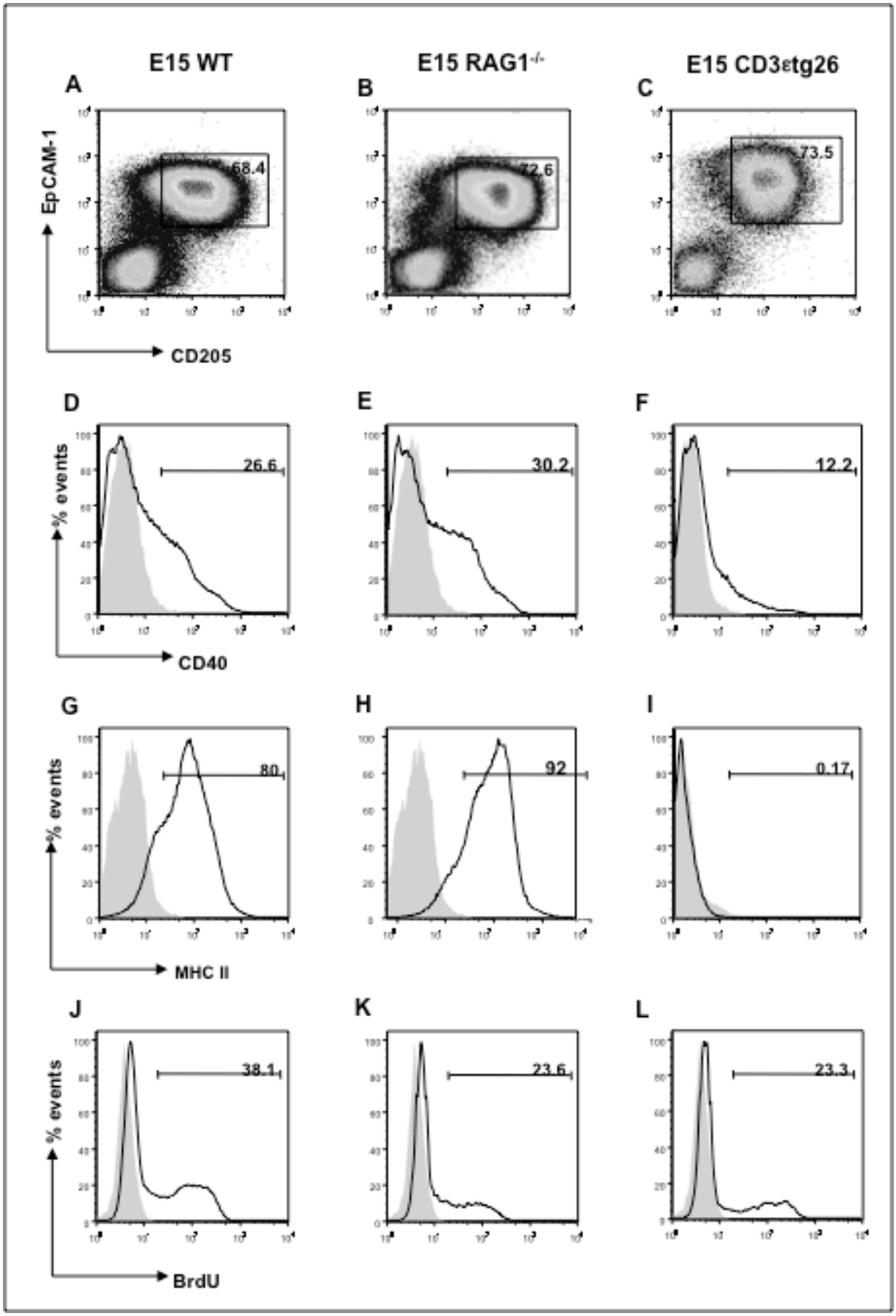
Proliferative status of EpCAM1$^+$ CD205$^+$ cTECs was also analysed in both mutant mice and interestingly the presence of thymocytes are not required for the proliferation of these cells as indicated by the incorporation of BrdU, albeit at lower levels compared to WT (figure 3.11J-L).

3.4 Discussion.

The development of cTECs is an essential step in the formation of a functional thymic microenvironment capable of supporting early stages of T cell development and positive selection of developing thymocytes. cTECs derive from common bTECs, which are known to also give rise to TEC cells of the medullary lineage (Rossi et al., 2006). By contrast to the advances made with regard to mTEC development (as outlined in section 1.3.6), mechanisms and stages of bTEC differentiation into cTECs have not previously been identified. Therefore, the aim of this chapter was to characterise cTEC development

Figure 3.11 Requirement for thymocyte crosstalk during later stages of cortical thymic epithelial cell development.

Freshly isolated E15 thymus lobes from WT, RAG1$^{-/-}$ and CD3εtg26 mice were enzymatically digested to obtain cell suspensions of thymic stromal cells. CD40 and MHC II expression and BrdU incorporation was measured in gated CD45^{-} EpCAM-1^{+} CD205^{+} cortical cells (A-C). Histograms (D-L) shown are generated by gating on EpCAM-1^{+} CD205^{+} cTECs depleted of CD45^{+} cells using magnetic beads prior to staining. Shaded grey areas represent the negative controls and numbers in histograms indicate percentage of cells within the gates. Data representative of at least three separate experiments.


E15 WT
E15 RAG1-/-
E15 CD3εtg26
A
B
C
EpCAM-1
CD205
68.4
72.6
73.5
D
E
F
% events
CD40
26.6
30.2
12.2
G
H
I
% events
MHC II
80
92
0.17
J
K
L
% events
BrdU
38.1
23.6
23.3

downstream of bipotent progenitors and to identify molecular and cellular regulators of this pathway. Based on the data presented in this chapter, we now propose a new model of cTEC development as well as providing novel evidence for the existence of a cTEC progenitor (figure 3.12 summarises the data presented in this chapter as a proposed model of cTEC development).

In our study we used the endocytic receptor CD205 to identify cells of the cortical lineage. CD205 is also expressed by DCs, which shares a common feature with cTECs in their ability to present MHC-bound peptides to T cells. However, by contrast to DCs, instead of inducing T cell activation or negative selection, cTECs induce positive selection. Expression of CD205 by cTECs reflects their functional specialisation as efficient mediators of positive selection. By employing an ontogenetic approach, we have identified a previously unreported population of cortical epithelial cells expressing CD205 in the early embryo. These $CD205^+$ cTECs in the early embryo differ from more mature $CD205^+$ cTECs with regard to their expression of the co-stimulatory molecule CD40 and MHC II. Moreover, these immature $CD205^+$ cortical cells in the early embryo have lower levels of expression of the cortical associated genes β5T and cathepsin L and are highly enriched for proliferating cells. These findings provide convincing evidence for a possible precursor-product relationship where mature $CD205^+$ cTECs develop from immature $CD205^+$ cTECs. Herein, we propose that $CD205^+$ cells in the early embryo represent a cTEC progenitor population that develops downstream of bTEC progenitors and later develops into mature cTECs.

It was previously thought that at day 12 of embryonic gestation, TEC cells are homogenous and mostly represent cells at the bipotent stage of development, which have not yet differentiated towards a cTEC and mTEC lineage (Klug et al., 2002; Rossi et al., 2006). Interestingly, in our ontogeny experiment we observed the presence of $CD205^+$ cTECs at E12

of development, which correlated with the expression of β5T, thereby reflecting heterogeneity at this early stage of thymus development. Moreover, preliminary experiments in our lab have demonstrated further heterogeneity with regard to expression of the cTEC marker ly51 with the presence of a population of $Ly51^{+}$ $CD205^{-}$ and $Ly51^{-}$ $CD205^{+}$ cells (data not shown). Collectively, this data implies that the cTEC lineage appears during thymus ontogeny earlier than previously thought. Experiments are currently underway in our lab to investigate if the appearance of the cTEC lineage occurs before E12 by dissecting out the pharyngeal pouch, containing the thymus rudiment, from E11 FoxN1-GFP transgenic mice and looking for CD205 expression.

We have also demonstrated that the initial appearance of $CD205^{+}$ cTECs expressing β5T at E12 occurs in a FoxN1 dependent manner. Nude mice, which have a deficiency in the FoxN1 gene, displayed a notable absence of the cTEC lineage at E12 and this remained through to E15. This finding confirms the notion that FoxN1 deficient TECs are likely to represent bTECs that are unable to differentiate into the cortical and medullary lineages (Bleul et al., 2006).

Whilst a normal programme of T cell development is thought to play a role in cTEC development (Hollander et al., 2006), it was previously unclear which thymocyte subsets are involved and the stages of cTEC development they regulate. Our findings demonstrate for the first time the stage specific requirement for thymocyte crosstalk. In our study we used CD3εtg26 mice, which due to over-expression of human CD3ε gene, have a block at the earliest stage in T cell development ($CD4^{-}$ $CD8^{-}$ $CD44^{+}$ $CD25^{-}$, DN1) (Hollander et al., 1995). Previous studies have shown that these mice have a small thymus with severe abnormalities in cortical and medullary development and organisation and a poor demarcation between these two regions (Hollander et al., 1995; Wang et al., 1994; Wang et al., 1995). In our study we

also looked at RAG1 deficient mice where T cell development proceeds to a later stage ($CD4^-$ $CD8^-$ $CD44^-$ $CD25^+$, DN3). It has previously been shown that the thymus in these mice show a relatively well developed cortex but abnormal medullary architecture (Wang et al., 1995). In the present study we demonstrate that thymocyte derived signals are not required for the initial development of the cortex, as illustrated by the normal expression of $CD205^+$ cells in mice with perturbed thymocyte development (both $RAG1^{-/-}$ and CD3εtg26 mice) and this is in line with previous reports that cTEC development can occur in the absence of a normal programme of T cell development (Gray et al., 2006). However, further analysis of the $CD205^+$ population revealed that DN1-DN3 thymocytes are required for the further maturation and/or maintenance of cTECs as defined by the lack of CD40 and MHC II expression in mice with a block at the earliest stage of T cell development (CD3εtg26 mice). However, it is interesting to note that TECs were still able to undergo proliferation, although to a lesser extent than WT, suggesting a normal programme of T cell development is not essential for the proliferation of developing cTECs. It is currently unclear precisely how DN thymocytes may influence cTEC development. However, it is known that DN cells play a role in cTEC remodelling and for the formation of the typical three-dimensional reticular arrangement of cTECs (van Ewijk et al., 2000).

The failure of CD3εtg26 mice, to acquire CD40 expression on the cortical $CD205^+$ cells and the small proportion of $CD40^+$ medullary cells in the early thymus and organ cultured thymus of these mice demonstrates the importance of thymocyte derived signals in CD40 expression. CD40 belongs to a family of TNF receptors and signals via the NFκB pathway to mediate mTEC development (Gray et al., 2006). CD40 binds to CD40L expressed by single positive CD4 (SP4) thymocytes, which induce signals for mTEC development by possibly regulating $AIRE^+$ mTEC turnover and/or survival (White et al.,

2008). Whilst our study shows that CD40 is induced on cTECs and thymocyte derived signals are important for this, it is not clear what role CD40 signalling has in the cortex with no previous reports of severe cTEC abnormalities in $CD40^{-/-}$ mice having been identified so far (Akiyama et al., 2008).

This study also demonstrates for the first time that different stages during cTEC development have different proliferative capacities. Progenitor populations rely on their proliferation for their maintenance and phenotypic differentiation. Immature $CD205^{+}$ cTECs are a rapidly proliferating population of cells, whereas more mature $CD205^{+}$ cTECs do not have any proliferative capacity. Whilst we show that TEC proliferation is thymocyte independent, it is currently unclear what is regulating this proliferation. However, mesenchyme could be a likely candidate as studies have shown that these cells provide fibroblast growth factors such as FGF7 and 10 to induce proliferation of TECs at E12 of development (Jenkinson et al., 2003).

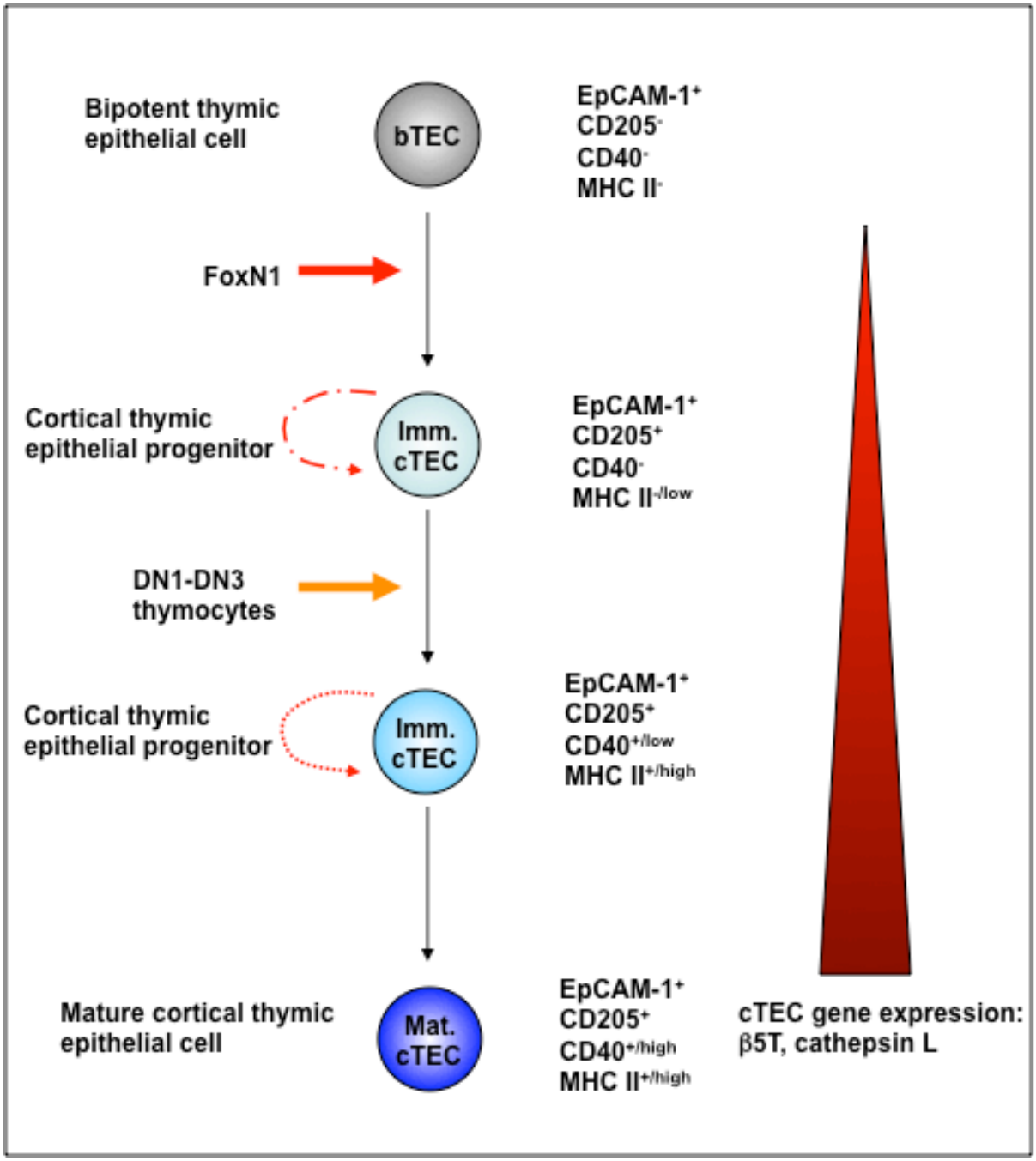
In summary, the data presented in this chapter defines stages in cTEC development and identifies important checkpoints in the development of the cortical lineage. This data should help in understanding how these cells become specialised in their ability to support positive selection.

Figure 3.12 A New Model of Cortical Thymic Epithelial Cell Development.

Figure summarises data presented in this chapter to provide a new model of cortical thymic epithelial cell (cTEC) development where a bipotent thymic epithelial cell (bTEC) develops into a mature cTEC via cTEC progenitor populations expressing CD205.

Circular arrows indicate levels of proliferation. Red and orange arrows indicates requirement for FoxN1 and thymocytes respectively. Also indicated is the increase in expression of cTEC genes through cTEC development.

Imm, immature, mat, mature.


Bipotent thymic epithelial cell
bTEC
EpCAM-1+
CD205-
CD40-
MHC II-
FoxN1
Cortical thymic epithelial progenitor
Imm. cTEC
EpCAM-1+
CD205+
CD40-
MHC II-/low
DN1-DN3 thymocytes
Cortical thymic epithelial progenitor
Imm. cTEC
EpCAM-1+
CD205+
CD40+/low
MHC II+/high
Mature cortical thymic epithelial cell
Mat. cTEC
EpCAM-1+
CD205+
CD40+/high
MHC II+/high
cTEC gene expression:
β5T, cathepsin L

CHAPTER 4

ESTABLISHMENT OF THYMIC MICROENVIRONMENTS AND A ROLE FOR DOCK2 IN THE THYMUS

4.1 Introduction.

Intra-thymic development of T cells is highly stringent and is defined by the migration of developing thymocytes through discrete regions of the thymus where immature T cell precursors receive signals from thymic stromal cells for differentiation, survival and selection. The stratified positioning of thymocyte subsets within the thymus forms distinct and specialised thymic microenvironments. The localisation of double positive (DP) thymocytes in the cortex is essential to support positive selection of developing thymocytes via their low affinity interactions with self-peptide/MHC complexes presented by cortical thymic epithelial cells (cTECs) (Anderson and Jenkinson, 2001). By contrast, migration and localisation of single positive (SP) thymocytes to the medulla is necessary to support the later stages of T cell development where potentially auto-reactive T cells are deleted via a process of negative selection mediated at least in part by medullary thymic epithelial cells (mTECs) (Takahama, 2006). Despite these clearly defined roles for thymocyte localisation to the cortex and medulla, the importance of the subcapsular zone (SCZ) during T cell development is yet to be fully defined. The SCZ is the region that lies directly adjacent to the capsule and is known to house developing thymocytes at the double negative 3 (DN3) stage of development (defined as $CD4^-$ $CD8^-$ $CD25^+$ $CD44^-$) (Lind et al., 2001). This region provides a suitable microenvironment to support proliferation of DN cells emerging from β-selection and transition to the DP stage of T cell development.

The signals that establish the formation of the SCZ and other thymic microenvironments are yet to be fully characterised. Many studies have now reported that chemokines play a role in directing the intra-thymic trafficking of thymocytes through the SCZ, cortex and medulla (Takahama, 2006). Studies on mice deficient for certain chemokine-chemokine receptor pairs reveal perturbed thymocyte migration as a result. In

particular, CXCL12 and its receptor CXCR4 has been identified as essential for the proper localisation of DN cells in the cortex (Plotkin et al., 2003). Similarly, CCL25 and its receptor CCR9 are known to be important for the localisation of DN3 cells to the SCZ (Benz et al., 2004). However, later stages of T cell development are known to involve CCL19 and CCL21 signalling via their receptor CCR7 and this has been identified as essential for cortex to medulla migration of developing thymocytes (Ueno et al., 2004).

For cells to chemotax, they must first acquire a polarised morphology to form a leading lamellipodium and this requires a complex intracellular signalling cascade (Reif and Cyster, 2002). Chemokine receptors signal via heterotrimeric G-protein-coupled receptors. When chemokines bind to their receptors, a variety of signalling pathways are initiated that activate the Rho family of GTPases, in particular Rac, to drive membrane polarisation and cytoskeletal changes, essential for cell migration (Kunisaki et al., 2006). The Rac protein is a key regulator of cell motility by controlling F actin polymerisation and lamellipodia formation (Nombela-Arrieta et al., 2007). DOCK2 (dedicator of cytokinesis 2) is a novel member of the CDM family of scaffold proteins (Reif and Cyster, 2002). DOCK2 is a haematopoietic cell specific protein that functions as a guanine exchange factor for Rac and is a central regulator of chemokine-mediated Rac-activation and cytoskeletal reorganisation in lymphocytes, neutrophils and a small fraction of DCs (Fukui et al., 2001; Gotoh et al., 2008; Kunisaki et al., 2006). However, there is evidence to suggest that phosphoinositide-3-kinase (PI3K) isoforms are involved in DOCK2 independent chemokine-induced migration (Nombela-Arrieta et al., 2004). Figure 4.1 represents a simplified scheme for the role of DOCK2 during chemokine-induced lymphocyte migration.

In the absence of DOCK2, chemokine induced F actin polymerisation and short-term homing are disrupted in T and B cells (Nombela-Arrieta et al, 2007). It has also been reported that mice deficient in DOCK2 exhibit impaired T lymphocyte migration in response

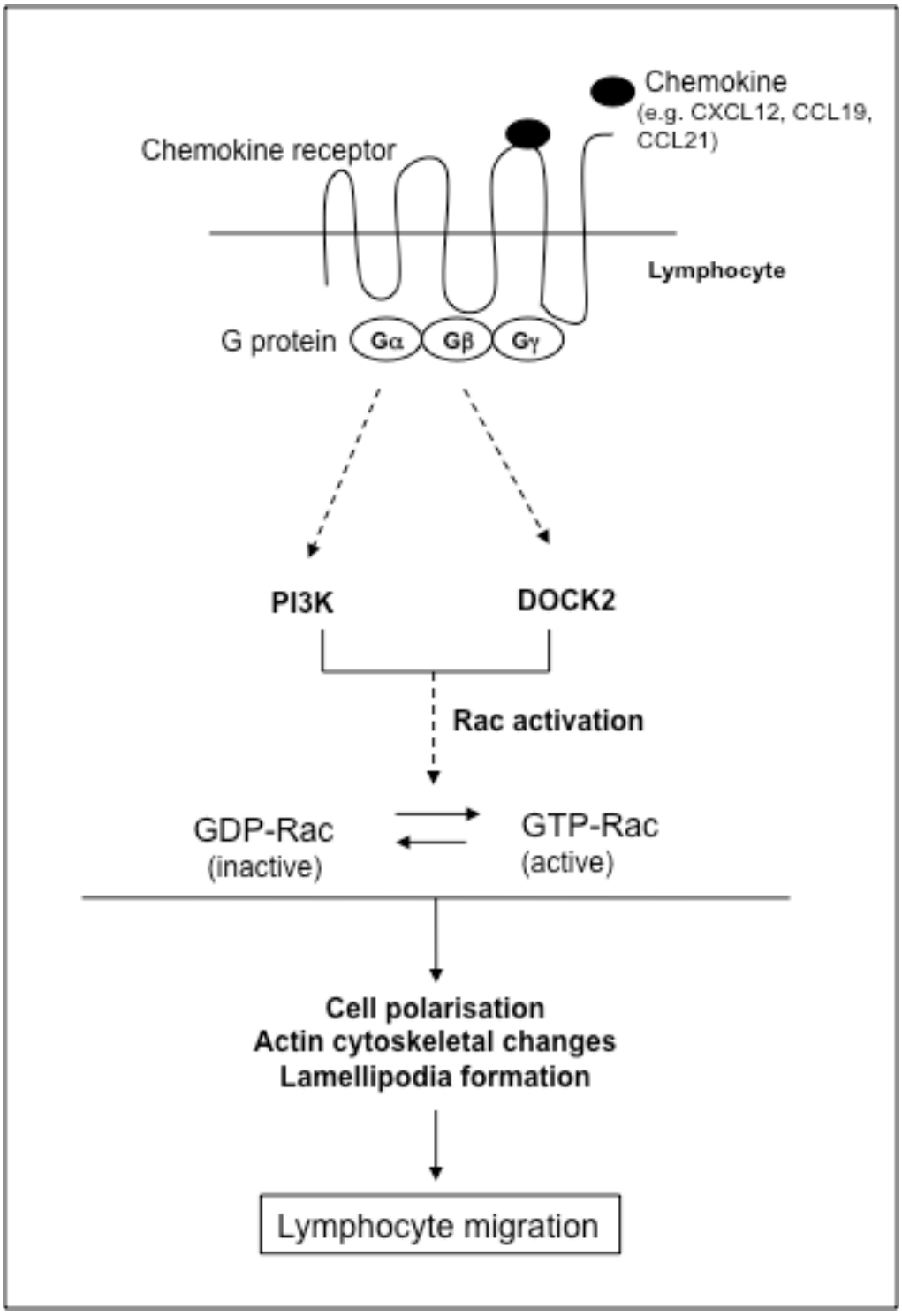
to several homeostatic chemokines including CXCL12, CCL19 and CCL21 (Fukui et al., 2001; Gotoh et al., 2008; Shulman et al., 2006), indicating a role for DOCK2 downstream of chemokine receptor signalling. This defect is manifested in the secondary lymphoid organs, which have abnormal architectures in DOCK2$^{-/-}$ mice (Fukui et al., 2001). These mice exhibit severe atrophy in T and B cell areas in all secondary lymphoid organs due to defective homing of DOCK2$^{-/-}$ lymphocytes to the spleen and lymph nodes. It has also been shown that the number of splenocytes and CD4^{+} T cells in the lymph nodes and thymus were markedly reduced in DOCK2$^{-/-}$ mice (Fukui et al., 2001). Interestingly, this study also reported that SP4 and SP8 cells were abnormally distributed throughout the thymic cortex as small patches. Whilst the phenotype of DOCK2$^{-/-}$ mice has been relatively well studied with relation to the secondary lymphoid organs, the precise role of DOCK2 in the thymus is yet to be fully characterised. Furthermore, previous studies have not analysed the nature of the SCZ in DOCK2$^{-/-}$ mice. Thus, due to the importance of DOCK2 as a central regulator of chemokine induced lymphocyte migration, it was of interest in this study to investigate the role of DOCK2 during T cell development and establishment of thymic microenvironments.

Therefore, the aims and objectives of this chapter are as follows;

- To study the establishment of thymic microenvironments with particular emphasis on the SCZ.
- To employ a method to study the thymic anatomical compartmentalisation of chemokines known to be important during T cell development.
- To study the requirement for DOCK2 during T cell development and organisation of thymic microenvironments with a view to further understand chemokine-induced migration of thymocytes and their role in the establishment of thymic microenvironments.

Figure 4.1 A Schematic Representation of the Role for DOCK2 During Chemokine Induced Lymphocyte Migration.

Secreted chemokine binds to G protein coupled chemokine receptors on the surface of lymphocytes initiating a chemokine-signalling cascade that activates Rac. A phosphatidylinositol-3-OH kinase (PI3K) dependent pathway exists along with a PI3K independent DOCK2 pathway. DOCK2 functions downstream of chemokine-chemokine receptor engagement to activate Rac, which subsequently initiates cell polarisation, actin cytoskeletal rearrangements and lamellipodia formation essential for cell migration. For simplicity, potential intermediates in the signalling pathways are not shown.


Chemokine
(e.g. CXCL12, CCL19, CCL21)
Chemokine receptor
Lymphocyte
G protein
Gα
Gβ
Gγ
PI3K
DOCK2
Rac activation
GDP-Rac
(inactive)
GTP-Rac
(active)
Cell polarisation
Actin cytoskeletal changes
Lamellipodia formation
Lymphocyte migration

4.2 Specialised Materials and Methods.

4.2.1 Laser Capture Microdissection.

In order to study the anatomical compartmentalisation of chemokines within the thymus, a laser capture microdissection (LCM) technique was employed. This technique avoids the technical difficulties sometimes associated with measuring chemokine expression by conventional methods such as flow cytometry and confocal microscopy. Therefore, LCM is a more useful technique, which allows for the investigation of gene expression within specified areas of tissue.

Figure 4.2 summarises the overall process of LCM. 5μm thick sections of frozen adult thymus were cut and mounted onto membrane covered nuclease free slides (PALM). Slides were allowed to dry for 30 minutes before fixation in acetone (Baker) for 20 minutes. Following fixation, slides were stored at -79°C until required. Slides were removed from -79°C and allowed to air dry for 1 minute before staining. In order to visualise the tissue sections and identify regions, slides were stained with the nuclear dye cresyl violet (figure 4.2A). Cresyl violet-stained thymus sections identifies cortical areas as a darker violet compared to the medulla. The cresyl violet solution was prepared by dissolving the cresyl violet acetate powder (Sigma) in absolute molecular biology grade ethanol (Sigma) at a 1% W/V ratio. This was then placed on a rotor and allowed to dissolve overnight. After the overnight incubation, the cresyl violet solution was filtered to remove any precipitate. Prior to staining, slides were dipped sequentially into 100, 70 and 50% solutions of absolute molecular biology grade ethanol. Slides were immediately stained with the cresyl violet solution for approximately 6 minutes. Slides were then washed by sequential dipping in the ethanol solutions in the reverse order (i.e. 50, 70 and 100%). All dilutions of ethanol were

prepared in BPC grade water (Sigma). Slides were left to air dry for 1 minute before microdissection. All equipment and solutions were kept RNase free and incubations were kept to a minimum to ensure negligible RNA degradation.

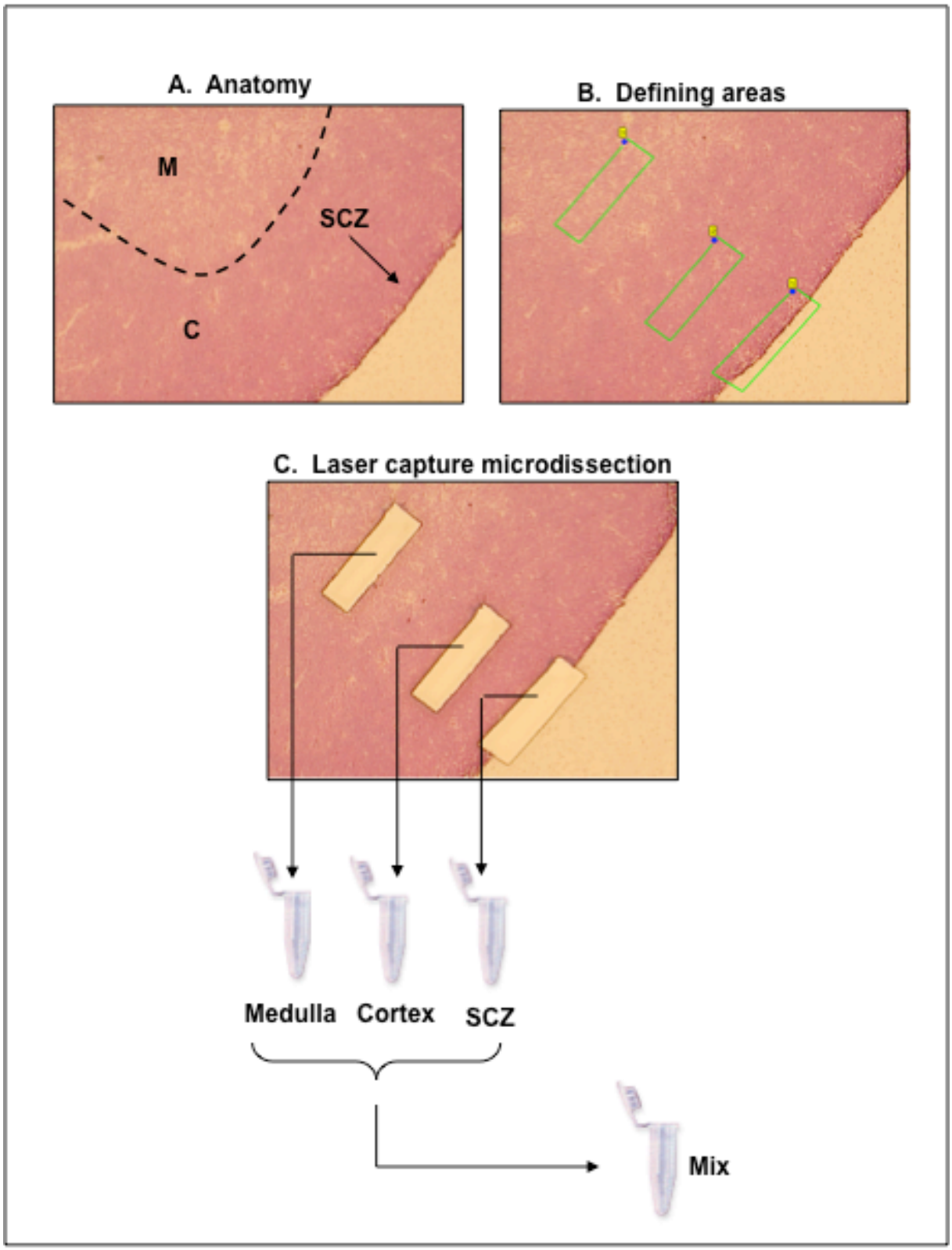
Using a laser microdissecting microscope Microbeam HT (Palm Microlaser Technologies, Bernried, Germany) sections of subcapsular, cortical and medullary areas were microdissected. Using the microscope software, circles or boxes (sizes of which were kept consistent throughout) were drawn within cortical and medullary regions (figure 4.2B). For the SCZ, boxes (sizes of which were kept consistent throughout) were drawn in the approximated region directly beneath and including the capsule. This allows for the precise identification and isolation of these specific areas of interest with negligible contamination from adjacent cells. Microdissected sections were transferred by laser pressure catapulting into the caps of 0.5ml RNase free eppendorffs containing 9μl of lysis buffer (figure 4.2C). Multiple sections (approximately 30) were microdissected from each region of interest and pooled into three separate tubes. As a positive control, approximately 10 sections of equal size were microdissected from each region and pooled into one tube (mix sample). Following microdissection, tubes were microfuged in a temperature controlled microfuge at 4°C to allow the lysis buffer containing the dissected tissue sections to transfer from the cap of the tube to the bottom. Tubes were stored at -79°C before mRNA extraction. Quantitative RT-PCRs were carried out in collaboration with S. Parnell, University of Birmingham (as detailed in section 2.8). Ct values were normally obtained using standard curves from cDNA from FTOCs. Relative expression values for samples normalised to β-actin were obtained.

4.2.2 Statistical Analysis.

All data was analysed by using the unpaired student t test using GraphPad software. P values below 0.05 were considered significant.

Figure 4.2 Laser Capture Microdissection.

Cresyl violet stained sections of adult WT thymus reveals the cortex as a darker violet compared to the medulla (A). Using the LCM software, boxes were drawn around the approximated region of the subcapsular zone (SCZ) and within the cortex and medulla (B). These areas were then microdissected and transferred to separate tubes by laser pressure catapulting (C). As a positive control, microdissected sections from each region were pooled into one tube (mix).


A. Anatomy
M
SCZ
C
B. Defining areas
C. Laser capture microdissection
Medulla
Cortex
SCZ
Mix

4.3 Results.

4.3.1 Thymocyte Subsets Are Localised in Defined Regions of the Adult Thymus.

To identify the anatomical location of the different thymocyte subsets, thymus sections from a 4-6 week old mouse was stained for confocal microscopy analysis for CD25 to allow for identification of DN cells (CD25 is expressed on both DN2 and DN3 subsets) and CD4 and CD8 to allow for identification of DP and SP cells. Defined regions of subcapsular, cortex and medulla containing thymocytes at different stages of development were identified (figure 4.3A-C). The thymus has a subcapsular zone, the area directly beneath the capsule, containing DN thymocytes that tend to be at the DN3 stage of development, as identified by the expression of CD25 (figure 4.3A). The cortex provides an environment for DP thymocytes, as identified by expression of both CD4 and CD8 (figure 4.3A). The medulla constitutes thymocytes at the SP stage of development. I.e. mature $CD4^+$ $CD8^-$ or $CD4^-$ $CD8^+$ cells (figure 4.3B and C). Cortical and medullary regions were identified on the basis of DAPI staining (not shown).

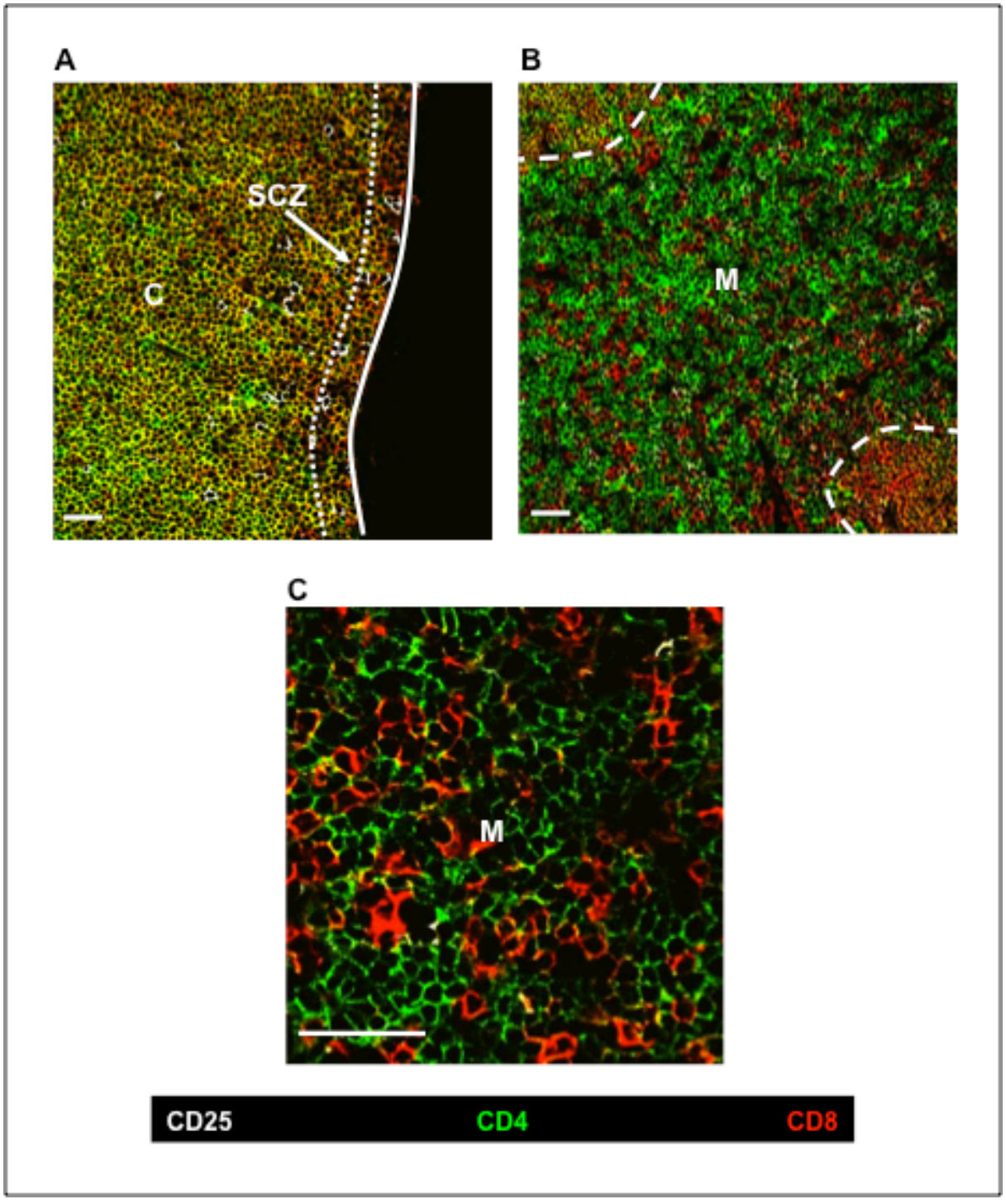
4.3.2 The Emergence of the Subcapsular Zone During Thymus Ontogeny.

Due to the importance of the SCZ for supporting localisation of DN3 cells, which are defined as $CD4^-$ $CD8^-$ $CD25^+$ $CD44^-$, we aimed to identify the emergence of this region during thymus ontogeny. Sections of thymus dissected from embryos at day 14, 16 and 18, a day 1 neonate and a 4-6 week old adult were stained for CD25, CD4 and CD8 for confocal microscopy analysis (figure 4.4). At E14 and E16, there is a distinct absence of CD4 and CD8 expressing cells (figure 4.4A and B).

Figure 4.3 Thymocyte Subsets Are Localised in Defined Regions of the Adult Thymus.

Frozen thymus sections from a 4-6 week old WT mouse was stained for CD25 (identifies double negative thymocytes) and CD4 and CD8 (identifies double positive and single positive thymocytes) (4.3A-C).

The solid line denotes the edge of the tissue, the dotted line denotes the approximated region of the subcapsular zone and the dashed line represents the corticomedullary junction. Bar indicates scale of 50μm. SCZ, subcapsular zone, C, cortex, M, medulla.


A
SCZ
C
B
M
C
M
CD25
CD4
CD8

At these stages of thymus development, the thymus constitutes thymocytes at the DN stage of development with the $CD25^+$ cells scattered throughout the thymus. However, by E18 the appearance of $CD4^+$ $CD8^+$ (DP) cells was identified (figure 4.4C). Most striking was the observation that the $CD25^+$ DN cells are beginning to migrate outwards towards the capsule forming an elementary SCZ microenvironment where the DP cells were typically absent (figure 4.4D). A day 0 thymus mostly constitutes DP cells located in the cortex and an appearance of small medullary patches containing $CD4^+$ $CD8^-$ (SP4) and $CD4^-$ $CD8^+$ (SP8) cells were also observed (figure 4.4E). A clearly defined SCZ has now formed providing a microenvironment for the $CD25^+$ DN3 cells to localise (figure 4.4F). It is particularly interesting to note that similar to E18, DP cells remain largely absent from the SCZ, which mostly constitutes $CD25^+$ DN3 cells. This pattern of DN3 localisation to the SCZ does not remain as conserved in the adult thymus (figure 4.4G and H). In contrast to E18 and day 0, the SCZ in the adult is not as clearly defined and appears to occupy a smaller fraction of the thymus. In further contrast to E18 and day 0, DP cells were observed in this region along with the $CD25^+$ DN3 cells.

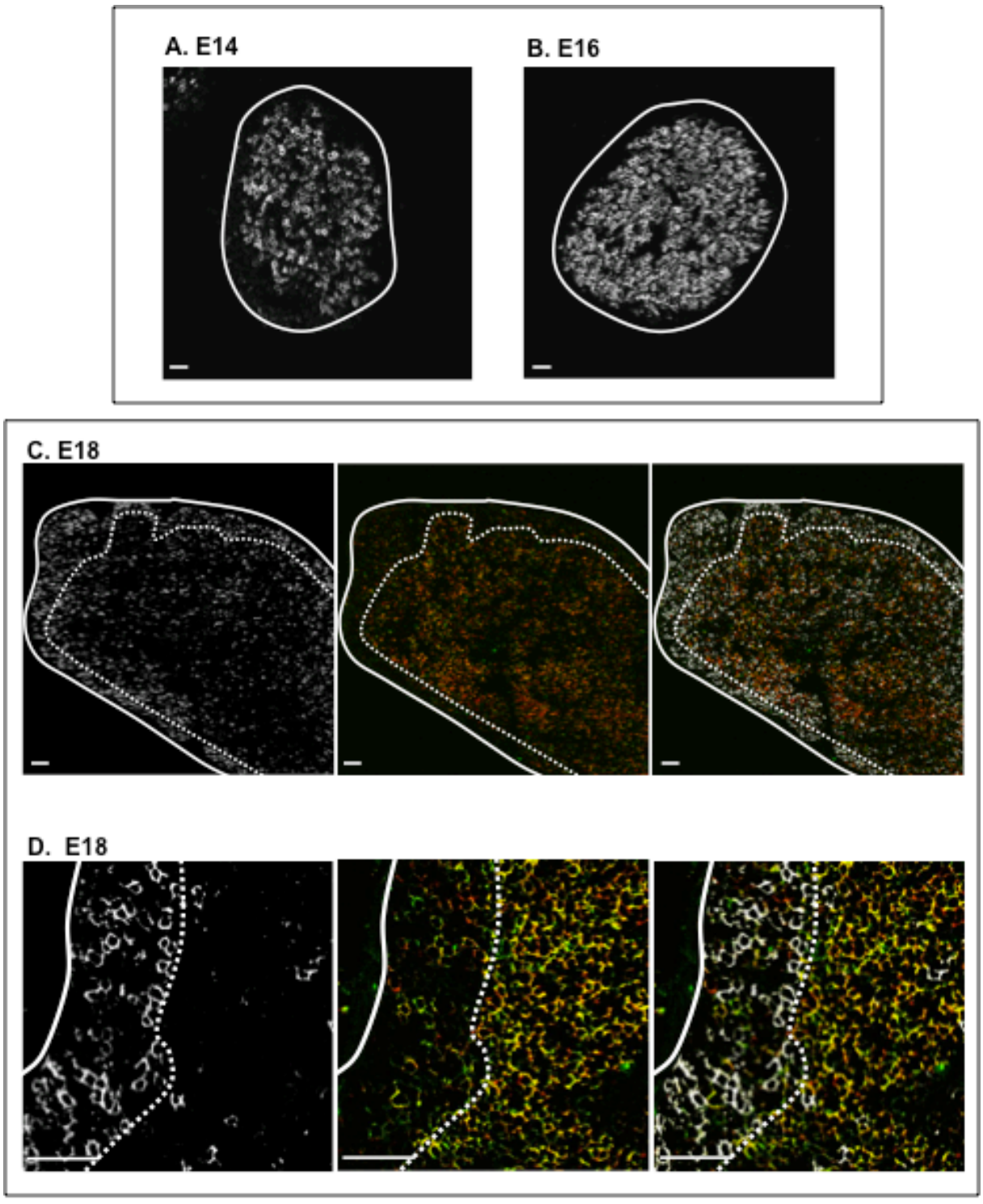
4.3.3 Laser Capture Microdissection as a Method to Study Gene Expression Within Specified Areas of the Thymus.

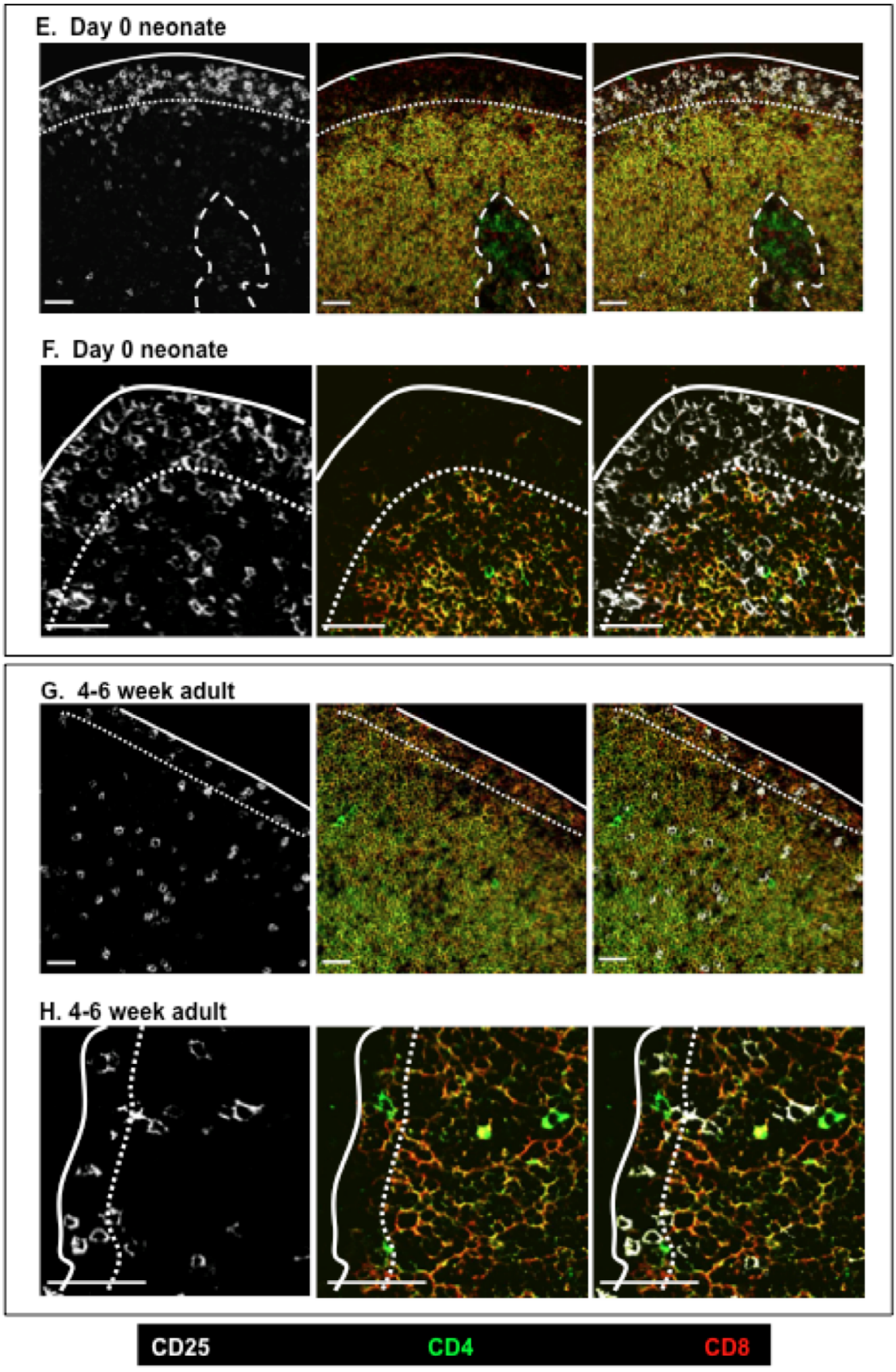
Thus far we have established that distinct microenvironments are present within the thymus and that these regions develop during ontogeny. To investigate the role of chemokines in organising thymic microenvironments, a laser capture microdissection (LCM) technique was employed. To evaluate the effectiveness of using LCM to study gene expression within specified areas of the thymus, we initially looked at the expression of the autoimmune regulator gene (AIRE) within microdissected regions of SCZ, cortex and

Figure 4.4 The Emergence of The Subcapsular Zone During Thymus Ontogeny.

Frozen sections of WT thymus dissected from mice at the indicated ages of development were stained for CD25, CD4 and CD8. Distribution of thymocytes at different stages of T cell development is shown as defined by the differential expression of the above markers (A-H).

Low power (A-C, E and G) and high power images are shown (D, F, H). The solid line denotes the edge of the tissue, the dotted line is the approximated region of subcapsular zone and the dashed line represents the corticomedullary junction. Bar indicates scale of 50μm.


A. E14
B. E16
C. E18
D. E18
CD25
CD4
CD8


E. Day 0 neonate
F. Day 0 neonate
G. 4-6 week adult
H. 4-6 week adult
CD25
CD4
CD8

medullary region of the thymus (usually associated with keratin 5^+ mTECs) and not the cortex. To confirm this by LCM, dissected regions of SCZ, cortex and medulla of the thymus were converted to cDNA and the expression of AIRE was examined by quantitative RT-PCR. A mix sample containing pooled sections from all regions was used as a positive control. Relative gene expression was calculated for samples normalised to the housekeeping gene β-actin. As expected, AIRE expression was found in the positive control mix sample and in the medulla, with undetectable expression levels in the SCZ and cortex (figure 4.5B).

4.3.4 Distinct Regions of the Thymus Reveal Differential Gene Expression Patterns of Chemokines.

Section 4.3.3 establishes the success of LCM as a method for studying gene expression in various thymus areas. This technique was then employed to determine niche specific expression of chemokines and chemokine receptors that are known to be important during T cell development. Quantitative RT-PCR was carried out on cDNA obtained from laser microdissected areas of SCZ, cortex and medulla from adult thymus (6-8 weeks old). A mix sample containing pooled sections from all regions was used as a positive control. Relative gene expression was calculated for samples normalised to β-actin. Data was initially obtained by semi-quantitative PCR (data not shown) before confirmation by quantitative RT-PCR.

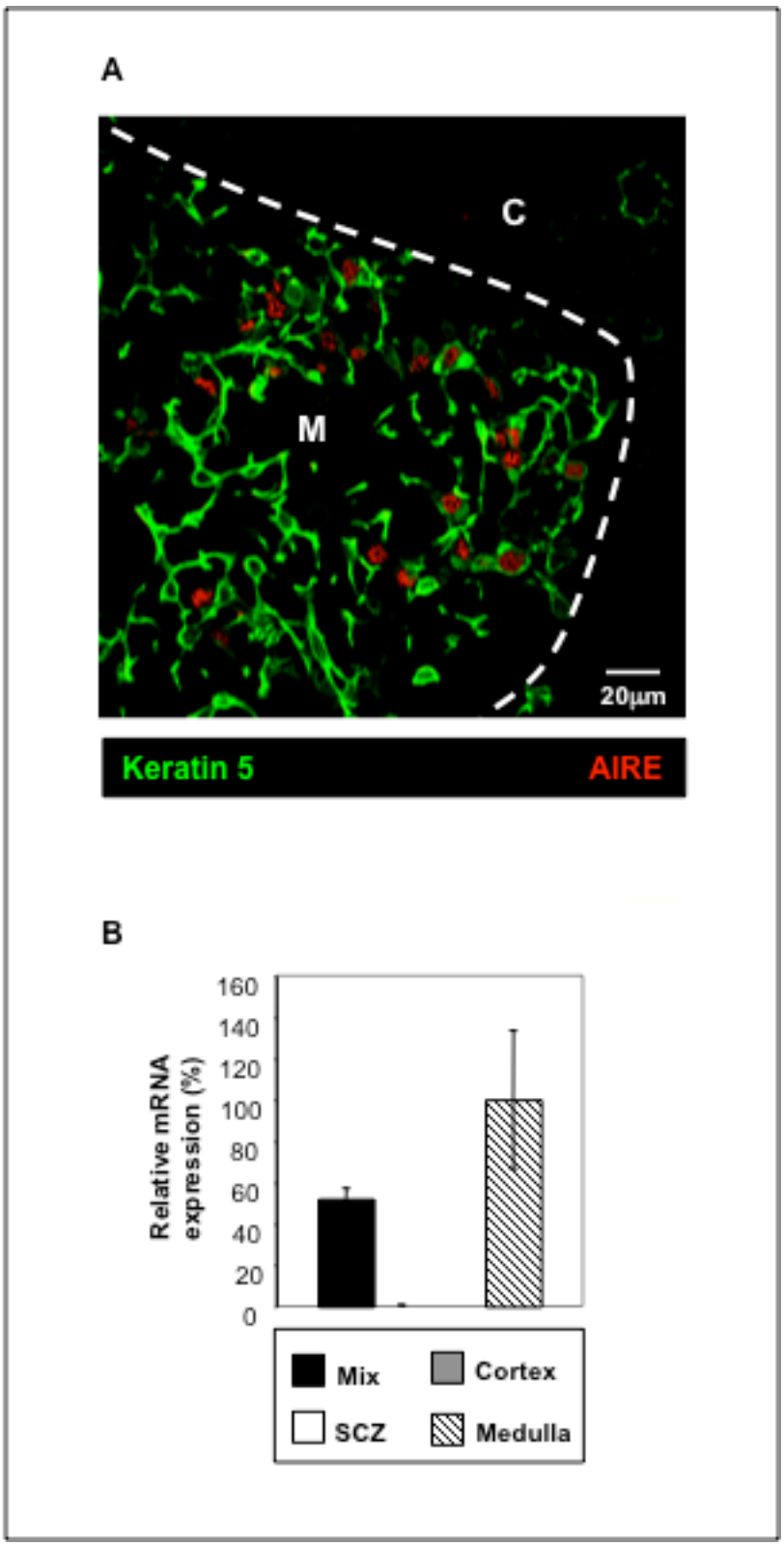
CCL25 (TECK) has previously been known to act on immature DP cells and mature SP cells. In the present study CCL25 was found to be located throughout the thymus but most

Figure 4.5 Laser Capture Microdissection as a Method to Study Gene Expression Within Specified Areas of the Thymus.

Frozen sections of adult thymus were stained for the medullary specific epithelial cell marker keratin 5 and the autoimmune regulator AIRE for confocal analysis (A).

Scale of image is indicated. C, cortex, M, medulla. Image courtesy of S. Rossi, University of Birmingham.

Quantitative RT-PCR was carried out on cDNA obtained from laser microdissected areas of subcapsular zone (SCZ), cortex and medulla to determine AIRE expression in each of the areas. Relative mRNA expression of AIRE normalised to β-actin is shown (B). PCRs were carried out in duplicates and error bars indicate the standard error of the mean. Data representative of two distinct experiments.


A
C
M
20µm
Keratin 5
AIRE
B
Relative mRNA expression (%)
160
140
120
100
80
60
40
20
0
Mix
Cortex
SCZ
Medulla

interestingly was predominantly expressed in the SCZ (figure 4.6A). The receptor for CCL25 is CCR9 and expression of which was highest in the cortex and SCZ with minimal levels found in the medulla (figure 4.6B). CXCL12 (SDF-1) preferentially attracts DN cells and DP cells and its receptor CXCR4 is known to be highly expressed in these cells. Interestingly, in this study CXCL12 was expressed predominantly in the SCZ, with minimal expression levels in the cortex and medulla (figure 4.6C). Similarly, expression of the receptor for CXCL12, CXCR4 was highest in the SCZ but also the cortex (figure 4.6D). Low levels of expression were found in the medulla. By contrast to the expression of CCL25, CXCL12 and their respective receptors, the expression patterns of the chemokines CCL19 and CCL21 were much different. Both chemokines have been reported to be important in the later stages of T cell development and are chemoattractive for SP thymocytes (Ueno et al., 2004). In line with this, we confirmed that CCL19 and CCL21 were present exclusively in the medulla with almost undetectable levels in the SCZ and cortex (figure 4.6E and F). By comparison, expression of the receptor for both CCL19 and CCL21, CCR7 was predominant in the medulla, with low-level expression in the cortex and undetectable expression levels in the SCZ (figure 4.6G).

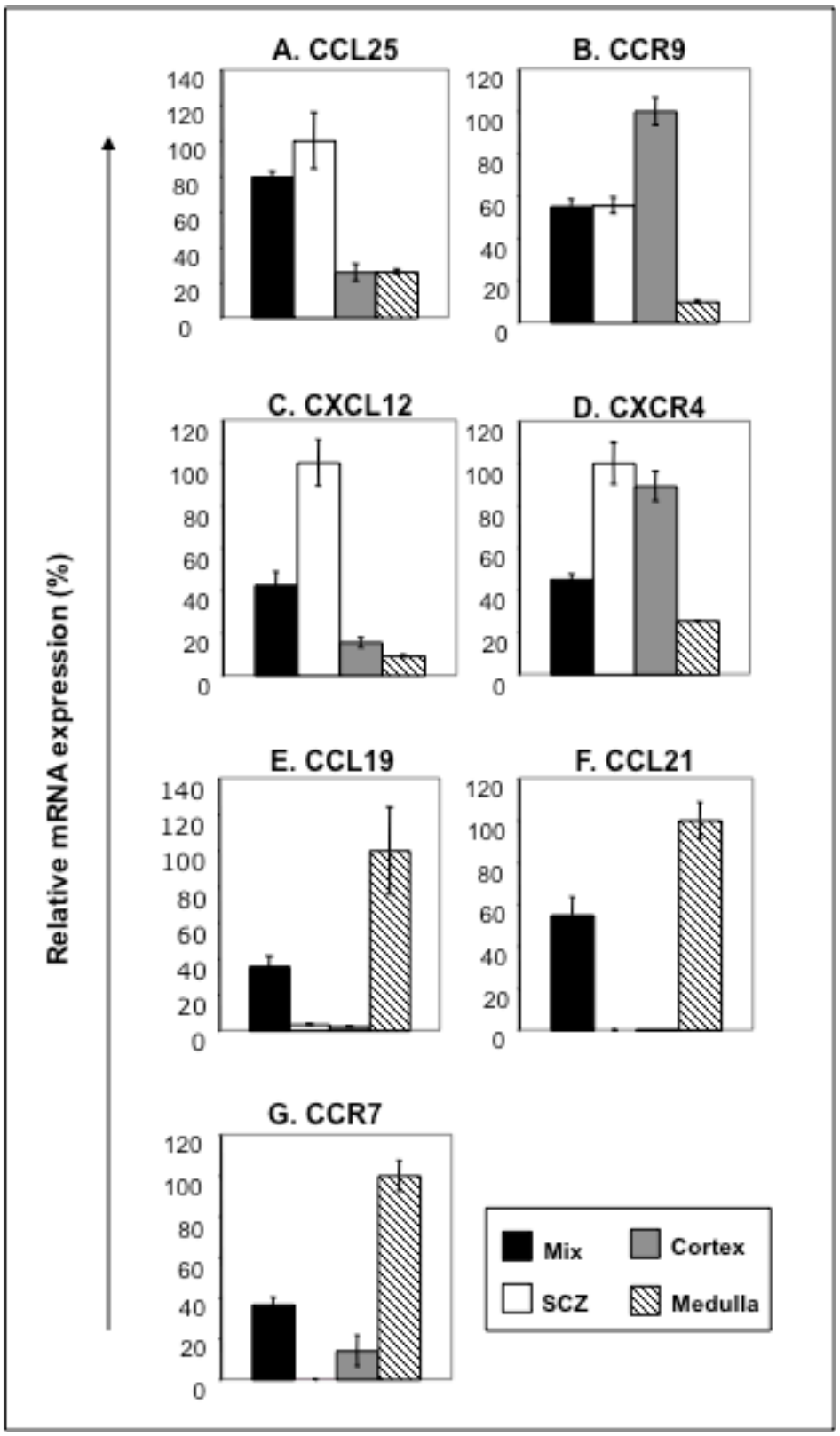
To summarise, data presented in this section confirms that chemokine-chemokine receptors associated with particular stages of T cell development are expressed in defined regions of the thymus.

Figure 4.6 Distinct Regions of the Thymus Reveal Differential Gene Expression Patterns of Chemokines.

Subcapsular, cortical and medullary regions were laser microdissected from frozen sections of WT adult thymus (6-8 weeks old). As a positive control, microdissected sections from all regions were pooled into a single tube (mix).

Quantitative RT-PCR was carried out for the indicated chemokines and their receptors. Relative mRNA expression of indicated chemokine/chemokine receptors normalised to β-actin is shown (A-G).

PCRs were carried out in duplicate and error bars indicate standard error of the mean.


A. CCL25
B. CCR9
C. CXCL12
D. CXCR4
E. CCL19
F. CCL21
G. CCR7
Relative mRNA expression (%)
Mix
Cortex
SCZ
Medulla

4.3.5 Abnormal Localisation of Double Positive Thymocytes to the Subcapsular Zone in CCR9$^{-/-}$ Neonatal Mice.

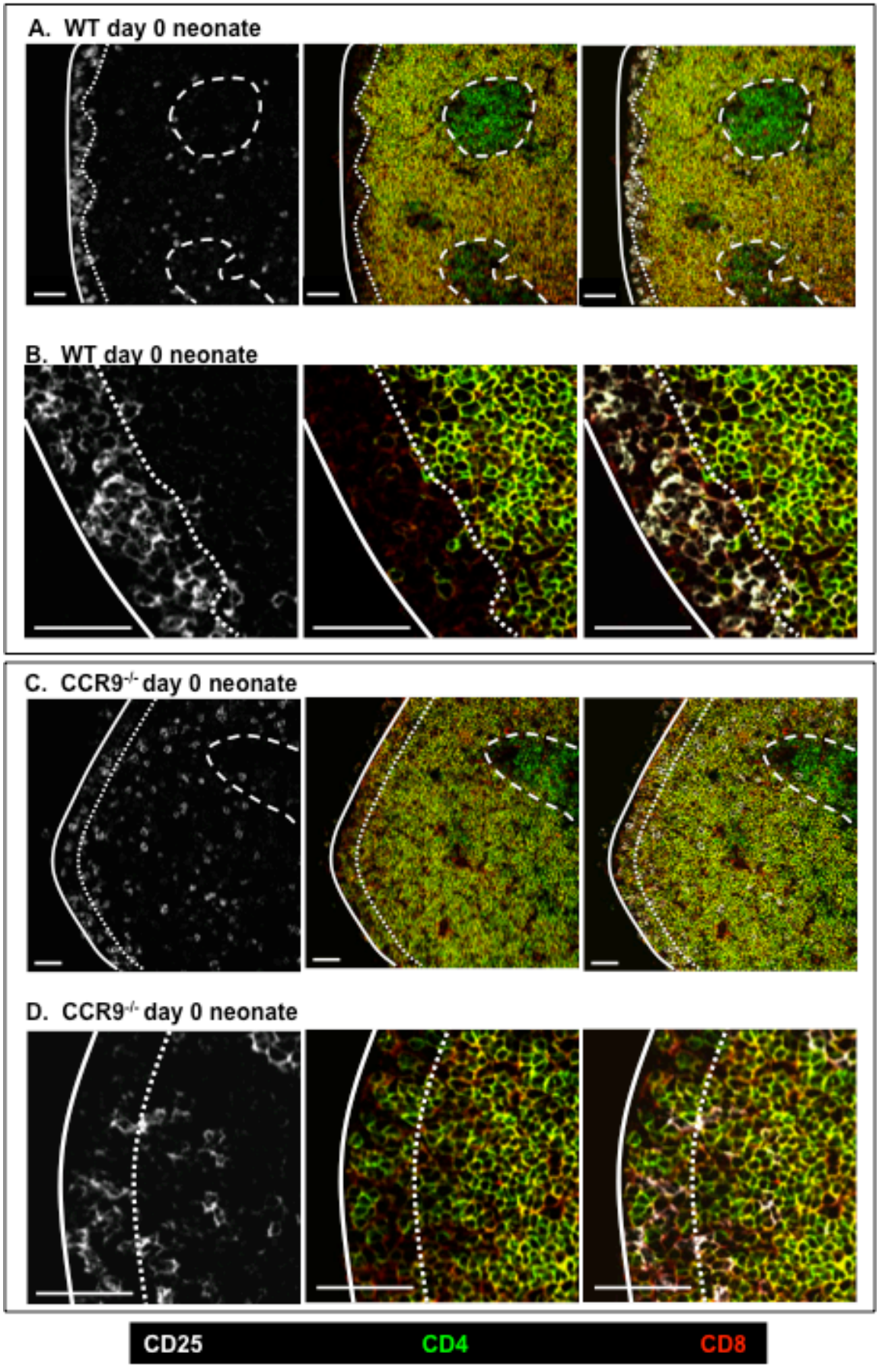
So far we have demonstrated that thymocytes at different stages of T cell development occupy different areas of the thymus and these areas constitute specific chemokine distribution. Therefore, with regard to the SCZ we were interested in whether deficiency in chemokines could result in perturbed localisation of thymocytes to the SCZ. In particular, we studied mice deficient for CCR9. A previous study has demonstrated that CCR9$^{-/-}$ adult mice have abnormal distribution of CD25^{+} DN3 cells, away from the SCZ (Benz et al., 2004). We aimed to extend this study by looking to see if deficiency in CCR9 also leads to perturbed DP cell distribution in the SCZ of both adult and neonatal mice. Thymus sections from 2 week old and a day 0 (day of birth) WT and CCR9$^{-/-}$ mice were stained for confocal microscopy analysis for CD25, CD4 and CD8 (figure 4.7). As illustrated in figure 4.7A and B, CD25^{+} DN3 cells commonly occupy the subcapsular region of the thymus in a day 0 neonate, with DP cells typically absent. However, in CCR9$^{-/-}$ day 0 mice there is a dramatic change in the cellular composition of the SCZ (figure 4.7C and D). Whilst some CD25^{+} cells are still able to localise to the SCZ, more CD25^{+} cells can be observed in the cortex compared to WT day 0. Most importantly, DP cells that are distinctly absent in a WT day 0 thymus were aberrantly located in the SCZ of the CCR9$^{-/-}$ day 0 thymus.

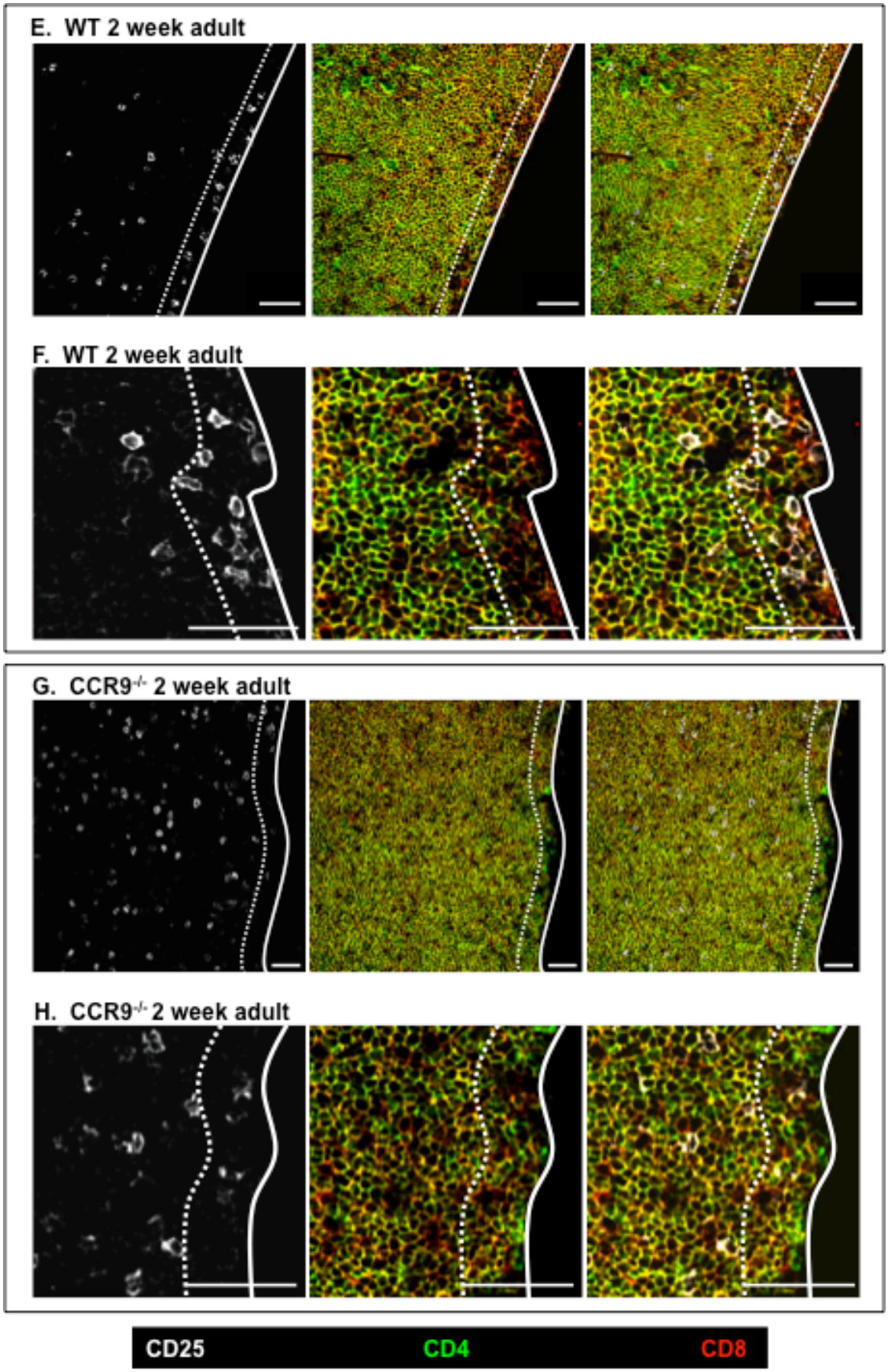
In a WT adult thymus, localisation of DP cells to the SCZ correlates with fewer CD25^{+} cells localising to this region resulting in a less defined SCZ (figure 4.7E and F). In a CCR9$^{-/-}$ adult, fewer CD25^{+} cells localise to the SCZ with more cells distributed throughout the cortex. (figure 4.7G and H). By comparison to a WT adult and a day 0 CCR9$^{-/-}$ thymus, DP cells are located in the SCZ of a CCR9$^{-/-}$ thymus.

Figure 4.7 Abnormal Localisation of Double Positive Thymocytes to the Subcapsular Zone in CCR9$^{-/-}$ Mice.

Frozen sections of thymus dissected from day 0 and 2 week adult WT and CCR9$^{-/-}$ mice were stained for CD25, CD4 and CD8. Distribution of thymocytes at different stages of T cell development is shown (A-H). Low power (A, C, E and G) and high power (B, D, F and H) images for each mouse analysed are shown.

The solid line denotes the edge of the tissue, the dotted line is the approximated region of subcapsular zone and the dashed line represents the corticomedullary junction. Bar indicates scale of 50μm.


A. WT day 0 neonate
B. WT day 0 neonate
C. CCR9-/- day 0 neonate
D. CCR9-/- day 0 neonate
CD25
CD4
CD8


E. WT 2 week adult
F. WT 2 week adult
G. CCR9-/- 2 week adult
H. CCR9-/- 2 week adult
CD25
CD4
CD8

4.3.6 DOCK2, An Important Regulator of Chemokine Induced Cell Migration, Is Expressed By Thymocyte Subsets.

DOCK2 is a novel haematopoietic specific protein that is thought to play an important role in chemokine-induced lymphocyte migration by functioning downstream of chemokine receptors including CCR7 and CXCR4. The precise role of DOCK2 in the thymus has not been fully characterised. Thus, in the final part of this chapter, we aim to define the role that DOCK2 may have during thymocyte development and organisation of thymic microenvironments.

Firstly, we sought to determine which thymocyte subsets express DOCK2 (figure 4.8). Thymocytes from a 4-6 week old WT thymus were stained for TCR, CD4 and CD8. Cells were MoFlo sorted for TCR^- DN cells ($CD4^-$ $CD8^-$), TCR^{low} DP cells ($CD4^+$ $CD8^+$), TCR^{high} SP4 and SP8 cells ($CD4^+$ $CD8^-$ and $CD4^-$ $CD8^+$). Quantitative RT-PCR for DOCK2 shows that DOCK2 is expressed in all thymocyte subsets, with DP and SP4 cells showing the highest level of expression (figure 4.8A). To determine expression of DOCK2 in different DN subsets, thymocytes from embryonic day 15 thymus were stained for CD25 and CD44. Cells were MoFlo sorted for DN1 cells ($CD25^-$ $CD44^+$), DN2 cells ($CD25^+$ $CD44^+$) and DN3 cells ($CD25^+$ $CD44^-$). Quantitative RT-PCR for DOCK2 shows that DOCK2 is expressed by DN1-DN3 thymocytes, with DN3 cells showing the highest level of expression (figure 4.8B).

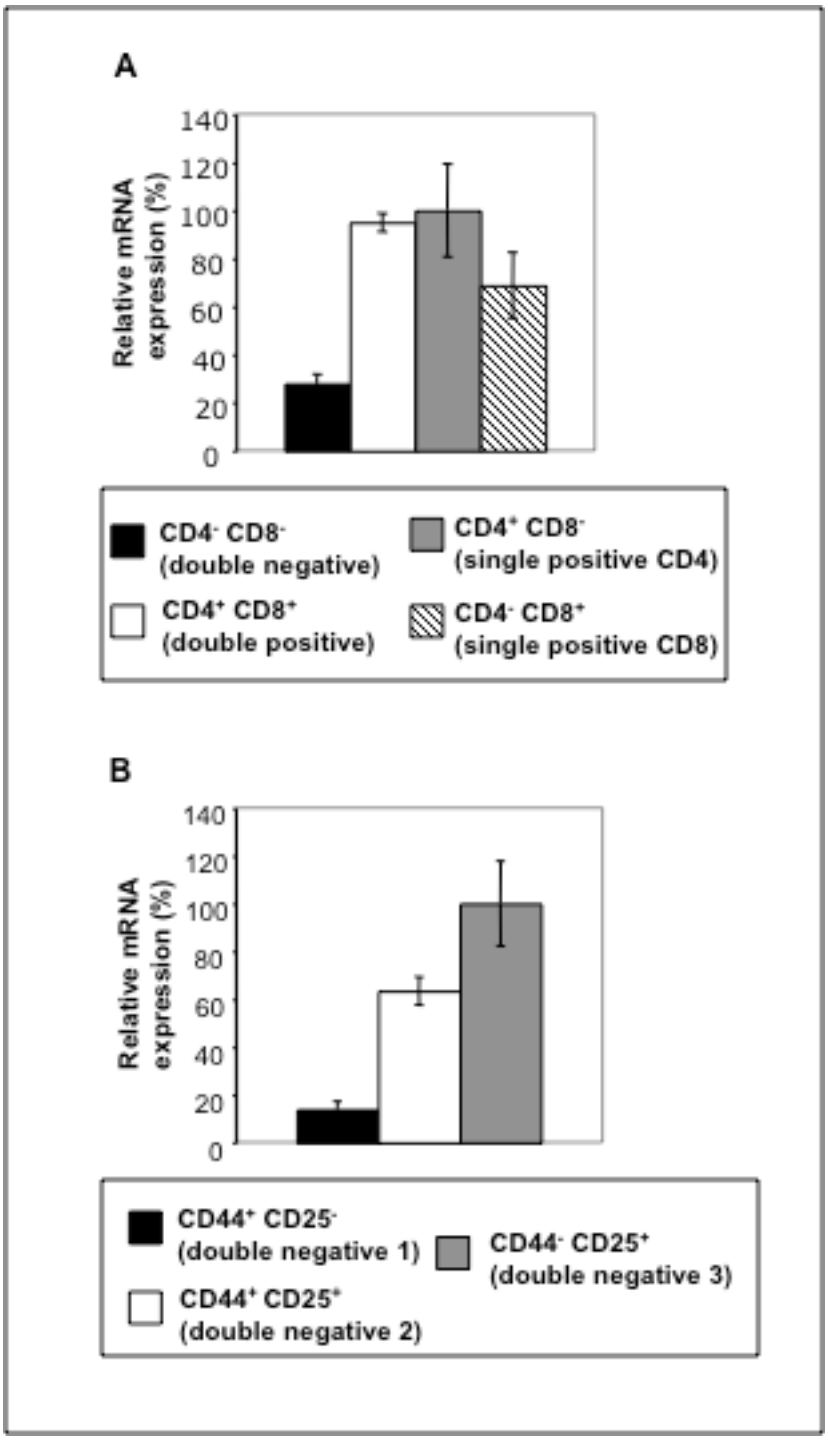
4.3.7 Quantification of Thymocyte Subsets In $DOCK2^{-/-}$ Mice.

Following our identification of DOCK2 expression on various thymocyte subsets, we then went on to determine the effect DOCK2 deficiency may have during T cell development by quantifying numbers and proportions of thymocyte subsets in $DOCK2^{-/-}$ mice (figure 4.9).

Figure 4.8 DOCK2, An Important Regulator of Chemokine Induced Cell Migration, Is Expressed On Thymocyte Subsets.

Thymocytes from a WT adult were cell sorted for TCR^- DN cells ($CD4^-$ $CD8^-$), TCR^{low} DP cells ($CD4^+$ $CD8^+$), TCR^{high} SP4 and SP8 cells ($CD4^+$ $CD8^-$ and $CD4^-$ $CD8^+$). Thymocytes from E15 thymus were cell sorted for DN1 cells ($CD25^-$ $CD44^+$), DN2 cells ($CD25^+$ $CD44^+$) and DN3 cells ($CD25^+$ $CD44^-$). Relative mRNA expression of DOCK2 normalised to β-actin on the indicated thymocyte subsets are shown (A and B).

PCRs were carried out in triplicate and error bars indicate standard error of the mean. Data representative of two distinct sorting experiments.


A
Relative mRNA expression (%)
140
120
100
80
60
40
20
0
CD4- CD8-
(double negative)
CD4+ CD8-
(single positive CD4)
CD4+ CD8+
(double positive)
CD4- CD8+
(single positive CD8)
B
Relative mRNA expression (%)
140
120
100
80
60
40
20
0
CD44+ CD25-
(double negative 1)
CD44- CD25+
(double negative 3)
CD44+ CD25+
(double negative 2)

Thymocytes were obtained from eight 6-8 week old WT and DOCK2$^{-/-}$ mice. Absolute numbers of thymocytes from each mouse was analysed (figure 4.9A). Deficiency in DOCK2 results in a pronounced and significant reduction in thymus cellularity compared to WT mice ($p=0.001$). However, despite this marked reduction in thymus cellularity, T cell development appeared to proceed normally in DOCK2$^{-/-}$ mice compared to WT mice with all four major thymocyte subsets present (figure 4.9B and C). Further analysis of proportions (figure 4.9D-G top panels) and actual cell numbers (figure 4.9D-G lower panels) of each thymocyte subset from WT and DOCK2$^{-/-}$ mice was carried out. Data reveals interesting differences in some of the subsets. The proportions of DN cells (defined as CD4^{-} CD8^{-}) in DOCK2$^{-/-}$ mice were significantly higher compared to WT mice ($p=0.009$), with some DOCK2$^{-/-}$ mice showing more than double the proportions of DN cells (figure 4.9D top graph). Proportions of DP cells (defined as CD4^{+} CD8^{+}) in DOCK2$^{-/-}$ mice were slightly but significantly reduced ($p=0.02$) (figure 4.9E top graph) and proportions of SP4 cells (defined as CD4^{+} CD8^{-}) in DOCK2$^{-/-}$ mice were also significantly reduced ($p=0.0007$) (figure 4.9F top graph). Collectively this data shows that whilst DOCK2 appears to be important during some stages of T cell development, overall it is not essential for normal T cell development to occur.

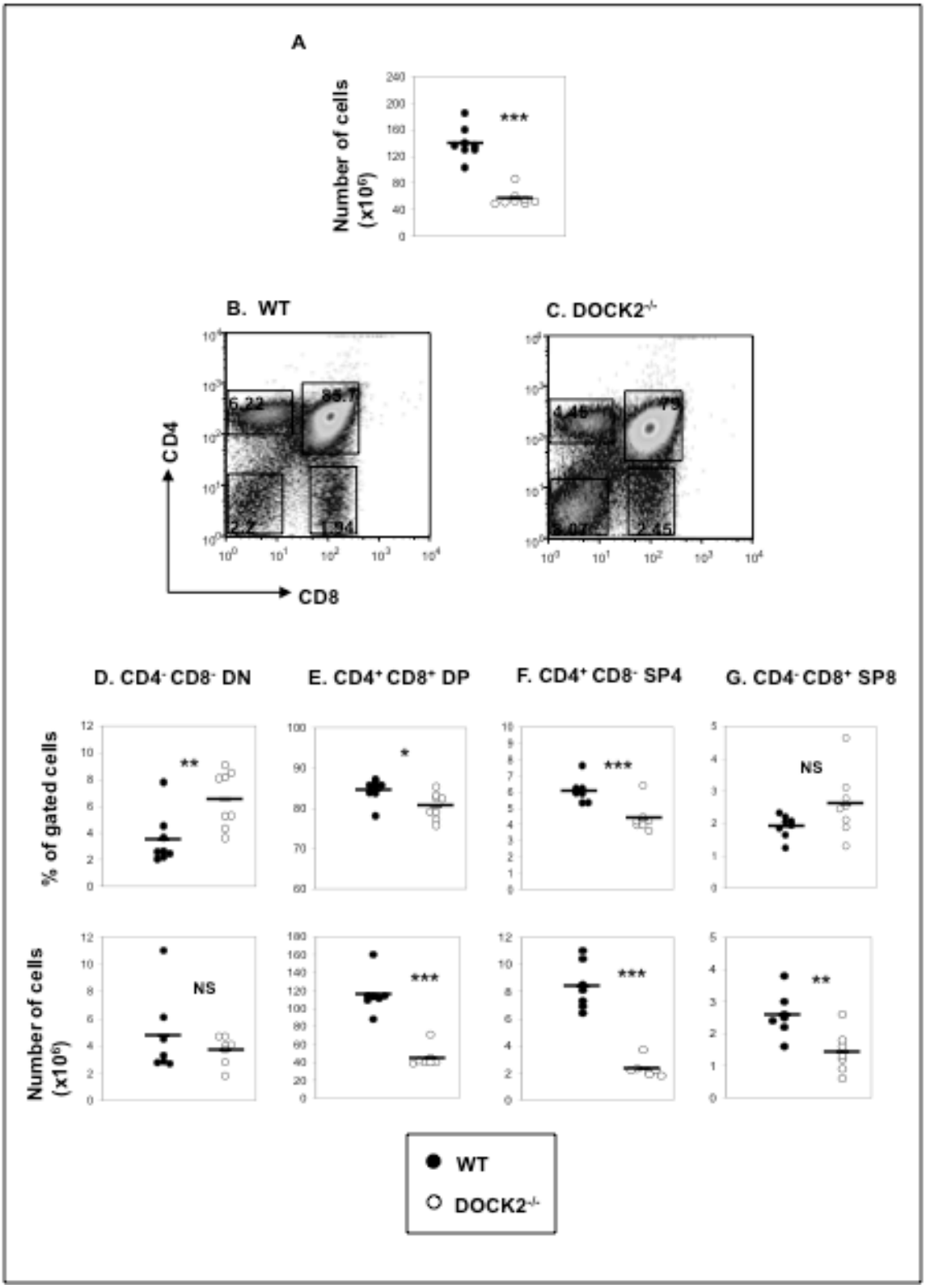
4.3.8 Quantification of Double Negative Thymocyte Subsets In DOCK2$^{-/-}$ Mice.

In the previous section we demonstrate a possible role for DOCK2 during the DN stage of T cell development as evidenced by the significant increase in DN proportions in DOCK2$^{-/-}$ mice compared to WT. We went on to investigate this further by quantifying numbers and proportions of the different DN subsets in DOCK2$^{-/-}$ mice (figure 4.10). Thymocytes from three 6-8 week old WT and DOCK2$^{-/-}$ mice were stained for a cocktail of lineage markers that included CD11b (macrophage marker), B220 (B cell marker), LY-6G (erythroid cell marker)

Figure 4.9 Quantification of Thymocyte Subsets In DOCK2$^{-/-}$ Mice.

Thymocytes were obtained from eight 6-8 week old WT and DOCK2$^{-/-}$ mice and stained for CD4 and CD8. Absolute thymus cellularity is shown (A). Thymocytes subsets present, based on the differential expression of CD4 and CD8, in WT and DOCK2$^{-/-}$ are shown (B and C). Percentages of cells within gated areas are indicated.

Proportions of the indicated thymocyte subsets are shown (D-G top panels) and actual cell numbers of each thymocyte subset was calculated (D-G lower panels). Each point represents one mouse. Horizontal bars represent the calculated mean values. Levels of significance are shown. (NS, not significant, *, $p<0.05$, **, $p<0.01$, ***, $p<0.001$).


A
Number of cells (x10⁶)
***
B. WT
C. DOCK2-/-
CD4
CD8
6.22
85.7
2.2
1.94
4.46
2.45
D. CD4- CD8- DN
E. CD4+ CD8+ DP
F. CD4+ CD8- SP4
G. CD4- CD8+ SP8
% of gated cells
Number of cells (x10⁶)
**
*
***
NS
WT
DOCK2-/-

and CD4 and CD8 (T lineage markers) along with the DN markers CD25 and CD44. By gating on lineage negative (lin^-) cells, CD25 and CD44 expression was analysed on true DN thymocytes in WT and $DOCK2^{-/-}$ mice (figure 4.10A and B). Differential expression of CD25 and CD44 on WT lin^- thymocytes revealed the presence of four distinct DN subsets. DN1 cells were identified as $CD25^-$ $CD44^+$, DN2 cells as $CD25^+$ $CD44^+$, DN3 cells as $CD25^+$ $CD44^-$ and DN4 cells as $CD25^-$ $CD44^-$. Analysis of these subsets in $DOCK2^{-/-}$ mice revealed that DOCK2 is not required for differentiation through the DN stages as illustrated by the presence of all four subsets in these mice (figure 4.10B). However, further analysis of proportions and actual cell numbers of each DN thymocyte subset from WT and $DOCK2^{-/-}$ mice revealed some differences. Surprisingly, despite DN1 cells having low expression of DOCK2 (figure 4.8B), proportions of DN1 cells were significantly reduced in $DOCK2^{-/-}$ mice compared to WT ($p=0.02$) (figure 4.10C top graph). Interestingly, a significant increase in DN3 proportions was observed in $DOCK2^{-/-}$ mice compared to WT ($p=0.01$) (figure 4.10E top graph) and this correlated with a significant reduction in proportions of DN4 cells in $DOCK2^{-/-}$ mice compared to WT ($p=0.03$) (figure 4.10F top graph). This suggests a potential block at the DN3-DN4 stage. Calculation of actual lin^- DN1-4 cells did not reveal any significant differences between $DOCK2^{-/-}$ and WT mice (figure 4.10C-F lower graphs).

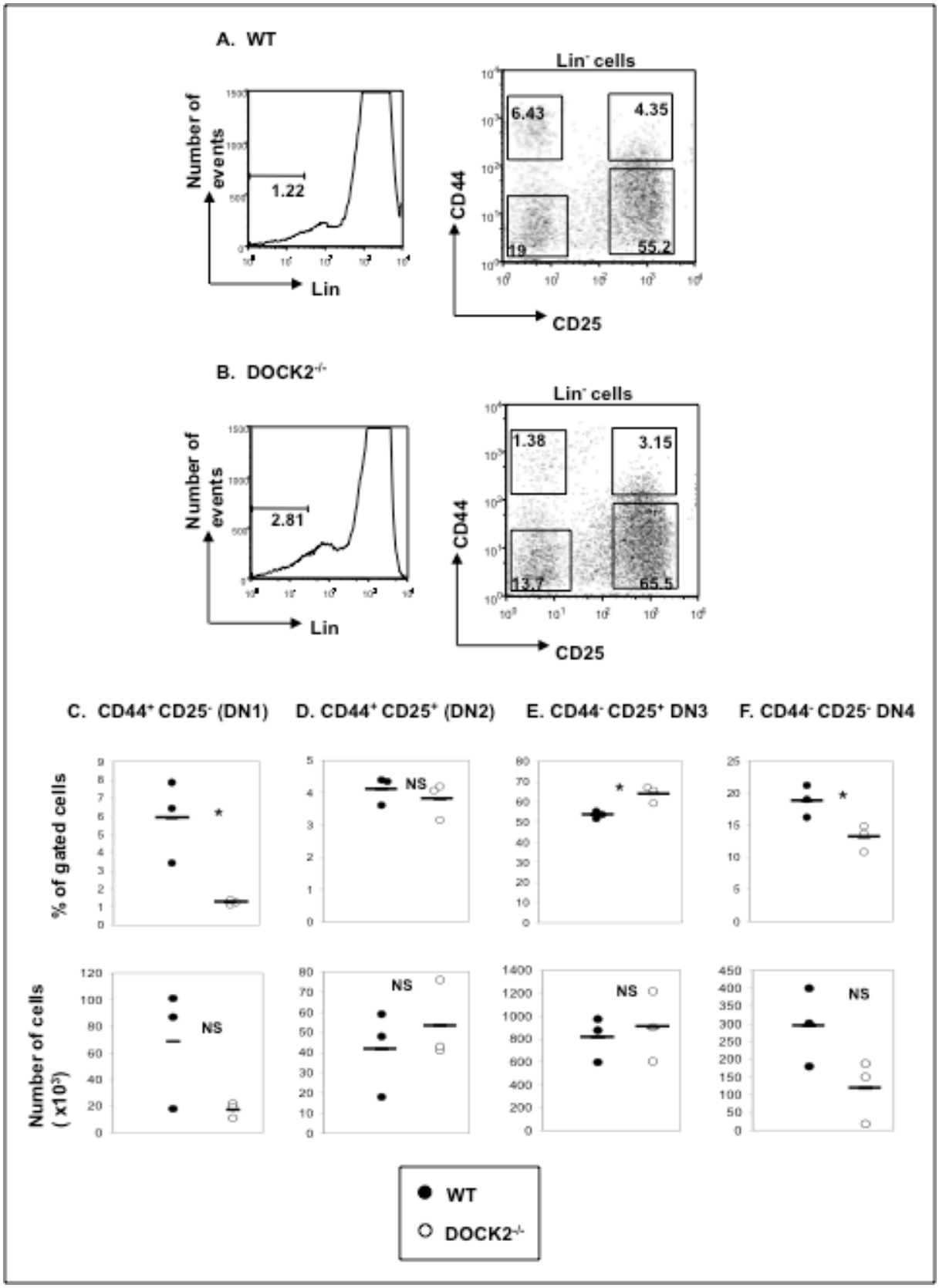
4.3.9 Quantification of Immature Single Positive Cells in $DOCK2^{-/-}$ Mice.

In the previous section, a potential block at the DN3-DN4 stage was observed. Cells at this stage of development are beginning to assemble the pre-TCR and acquire CD4 and CD8 to become DP thymocytes. This intermediate stage of development is defined by immature single positive (ISP) cells that have low surface level expression of TCRβ and are $CD8^+$.

Figure 4.10 Quantification of Double Negative Thymocyte Subsets in DOCK2$^{-/-}$ Mice.

Thymocytes were obtained from three 6-8 week old WT and DOCK2$^{-/-}$ mice and stained for a cocktail of lineage markers and the DN markers CD25 and CD44. Gating on lineage^{-} (lin^{-}) cells in both WT and DOCK2$^{-/-}$ mice shows different DN subsets based on the differential expression of CD25 and CD44 (A and B). Percentages of cells within gated areas are indicated.

Proportions of the indicated DN thymocyte subsets are shown (C-F top panels) and actual cell numbers of each DN thymocyte subset was calculated (C-F lower panels). Each point represents one mouse. Horizontal bars represent the calculated mean values. Levels of significance are shown (NS, not significant, *, $p<0.05$).


A. WT
Lin⁻ cells
Number of events
1.22
Lin
6.43
4.35
19
55.2
CD44
CD25
B. DOCK2⁻/⁻
Lin⁻ cells
Number of events
2.81
Lin
1.38
3.15
13.7
65.5
CD44
CD25
C. CD44⁺ CD25⁻ (DN1)
D. CD44⁺ CD25⁺ (DN2)
E. CD44⁻ CD25⁺ DN3
F. CD44⁻ CD25⁻ DN4
% of gated cells
Number of cells (x10³)
NS
*
WT
DOCK2⁻/⁻

Numbers and proportions of CD8 ISP cells were quantified in four 6-8 week old WT and DOCK2$^{-/-}$ mice (figure 4.11). Thymocytes were stained for TCRβ, CD4 and CD8 and ISP cells were identified as CD8^{+} TCRβ^{low} (figure 4.11A and B). Proportions of CD8 ISP cells appeared to be marginally higher in DOCK2$^{-/-}$ mice compared to WT (figure 4.11C). However, this was not found to be significant (p=0.08). Calculation of actual ISP cell numbers revealed no significant differences between WT and DOCK2$^{-/-}$ mice (p=0.5) (figure 4.11D).

4.3.10 Quantification of Mature Thymocyte Subsets in DOCK2$^{-/-}$ Mice.

To clarify if DOCK2 has a role in the generation of mature T cells, thymocytes from four 6-8 week old WT and DOCK2$^{-/-}$ mice were stained for TCRβ, CD4 and CD8. Numbers and proportions of mature CD4 and CD8 thymocytes were analysed in WT and DOCK2$^{-/-}$ mice (figure 4.12). Analysis shows that DOCK2 is not essential for the generation of mature CD4^{+} and CD8^{+} thymocytes when compared to WT mice (figure 4.12A and B). However, analysis of the total proportions of mature thymocytes as defined by high expression of TCRβ revealed that DOCK2$^{-/-}$ mice have a significant reduction of these cells (p=0.003) (figure 4.12C top graph). Calculation of actual mature thymocytes also reveals a significant reduction (p=0.0001) of these cells in DOCK2$^{-/-}$ mice (figure 4.12C lower graph). Surprisingly, despite this marked reduction of total TCRβ^{high} cells in DOCK2$^{-/-}$ mice, proportions of mature CD4 and CD8 thymocytes, defined as TCRβ^{high} CD4^{+} or TCRβ^{high} CD8^{+} respectively, were not significantly altered (mature CD4^{+}, p=0.1, mature CD8^{+}, p=0.4). However, calculation of actual numbers of mature CD4 and CD8 thymocytes reveals

Figure 4.11 Quantification of Immature Single Positive Cells in DOCK2$^{-/-}$ Mice.

Thymocytes were obtained from four 6-8 week old WT and DOCK2$^{-/-}$ mice were stained for TCRβ, CD4 and CD8. Immature single positive (ISP) CD8 cells, defined as CD8^{+} TCRβ^{low}, are shown for both WT and DOCK2$^{-/-}$ mice (A and B). Percentages of cells within gated areas are indicated.

Proportions and actual cell numbers of CD8 ISP cells are shown (C and D). Each point represents one mouse. Horizontal bars represent the calculated mean values. Levels of significance are shown (NS, not significant).

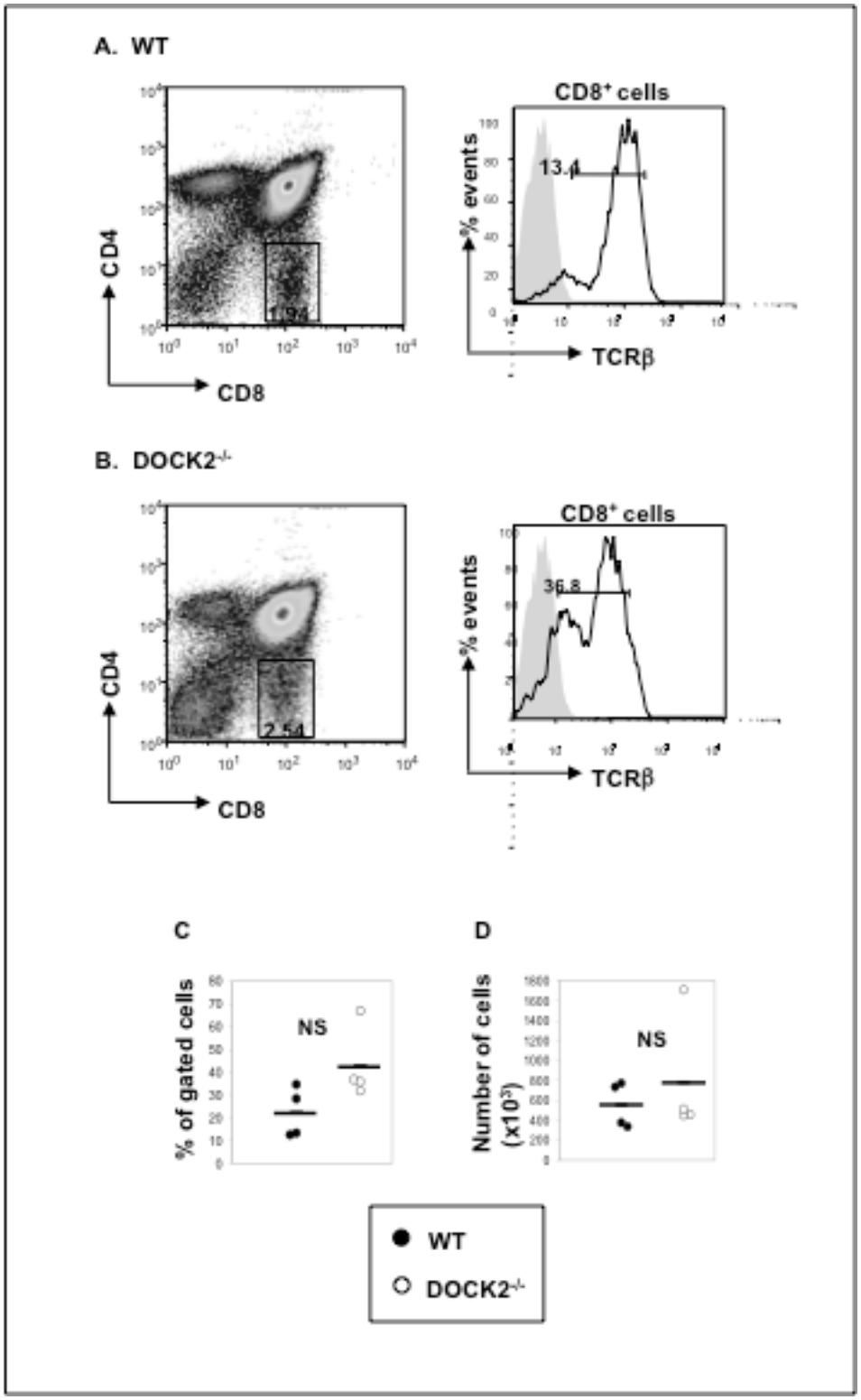
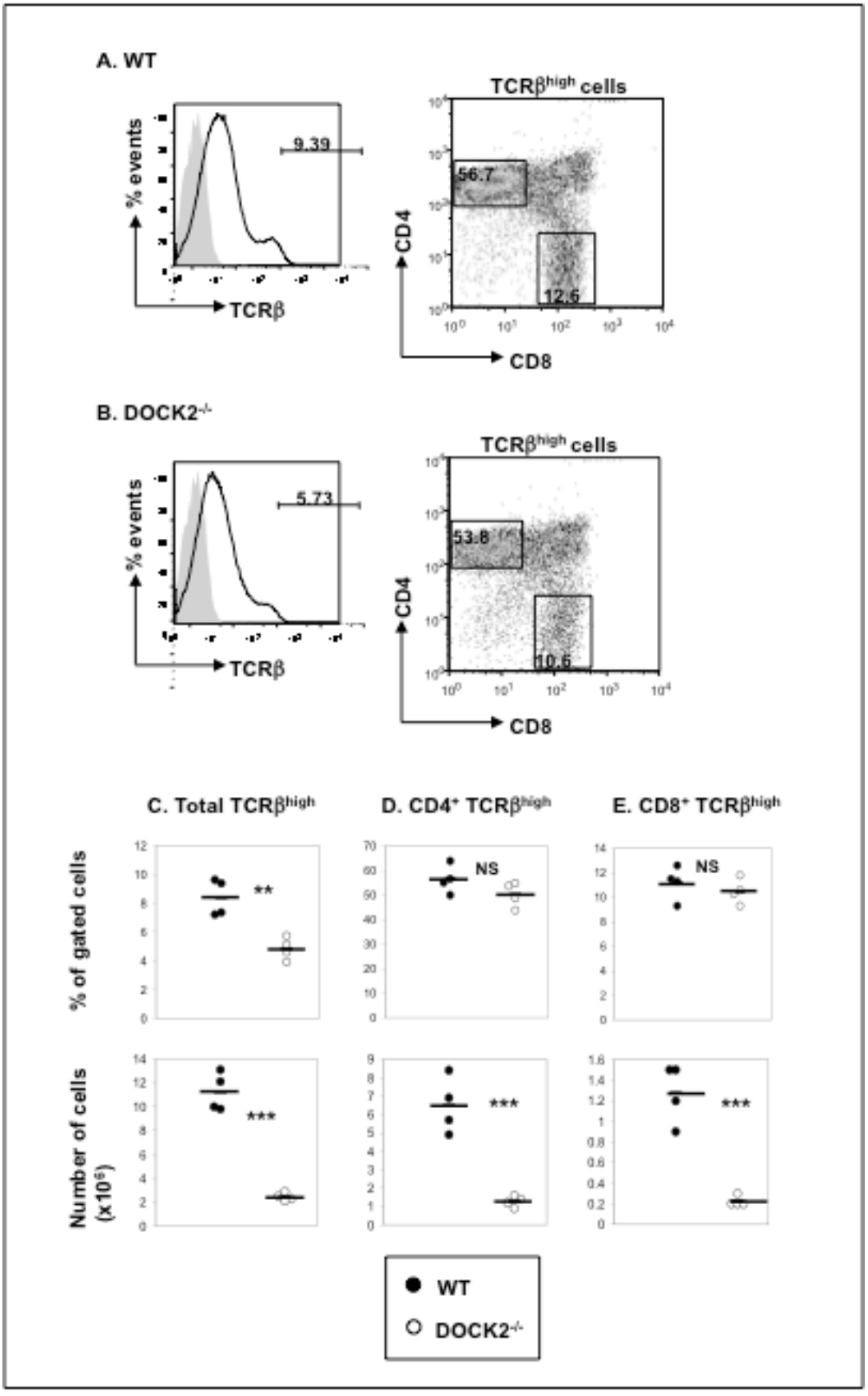

A. WT
CD4
CD8
1.94
CD8+ cells
% events
13.4
TCRβ
B. DOCK2-/-
CD4
CD8
2.54
CD8+ cells
% events
36.8
TCRβ
C
% of gated cells
NS
D
Number of cells (x10³)
NS
WT
DOCK2-/-

Figure 4.12 Quantification of Mature Thymocyte Subsets In DOCK2$^{-/-}$ Mice.

Thymocytes were obtained from four 6-8 week old WT and DOCK2$^{-/-}$ mice and stained for TCRβ, CD4 and CD8. Mature CD4^{+} and CD8^{+} thymocyte subsets present within the TCRβ^{high} population, in WT and DOCK2$^{-/-}$ are shown (A and B). Percentages of cells within gated areas are indicated.

Proportions of the indicated thymocyte subsets are shown (C-E top panels) and actual cell numbers of each subset was calculated (C-E lower panels). Each point represents one mouse. Horizontal bars represent the calculated mean values. Levels of significance are shown (NS, not significant, **, $p<0.01$, ***, $p<0.001$).


A. WT
TCRβhigh cells
% events
9.39
TCRβ
56.7
12.6
CD4
CD8
B. DOCK2-/-
TCRβhigh cells
% events
5.73
TCRβ
53.8
10.6
CD4
CD8
C. Total TCRβhigh
D. CD4+ TCRβhigh
E. CD8+ TCRβhigh
% of gated cells
**
NS
NS
Number of cells (x10^6)
***
***
***
WT
DOCK2-/-

significant reductions of both subsets in DOCK2$^{-/-}$ mice (mature CD4, p=0.0005, mature CD8, p=0.0004). (figure 5.12D and E lower graphs).

4.3.11 Composition of Thymic Microenvironments in DOCK2$^{-/-}$ Mice.

To determine whether DOCK2$^{-/-}$ mice have abnormal cortex and medullary architectures in the thymus, frozen thymic tissue from adult WT and DOCK2$^{-/-}$ mice were stained for CD4, CD8 and the medullary marker keratin 5 for confocal microscopy analysis (figure 4.13 and 4.14). Tile scans at x10 magnification were taken of the entire thymus for three 4-6 week old WT and DOCK2$^{-/-}$ mice (figure 4.13A and B show representative tile scan images of WT and DOCK2$^{-/-}$ mice). Cortex and medullary regions were identified on the basis of keratin 5 expression and DAPI staining (DAPI not shown). Data shows that whilst a WT thymus has clearly defined and contiguous areas of medulla containing SP4 and SP8 cells, DOCK2$^{-/-}$ mice show smaller and more numerous areas of medulla distributed throughout the cortex. The size (μm x μm) of the medullary regions was quantified in both WT and DOCK2$^{-/-}$ mice using the LSM software by measuring the total area of the thymus and the medullary regions. Size of the medullary areas was calculated as a percentage of the total thymus area (figure 4.13C). Data shows that DOCK2$^{-/-}$ mice appear to have smaller areas of medulla compared to WT mice. However, this was not found to be significant (p=0.4) and clearly similar analysis will need to be carried out on more mice.

Despite the apparent abnormal medullary architecture in DOCK2$^{-/-}$ mice, thymocyte composition of both the cortex and medulla was normal (figure 4.14). Comparable to a WT thymus (figure 4.14A and C), DP cells were correctly located in the cortex and SP cells were still able to localise to the medulla (figure 4.14B and D) in the thymus of DOCK2$^{-/-}$ mice.

Figure 4.13 DOCK2$^{-/-}$ Mice Have Abnormal Medullary Architecture in the Thymus.

Frozen tissues sections of three 6-8 week old adult WT and DOCK2$^{-/-}$ mice were stained for CD4, CD8 and keratin 5 for confocal analysis. Tile scans taken at x10 magnification of the entire thymus from one WT and DOCK2$^{-/-}$ mouse are shown (A and B). Solid line denotes the edge of the tissue; dashed line denotes the corticomedullary junction. Bar indicates scale of 500μm.

The size (μm x μm) of the medullary regions of each mouse, calculated as a percentage of the total thymus area (μm x μm), is shown (C). Each point represents one mouse. Levels of significance shown (NS, not significant).

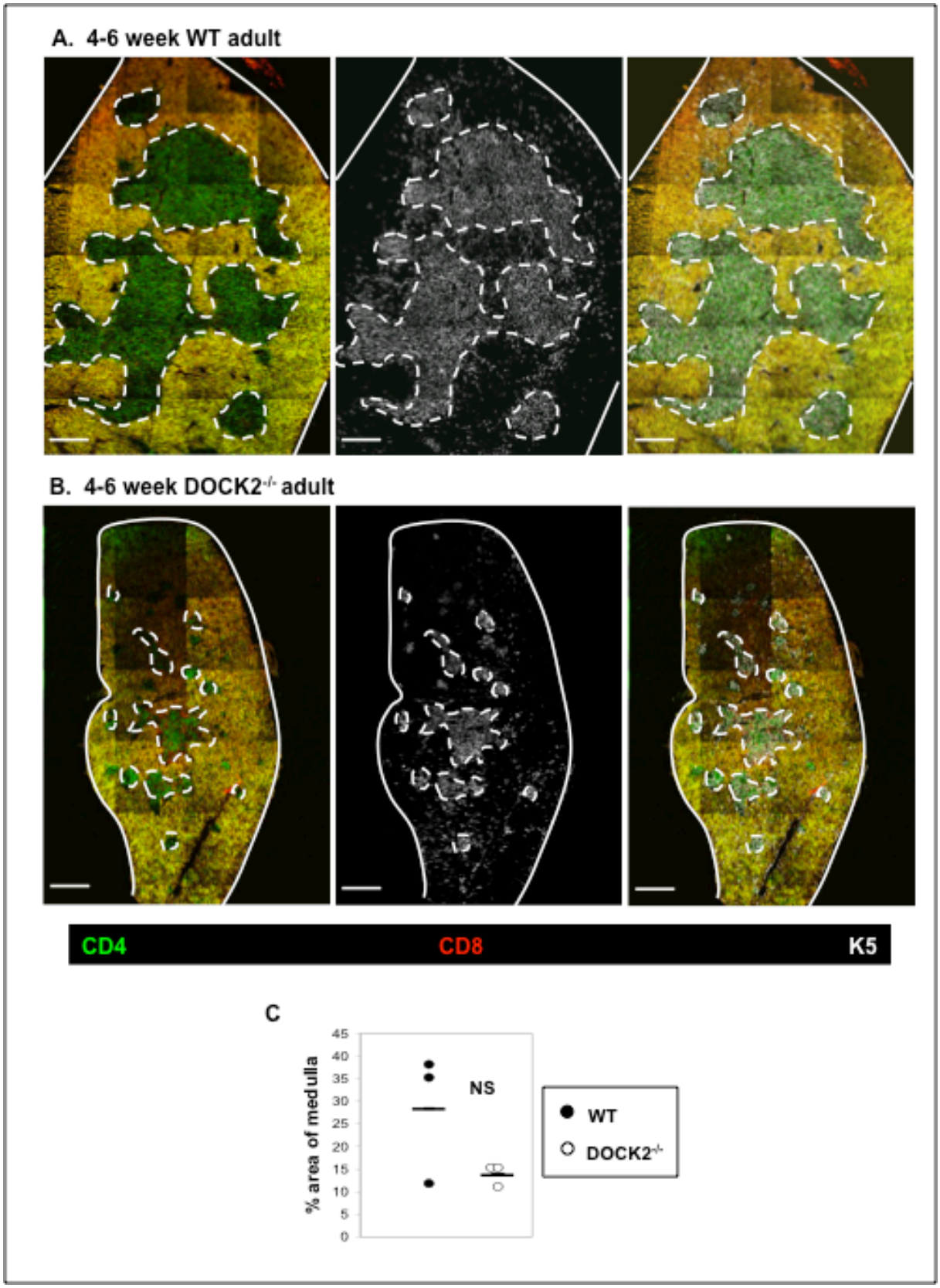
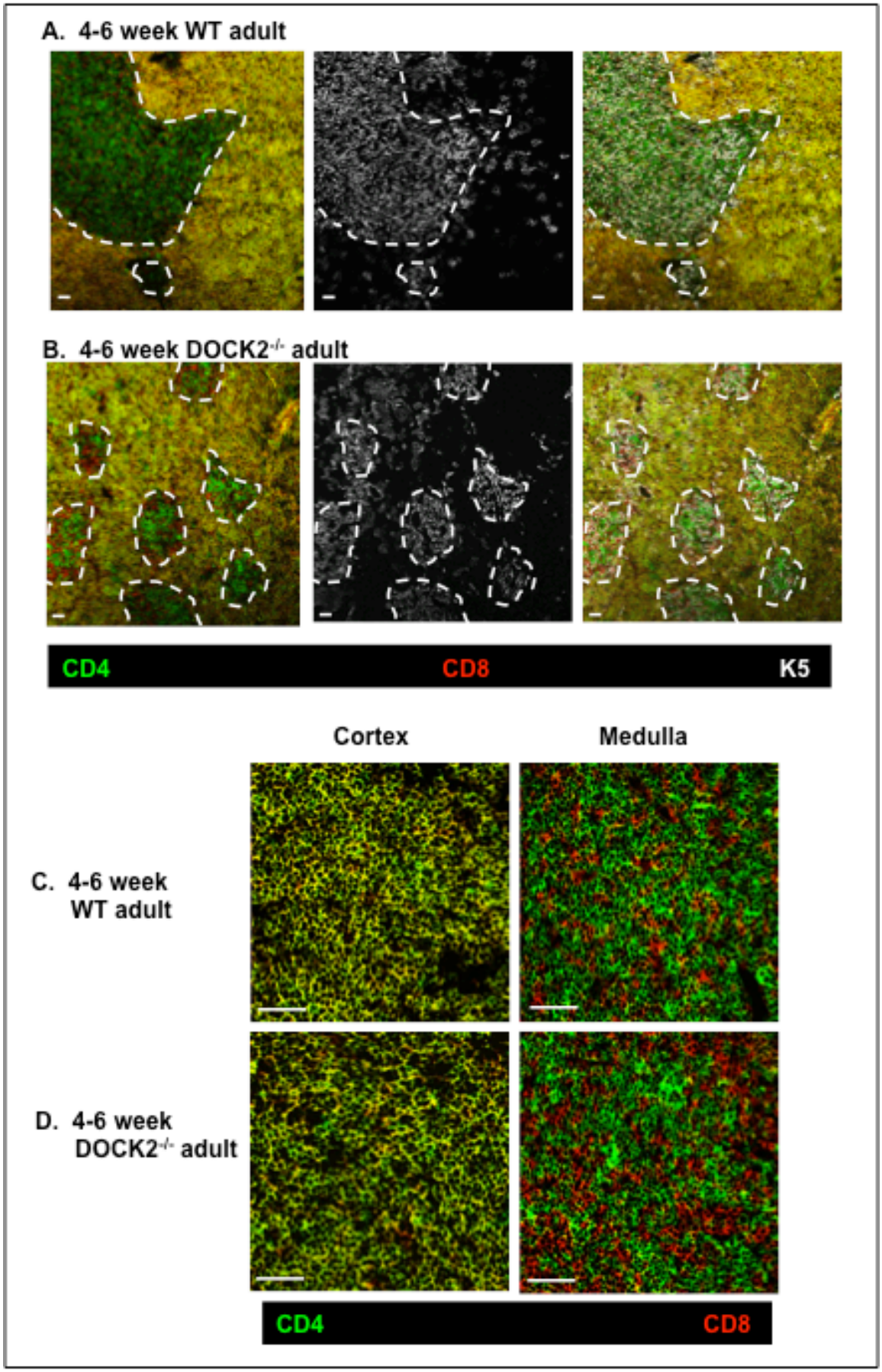

A. 4-6 week WT adult
B. 4-6 week DOCK2-/- adult
CD4
CD8
K5
C
% area of medulla
45
40
35
30
25
20
15
10
5
0
NS
WT
DOCK2-/-

Figure 4.14 DOCK2$^{-/-}$ Mice Have Normal Thymocyte Distribution.

Frozen sections of thymus from 6-8 week old WT and DOCK2$^{-/-}$ mice were stained for CD4, CD8 and keratin 5. Low power images for each mouse show thymocyte distribution in the cortex and medulla (as identified on the basis of keratin 5 expression) (A and B). High power images of cortex and medulla in both mice are also shown (C and D).

Dashed line denotes the corticomedullary junction. Bar indicates scale of 50μm.


A. 4-6 week WT adult
B. 4-6 week DOCK2-/- adult
CD4
CD8
K5
Cortex
Medulla
C. 4-6 week
WT adult
D. 4-6 week
DOCK2-/- adult
CD4
CD8

Furthermore, co-expression of keratin 5 in the SP4 and SP8 patches that were scattered throughout the cortex in the DOCK2$^{-/-}$ thymus confirmed these patches as medullary regions and not abnormally located SP cells in the cortex.

4.3.12 Localisation of CD25^{+} Cells in the Thymus of DOCK2$^{-/-}$ Neonatal and Adult Mice.

To investigate CD25^{+} cell localisation to the SCZ in DOCK2$^{-/-}$ mice, frozen tissue sections of thymus from of 6-8 week old day 3 neonatal WT and DOCK2$^{-/-}$ mice were stained for CD25, CD4 and CD8 for confocal analysis (figures 4.15 and 4.16). Numbers of CD25^{+} cells in subcapsular, cortical and medullary regions were quantified. The SCZ was identified by drawing a box around the approximated region directly beneath and including the capsule. This was kept consistent throughout. Cortex was identified as the region containing DP cells, whereas the medulla was identified as the region containing SP cells. Additionally, cortex and medulla was also identified on the basis of DAPI staining (not shown). Boxes were drawn around these areas. CD25^{+} cells were counted in 3-5 areas of subcapsular, cortex and medulla for three separate adult and neonatal WT and DOCK2$^{-/-}$ thymi. Number of cells was divided by the measured area of the drawn boxes (μm x μm). Numbers of cells per μm^2 were multiplied by 10^6 to give numbers of cells per mm^2. Mean values were calculated for the 3-5 areas of SCZ, cortex and medulla for each mouse and number of cells per mm^2 was plotted (figure 4.15G and 4.16G).

DOCK2 deficiency in neonatal mice, by comparison to WT (figure 4.15A-C), did not appear to have any significant detrimental effects on CD25^{+} cell distribution as these cells were found in their normal location in the SCZ with few cells found in the cortex and medulla (figure 5.15D-F). Also DP cells were not mislocalised in the SCZ, which is in contrast to

CCR9$^{-/-}$ mice, which already show DN and DP localisation defects at the neonatal stage.

Surprisingly, differences in CD25^{+} cell distribution become more apparent in the DOCK2$^{-/-}$ adult (figure 4.16). Whilst the CD25^{+} cells still retained the ability to localise to the SCZ (figure 4.16D), the numbers of these cells were increased in the cortex of DOCK2$^{-/-}$ adult mice (figure 4.16E) compared to WT (figure 4.16B) and calculation of numbers of CD25^{+} cells revealed that this increase was significant (p=0.002) (figure 4.16G). Furthermore, these CD25^{+} cells were not CD4^{+} or CD44^{+}, confirming that these cells are not Tregs or DN2 cells and are in fact DN3 cells (data not shown). No significant differences observed in CD25^{+} cell distribution in SCZ (p=0.5) and medulla (p=0.4) between WT and DOCK2$^{-/-}$ mice.

4.3.13 Increased proportions of CD25^{+} cells in DOCK2$^{-/-}$ mice.

Data in section 4.3.12 indicates mislocalisation of CD25^{+} cells in the cortex of DOCK2$^{-/-}$ mice. As numbers of CD25^{+} cells in the SCZ and medulla were comparable between the DOCK2$^{-/-}$ and WT mice, this would suggest that DOCK2$^{-/-}$ mice will have an increase in total proportions of CD25^{+} cells compared to WT. Thymocytes from three WT and DOCK2$^{-/-}$ mice were stained for CD25 and proportions and total numbers of CD25^{+} cells were analysed (figure 4.17). Data shows that DOCK2$^{-/-}$ mice have significantly increased proportions of CD25^{+} cells compared to WT (p=0.0001) (figure 4.17C). Calculation of actual CD25^{+} cells revealed no significant differences between WT and DOCK2$^{-/-}$ mice (p=0.5) (figure 4.17D).

Figure 4.15 Normal Localisation of $CD25^+$ Cells to the SCZ in $DOCK2^{-/-}$ Neonatal Mice.

Frozen sections of thymus from 3 neonatal (day 3) WT and $DOCK2^{-/-}$ mice were stained for CD25, CD4 and CD8. Representative images of $CD25^+$ cell distribution in SCZ, cortex and medulla are shown in both WT and $DOCK2^{-/-}$ mice (A-F).

Numbers of $CD25^+$ cells in each area was quantified by counting cells in 3-5 areas of SCZ, cortex and medulla from 3 WT and $DOCK2^{-/-}$ mice. Number of cells was divided by the area of these regions (μm x μm). Number of cells per μm^2 was multiplied by 10^6 to give number of cells per mm^2. Plot shows the mean number of cells per mm^2 calculated from the 3-5 areas of SCZ, cortex and medulla.

Each point represents one mouse. Horizontal bars indicate the calculated mean. Levels of significance shown (NS, not significant). (Note: only 2 points are shown for $DOCK2^{-/-}$ medulla as the third mouse did not have large enough areas of medulla to allow for quantification).

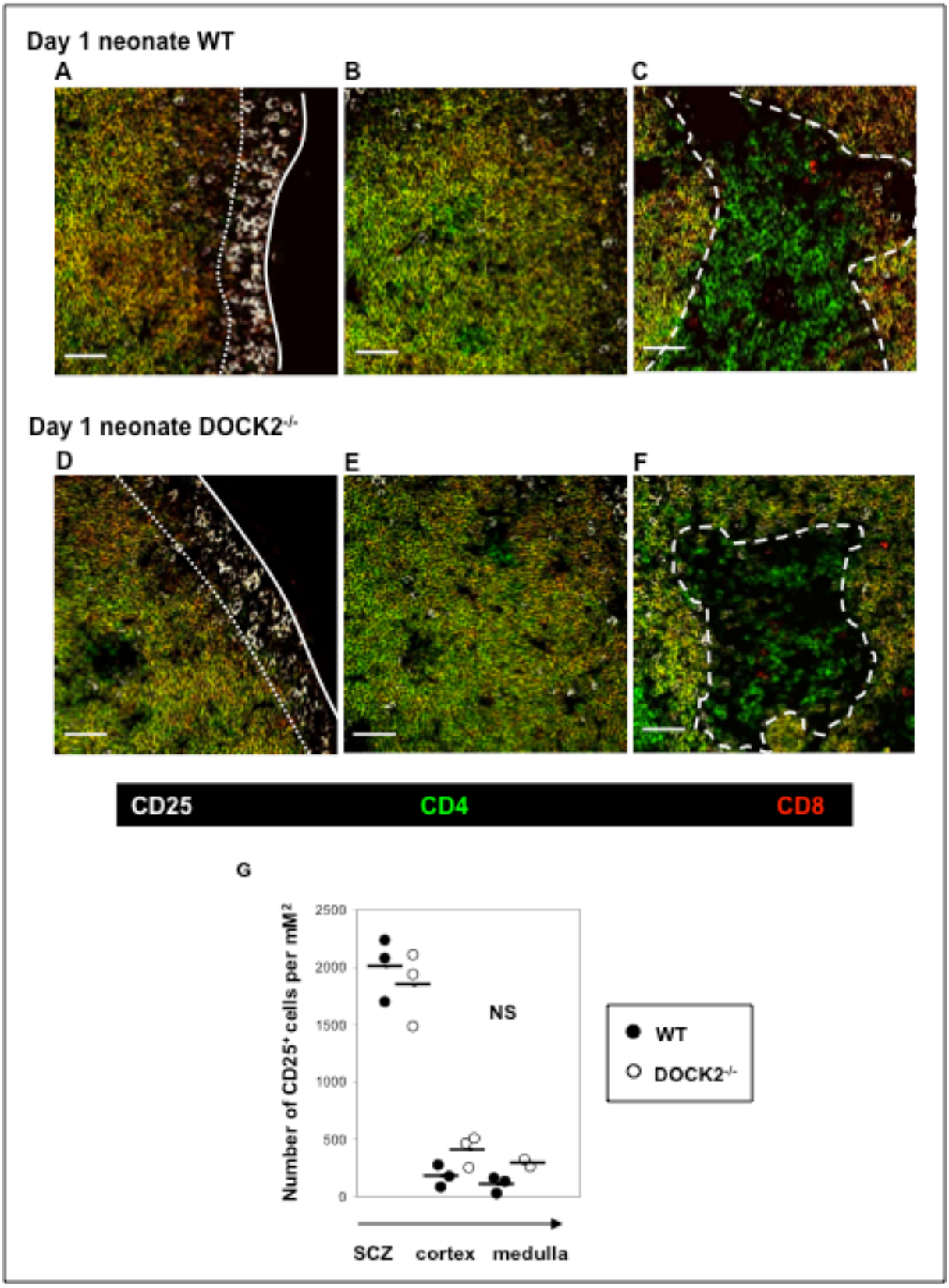

Day 1 neonate WT
A
B
C
Day 1 neonate DOCK2-/-
D
E
F
CD25
CD4
CD8
G
Number of CD25+ cells per mM2
2500
2000
1500
1000
500
0
NS
WT
DOCK2-/-
SCZ
cortex
medulla

Figure 4.16 Abnormal Localisation of CD25^{+} Cells in the Cortex of DOCK2$^{-/-}$ Adult Mice.

Frozen sections of thymus from 3 adult (6-8 week old) WT and DOCK2$^{-/-}$ mice were stained for CD25, CD4 and CD8. Representative images of CD25^{+} cell distribution in SCZ, cortex and medulla are shown in both WT and DOCK2$^{-/-}$ mice (A-F).

Numbers of CD25^{+} cells in each area was quantified by counting cells in 3-5 areas of SCZ, cortex and medulla from 3 WT and DOCK2$^{-/-}$ mice. Number of cells was divided by the area of these regions (μm x μm). Number of cells per μm^2 was multiplied by 10^6 to give number of cells per mm^2. Plot shows the mean number of cells per mm^2 calculated from the 3-5 areas of SCZ, cortex and medulla.

Each point represents one mouse. Horizontal bars indicate the calculated mean. Levels of significance shown (NS, not significant, **, $p<0.01$).

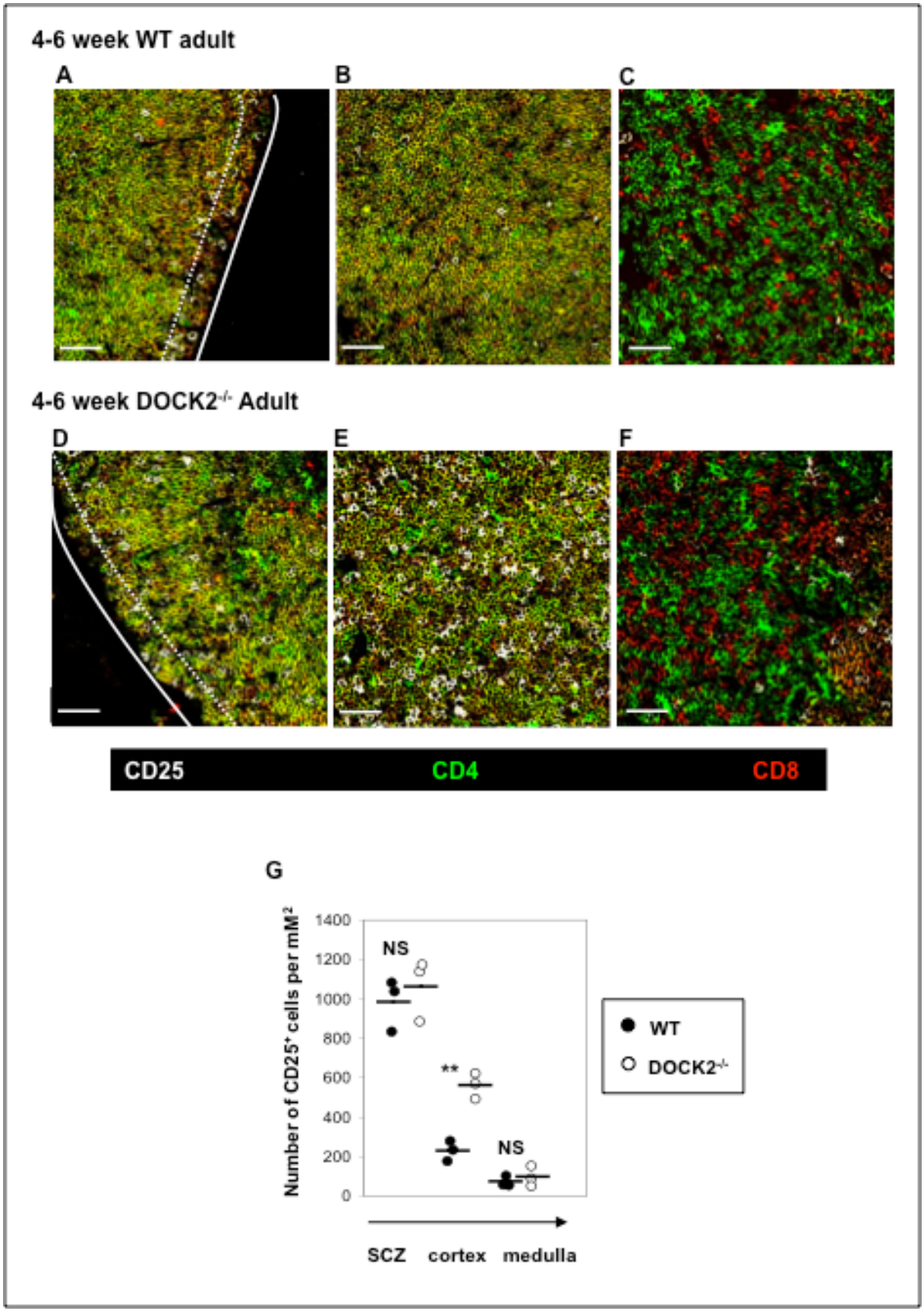
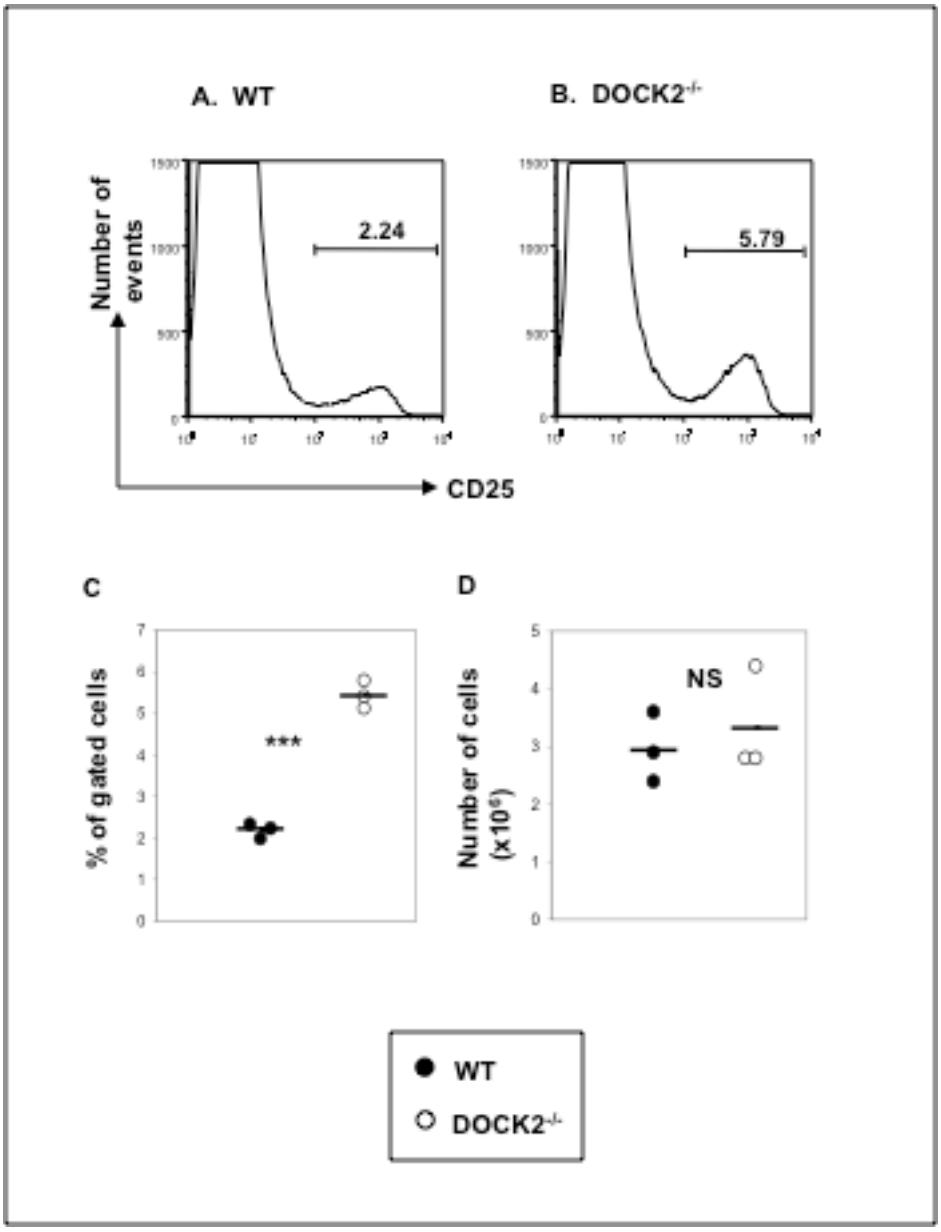

4-6 week WT adult
A
B
C
4-6 week DOCK2-/- Adult
D
E
F
CD25
CD4
CD8
G
Number of CD25+ cells per mM2
1400
1200
1000
800
600
400
200
0
NS
**
NS
WT
DOCK2-/-
SCZ
cortex
medulla

Figure 4.17 Increased Proportions of CD25^{+} Cells in DOCK2$^{-/-}$ Mice.

Thymocytes from three adult WT and DOCK2$^{-/-}$ mice were stained for CD25 for flow cytometry analysis. Histograms show CD25 expression within live-gated cells from WT (A) and DOCK2$^{-/-}$ (B) mice. Percentages of cells within gated areas are indicated.

Proportions of CD25^{+} cells are shown (C) and actual CD25^{+} cell numbers was calculated (D).

Each point represents one mouse. Horizontal bars represent the calculated mean values. Levels of significance are shown. (NS= not significant, ***p<0.001).


A. WT
B. DOCK2-/-
Number of events
2.24
5.79
CD25
C
D
% of gated cells
***
Number of cells (x10^6)
NS
WT
DOCK2-/-

4.4 Discussion.

4.4.1 Establishment of the Subcapsular Zone and A Role For CCR9 Signalling.

T cell development is defined by the ordered migration of developing thymocytes through distinct thymic niches. Whilst the migration of thymocytes to cortical and medullary regions are known to be associated with positive and negative selection respectively, the importance of thymocyte homing to the SCZ remains speculative. The SCZ is the area that lies directly adjacent to the mesenchymal cell derived capsule surrounding the thymus. This region is thought to provide a suitable microenvironment to support the differentiation of DN3 cells. Pre-TCR signalling defines this stage of T cell development, where rapidly proliferating cells undergoing TCR gene rearrangements emerge from a process of β-selection to begin their transition to the DP stage of T cell development (Lind et al., 2001). Thus, identifying the signals that govern SCZ formation is crucial to our understanding of an important early checkpoint in T cell development. This study aimed to elucidate the establishment of the SCZ during ontogeny and highlight the role for chemokine directed migration.

In the present study, $CD25^+$ DN cells were scattered throughout the early embryonic thymus (E14-E16) and localisation to a subcapsular niche was distinctly absent. However, during late embryonic gestation (E18) when DP cells are beginning to develop, establishment of the SCZ occurs providing a niche for $CD25^+$ DN3 cells to localise. By postnatal day 0 a clearly defined SCZ containing $CD25^+$ cells was present and most strikingly, DP cells located in the cortex were distinctly absent from the SCZ at both E18 and day 0. The high proliferative burst associated with DN to DP transition may generate a physical force that helps to propel newly generated DP cells out of the SCZ, possibly explaining the absence of DP cells in this region. In a 6-8 week old adult thymus the SCZ appeared less defined with

fewer $CD25^+$ cells localising to this region and DP cells clearly present. Previous reports highlight the importance of chemokine directed migration in regulating the complex trafficking of thymocytes (Takahama, 2006) and in line with this our study demonstrates compartmentalisation of chemokines in discrete thymus regions. By employing a laser capture microdissection (LCM) technique, we identified that expression of CXCL12, CCL25 and their respective receptors CXCR4 and CCR9 were mostly confined to the SCZ and cortex, whereas expression of CCL19, CCL21 and their receptor CCR7 were almost exclusive to the medulla. This differential chemokine expression pattern suggests that chemokine induced migration is important in regulating the stratified positioning of thymocytes. This data is in line with Ueno et al (2004) who report the importance of CCR7 signalling in regulating cortex to medulla migration of SP thymocytes and those who report the importance of CCR9 and CXCR4 signalling in regulating DN localisation in the cortex and SCZ (Benz et al., 2004; Lind et al., 2001; Plotkin et al., 2003). However, in the context of this study it was of interest to study the requirement for CCR9-CCL25 signalling during thymocyte trafficking to the SCZ.

In $CCR9^{-/-}$ adults $CD25^+$ cells were found scattered throughout the cortex and failed to localise to the SCZ, in accordance with Benz et al (2004) who suggest that localisation of $CD25^+$ DN2/3 cells in the adult is dependent on CCR9 signalling. However, the present study extends previous findings by demonstrating a more dramatic difference in thymocyte localisation to the SCZ in $CCR9^{-/-}$ neonates. In contrast to WT neonates, $CCR9^{-/-}$ neonates had fewer $CD25^+$ cells localising to the SCZ but most importantly displayed perturbed localisation of DP cells to the SCZ. Thus, these mice resembled a WT adult with regard to their thymocyte composition in the SCZ. This data potentially suggests a different or a more complex role for CCR9 signalling during thymocyte localisation to the SCZ, than previously

thought. Rather than acting on $CD25^+$ cells, CCL25 could be regulating localisation of DP cells instead. Norment et al (2000) report that CCR9 expression is up-regulated on DN cells following assembly of the pre-TCR as DN cells undergo transition to the DP stage. Furthermore, initiation of the pre-TCR signal correlated with a higher migratory capacity of thymocytes in response to CCL25. In view of this, our data suggest that in a WT neonatal thymus, DN cells that have migrated to the SCZ (possibly in response to CXCL12) undergo a pre-TCR signal and up-regulate expression of CCR9 and the CD4 and CD8 co-receptors before moving out of the SCZ into the cortex in response to CCL25. However, in the absence of CCR9, DP cells emerging from pre-TCR signalling are unable to receive a CCL25 signal so preventing their migration out of the SCZ and into the cortex, potentially explaining why DP cells accumulate in the SCZ of $CCR9^{-/-}$ neonates. Clearly, in these mice a majority of DP cells can still localise to the cortex and this may reflect either a compensatory mechanism for DP cell localisation in the absence of CCR9 or the fact that all DP cells cannot physically occupy such a small region of the thymus. In further support of our hypothesis, Uehara et al (2006) show that the premature expression of CCR9 in DN thymocytes (i.e. prior to receiving a pre-TCR signal) leads to the accumulation of $CD25^+$ cells in the cortex. Whilst they suggest that this retention of DN cells in the cortex is due to failure of DN cells to migrate towards the SCZ, data in the present study may alternatively suggest that early CCR9 signalling could result in a premature migration signal to the $CD25^+$ cells, such that these cells exit the SCZ before completing pre-TCR signalling and transition to the DP stage.

In this study a striking difference in DP cell localisation to the SCZ was observed in WT neonates compared to adults. In light of the finding that $CCR9^{-/-}$ neonates resemble WT adults, one can speculate that a differential requirement for CCR9 signalling exists between the neonate and an adult. Certainly, the requirement for chemokine signalling is already

known to differ between the fetal and post-natal stages during thymus development. Thus, it is possible that DP cells in a WT adult do not require CCL25 signalling to move out of the SCZ. Reports suggest that whilst late stage DN cells have low levels of CCR9, a small proportion of DN2/3 cells do have the capacity to migrate in response to CCL25 signalling (Uehara et al., 2002; Wurbel et al., 2000). This may explain why in the WT adult a proportion of $CD25^{+}$ DN cells do localise to the SCZ and why deficiency in CCR9 results in an almost complete failure of $CD25^{+}$ cells to localise to the SCZ as demonstrated in this study and by Benz et al (2004). Alternatively, differences observed in DP cell localisation to the SCZ in the adult and neonate may be explained by an increased efficiency in DN cell differentiation, where adult DN cells are faster at becoming DP cells and so have already undergone pre-TCR signalling and expression of the co-receptors before reaching the SCZ.

Given that T cell development is characterised by the ordered migration of each substage through the distinct regions of the thymus, it could be presumed that impaired migration of DN or DP thymocytes into or out of the SCZ may lead to a defect in pre-TCR signalling and a subsequent block in the DN-DP stage. However, perturbed localisation of DP and SP cells was not observed in $CCR9^{-/-}$ neonates and adults in this study. Furthermore, previous studies did not demonstrate reduced proportions of DP cells or abnormal T cell development overall in $CCR9^{-/-}$ mice (Benz et al., 2004; Wurbel et al., 2001). Such findings indicate that localisation of thymocytes to the SCZ may not be an essential requirement for early T cell development. This fits with the notion that the pre-TCR complex regulates T cell development in a ligand independent manner, which also argues against the need for a 'physical niche' for DN to DP cell transition (Irving et al., 1998; Yamasaki et al., 2006). However, a more recent study finds that when $CCR9^{-/-}$ DN cells were placed in a competitive environment with WT DN cells, they were impaired in their ability to generate DP compared

with WT cells (Svensson et al., 2008). This supports the notion that pre-TCR induced CCR9 expression may have a non-redundant role during pre-TCR signalling and transition to the DP stage. Furthermore, this study also demonstrated that CCR9$^{-/-}$ DP cells were disadvantaged in their ability to generate SP4 cells when in competition with WT DP cells.

To summarise, not only do our findings reinforce the view that CCR9-CCL25 signalling is important in the localisation of thymocytes to the SCZ, a novel finding that CCR9-CCL25 may also have a role in DP cell localisation in the SCZ was also reported.

4.4.2 The Importance of DOCK2 During T Cell Development and Organisation of Thymic Microenvironments.

In line with previous data indicating a role for chemokines in thymocyte localisation, T cell development and compartmentalisation was also analysed in DOCK2$^{-/-}$ mice, which have been reported to have poorly characterised abnormalities in T cell development and thymocyte localisation.

As evidenced by our quantitative RT-PCR data, DOCK2 gene expression is found in all thymocyte subsets, with DP and SP4 cells expressing the highest levels. This suggests an important role for DOCK2 during these stages of T cell development, as will be discussed below. Furthermore, DOCK2 expression was present in all DN subsets, with DN3 cells showing the highest level. Contrary to flow cytometry data showing that DOCK2 deficiency leads to a significant reduction in proportions of DN1 cells, these particular cells express low levels of DOCK2. To explain this apparent discrepancy, it is currently unclear if DOCK2 is expressed by all DN1 subsets including DN1a, b and c (as identified by Porritt et al., 2004) and whether all these subsets are present in our sorted populations.

Whilst it is unclear if DOCK2 has a chemokine independent function, the fact that it mediates lymphocyte migration by functioning downstream of CCR7 and CXCR4 signalling prompted us to determine if deficiency in DOCK2 would lead to abnormalities in T cell development and thymus organisation. The present study reports that deficiency in DOCK2 leads to a severe reduction in thymus cellularity. However, T cell development still occurs in a normal manner with the presence of all substages of T cell development and mature subsets of $CD4^+$ and $CD8^+$ T cells in the thymus. Further analysis revealed that proportions of DN cells were increased in $DOCK2^{-/-}$ mice compared to WT, along with decreased proportions of DP and SP4 cells. Observations of reduced thymic cellularity and increased proportions of DN cells are in accordance with previous claims (Fukui et al., 2001). Due to this observed increase in DN proportions, the effect DOCK2 deficiency has on the development of thymocytes through the DN stages was investigated. Increased proportions of DN3 cells and a reduction in DN4 proportions were observed and this correlated with a marginal increase in immature single positive (ISP) $CD8^+$ cell proportions, which lie between the DN to DP stage of T cell development. Pre-TCR signalling and subsequent development to the DP stage defines this important stage of T cell development. These findings differ from what has been reported in $CCR7^{-/-}$ mice and mice deficient for the CCR7 ligands CCL19 and CCL21 (*plt/plt* mice), which show increased DN1 cells and DN1-2 cells (a transitional substage between the DN1 and DN2 stage), along with a decrease in DN3 and DN4 cells (Ueno et al., 2004). Observations of increased ISP cells in the present study tentatively suggest a block during the DN3-4 stage and a possible role for DOCK2 during pre-TCR signalling. Indeed these findings correlate with our observations that deficiency in DOCK2 in adult mice leads to accumulation of $CD25^+$ cells in the cortex. A study by Sanui et al (2003) demonstrates that in the absence of DOCK2, TCR polarisation and clustering is disrupted during immunological

synapse formation (IS). They conclude that DOCK2 is downstream regulator of TCR signalling and is essential for TCR mediated activation of Rac, thus implicating an important role for DOCK2 in the formation of the IS. In the context of the present study, if DOCK2 is also an important regulator of pre-TCR signalling, DN cells that have accumulated in the cortex of DOCK2$^{-/-}$ mice have failed to receive a pre-TCR signal and so are blocked in their ability to give rise to DP cells. Certainly if this scenario was true then this may account for the observed increase in DN proportions and decreased DP proportions.

Our observations of CD25^{+} cell accumulation in the cortex was only apparent in adult DOCK2$^{-/-}$ mice and was similar to our observations of CD25^{+} cell accumulation in the cortex of CCR9$^{-/-}$ adult mice. However, by contrast to CCR9$^{-/-}$ adults, a large fraction of CD25^{+} cells could still accumulate in the SCZ of DOCK2$^{-/-}$ mice, suggesting the existence of a DOCK2 independent pathway. This data is also in contrast to CCR7$^{-/-}$ and *plt/plt* mice, which have less abundant CD25^{+} DN3 cells in the outer cortex and SCZ and an accumulation of DN2 cells at the cortico-medullary junction (Ueno et al., 2004).

In the present study reduced proportions SP4 cells in DOCK2$^{-/-}$ mice were observed. A previous study has reported that DOCK2 deficiency leads to reduced efficacy of positive selection (Sanui et al., 2003). Collectively, this data may suggest a reduced ability of DOCK2$^{-/-}$ DP cells to form stable interactions with thymic epithelial cells (TECs). Indeed preliminary experiments in the lab using thymocyte-epithelial cell conjugate assays where cell-cell contact is dependant on TCR-MHC interactions, suggest that DOCK2$^{-/-}$ DP cells are less able to form stable conjugates with TECs compared to WT DP cells (Will Jenkinson, unpublished data). This suggests a possible role for DOCK2 during initial stages of positive selection.

In this study we also show that deficiency in DOCK2 leads to abnormal medullary architecture, where medullary regions are reduced in size and are scattered throughout the cortex as small patches. This suggests an important role for DOCK2 in the development of normal medullary regions. The abnormalities we observe in the DOCK2$^{-/-}$ mouse are in accordance to those seen in mice deficient for CCR7 and *plt/plt* mice, which also display small and sparsely distributed medullary regions throughout the cortex (Ueno et al., 2004). However, most importantly we show that despite the abnormal medullary architecture in DOCK2$^{-/-}$ mice there is still normal accumulation of SP thymocytes in the medulla and not the in cortex, which is in contrast to a previous study that reported mislocalisation of SP cells in the cortex of DOCK2$^{-/-}$ mice (Fukui et al., 2001). This apparent discrepancy can be explained by the fact the in our study we used the medullary marker keratin 5 to characterise the precise location of these SP4 and SP8 patches. In CCR7$^{-/-}$ mice, accumulation of SP cells in the medulla is poor suggesting that CCR7 signals are essential for guiding SP cells through the medulla and is essential for maintaining proper thymic morphology (Ueno et al., 2004). Clearly, if DOCK2 is functioning downstream of CCR7 signalling then this may account for the abnormalities observed in the medulla in our study. With regard to the secondary lymphoid organs T and B cell segregation is absent in *plt/plt* mice and this is in contrast to DOCK2$^{-/-}$ mice (Reif and Cyster, 2002). This suggests that CCR7 can signal in a DOCK2 independent manner and this could potentially explain why the thymic abnormalities we observe are not quite as severe as those observed in the CCR7$^{-/-}$ mice. Furthermore, Ueno et al (2004) report that the phenotype of the CCR7$^{-/-}$ mouse becomes more aggravated with age (8 months). So whilst we see modest detrimental effects on T cell development in DOCK2$^{-/-}$ mice that are 6-8 weeks old, we may see more pronounced defects if were to investigate T cell development in older mice.

The study by Fukui et al (2001) also suggest that DOCK2$^{-/-}$ mature thymocytes, that are failing to respond to CCL19, have a thymic emigration defect, which accounts for the T lymphocytopenia they observed in the periphery of the DOCK2$^{-/-}$ mice. However, in our study we did not observe increased proportions of mature CD4^{+} or CD8^{+} T cells in DOCK2$^{-/-}$ mice, suggesting no defects in thymocyte export. Clearly, this should be investigated further by analysing proportions of recent thymic emigrants based on expression of CD62L and Qa-2, which are indicative of the most mature SP cells. Indeed, preliminary data in this study shows increased numbers of Qa-2^{+} CD4^{+} thymocytes in the thymus of DOCK2$^{-/-}$ mice compared to WT (data not shown) indicating that there may indeed be an emigration defect in DOCK2$^{-/-}$ mice.

This study shows that a majority of DOCK2$^{-/-}$ thymocytes clearly still retain the ability to respond to chemokines as evidenced by the overall normal organisation of DP and SP cells. This may suggest the existence of an alternative pathway that operates in the absence of DOCK2 or the chemokines (CCL19, CCL21 and CXCL12). Whether this alternative pathway operates in the presence of DOCK2, as is the case in WT mice, or if this pathway becomes activated in the absence of DOCK2, requires further investigation. Molecules, other than DOCK2, may exist that might be involved in relaying CCR7 and CXCR4 mediated signals to induce cell migration. These may include the structurally related CDM proteins DOCK180, DOCK10 and DOCK11, which are all known to be expressed by lymphocytes (Gotoh et al., 2008; Yelo et al., 2008) or even PI3K, which is known to be important for lymphocyte migration (Okkenhaug and Vanhaesebroeck, 2003). Mice deficient for both DOCK2 and the above-related CDM proteins may help to understand this pathway further. Indeed, a recent study has used mice doubly deficient for DOCK2 and DOCK180 and found

reduced pre-vascular colonisation of the fetal thymus, shedding further light on the role of these pathways in the thymus (Lei et al., 2009).

To summarise, DOCK2 may be important for the migration and development of progenitors undergoing pre-TCR signalling as well as proper maintenance of thymus morphology. However, T cell development still occurs in the absence of DOCK2 suggesting that chemokine signalling via DOCK2 is not a prerequisite for normal T cell development but may be important for optimal development of mature T cells with a diverse TCR repertoire. Certainly, competitive bone marrow transfer experiments (similar to those carried out by Svensson et al, 2008) where DOCK2$^{-/-}$ thymocytes are placed in direct competition with WT thymocytes will further elucidate the role of DOCK2 during T cell development.

CHAPTER 5

PHENOTYPIC ANALYSIS OF MEDULLARY THYMIC EPITHELIAL CELLS AND THE THYMIC MESENCHYME

5.1 Introduction.

Following the identification of a bipotent thymic epithelial cell (bTEC) progenitor that can differentiate into mature cortical and medullary epithelium (Bleul et al., 2006; Rossi et al., 2006), subsequent studies have begun to provide a more detailed account of the events leading to the differentiation of mature mTECs. Namely, the identification of heterogeneous subsets within the mTEC lineage has been valuable. The two major subsets of mTECs known to exist consist of an $mTEC^{low}$ subset defined as $CD80^{-}$ $AIRE^{-}$ MHC II^{low} and an $mTEC^{high}$ subset defined as $CD80^{+}$ $AIRE^{+}$ MHC II^{high} (Derbinski et al., 2005; Gray et al., 2007a; Rossi et al., 2007b). According to the terminal differentiation model, these subsets are thought to share a precursor-product relationship where mature $mTEC^{high}$ cells that express a wide array of tissue restricted antigens (TRAs) are derived from less mature $mTEC^{low}$ cells that express a limited array of TRAs (Derbinski et al., 2005; Yano et al., 2008). Whilst the medullary epithelium is known to be essential for establishing immunological tolerance via the deletion of autoreactive thymocytes, the underlying signalling pathways and mechanisms that regulate mTEC development are yet to be fully defined. However, RANKL signals derived from thymic lymphoid tissue inducer cells (LTi) and/or SP4 thymocytes have been identified as being important in regulating the development of $CD80^{+}$ $AIRE^{+}$ mTECs (Akiyama et al., 2008; Hikosaka et al., 2008; Rossi et al., 2007b). Thus, analysis of the mechanisms that regulate the differentiation of mTECs, particularly the subset expressing AIRE is essential to gain a better understanding of how central tolerance is regulated. Whilst expression of co-stimulatory molecules such as CD80 on mTECs is important for negative selection, the possible roles of other co-stimulatory markers are undefined. The discovery of differential expression of the programmed death-1 (PD-1) ligands PD-L1 (on cortex and medulla) and PD-L2 (medulla only) within the thymus has further highlighted the heterogeneity of TECs

with regard to expression of co-stimulatory molecules (Liang et al., 2003). Due to the importance of PD-1 signalling to deliver inhibitory signals to T cells to mediate peripheral T cell tolerance and the up-regulation of this receptor during positive selection (Keir et al., 2008; Sharpe et al., 2007), it was thus of interest in this study to investigate the phenotype of mTECs with regard to PD-L1 and PD-L2 expression with a view to extend current knowledge on the role of PD-1 in the thymus.

In the final part of this chapter, we also aim to investigate non-epithelial thymic stromal cells, which mostly comprise of mesenchymal cells that constitute the capsule, septae and the stromal cell network including fibroblasts. Mesenchymal cells are known to play a crucial role in TEC proliferation in the fetal thymus through the provision of fibroblast growth factors (FGFs) (Jenkinson et al., 2003). However, the precise role of the mesenchyme in the post-natal and adult thymus remains to be determined. Despite the identification of subsets of mesenchyme expressing Ly51 (the so called 'cortical mesenchyme') (Muller et al., 2005) and the fibroblast marker MTS15 (Gray et al., 2007b), knowledge about the precise phenotype and heterogeneity of these cells is limited. Certainly, to begin to understand the developmental origins and thymus specific functions of these cells, elucidating the phenotype of these cells and identifying mesenchyme specific markers is vital.

Thus the aims of this chapter are as follows:

- Analyse co-stimulatory molecule expression in cTECs and distinct mTEC subsets.
- Analyse the role of RANK signalling in mTEC development.
- Define heterogeneity within non-epithelial thymic stromal cells by identifying mesenchyme specific markers and carrying out a genotypic analysis of such cells.

5.2 Specialised Methods.

5.2.1 Addition of Anti-RANK to Fetal Thymus Organ Cultures.

Day 15 embryonic lobes were placed in fetal thymus organ culture (FTOC) with or without 2-dguo to eliminate lymphoid cells as described in section 2.4. After 7 days in culture half the 2-dguo FTOC lobes were placed on nucleopore filters in centre well organ culture dishes (BD) containing 200µl of DMEM, whereas the remaining half were placed in dishes containing 200µl of DMEM supplemented with 10µg/ml anti-RANK (R&D systems). After 4 days, lobes were removed from culture and treated for flow cytometry analysis.

5.2.2 Intracellular AIRE Staining Protocol for Flow Cytometry.

AIRE is a nuclear protein and so an intracellular staining protocol (ebioscience) was employed to analyse AIRE expression in FTOC lobes. AIRE staining was carried out in collaboration with A.White, University of Birmingham. Stromal cell suspensions that were CD45 depleted using dynal beads (described in section 2.5.3) were surface stained with the relevant antibodies as described in section 2.6. Following surface stain, cells were washed in 200µl IC fixation buffer and vortexed before incubation at 4°C for approximately 40 minutes. Cells were then washed twice with 1ml of perm wash buffer (diluted 1:10 in dH_20). Washed cells were then incubated for 20 minutes with anti-AIRE diluted in perm wash buffer. Cells were washed twice again as above before resuspension in PBS.

5.3 Results.

5.3.1 Defining Heterogeneity in Cortical and Medullary Thymic Epithelial Cells.

To identify heterogeneity within TECs and to emphasise differences between the cortical and medullary lineages, thymus lobes dissected from embryos at day 15 of gestation were placed in FTOC for 7 days. Suspensions of thymic stromal cells were stained for CD45, EpCAM-1, Ly51 and a panel of stromal cell markers. Expression of the indicated stromal cell markers were analysed on cTECs, identified as $CD45^-$ $EpCAM\text{-}1^+$ $Ly51^+$ (figure 5.1) and on mTECs, identified as $CD45^-$ $EpCAM\text{-}1^+$ $Ly51^-$ (figure 5.2).

Analysis of cTECs revealed that a majority of these cells lack expression of the co-stimulatory molecule CD80 (B7.1) and are distinctly negative for the expression of AIRE (figure 5.1B and C). By contrast, MHC II was expressed by a majority of cTECs (figure 5.1D). A majority of cTECs were found to express PD-L1 and PD-L2 (figure 5.1E and F). Similarly, gp38, which is expressed by stromal cells in T cell dependent areas of peripheral lymphoid tissues, was also expressed by a majority of cTECs (figure 5.1G). The cell adhesion molecules VCAM-1 and ICAM-1 were also expressed by a majority of cTECs (figure 5.1H and I). Expression of PDGFRα on cTECs was distinctly absent (figure 5.1J).

Analysis of mTECs revealed a contrasting expression profile of the above stromal cell markers. Expression of CD80 was heterogeneous with the presence of a $CD80^-$ population and a small population of $CD80^+$ cells (figure 5.2B). Expression of AIRE was restricted to a very small proportion of mTECs (figure 5.2C). By contrast to the uniform high levels of MHC II expression on cTECs, expression on mTECs was more heterogeneous with the presence of mTECs expressing low to intermediate levels of MHC II (MHC II^{low}) and high levels of MHC II (MHC II^{high}) (figure 5.2D). By comparison to cTECs, PD-L1, gp38 and ICAM-1 were expressed by a majority of mTECs (figure 5.2E, G and I). Most interestingly,

Figure 5.1 Phenotype of Cortical Thymic Epithelial Cells.

Thymus lobes dissected at day 15 of gestation from WT mouse embryos were placed in fetal thymus organ culture (FTOC) for 7 days. Following culture, lobes were enzymatically digested to obtain suspensions of thymic stromal cells. Cells were stained for flow cytometry for CD45, EpCAM-1, Ly51 and the indicated stromal cell markers. Expression profile of EpCAM-1 and Ly51 is shown on $CD45^-$ cells (A). Histograms show expression of the indicated stromal cell markers on cortical thymic epithelial cells (cTECs) defined as $CD45^-$ $EpCAM\text{-}1^+$ $Ly51^+$ (B-J).

Shaded grey areas represent the negative control. Percentages of cells within gated areas are indicated. Data representative of at least 2 experiments.

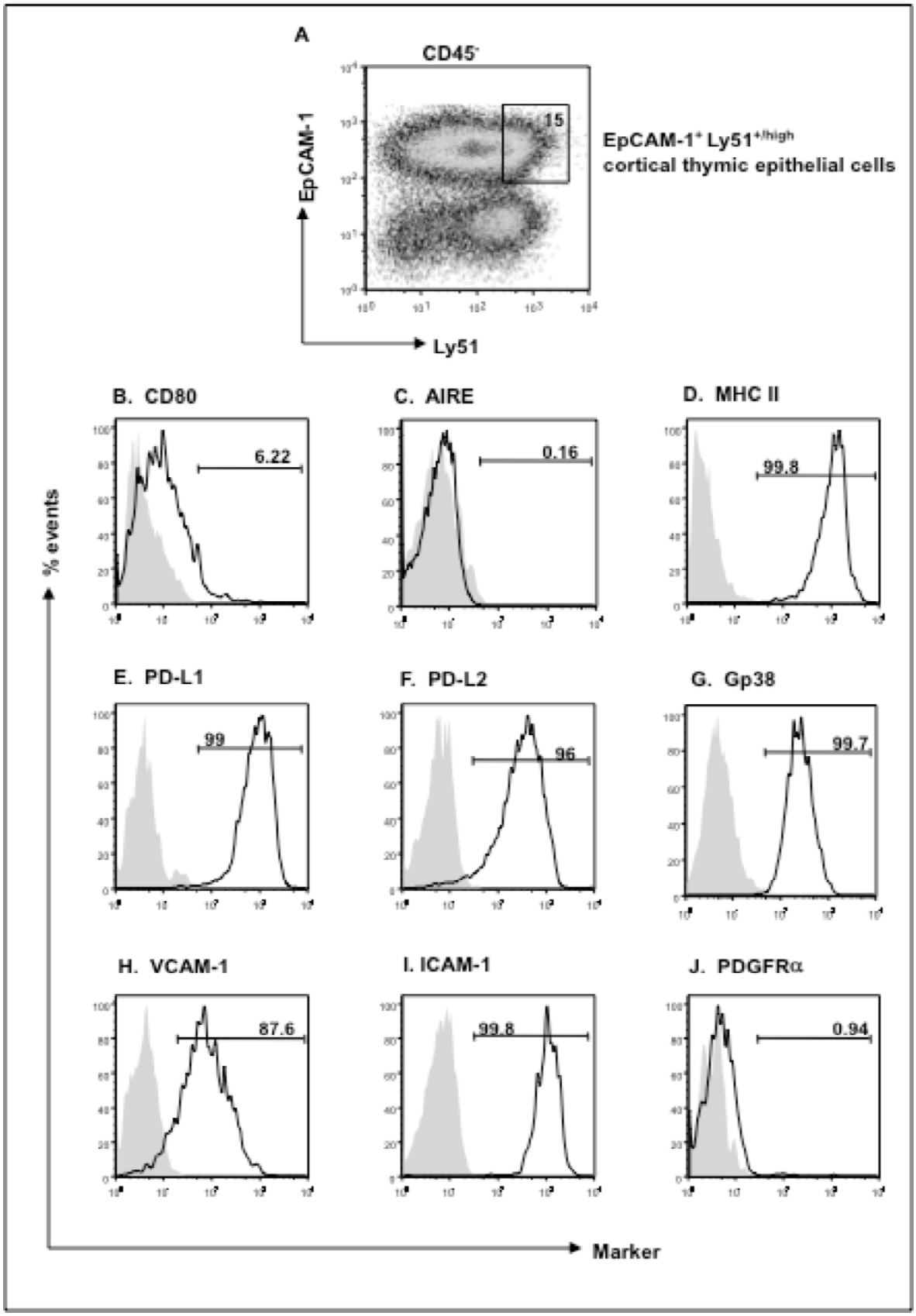
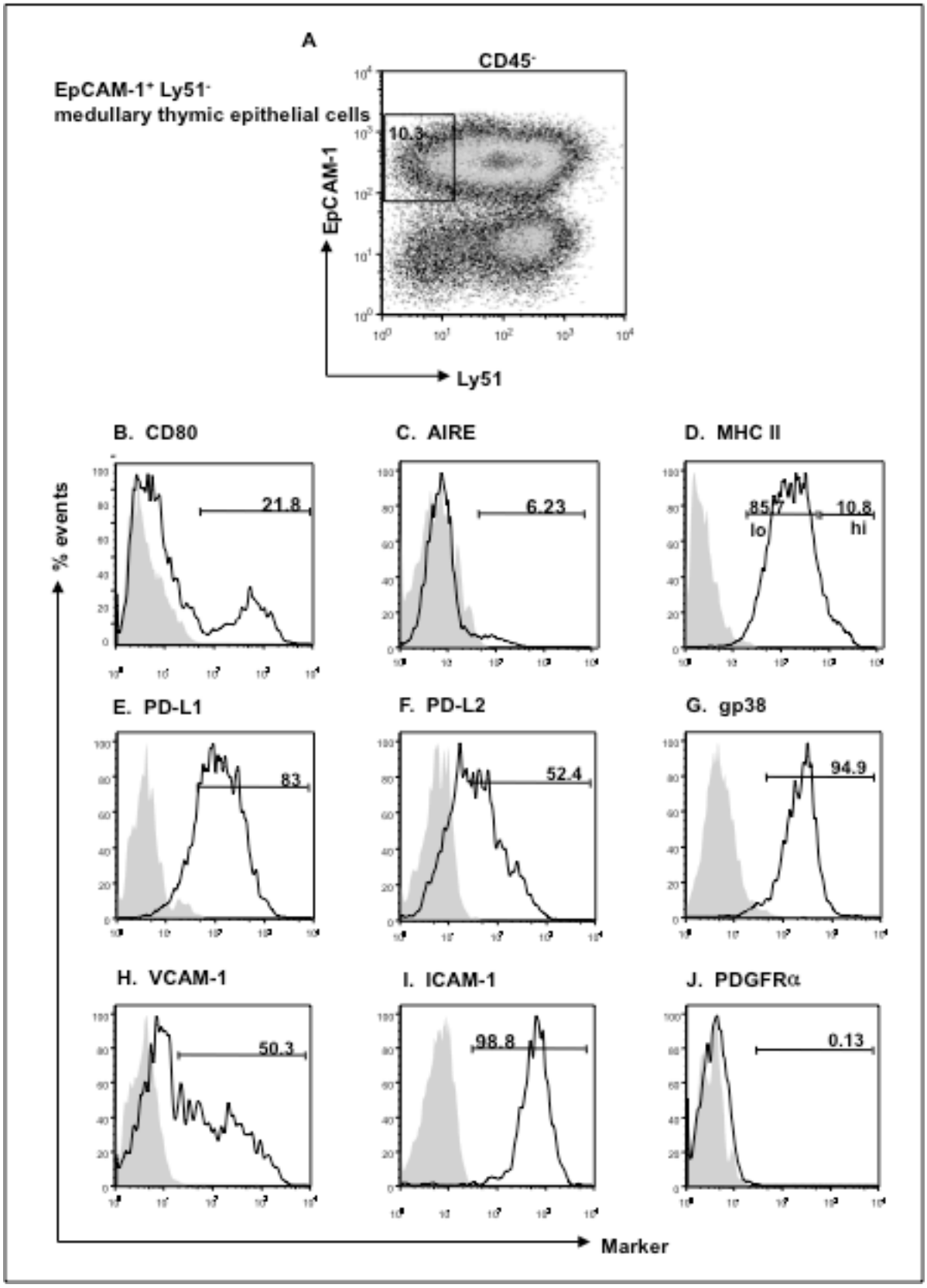

A
CD45-
EpCAM-1
Ly51
15
EpCAM-1+ Ly51+/high
cortical thymic epithelial cells
B. CD80
6.22
C. AIRE
0.16
D. MHC II
99.8
E. PD-L1
99
F. PD-L2
96
G. Gp38
99.7
H. VCAM-1
87.6
I. ICAM-1
99.8
J. PDGFRα
0.94
% events
Marker

Figure 5.2 Phenotype of Medullary Thymic Epithelial Cells.

Thymus lobes dissected at day 15 of gestation from WT mouse embryos were placed in fetal thymus organ culture (FTOC) for 7 days. Following culture, lobes were enzymatically digested to obtain suspensions of thymic stromal cells. Cells were stained for flow cytometry for CD45, EpCAM-1, Ly51 and the indicated stromal cell markers. Expression profile of EpCAM-1 and Ly51 is shown on $CD45^-$ cells (A). Histograms show expression of the indicated stromal cell markers on medullary thymic epithelial cells (mTECs) defined as $CD45^-$ $EpCAM\text{-}1^+$ $Ly51^-$(B-J).

Shaded grey areas represent the negative control. Percentages of cells within gated areas are indicated. Data representative of at least 2 experiments.


A
CD45-
EpCAM-1+ Ly51-
medullary thymic epithelial cells
10.3
EpCAM-1
Ly51
B. CD80
21.8
C. AIRE
6.23
D. MHC II
85.7
10.8
lo
hi
% events
E. PD-L1
83
F. PD-L2
52.4
G. gp38
94.9
H. VCAM-1
50.3
I. ICAM-1
98.8
J. PDGFRα
0.13
Marker

in contrast to cTECs, PD-L2 and VCAM-1 expression was more heterogeneous with approximately only half of mTECs expressing these two molecules (figure 5.2F and H). Expression of PDGFRα on mTECs was distinctly absent (figure 5.2J).

The table in figure 5.3 summarises the proportion of cTECs and mTECs expressing the named stromal cell markers.

5.3.2 Expression of PD-L1 and PD-L2 Does Not Discriminate Between mTEC^{low} and mTEC^{high} Cells.

The precise roles of the PD-1 ligands PD-L1 and PD-L2 in the thymus are unclear and thus in this chapter we aimed to extend current knowledge on the phenotype of mTECs expressing these ligands. As demonstrated in figure 5.2D, mTECs can be divided on the basis of varying levels of MHC II expression, with low level expression associated with mTEC^{low} cells and high level expression associated with mTEC^{high} cells. To determine if PD-L1 and PD-L2 can distinguish between these two cells types, expression was analysed on mTEC^{low} and mTEC^{high} cells and this was compared to CD80 and AIRE expression on these two mTEC subsets (figure 5.4). CD45 depleted stromal cell suspensions obtained from 7 day FTOC lobes were stained for Ly51, MHC II and either CD80, AIRE, PD-L1 or PD-L2. MHC II and EpCAM-1 expression on Ly51^{-} cells (figure 5.4A) revealed two populations of mTECs expressing low to intermediate (mTEC^{low}) and high levels of MHC II (mTEC^{high}) (figure 5.4B). Analysis revealed that as expected, CD80 and AIRE expression was restricted to the mTEC^{high} subset (figure 5.4C and D). By contrast, expression of PD-L1 and PD-L2 was found on both mTEC^{low} and mTEC^{high} cells (figures 5.4E and F).

Figure 5.3 Summary of the Phenotype of Cortical and Medullary Epithelial Cells.

Table summarises data from figures 5.1 and 5.2. Proportion (% of gated cells) of cortical and medullary epithelial cells expressing the indicated stromal cell markers are shown.

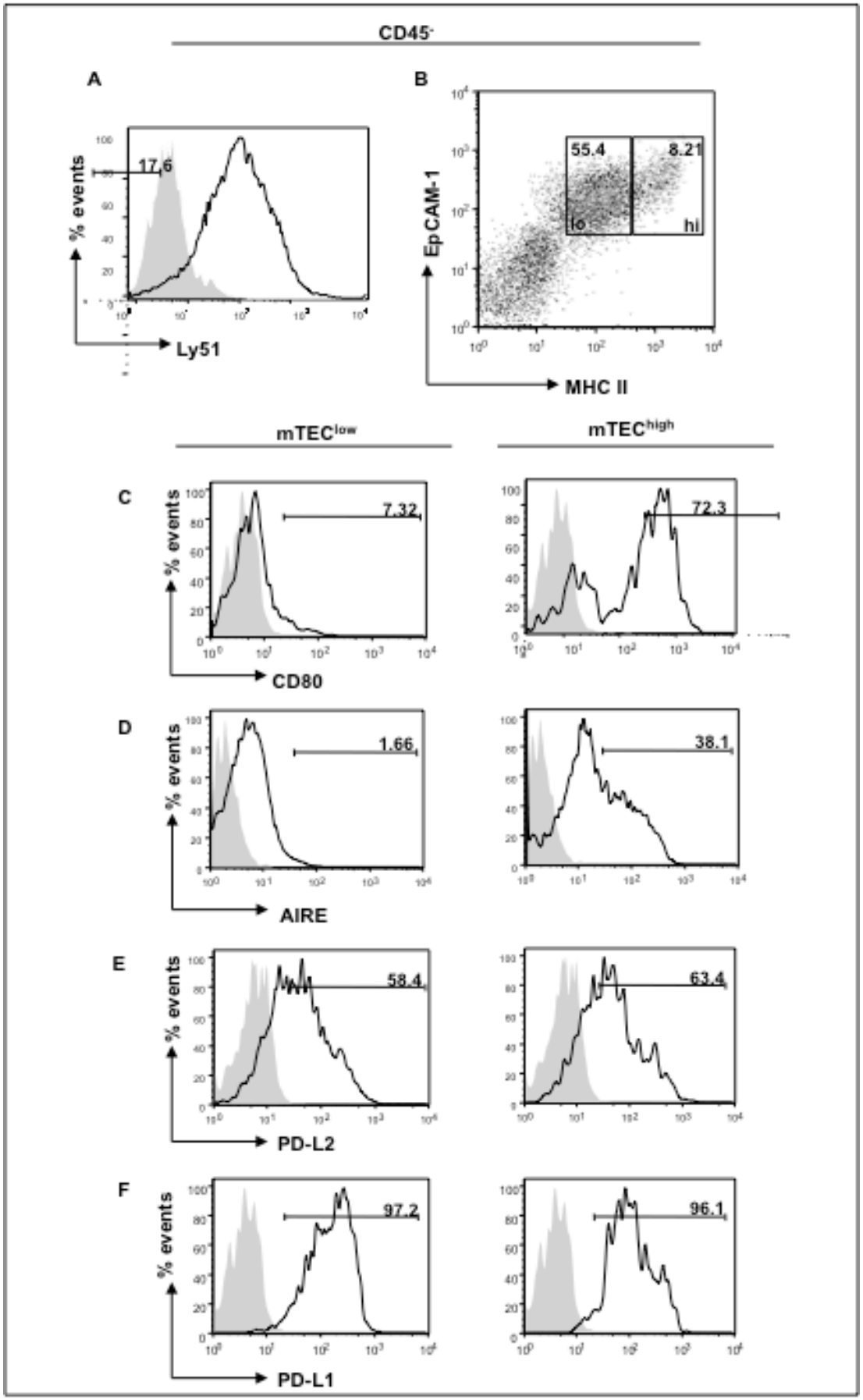
Key: -, 0-5%, +, 6-20%, ++, 21-70%, +++, 71-100%.

Marker	Cortical thymic epithelial cells (cTEC)	Medullary thymic epithelial cells (mTEC)
CD80	+	++
MHC II	+++	+++
PD-L1	+++	+++
PD-L2	+++	++
VCAM-1	+++	++
ICAM-1	+++	+++
AIRE	-	+
Gp38	+++	+++
PDGFRα	-	-

Figure 5.4 Expression of PD-L1 and PD-L2 Does Not Discriminate Between mTEC^{low} and mTEC^{high} Cells.

Thymus lobes dissected at day 15 of gestation from WT mouse embryos were placed in fetal thymus organ culture (FTOC) for 7 days. Following culture, lobes were enzymatically digested to obtain suspensions of thymic stromal cells. Cells were CD45 depleted and stained for Ly51, EpCAM-1 and MHC II. By gating on Ly51^{-} cells (A), EpCAM-1 and MHC II expression was analysed (B). Histograms show CD80, AIRE, PD-L1 and PD-L2 expression on gated mTEC^{low} cells defined as EpCAM-1^{+} MHC II^{low} (C-F left panels) and mTEC^{high} cells defined as EpCAM-1^{+} MHC II^{high} (C-F right panels).

Shaded grey areas represent the negative control. Percentages of gated cells are shown. Data representative of 3 experiments.


CD45-
A
17.6
% events
Ly51
B
55.4
8.21
lo
hi
EpCAM-1
MHC II
mTEClow
mTEChigh
C
7.32
72.3
% events
CD80
D
1.66
38.1
% events
AIRE
E
58.4
63.4
% events
PD-L2
F
97.2
96.1
% events
PD-L1

5.3.3 A Role for Thymic Crosstalk and RANK-RANKL Signalling During mTEC Development.

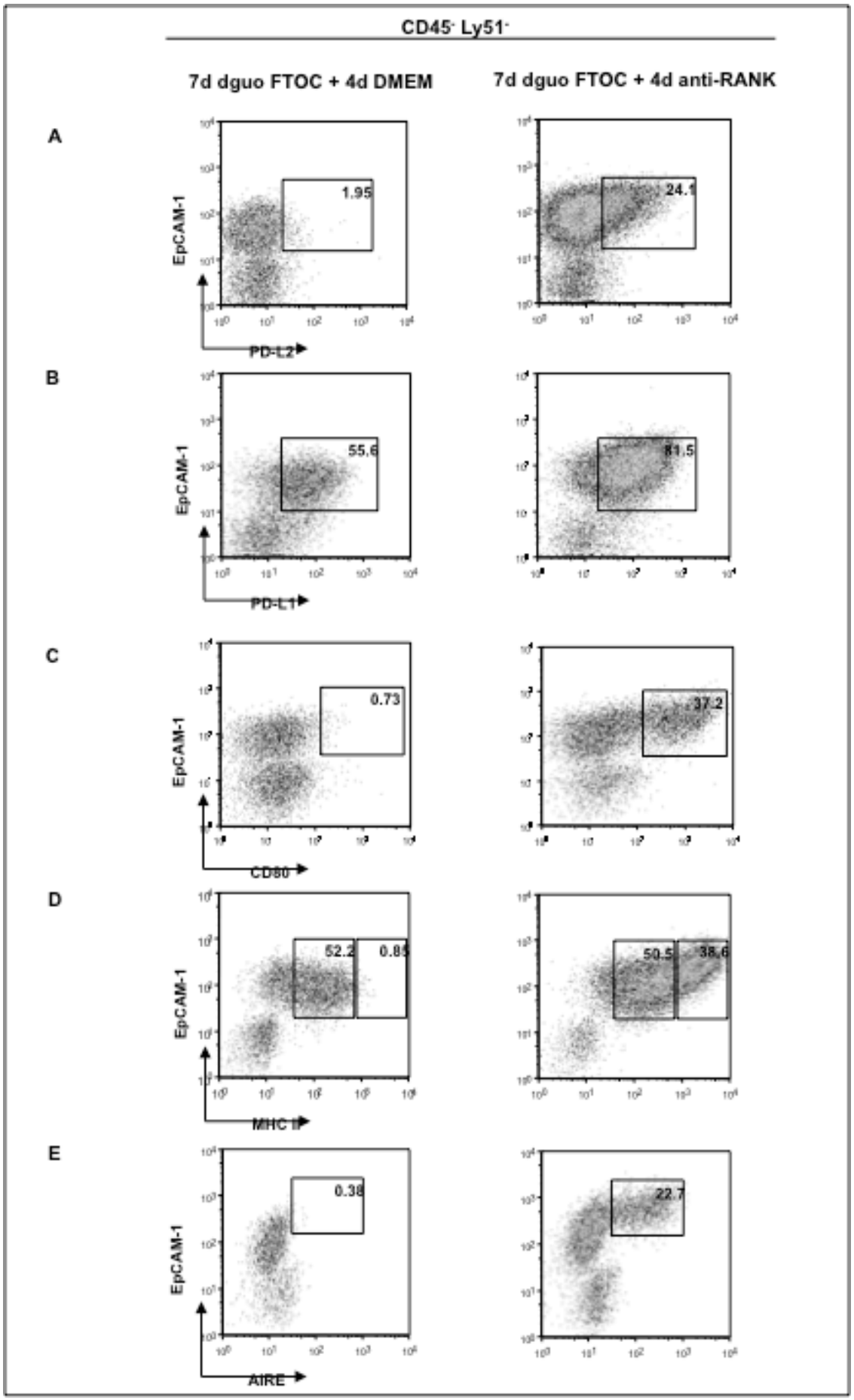
As thymic crosstalk is essential for the development of the thymic microenvironments, we then sought to determine if haematopoietic cells are important for the development of mTEC subsets and subsequently to identify if RANK-RANKL signalling is involved in this. E15 lobes placed in 2-dGuo FTOC for 7 days to deplete lymphoid cells were cultured with or without an agonistic antibody against RANK for a further 4 days. Expression of PD-L1, PD-L2, CD80, AIRE and MHC II was analysed on $CD45^{-}$ $Ly51^{-}$ EpCAM-1^{+} mTECs obtained from 7 day 2-dGuo FTOCs cultured for 4 days in DMEM or anti-RANK (figure 5.5). As a positive control, 11 day FTOCs were analysed for the expression of the above markers (data not shown).

Figure 5.5A-E shows expression of the indicated markers on mTECs, when haematopoietic cells are absent. mTECs expressing PD-L2, CD80 and AIRE were totally absent, indicating the importance of thymic crosstalk for the development of these mTEC populations (figure 5.5A, C and E left panel). Most importantly, treatment of 7d 2-dguo FTOCs with anti-RANK induced the appearance of these mTECs expressing PD-L2, CD80 and AIRE after 4 days in culture (figure 5.5A, C, and E right panel). By contrast, haematopoietic cells were not essential for the development of mTECs expressing PD-L1 lymphocytes (figure 5.5B left panel). However, addition of anti-RANK induced further development of PD-$L1^{+}$ mTECs (figure 5.5B right panel). Interestingly, absence of haematopoietic cells was important for the development of $mTEC^{high}$ cells as evidenced by the absence of mTECs expressing high levels of MHC II (figure 5.5D left panel). Addition of anti-RANK induced the development of $mTEC^{high}$ cells expressing high levels of MHC II (figure 5.5D right panel).

Figure 5.5 A Role for Thymic Crosstalk and RANK-RANKL Signalling During mTEC Development.

E15 lobes placed in fetal thymus organ culture (FTOC) with 2-dGuo for 7 days, were subsequently cultured in the presence or absence of anti-RANK for a further 4 days. Following culture, lobes were enzymatically digested to obtain suspensions of thymic stromal cells. Cells were stained for CD45, EpCAM-1, Ly51 and the indicated markers. Plots show PD-L1, PD-L2, CD80, MHC II and AIRE expression on $CD45^-$ $Ly51^-$ $EpCAM\text{-}1^+$ mTECs from 7 day 2-dGuo FTOC cultured in the presence (A-E right panels) and absence (A-E left panels) of anti-RANK.

AIRE data courtesy of A. White, University of Birmingham. Percentages of cells within gated areas are indicated. Data representative of at least 2 experiments.


CD45- Ly51-
7d dguo FTOC + 4d DMEM
7d dguo FTOC + 4d anti-RANK
A
B
C
D
E
EpCAM-1
PD-L2
PD-L1
CD80
MHC II
AIRE
1.95
24.1
55.6
81.5
0.73
37.2
52.2
0.85
50.5
38.6
0.38
22.7

5.3.4 PD-1, the Receptor for PD-L1 and PD-L2, is Expressed On Thymocyte Subsets.

Having demonstrated that PD-L1 and PD-L2 are expressed on TECs, it was of interest to determine the expression of their receptor PD-1 on different thymocyte subsets. Thymocytes obtained from a 4-6 week old adult WT mouse were stained for CD4, CD8, TCRβ and PD-1 (figure 5.6). PD-1 expression was analysed on different thymocyte subsets defined by the differential expression of CD4 and CD8. Analysis revealed that PD-1 was expressed mostly on DN ($CD4^-$ $CD8^-$) (figure 5.6B) and SP4 ($CD4^+$ $CD8^-$) thymocytes (figure 5.6D). Expression of PD-1 on DP ($CD4^+$ $CD8^+$) (figure 5.6C) and SP8 ($CD4^-$ $CD8^+$) (figure 5.6E) thymocytes was minimal. PD-1 expression was also analysed on mature thymocytes expressing high levels of TCRβ (figure 5.6F). Data revealed that a proportion of mature SP4 cells expressed PD-1 (figure 5.6G), with very few mature CD8 cells expressing PD-1 (figure 5.6H).

To further analyse expression of PD-1 on thymocyte subsets, adult thymocytes were also stained for CD69, which is an activation marker up-regulated following sustained TCR-MHC interactions on developing thymocytes. PD-1 expression was analysed on thymocyte subsets expressing high levels of CD69 (figure 5.7A). Data reveals that expression of PD-1 expression is induced on DP thymocytes following TCR-MHC interactions (figure 5.7B). PD-1 was also expressed on a proportion of $CD69^+$ $CD4^+$ (figure 5.7C) and $CD69^+$ $CD8^+$ (figure 5.7D) thymocytes. Furthermore, by subdividing $CD4^+$ thymocytes on the basis of CD25 expression (figure 5.7E), PD-1 expression was found mostly on thymic Tregs defined as $CD4^+$ $CD25^+$ (figure 5.7G) as well as a smaller proportion of non-Tregs defined as $CD4^+$ $CD25^-$ (figure 5.7F).

Figure 5.6 PD-1 is Expressed On Various Thymocyte Subsets.

Thymocytes from a 4-6 week old adult WT mouse were stained for CD4, CD8, TCRβ and PD-1. Different thymocyte subsets are defined by the differential expression of CD4 and CD8 (A). Mature thymocytes expressing high levels of TCRβ are also shown (F). Histograms show expression of PD-1 on the various gated thymocyte subsets (B-E and G and H).

Shaded grey areas represent the negative control. Percentages of cells within gated areas are indicated. Data representative of 2 experiments. DN, double negative, DP, double positive, SP4, single positive CD4, SP8, single positive CD8.

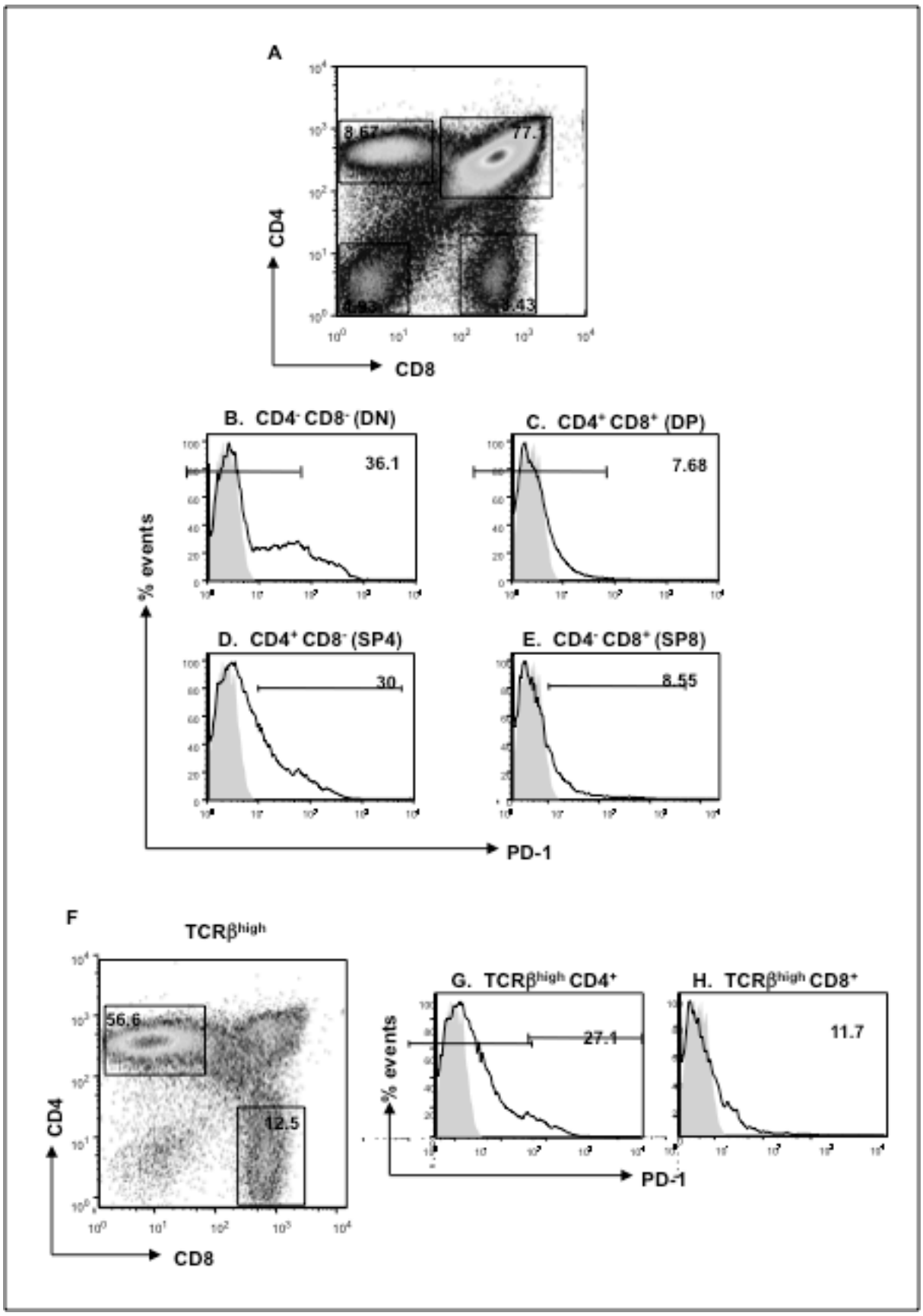
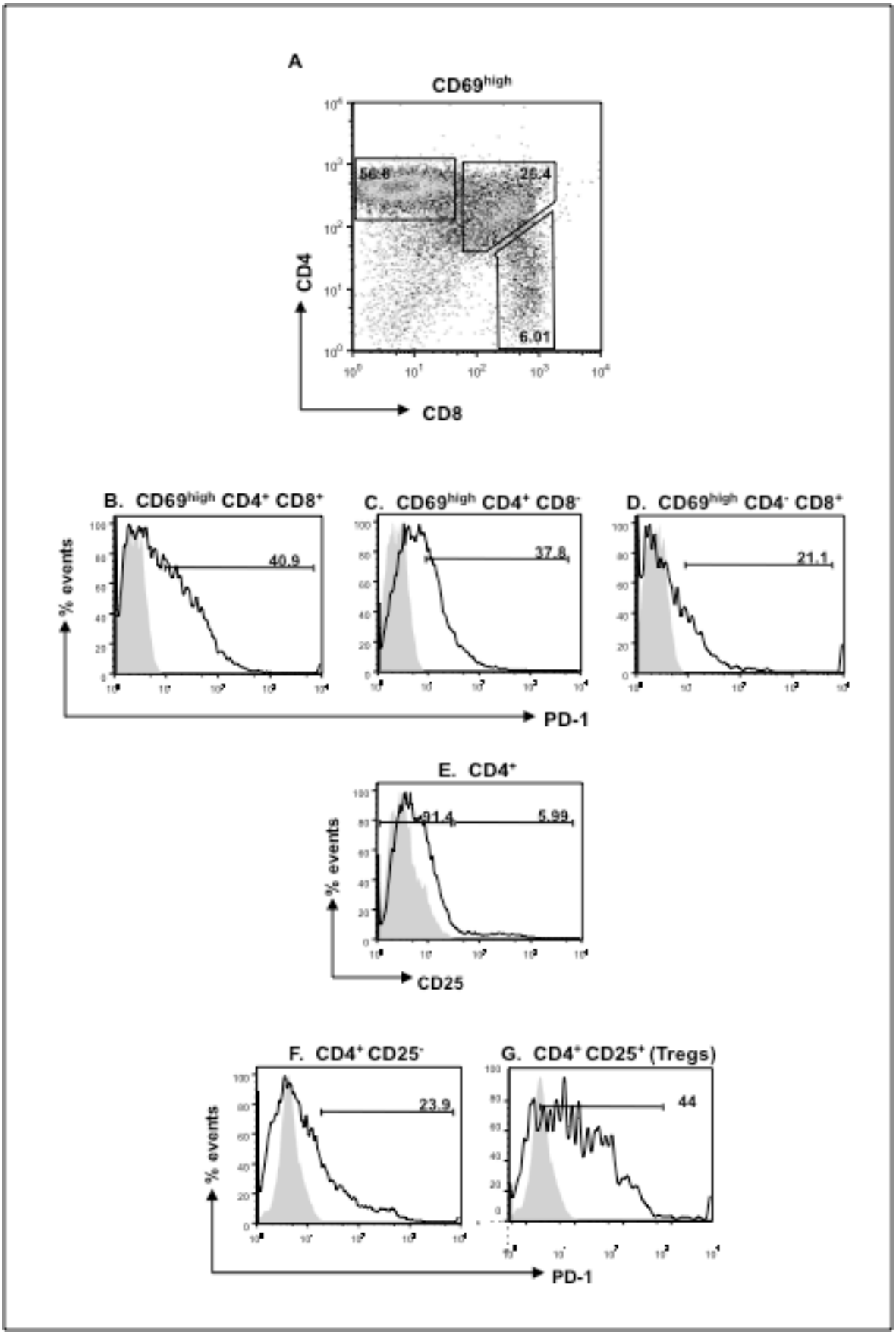

A
8.67
77.1
4.83
3.43
CD4
CD8
B. CD4- CD8- (DN)
36.1
C. CD4+ CD8+ (DP)
7.68
% events
D. CD4+ CD8- (SP4)
30
E. CD4- CD8+ (SP8)
8.55
PD-1
F
TCRβhigh
56.6
12.5
CD4
CD8
G. TCRβhigh CD4+
27.1
H. TCRβhigh CD8+
11.7
% events
PD-1

Figure 5.7 PD-1 is Expressed On Activated Thymocytes and Tregs.

Thymocytes from a 4-6 week old adult WT mouse were stained for CD4, CD8, CD69 and PD-1. Activated thymocyte subsets defined by the expression of CD4 and CD8 on $CD69^{high}$ cells are shown (A). Histograms show PD-1 expression on the different thymocyte subsets (B-D). Thymocytes were also stained for CD25 with CD4 (E). Expression of PD-1 on gated populations is shown (F and G).

Shaded grey areas represent the negative control. Percentages of cells within gated areas are indicated. Data representative of 2 experiments.


A
CD69high
56.8
26.4
6.01
CD4
CD8
B. CD69high CD4+ CD8+
40.9
% events
PD-1
C. CD69high CD4+ CD8-
37.8
D. CD69high CD4- CD8+
21.1
E. CD4+
91.4
5.99
% events
CD25
F. CD4+ CD25-
23.9
% events
PD-1
G. CD4+ CD25+ (Tregs)
44

To summarise, data in this chapter so far highlights the heterogeneity of mTEC subsets with particular regard to expression of co-stimulatory molecules and moreover shows the essential role of thymic crosstalk in the development of these subsets.

5.3.5 Phenotypic Characterisation of the Thymic Mesenchyme.

In the final part of this chapter we aimed to further define the phenotype of mesenchymal cells in the thymus. Stromal cells obtained from 7 day FTOC lobes were stained for CD45, EpCAM-1 and a panel of stromal cell markers (figure 5.8). Mesenchymal cells were identified as $CD45^-$ $EpCAM\text{-}1^-$ (figure 5.8A). Analysis of CD80, AIRE, MHC II, PD-L1 and PD-L2 expression revealed an absence or a low proportion of mesenchymal cells expressing these molecules (figure 5.8B-F). Analysis of VCAM-1, ICAM-1 and PDGFRα indicated heterogeneous expression within the mesenchyme (figure 5.8H-J). Interestingly, in contrast to the above named markers, gp38 was expressed on a majority of mesenchymal cells with the presence of cells expressing high levels of gp38 (figure 5.8G). The table in figure 5.9 summarises the proportion of mesenchymal cells expressing the indicated stromal cell markers.

5.3.6 Defining Heterogeneity Within the Thymic Mesenchyme.

Having analysed the phenotypic profile of the mesenchyme, we then sought to define this population further by focusing on the expression of gp38 and PDGFRα (figure 5.10). CD45 depleted stromal cells obtained from 7 day FTOCs were stained for EpCAM-1, Ly51, gp38 and PDGFRα (figure 5.10A). Analysis of PDGFRα, gp38 and Ly51 expression on $CD45^-$ $EpCAM\text{-}1^-$ cells revealed distinct populations of mesenchyme including a $gp38^+$

Figure 5.8 Phenotypic Characterisation of the Thymic Mesenchyme.

Thymus lobes dissected at day 15 of gestation from wildtype (WT) mouse embryos were placed in fetal thymus organ culture (FTOC) for 7 days. Following culture, lobes were enzymatically digested to obtain suspensions of thymic stromal cells. Cells were stained for flow cytometry for CD45, EpCAM-1 and the indicated stromal cell markers. Expression profile of CD45 and EpCAM-1 cells is shown (A). Histograms show expression of the indicated stromal cell markers on thymic mesenchymal cells defined as $CD45^-$ $EpCAM\text{-}1^-$ (B-J).

Shaded grey areas represent the negative control. Percentages of cells within gated areas are indicated. Data representative of at least 2 experiments.

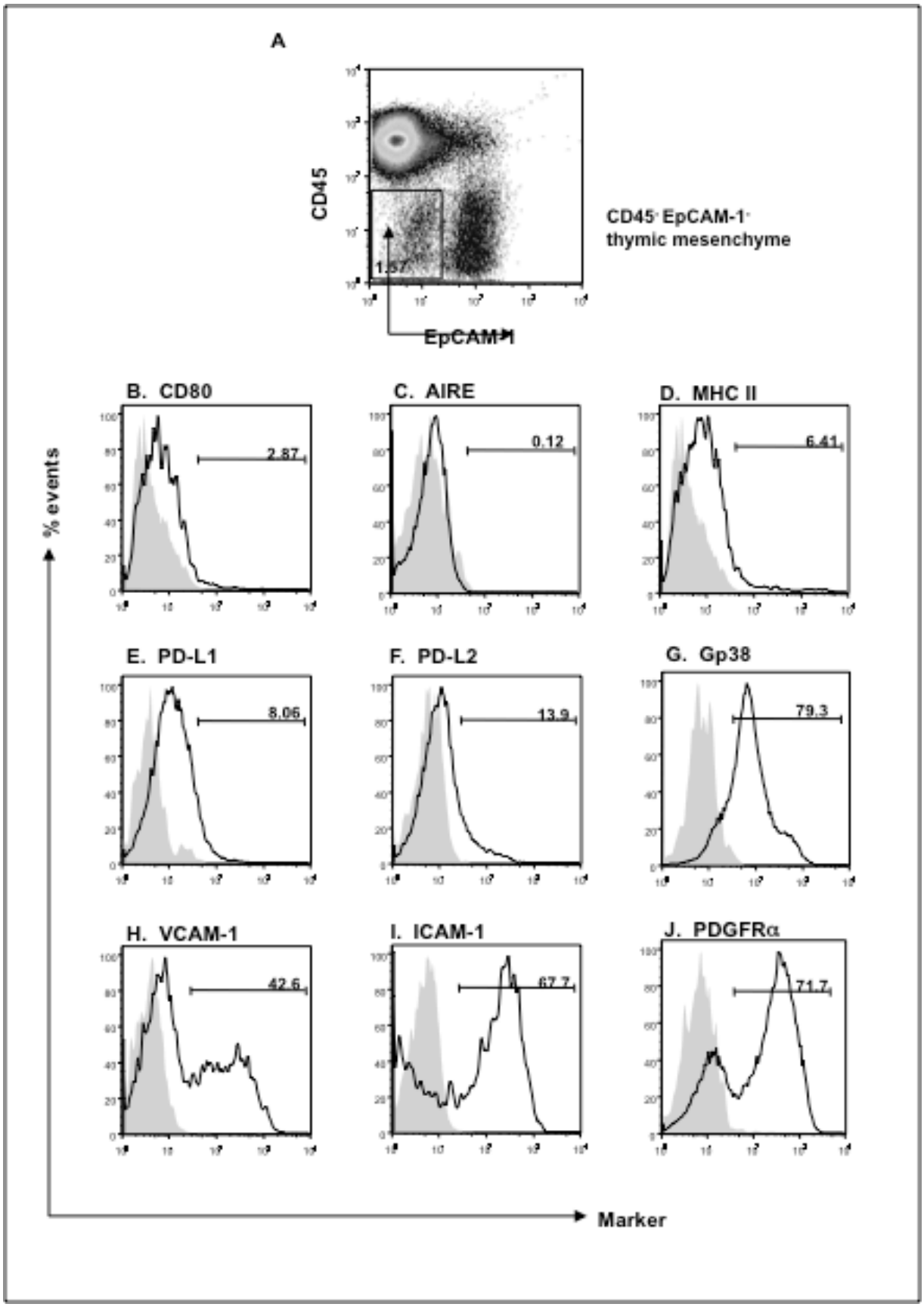

A
CD45
EpCAM-1
1.57
CD45- EpCAM-1+
thymic mesenchyme
B. CD80
2.87
C. AIRE
0.12
D. MHC II
6.41
E. PD-L1
8.06
F. PD-L2
13.9
G. Gp38
79.3
H. VCAM-1
42.6
I. ICAM-1
67.7
J. PDGFRα
71.7
% events
Marker

Figure 5.9 Summary of the Phenotype of the Thymic Mesenchyme.

Table summarises data from figure 5.8. Proportion (% of gated cells) of thymic mesenchymal cells expressing the indicated stromal cell markers is shown.

Key: -, 0-5%, +, 6-20%, ++, 21-70%, +++, 71-100%.

Marker	Thymic mesenchymal cells
CD80	-
MHC II	+
PD-L1	+
PD-L2	+
VCAM-1	++
ICAM-1	++
AIRE	-
Gp38	+++
PDGFRα	+++

PDGFRα^+ population of cells, mostly expressing Ly51 (figure 5.10B), a gp38^{low} PDGFRα^- population, with some cells expressing Ly51 (figure 5.10C) and finally a gp38^{high} PDGFRα^- population not expressing Ly51 (5.10D).

5.3.7 Genotypic Characterisation of the Thymic Mesenchyme.

Following our identification of different sub-populations of mesenchyme, we then aimed to determine gene expression profile of these mesenchymal cells (Note: suitable cDNA yields were obtained only from the gp38$^+$ PDGFRα^+ subset). Stromal cell suspensions from 7 day FTOC lobes were cell sorted for CD45$^-$ EpCAM-1$^-$ gp38$^+$ PDGFRα^+ cells. Gene expression in these cells was compared to cells sorted for total epithelium (CD45$^-$ EpCAM-1$^+$) and total mesenchyme (CD45$^-$ EpCAM-1$^-$) (figure 5.11). Quantitative RT-PCR was carried out for a panel of stromal cell associated genes. As expected, FoxN1 was specifically expressed by the epithelium and not in the mesenchyme populations demonstrating the purity of the sort (figure 5.11A). Expression of the chemokines CCL21 and CCL19 was mostly restricted to the epithelium (figure 5.11B and C). By contrast, fibroblast growth factors (FGF) 7 and 10, were restricted to the mesenchyme populations (figure 5.11E and F) whereas the receptor for FGF7 and 10, FGFR2 was predominantly expressed in the epithelium (figure 5.11D).

Figure 5.10 Defining Heterogeneity Within the Thymic Mesenchyme.

CD45 depleted stromal cell suspensions from E15 lobes placed in fetal thymus organ culture (FTOC) for 7 days were stained for EpCAM-1, Ly51, gp38 and PDGFRα. Gp38 and PDGFRα profile on $CD45^-$ $EpCAM\text{-}1^-$ cells is shown (A). Histograms show Ly51 expression on gated populations.

Shaded grey areas represent the negative control. Percentages of cells within gated areas are indicated. Data representative of 3 experiments.

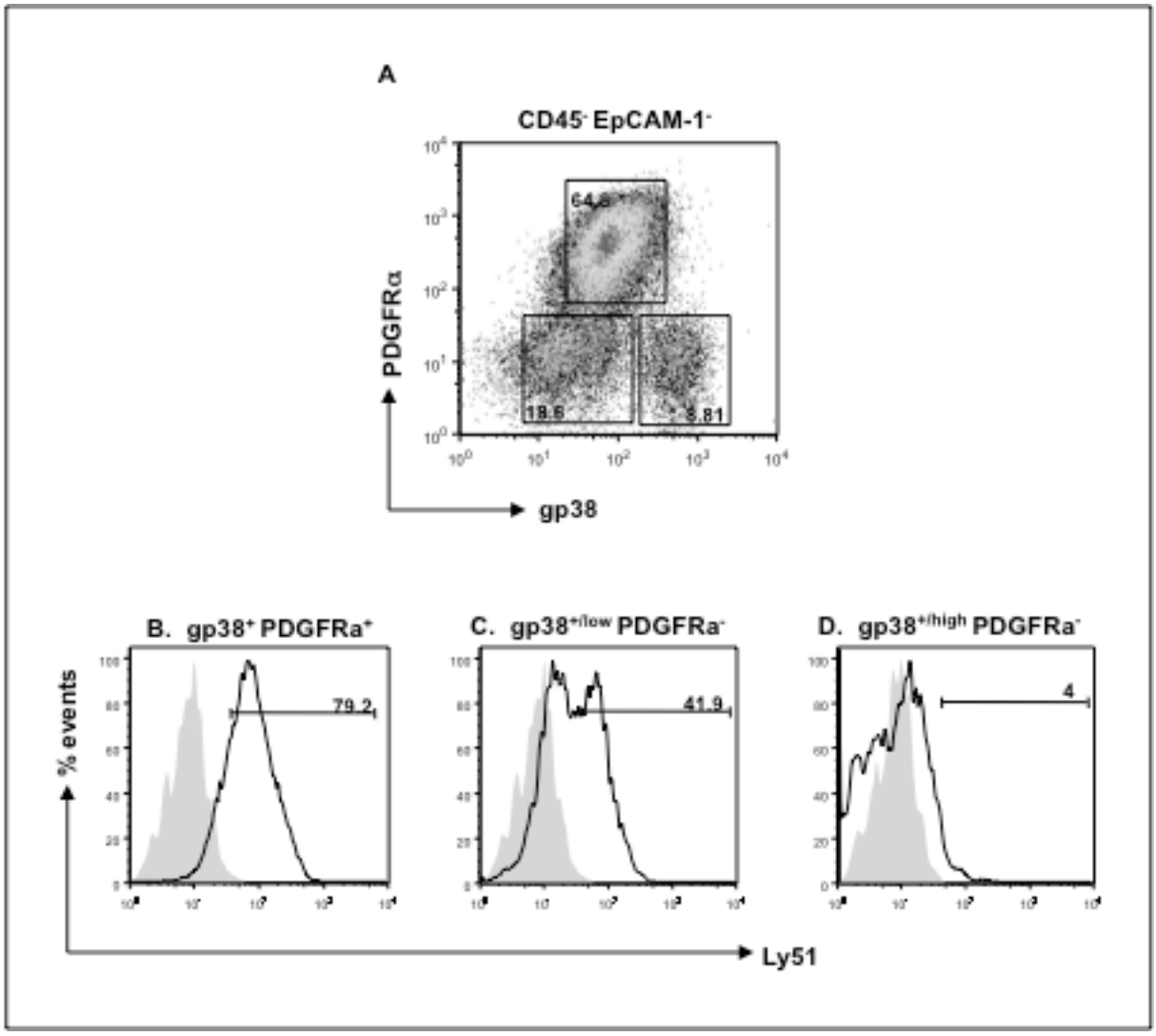
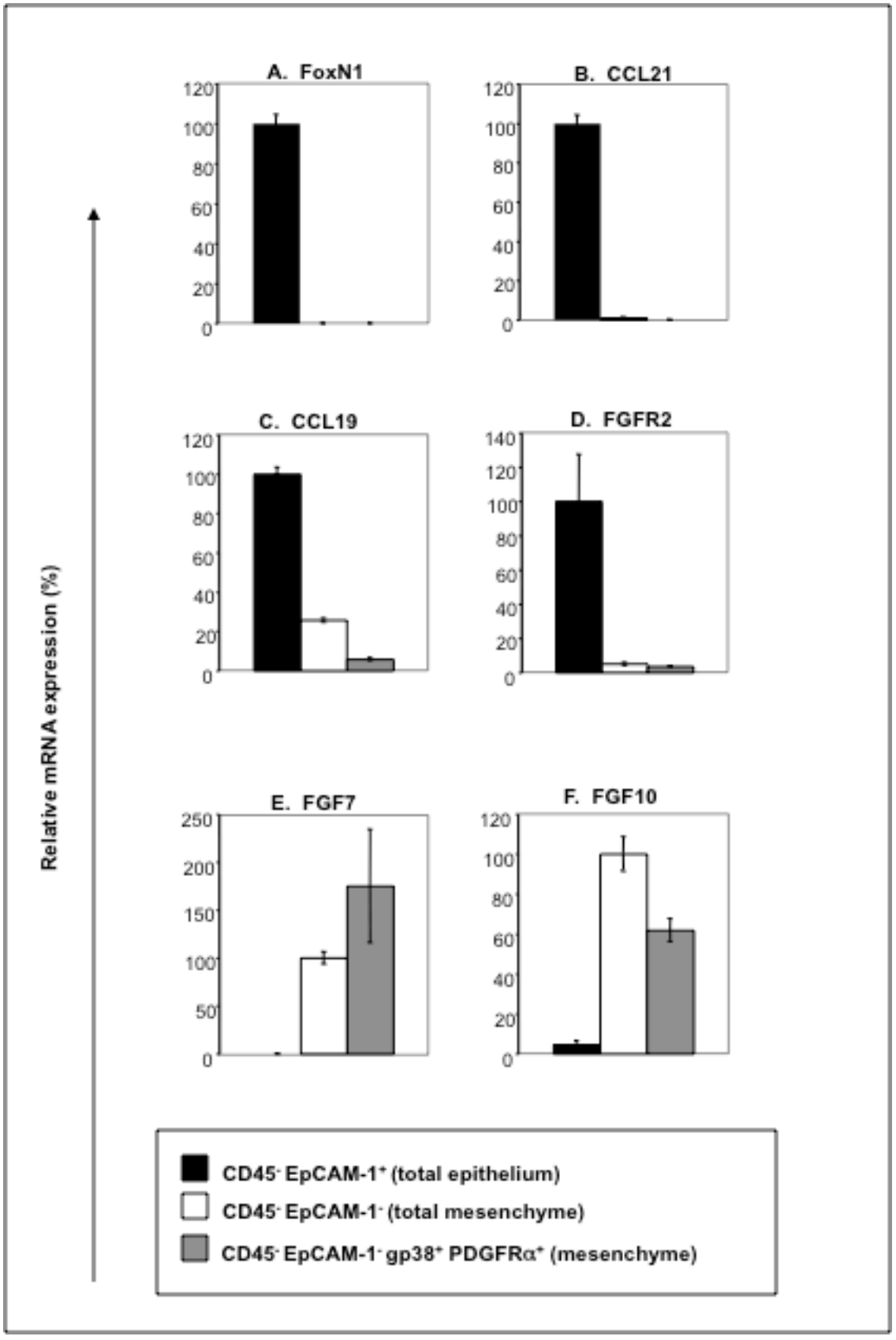

A
CD45- EpCAM-1-
PDGFRα
gp38
64.6
18.6
3.81
B. gp38+ PDGFRa+
79.2
C. gp38+/low PDGFRa-
41.9
D. gp38+/high PDGFRa-
4
% events
Ly51

Figure 5.11 Genotypic Characterisation of the Thymic Mesenchyme.

E15 lobes placed in fetal thymus organ culture (FTOC) for 7 days were cell sorted for total epithelial cells defined as $CD45^-$ $EpCAM\text{-}1^+$ and total mesenchymal cells defined as $CD45^-$ $EpCAM\text{-}1^-$. 7 day FTOC lobes were also sorted for a sub-population of mesenchymal cells defined as $CD45^-$ $EpCAM\text{-}1^-$ $gp38^+$ $PDGFR\alpha^+$. Quantitative RT-PCR was carried out for the indicated genes on the three populations of cells. Relative mRNA expression of the indicated genes normalised to β-actin is shown (A-F).

PCRs were carried out in triplicate and error bars indicate standard error of the mean. Data representative of two distinct sorting experiments.


A. FoxN1
B. CCL21
C. CCL19
D. FGFR2
E. FGF7
F. FGF10
Relative mRNA expression (%)
CD45- EpCAM-1+ (total epithelium)
CD45- EpCAM-1- (total mesenchyme)
CD45- EpCAM-1- gp38+ PDGFRα+ (mesenchyme)

5.4 Discussion.

5.4.1 Defining Medullary Epithelial Cell Subsets.

The identification of heterogeneous subsets within the mTEC lineage has advanced the understanding of the mechanisms and developmental pathways leading to the differentiation of mature mTECs. However, the extent of mTEC heterogeneity and the lineage relationships between mTEC subsets are yet to be fully defined. Clearly, a further understanding of how mTECs differentiate and the signalling pathways directing this will help to determine the basis for such mTEC heterogeneity. In view of this, experiments in this chapter aimed to phenotypically define the diversity of mTEC subsets.

Data in this chapter emphasizes some of the differences between epithelial cells in the cortex and medulla with particular regard to the variable expression of CD80 and the autoimmune regulator AIRE. Such differences between the cortex and the medulla are likely to be a reflection of the tolerogenic capacity of mTECs. However, the expression of CD80, PD-L1 and PD-L2 (and CD40 as shown in chapter 3) on both mTECs and cTECs (albeit at varying levels) reflects the ability of both lineages to deliver co-stimulatory signals to developing thymocytes. This data is in line with Gray et al (2006) who demonstrate that cTEC and mTEC subsets have the capacity to stimulate T cells *in vitro* at levels comparable to that of DCs. PD-L1 and PD-L2 are known to deliver inhibitory signals through their receptor PD-1 to regulate peripheral T cell activation and tolerance (Keir et al., 2008). These inhibitory signals delivered by PD-1 have been demonstrated in PD-$1^{-/-}$ mice, which display autoimmune symptoms implicating this pathway in regulating peripheral tolerance (Nishimura et al., 1999). Thus, our findings that cTECs broadly express both of these ligands may add support to the notion that the cortex has the capacity to support negative selection in the thymus (Goldman et al., 2005; McCaughtry et al., 2008).

Our findings of broad PD-L1 expression across the thymus are in accordance with previous studies (Brown et al., 2003; Liang et al., 2003). However, whilst both these studies suggest that PD-L2 expression is restricted to the medulla, data in this chapter shows that PD-L2 is actually expressed by both mTECs and cTECs. A possible explanation for this discrepancy maybe explained by the fact that Liang et al (2003) and Brown et al (2003) analysed PD-L2 expression by histology and not flow cytometry, which may be a more sensitive technique to detect PD-L2 expression in the cortex. Interestingly, in contrast to PD-L1, not all mTECs expressed PD-L2 possibly suggesting separate roles for these two ligands in the thymus.

As expected, data in this study shows that expression of CD80 and AIRE are restricted to the $mTEC^{high}$ lineage and this is in line with data that suggests this particular subset is more responsible for TEC-mediated tolerance (Gray et al., 2006). However, such expression patterns did not extend to the PD-1 ligands as in our study both PD-L1 and PD-L2 did not distinguish between $mTEC^{low}$ and $mTEC^{high}$ subsets. It is important to note that not all $mTEC^{high}$ cells express CD80 and AIRE and it has previously been shown that PD-L1 is expressed on both $AIRE^{-}$ and $AIRE^{+}$ $mTEC^{high}$ subsets (Gray et al., 2007a). It is currently unclear if PD-L2 shares a similar pattern and certainly future experiments that determine if PD-L2 is expressed on $AIRE^{+}$ mTECs would help to further define these subsets.

A role for thymic crosstalk in the development of the medulla has been well established (Shores et al., 1991; van Ewijk et al., 1994). Potential mediators of thymic cross-talk induced differentiation of TECs have focused on components of NF-κB signalling pathway (e.g. RelB, TRAF6 and LTβ), activation of which is required for the development of mTECs (Akiyama et al., 2005; Boehm et al., 2003; Burkly et al., 1995; Gray et al., 2006). A role for receptor activator of NF-κB (RANK) signalling has been well defined as essential for

the development of $CD80^+$ $AIRE^+$ mTECs (Akiyama et al., 2008; Rossi et al., 2007b). In the present study we confirm these findings by showing that stimulation of RANK induces the development of the $mTEC^{high}$ subset as evidenced by the induction of mTECs expressing CD80, AIRE and high levels of MHC II, which are typically missing in the absence of RANK signalling. However, data in this study shows that RANK signalling is also important for the development of mTECs expressing PD-L2 and to a lesser extent PD-L1. Our findings that RANK signalling mediates development of the $mTEC^{low}$ subset, which expresses PD-L2 but not AIRE or CD80, suggests that RANK signalling is not just restricted to the development of $AIRE^+$ mTECs but may also be required for overall normal development and organisation of the medulla. Our findings are consistent with studies showing that NF-κB signalling induces PD-L2 but not PD-L1 expression on peripheral DCs (Liang et al., 2003). It is important to note that overall, lack of thymic cross-talk and stimulation of RANK signalling did not appear to have a large effect on expression of PD-L1, PD-L2 or MHC II on cTECs (data not shown), which is in line with the knowledge that cTECs lack expression of RANK and consequently are unresponsive to RANKL.

LTi cells deliver RANKL signals and the important contribution of these cells to mTEC development has been highlighted in studies on mice deficient for retinoic acid receptor-related orphan receptor (ROR)γ, a regulator of LTi development (Eberl et al., 2004; Sun et al., 2000). These $ROR\gamma^{-/-}$ mice show reduced numbers of $AIRE^+$ mTECs. In the context of this study, it would be of future interest to determine if development of $PD\text{-}L2^+$ mTECs in these mice is perturbed.

Findings from a recent study demonstrate that the common γ chain cytokines (including IL-2, IL-7, IL-15 and IL-21), which are important for T cell proliferation and survival, directly induce PD-1 and its ligands on peripheral T cells (Kinter et al., 2008).

Future experiments could involve analysing common γ chain knockout mice to determine if expression of the PD-1 ligands is abnormal in TECs.

The broad expression of PD-L1 and PD-L2 in the thymus suggests an important role during T cell development. Thus, we aimed to determine the expression of their receptor PD-1 on thymocytes. Studies suggest a role for PD-1 as a negative regulator of TCR signalling in the thymus (Keir et al., 2005). In this study, they suggest that ligation of PD-1 with PD-L1 inhibits TCR-mediated signalling, thereby reducing the number of DP thymocytes that receive a TCR signal to induce maturation to the SP stage. Furthermore, this study also shows that PD-L1$^{-/-}$ mice have higher numbers of DP and SP4 thymocytes compared to WT. However, PD-L2$^{-/-}$ did not show any similar defects and did not appear to be required during positive selection, suggesting distinct roles for PD-L1 and PD-L2 in the thymus. Whilst such studies support our findings that PD-L1 is expressed in the cortex where positive selection occurs, it remains unclear what the precise role both PD-L1 and PD-L2 have in the medulla during T cell development.

Clearly, further studies that identify TEC subsets and reveal the molecular mechanism of TEC development and thymic microenvironment formation are essential to improve our understanding of the events mediating the generation of a self-tolerant T cell repertoire.

5.4.2 Defining Mesenchyme Heterogeneity.

In the final part of this chapter we turned our focus onto defining heterogeneity within the non-epithelial stromal cell compartment of the thymus, which mostly comprise of mesenchymal cells that constitute the capsule, septae, perivascular cells, and a network of fibroblasts. The thymic mesenchyme, particularly a subset expressing PDGFRα, is known to play an important role during early TEC development through the provision of fibroblast

growth factors (FGFs) (Jenkinson et al., 2003; Jenkinson et al., 2007b). However, these cells are not well characterised. Preliminary data in this study provides further evidence that the mesenchyme is heterogeneous. Data identifies three populations of mesenchyme based on the differential expression of PDGFRα and gp38. The most predominant population were the PDGFRα$^+$ gp38$^+$ cells, in which a high proportion of these cells expressed Ly51. Genotypic analysis of these cells revealed they lacked expression of genes associated with thymic epithelium but did express the mesenchyme associated growth factors FGF7 and 10. These cells are likely to represent the 'cortical mesenchyme' as reported by Muller et al (2005) who identified a subset of CD45$^-$ EpCAM$^-$ cells expressing Ly51 and FGF10. It would be of interest to determine if the PDGFRα$^+$ gp38$^+$ cells also express the fibroblast marker MTS15. A study identified a unique MTS15$^+$ population of fibroblasts in which a majority of these cells also co-expressed PDGFRα (Gray et al., 2007b). This study also found that MTS15$^+$ cells expressed FGF7 and FGF10, which corresponds to our data on the PDGFRα$^+$ gp38$^+$ cells. To determine if this gene expression is unique to the PDGFRα$^+$ gp38$^+$ population of mesenchyme then certainly further cell sorts of all identified mesenchyme populations in this study will need to be carried out. Clearly, other growth factors important for T cell development and TEC development should also be investigated, such as SCF, which is important for proliferation of early thymocytes (Rodewald et al., 1995) and IL-6, which has previously been identified as important for TEC proliferation (Meilin et al., 1995). Indeed data by Gray et al (2007b) have shown that MTS15$^+$ fibroblasts express both SCF and IL-6.

Our observation of a novel PDGFRα$^-$ gp38^{high} population that lacked Ly51 expression was interesting and clearly these cells require further characterisation to determine their function in the thymus. The precise origin of all identified populations in this study are unknown and ontogeny experiments which trace the lineage of these cells are likely to yield

useful information regarding these cell types. Furthermore, identifying the location of these cells within the thymic microenvironment would be useful in determining their functional roles during T cell development. Indeed there is evidence to suggest that MTS15^{+} fibroblasts, which were found to be located in SCZ, produce CXCL12 and so may be important in directing migration of DN cells toward the SCZ (Gray et al., 2007b).

Fibroblasts are known to support the differentiation of thymocytes beyond the DN2 stage through the provision of extracellular matrix molecules (Anderson et al., 1997; Anderson et al., 1993). Thus, future functional experiments could involve cell sorting the identified populations of mesenchyme and reaggregating them with DN2/3 thymocytes to determine if they can support the differentiation of these cells to the next stage of T cell development.

Whilst the LTβR pathway is known to be important for mTEC development, studies implicate a role for this pathway for development of the mesenchyme (Seach et al., 2008). Preliminary data in this study suggests that non-TECs have high levels of LTβR (data not shown) suggesting an important role for lymphotoxin signalling in the mesenchyme. Interestingly, Seach et al (2008) found reduced numbers of MTS15^{+} fibroblasts in LTα$^{-/-}$ and LTβ$^{-/-}$ mice. This prompts further investigation where future experiments could involve comparing cell sorted populations of PDGFRα^{+} gp38^{+} from WT and LTβR$^{-/-}$ mice to determine if LTβR$^{-/-}$ mesenchyme expresses abnormal levels of growth factors important for TEC or T cell development.

To conclude, whilst we have potentially identified novel populations of mesenchyme in the thymus, clearly further studies are warranted. Defining such heterogeneity will allow for further evaluation of the precise role the mesenchyme has alongside TECs and thymocytes in the thymus.

CHAPTER 6

GENERAL DISCUSSION

6.1 Background and Overall Aims of Study.

The development of a diverse and self-tolerant T cell repertoire is dependent on the unique structure of the thymus. The thymic stromal cell compartment is organised into distinct microenvironments with each mediating defined stages in T cell development. The thymic epithelial cells (TECs) represent the major stromal cell type of the various thymic regions and are essential during multiple stages of T cell development. Whilst epithelial cells of the cortical lineage impose self-MHC restriction on immature thymocytes via positive selection, epithelial cells of the medullary lineage aid in the deletion of autoreactive thymocytes via negative selection (Anderson et al., 2009). Thus, the complex trafficking of thymocytes through these different regions of the thymus enables developing thymocytes to receive essential signals from both cortical and medullary epithelial cells to induce their maturation. In turn, lymphostromal interactions are also thought to mediate the establishment and maintenance of mature thymic microenvironments via a process of thymic crosstalk (van Ewijk et al., 1994). Such bidirectional interactions between the various thymic stromal cells and haematopoietic cells are indispensable for thymus organogenesis. In contrast to the well-defined stages of T cell development, developmental steps and the signals that govern the establishment of thymic microenvironments remain largely undefined. In view of this, the overall aims of this study were to phenotypically characterise stages of thymic stromal cell development, with particular emphasis on the development of cortical thymic epithelial cells (cTECs), which prior to the start of this study was undefined. Furthermore, this study also set out to define molecular and cellular regulators of thymic stromal cell development. In addition, understanding the signals that induce the migration of thymocytes to appropriate intra-thymic niches for their further development was of equal importance in this study. With recent data suggesting a role for stromal cell-derived chemokines (Takahama, 2006), this

study also aimed to investigate the effect of chemokine-induced migration in the establishment of thymic microenvironments, with a particular focus on the poorly defined region of the subcapsular zone (SCZ). Thus, data within this thesis falls into two major themes, where chapters 3 and 5 deal with defining TEC development and chapter 5 focusing on the role of chemokine-mediated establishment of thymic microenvironments.

6.2 A Revised Model of Thymic Epithelial Cell Development.

Whilst stages in TEC development have yet to be fully elucidated, recent studies have begun to fill in gaps in our understanding of this process. Studies have discovered that cTECs and mTECs derive from a common bipotent TEC (bTEC) progenitor or stem cell (Bleul et al., 2006; Rossi et al., 2006). Moreover, the discovery of mTEC progenitors downstream of bTECs and the identification of a precursor-product relationship within this lineage have helped to delineate mechanisms of mTEC development (Hamazaki et al., 2007; Rodewald et al., 2001; Rossi et al., 2007b). However, due to a paucity of cTEC specific markers, events occurring downstream of bTECs leading to the differentiation of the cortical lineage have remained elusive and so attempts to isolate and study cTECs present an important challenge within the field. Thus, this prompted an ontogenetic approach to investigate steps and mechanisms regulating cTEC development downstream of bTECs. Using the endocytic receptor CD205, which is expressed within the cortex and not the medulla, we were able to track the development of cTECs from the earliest stage of thymus development through ontogeny in both WT mice and also transgenic mice to determine the signals involved in the development of the cortex. Furthermore, this approach allowed for the isolation of cTEC populations at various points in development to carry out a genotypic analysis. In addition, this study also set out to assess mTEC development with the aim of extending current

understanding of the development of this lineage. Data presented in chapters 3 and 5 regarding cTEC and mTEC development are summarised in figure 6.1 as a revised model of overall TEC development.

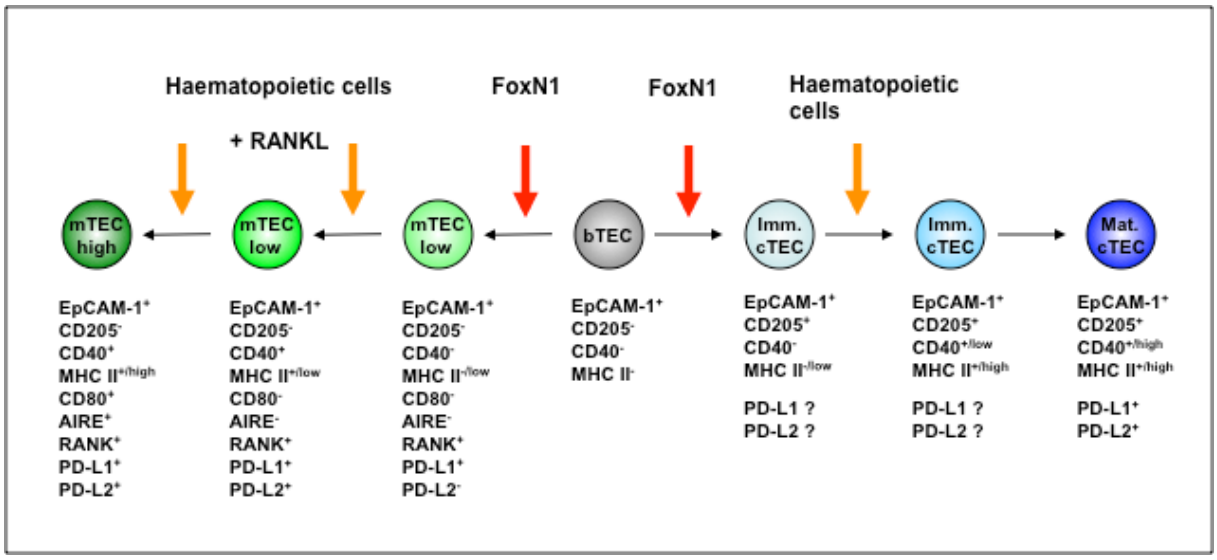
6.2.1 Development of the Cortex and a Role During Positive Selection.

This study identified a previously unreported cTEC population, defined as EpCAM-1^{+} $CD205^{+}$ $CD40^{-}$ MHC $II^{-/low}$, expressing the cTEC specific proteasome subunit β5T, during early thymus development. Importantly, emergence of these cells along with an mTEC population defined as EpCAM-1^{+} $CD205^{-}$ $CD40^{+}$, expressing mTEC associated genes such as AIRE and RANK, were dependent on the cell autonomous transcription factor FoxN1. This data confirms the notion that whilst FoxN1 is not required for initial establishment of the thymus rudiment, it is essential for specification of the cortical and medullary lineages (Blackburn et al., 1996; Bleul et al., 2006). Thus, FoxN1 deficient TECs are likely to represent bTEC progenitors arrested in their ability to differentiate into cortex and medulla. Consequently, FoxN1 deficient thymi remain in the adult as a cystic structure, unable to recruit lymphocyte progenitors (Itoi et al., 2001). It is currently unclear what signals regulate FoxN1 expression and activity, with some studies implicating a role for Wnt proteins, which belong to a family of secreted glycoproteins with diverse functions (Balciunaite et al., 2002; Hollander et al., 2006). Importantly, our data contradicts previous reports that TECs are homogenous in the early embryo, acquiring a specific cTEC or mTEC phenotype later in development (Bennett et al., 2002; Klug et al., 2002; Rossi et al., 2006). Instead, we demonstrate heterogeneity of TECs with an emergence of a defined cortical lineage as early as day 12 of gestation. Moreover, this study also identified a previously unreported stage

Figure 6.1 A Revised Model of Thymic Epithelial Cell Development.

Figure summarises data presented in chapters 3 and 5 to provide a revised model of thymic epithelial cell (TEC) development where a bipotent TEC progenitor develops into mature cortical epithelium and mature medullary epithelium via progenitor populations expressing the indicated markers. Red arrow indicates the requirement for FoxN1 and the orange arrow indicates the requirement for haematopoietic cells.

?, indicates unknown expression profile, imm, immature, mat, mature, bTEC, bipotent thymic epithelial cell, cTEC, cortical thymic epithelial cell, mTEC, medullary thymic epithelial cell.


Haematopoietic cells
+ RANKL
FoxN1
FoxN1
Haematopoietic cells
mTEC high
mTEC low
mTEC low
bTEC
Imm. cTEC
Imm. cTEC
Mat. cTEC
EpCAM-1+ CD205- CD40+ MHC II+/high CD80+ AIRE+ RANK+ PD-L1+ PD-L2+
EpCAM-1+ CD205- CD40+ MHC II+/low CD80- AIRE- RANK+ PD-L1+ PD-L2+
EpCAM-1+ CD205- CD40- MHC II-/low CD80- AIRE- RANK+ PD-L1+ PD-L2-
EpCAM-1+ CD205- CD40- MHC II-
EpCAM-1+ CD205+ CD40- MHC II-/low PD-L1 ? PD-L2 ?
EpCAM-1+ CD205+ CD40+/low MHC II+/high PD-L1 ? PD-L2 ?
EpCAM-1+ CD205+ CD40+/high MHC II+/high PD-L1+ PD-L2+

specific requirement for thymocytes during the development of the cortical lineage (discussed in section 6.2.3).

The unique ability of cTECs to support positive selection is poorly defined. However, the cortical epithelium is likely to possess unique cell surface molecules and antigen processing capabilities that promote positive selection rather than negative selection and these may be distinct from mTECs and APCs, which support negative selection. CD205 is also known to be expressed on a subset of peripheral DCs (Kraal et al., 1986) and it was previously reported that this endocytic receptor is associated with the capture and endocytosis of diverse carbohydrate bearing antigens to direct them to appropriate compartments for antigen processing and presentation to $CD4^+$ T cells (Jiang et al., 1995; Mahnke et al., 2000). The precise role that CD205 has in the thymic cortex is yet to be determined. A study shows an involvement of CD205 in the clearance of apoptotic thymocytes by cTECs *in vitro* (Small and Kraal, 2003). This data suggests a role for CD205 in the apoptosis of developing thymocytes that have failed to receive a positive selection signal. Thus, expression of CD205 on cTECs may reflect their functional capacity to support positive selection. However, the use of CD205 knockout mice has shown that DCs still have the capacity to endocytose dying cells, suggesting that CD205 may not be essential for this process (Iyoda et al., 2002). Certainly, investigating intra-thymic T cell development in such $CD205^{-/-}$ mice will help to understand the role of this receptor in the thymus.

This study has formally shown the existence of a cTEC progenitor downstream of bTEC progenitors and has identified novel checkpoints in the development of the cortical lineage. Further advances in the field have also emerged with the recent identification of β5T, which is expressed specifically by cTECs and is associated with the positive selection of $CD8^+$ T cells (Murata et al., 2007). Additionally, Gommeaux et al (2009) report that the

serine protease Prss16 is specifically expressed by cTECs and is important for the positive selection of $CD4^+$ T cells. These studies along with our findings will greatly advance the field of cTEC development and will help to begin to understand how cTECs become specialised in their ability to support positive selection. Moreover, the recently described antibody against the cTEC expressed Notch ligand delta-like 4 (Fiorini et al., 2008) should provide an important tool to study cTECs and to understand the pathways that underlie their development.

6.2.2 Development of the Medulla and a Role During Central Tolerance.

Medullary epithelium is indispensable for the induction of self-tolerance via the deletion of potentially autoreactive thymocytes by a process of negative selection. mTECs transcribe a wide spectrum of tissue restricted antigens under the control of the autoimmune regulator AIRE (Kyewski and Klein, 2006). Thus, elucidating the development of mTECs and in particular the subset expressing AIRE, is central to our understanding of how mTECs become specialised in their capacity to support negative selection and prevent autoimmune disease. This study extends current knowledge in the field by employing a phenotypic approach to analyse the heterogeneity of mTEC subsets. This study demonstrates further heterogeneity within the mTEC lineage based on the expression of co-stimulatory molecules such as the PD-1 ligands PD-L1 and PD-L2 and also CD80. Moreover, we further corroborate the requirement for thymic crosstalk for the development of these subsets (discussed in section 6.2.3). In particular, we demonstrate that not only is RANK-RANKL signalling important for development of the $AIRE^+$ $CD80^+$ $mTEC^{high}$ subset, as previously reported (Akiyama et al., 2008; Hikosaka et al., 2008; Irla et al., 2008; Rossi et al., 2007b), but this pathway is also involved in the development of the $mTEC^{low}$ subset, which does not

express AIRE or CD80 but does express PD-L2. Mice deficient for RANK show a marked absence of $AIRE^+$ mTECs and RANK deficiency in thymic stroma resulted in profound symptoms of autoimmunity such as inflammatory infiltrates in the liver and the presence of autoantibodies in the serum (Rossi et al., 2007b). Collectively, our data along with others emphasizes the central role of RANK signalling for the development and organisation of the thymic medulla to ensure establishment of self-tolerance.

Complex arrays of co-stimulatory signals are reported to be essential for negative selection of thymocytes. In particular, a study predicts that CD40 signalling induces other co-stimulatory molecules (including CD80 and CD86) to cooperatively regulate MHC II mediated negative selection (Li and Page, 2001). Furthermore, functional heterogeneity, such as varying levels of co-stimulatory molecules, within the mTEC subset has been reported to influence the outcome of deletion versus Treg cell fate specification (Aschenbrenner et al., 2007). In particular, reports suggest the B7/CD28 pathway is important for the development and homeostasis of Tregs, which are so important in the maintenance of peripheral tolerance (Salomon et al., 2000; Tai et al., 2005). To summarise, data in this thesis demonstrates the complexity of mTEC heterogeneity and this, together with previous reports, is likely to reflect the specialisation of the medullary microenvironment to impose self-tolerance. Indeed, findings from a recent study further highlight the unique nature of this region by showing that self-antigens can be transferred between DCs in the medulla and mTECs, thus widening the cellular basis for the induction of tolerance towards self-antigens (Koble and Kyewski, 2009).

6.2.3 A Role for Thymic Crosstalk.

In accordance with previous studies, data in this thesis highlights key similarities in the requirement for thymic crosstalk for the development of the cortex and medulla (Klug et

al., 1998; Naquet et al., 1999; Shores et al., 1991; van Ewijk et al., 2000). Our data suggests that the initial specification of bTEC progenitors into the cortical and medullary lineages is thymocyte independent; consistent with the notion that thymic crosstalk is not required for initial patterning and differentiation of fetal thymic epithelium (Jenkinson et al., 2005; Klug et al., 2002). However, whilst thymocytes are clearly not required for the initial establishment of these microenvironments, a developmental switch occurs where thymocyte derived signals are required for the further maturation and/or maintenance of epithelial regions. Our data shows that with regard to the cTEC lineage, further differentiation and acquisition of markers associated with functional maturation of cTECs (i.e. MHC II and CD40 expression) was dependent on the presence of thymocytes, suggesting a stage-specific requirement for thymic crosstalk during cTEC development. Furthermore, this study also extends current understanding on the development of $mTEC^{low}$ and $mTEC^{high}$ subsets by demonstrating a role for thymic crosstalk through the provision of RANK-RANKL signalling. Whilst thymic LTi cells provide RANKL during the fetal periods, both LTi and SP4 cells provide RANKL during the post-natal and adult stages (White et al., 2008). LTi cells providing TNFR ligands such as RANKL and LTα are also known to influence the maturation of microenvironments within secondary lymphoid organs via NF-κB mediated signalling (Cupedo et al., 2002). This common requirement for LTi derived signals is an example of the remarkable similarities between the thymic medulla and certain regions of the secondary lymphoid tissues such as the T cell zone of the spleen (Anderson et al., 2007; Derbinski and Kyewski, 2005).

Given the importance of thymic crosstalk for development of the cortex as described in this study, this raises the question of whether the ability to form the cortex is temporally restricted. Whilst mTEC development is plastic and can be restored by reconstituting adult T cell deficient mice with normal bone marrow derived precursors or peripheral T cells (Shores

et al., 1991; Surh et al., 1992); it was previously proposed that cortex development in CD3εtg26 mice, displaying a block at the earliest stage of T cell development, could be restored in fetal or early postnatal stages but not adult (Hollander et al., 1995). This suggested the thymic epithelium is rendered unreceptive to thymic crosstalk signals resulting in a developmental 'window' for cTEC differentiation. However, a recent study has found that TECs retain their receptivity to thymic crosstalk even after long periods of thymocyte absence (Roberts et al., 2009). In the context of this thesis, this has implications for current models of thymic crosstalk and also strategies aimed at restoring thymic function in patients following thymic involution or ablative therapy (as discussed in section 6.2.5).

Whilst it is difficult to distinguish the role of thymocytes for either maturation or maintenance of TECs, observations in this study shows that in contrast to CD3εtg26 mice, cTECs obtained from FTOCs depleted of all haematopoietic cells had normal levels of MHC II expression (data not shown). This indicates that thymic crosstalk is involved in the initial induction of MHC II expression on cTECs but is not required for maintenance of expression. The requirement for thymocytes during TEC development reflects the ability of T cells to influence the microenvironment they develop in by providing a feedback mechanism. However, the precise signals thymocytes provide to TECs remains speculative, with some studies reporting that DP and SP thymocytes generated in late fetal stages are able to provide growth factors akin to those provided by the mesenchyme during early fetal TEC development (Jenkinson et al., 2003), which may influence proliferation of TEC populations (Erickson et al., 2002). This could possibly explain why in the present study, levels of cTEC proliferation were slightly lower in thymocyte deficient mice compared to WT.

To summarise, the stage specific requirement for haematopoietic cells during cTEC and mTEC development highlights the essential role for thymic crosstalk at multiple stages of thymus development.

6.2.4 Future Directions.

Despite recent advancements in the field, many questions regarding TEC development remain unanswered. Whilst our model of TEC development suggests that bTECs undergo bifurcation into the cTEC and mTEC lineage, it is currently unclear what the precise sequence of development is. Indeed, our data may suggest that cortex develops before medulla as evidenced by the fact that appearance of $CD205^+$ cTECs preceded the emergence of $CD205^-$ $CD40^+$ mTECs. Additionally, studies have shown that development of the cortex is a prerequisite for medulla formation (Klug et al., 2002). It also remains unclear what signalling pathways cause bTECs to develop into either the cortical or medullary lineage. In other words, is it stochastic or regulated extrinsically? Such extrinsic signals are unlikely to be mesenchyme or thymocyte in origin as TECs at E12 of development can undergo cortical and medullary differentiation in the absence of mesenchyme (Jenkinson et al., 2003) or a normal programme of T cell development as demonstrated in our study and others (Jenkinson et al., 2003; Jenkinson et al., 2005).

It is currently unclear to what extent the $CD205^+$ cTEC progenitors identified in this study are committed to the cortical lineage. In other words, can $CD205^+$ cells in the early embryo give rise to mTECs? Future experiments could involve isolating these cells and reaggregating them with mismatched stromal cells to determine their fate. Additionally, whilst we propose that $CD205^+$ cTECs in the embryo are putative progenitors of more mature $CD205^+$ cTECs, we are yet to formally prove the existence of a precursor-product

relationship. Similar to experiments carried out on mTEC subsets (Rossi et al., 2007b), future experiments could involve purifying $CD205^+$ cTECs from an embryo and reaggregating them with mismatched stroma. Following culture, $CD205^+$ cells could be analysed for their expression of CD40 and MHC II. Furthermore, studies on mTECs have yielded interesting data on their lifespan and turnover capacity, with data suggesting that the turnover of $AIRE^+$ mTECs is two weeks (Gray et al., 2007a). Certainly future experiments should focus on employing similar strategies to determine the turnover capacity of cells at different stages of cTEC development.

Precise mechanisms regulating overall TEC development remain undefined. Current work in the lab aims to elucidate signals required for differentiation of TECs through the identification and manipulation of signalling pathways via a lentiviral gene delivery system. For example, such a tool could help target TEC associated genes such as AIRE and RANK to understand their development. Other target genes could include members of the Wnt family, which have been implicated in the cell fate of keratinocytes in the skin (Huelsken et al., 2001).

Whilst an understanding of TEC development contributes to the scientific understanding of thymus development, it may also have implications in a wider context, particularly in the development of strategies for thymic regeneration.

6.2.5 The importance of TEC Development in the Context of Developing Strategies For Thymic Regeneration.

The immune system undergoes an age-dependent deterioration in its ability to respond to infection and vaccination (Aw et al., 2009). Central to this is the gradual process of thymus involution, which correlates with a decline in thymic output (Berzins et al., 2002). The

thymus achieves its maximum size within the first year after birth and this is followed by a progressive decrease in functional epithelial microenvironments, which in humans is replaced by fatty tissue (George and Ritter, 1996). Hence, an ability to restore thymic microenvironments would aid in increasing thymic output with a view to combat the age-associated decline in the immune system. Studies have shown an age-dependent decline in cortical and medullary markers, accompanied by disorganised regions, thus affecting the signals given to thymocytes from the thymic microenvironments (Aw et al., 2009).

The potential to activate TEC progenitors in an adult thymus presents an attractive strategy to restore declining thymus function in the elderly or patients receiving therapy. Whether a bipotent or lineage restricted TEC progenitor exists within the post-natal and adult thymus, which acts as a stem cell pool with self-renewal capacity is currently unknown. The fact that the thymus undergoes atrophy would suggest an absence of a stem cell pool fitting with the notion that a functional thymus is only required during postnatal periods and early adulthood. However, studies have shown the ability to regenerate thymic microenvironments in mice through castration, implying that a stem cell population may exist in an adult thymus (Blackburn et al., 2002). Thus, in light of recent advances in TEC development, one could envisage a scenario where knowing the signals required to stimulate TEC stem cell expansion and differentiation *in situ* would further promote strategies aimed at thymic regeneration and subsequent peripheral T cell reconstitution in the elderly or immunocompromised patients (Blackburn et al., 2002).

6.3 Chemokine Mediated Establishment of Thymic Microenvironments.

T cell development is defined by the ordered migration of thymocytes through distinct regions of the thymus in order to receive essential signals for their differentiation and

selection. In the vascularised thymus, thymocyte progenitors enter at the CMJ before migrating towards the SCZ. This correlates with differentiation through the DN stage of T cell development where cells undergo β-selection, pre-TCR signalling and extensive proliferation (Prockop and Petrie, 2000). Following these events, emerging DP cells reverse their migration polarity moving outwards deeper into the cortex to undergo positive selection. Differentiation to the SP stage correlates with migration to the medulla where thymocytes undergo the final stages of maturation. Such microenvironments provide specialised and unique signals to support the multiple stages of T cell development. However, establishment of these microenvironments remain undefined. Defining the specialised conditions of these thymic microenvironments and in particular the signals that allow thymocytes to reach these regions is central to understanding T cell development.

Thus, this study aimed to characterise the role of chemokine-mediated establishment of thymic microenvironments, with a particular emphasis on the SCZ. Additionally, T cell development in DOCK2 deficient mice, where chemokine (namely CCR7 and CXCR4) signalling may be disrupted, was also investigated. This study demonstrates that the anatomic compartmentalisation of the thymus is established during ontogeny and remains relatively conserved through to the adult stages. Furthermore, by employing laser capture microdissection (LCM) this allowed for the isolation of cells defined by their specified locations. To characterise signals generated by stromal cells that cannot be identified phenotypically, but by their location in the thymus remains a challenge. Therefore, LCM was particularly useful for isolating the SCZ as there is currently no phenotypic marker that allows for the isolation and analysis of stromal cells in this region by flow cytometry. We were thus able to analyse the anatomical compartmentalisation of chemokines in different regions of the thymus. However, the technique of LCM has some limitations especially as isolated regions

will be 'contaminated' by haematopoietic cells that may 'dilute' the stromal cell derived signals. Gosink et al (2007) have optimised a method to electronically subtract gene expression in one or more components of a mixed cell population to reveal expression patterns of minor or difficult to isolate populations. Whether such a technique could be successfully applied to study stromal cell derived signals from microdissected regions of thymus remains to be determined. However, despite this limitation, LCM is a reliable technique to study populations of cells that can only be defined by their anatomical location. Indeed, LCM has been successfully applied in our lab to study if replication of murine CMV, which is known to cause severe destruction of the splenic T cell zone, occurs in the red pulp or white pulp stroma (Bekiaris et al., 2008). Furthermore, a recent study has employed LCM to isolate the 3rd pharyngeal pouch from mouse embryos and have identified a role for the transcription factor MafB in embryonic thymus development (Sultana et al., 2009). In the context of the present study, LCM could also be employed to study the establishment of thymic microenvironments throughout ontogeny. More specifically, establishment of the SCZ was observed to occur at E18. It would thus be of future interest to elucidate if a specific chemokine expression pattern already exists at this point of development. Furthermore, data in this thesis show that thymocyte migration to the SCZ is perturbed in CCR9$^{-/-}$ neonatal and adult mice and so it would be of equal interest to investigate if a similar scenario is present at E18.

Certainly any attempts to optimise LCM to isolate cells and/or regions from fluorescently labelled tissues will pave the way to study more specific areas of interest. Whilst this study primarily focused on chemokine related expression patterns within the intra-thymic niches and it would certainly be of future interest to employ LCM to study other genes

that may be important during T cell development such as members of the Wnt or Notch family.

Chemokine signalling is complex and so it is difficult to assess the precise roles of chemokines during thymocyte migration based solely on expression patterns. Nevertheless, by identifying the compartmentalisation of chemokines within the thymus and the expression of their receptors on thymocytes and the effect of knocked out chemokines is an important step in understanding this process. Data in this study supports current notion that intra-thymic regions can be characterised by differential expression patterns of chemokines (Petrie, 2003; Takahama, 2006). By paying particular attention to the poorly defined region of the SCZ we addressed what chemokines may be important in the establishment of this region. Importantly, by studying T cell development in DOCK2$^{-/-}$ mice, we can predict that in the absence of certain chemokines or signalling pathways, the thymus appears to have the capacity to initiate compensatory mechanisms that achieve normal T cell development.

This study, along with others clearly shows the importance of chemokines in directing thymocytes to specific niches. However, this poses the important question of how chemokines influence the migration of thymocytes. A long existing paradigm suggests that chemokines diffuse away from secreting cells to establish a chemokine gradient, where receptive cells away from the source detect the chemokine and move up the gradient (Vicker, 1989). However, whether this is the mechanism in place in the thymus is unclear. Studies suggest that rather than the presence of a chemokine gradient, T cells can respond to chemokines such as CXCL12 in the absence of a soluble chemokine gradient via the induction of cell polarisation and spatial re-orientation of the cell (Pelletier et al., 2000). However, the role of other factors that act in concert with chemokines in inducing thymocyte migration cannot be overlooked. Certainly the role of adhesion molecules such as integrins

have been implicated in cortex to SCZ migration (Petrie, 2003). For example, DN2 cells that are actively migrating towards the SCZ are known to express a variety of integrins that are thought to bind to certain ECM ligands. More specifically, laminin 5 is an extracellular matrix (ECM) molecule secreted by subcapsular epithelial cells and its receptors (e.g. $\alpha_6\beta_4$ integrin) are specifically up-regulated on DN3 cells (Kim et al., 2000). Such stromal elements present in the SCZ suggest that they may function during the DN stage of thymocyte development by providing a temporary anchorage site for these thymocytes, thereby maintaining the cells in the right environment for their survival and differentiation (Kim et al., 2000). Downregulation of this receptor on DP cells may explain the reversion of migration polarity. Chemokines interacting with ECM highlights the need to analyse migration in the context of the microenvironments in which they occur. Studies have shown that the stromal microenvironment of the SCZ tends to differ from the cortex and instead shares similar characteristics as the medulla (Farr et al., 1992).

The apparent requirement for specific niches for T cell development may not be absolute and this raises the longstanding question of whether thymocytes require specific niches to undergo T cell development, which is yet to be fully addressed. In the context of cortex to medulla migration, T cell maturation and export can occur in the absence of CCR7 mediated migration to the medulla (Kurobe et al., 2006). Certainly, recent data suggests that not only can the cortex support the clonal deletion of thymocytes self-reactive to ubiquitous self-antigens (McCaughtry et al., 2008) but the finding that thymocytes interact with DCs located in the cortex help to reinforce the view that thymocytes can undergo a significant amount of screening for self-reactivity prior to exposure to the medullary microenvironment (Ladi et al., 2008). However, the autoimmune symptoms observed in the study by Kurobe et al (2006), suggests that interaction of developing T cells with $AIRE^+$ mTECs is still required

to establish central tolerance to tissue restricted antigens. In the context of this study it would be of future interest to graft cortical epithelial cells (i.e. sorted on the basis of a $CD205^+$ phenotype) into an athymic host. Not only would such an experiment provide clues as to whether the medulla is derived from cortex, but also if this were not the case then would we see autoimmunity as result of failed negative selection?

Aberrations in chemokine or non-chemokine mediated migration of thymocytes may alter the maturation/migration processes of developing T cells, which may consequently alter the repertoire of the peripheral T cell pool. Indeed migratory disturbances have been observed in a disease context in mice infected with the protozoan parasite *Trypanosoma cruzi*, the causative agent of Chagas disease (Mendes-da-Cruz et al., 2006; Savino, 2006). Infected mice displayed increased deposition of ECM ligands (namely fibronectin and laminin) and chemokines (namely CXCL12) in the thymus. Such alterations correlated with the appearance of potentially auto-reactive thymus derived DP T cells in the peripheral organs of infected mice. Consequently, such reports provide further clues for understanding intra-thymic T cell development and for designing therapeutic strategies to target developing T cells in a disease context.

6.4 Concluding remarks

This thesis attempted to define the development and function of thymic microenvironments, with particular emphasis on characterising stages in cTEC development and the role of chemokine-mediated establishment of the SCZ. A major outcome of this work has been the definition of checkpoints in the development of the cortex, which is providing new insights into how the cortex acquires the ability to mediate positive selection. These

findings should provide the basis for developing future novel strategies for rejuvenating the thymus in immunocompromised patients. This will no doubt bring closer the prospect of exploiting the identified progenitor cells for increasing the longevity of the T cell repertoire.

Papers arising from this thesis

1. **Shakib S**, Desanti GE, Jenkinson WE, Parnell SM, Jenkinson EJ and Anderson G. Checkpoints in the Development of Thymic Cortical Epithelial Cells. (2009). *J Immunol* **182**, 130-137.

2. Bekiaris V, Timoshenko O, Hou TZ, Toellner K, **Shakib S**, Gaspal F, McConnell FM, Parnell SM, Withers D, Buckley CD, Sweet C, Yokoyama WM, Anderson G, Lane PJ. Ly49H+ NK cells migrate to and protect splenic white pulp stroma from murine cytomegalovirus infection. (2008). *J Immunol* **180**, 6768-76.

CHAPTER 7

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