Generation of a compartmentalised thymus organoid *in vitro* using fetal thymic epithelial progenitor cells

Julie Marie Sheridan

Thesis presented for the degree of Doctor of Philosophy

The University of Edinburgh

2007

Declaration

I declare that the work presented in this thesis is my own, except where otherwise stated.

Acknowledgements

I would like to thank my supervisor Clare Blackburn for giving me the opportunity to undertake this PhD and for being there to offer support, enthusiasm and most importantly money when I needed it. Thanks also go to the other members of the Blackburn lab for chat and help. Special thanks go to Natalie for the many, many hours of dissection together, Craig whose friendship and counsel have been a great asset, and Lucy, for the beers/wines/raspberry mules and of course the tea. Those that read this thesis will also be aware of what a huge thanks I owe to Carol Manson and the biomed unit staff, who have been saintly putting up with my never-ending requests. A similar debt I owe to Jan Vrana, who has sorted literally day and night for me. Also, I'd like to thank Sam for helpful discussion and thought-provoking statements.

I would have been unable to accomplish any of this without the love and support of my Mum and Mag. Mum, Les, Leslie, Jonathan and Gran: you are all great and I hope to see you more now that I have finished!

Abstract

The heterogeneous epithelial cell network of the thymus provides the microenvironments required for intrathymic T cell differentiation and repertoire selection. When grafted *in vivo* a population of fetal thymic epithelial cells, marked by the monoclonal antibodies MTS20 and MTS24, can generate all major thymic epithelial cell subtypes. Furthermore, the resultant thymic organoid can recruit T cell precursors and support their differentiation into mature $CD4^+$ and $CD8^+$ T cells. The work presented here evaluates the potential of $MTS20^+$ thymic epithelial progenitor cells to form the basis of a thymus equivalent *in vitro.*

To assess the ability of the $MTS20^+$ thymic epithelial progenitor cell population to support T cell differentiation *in vitro*, improvements were made to the established reaggregate fetal thymic organ culture (RFTOC) method that permitted the reliable generation and culture of TEPC-based RFTOC (TEPOC). Subsequent analysis demonstrated that both $MTS20^+$ and $MTS20^-$ fetal thymic epithelial cells were able to support the differentiation of αβ and γδ T cells and also supported the differentiation of several other haematopoietic populations including dendritic cells and thymic macrophages.

However, while immunofluorescence analysis showed that $MTS20^+$ cells were able to differentiate *in vitro* to generate all major thymic epithelial cell populations, only limited differentiation was observed in $MTS20^-$ cell-based cultures. Strikingly, the TEPOCs were able to form organised epithelial structures *in vitro,* characterised by clearly distinguished adjacent medullary and cortical areas. No evidence of organisation was seen in $MTS20^-$ cell-based cultures. Together, these data establish that $MTS20^+$ but not $MTS20^-$ thymic epithelial cells can generate functional *in vitro* thymus-equivalents that recapitulate the epithelial and haematopoietic landscape of the wild-type thymus. Furthermore, the unique organisational ability inherent within the TEPOCs may be useful as an *in vitro* model of the processes governing thymus organisation.

Table of Content

Abbreviations

BM – Bone marrow

BMP – Bone morphogenetic protein

CLP – Common lymphoid progenitor

CMJ – Cortio-medullary junction

cTEC – Cortical thymic epithelial cell

CTES – Clusters of thymic epithelial staining

DC – Dendritic cell

DETC – Dendritic epidermal T cell

DGS – DiGeorge's syndrome

DN – Double negative

DP – Double positive

ECM – Extra-cellular matrix

EGF – Epidermal growth factor

FACS – Fluorescence Activated Cell Sorting

FGF – Fibroblast growth factor

FTOC – Fetal thymic organ culture

HOS – High oxygen submersion

HPC – Haematopoietic progenitor cell

HSC – haematopoietic stem cell

ICN – Intracellular Notch domain

IFN – Interferon

IGF – Insulin-like growth factor

IL – Interleukin

LT - Lymphotoxin

LTβR – Lymphotoxin-β receptor

mAb – Monoclonal antibody

MDC – Macrophage-derived chemokine

MHC – Major histocompatibility complex

MLR – Mixed lymphocyte reaction

mTEC – Medullary thymic epithelial cell

NCC – Neural crest cell

NK – Natural killer cell

NKT – Natural killer T cell

RAG – Recombinase activating gene

RFTOC – Reaggregated fetal thymic organ culture

SCID - Severe combined immunodeficiency

SDF-1 – Stromal-derived factor-1

Shh – Sonic hedgehog

SP – Single positive

TCR - T cell receptor

TEC – Thymic epithelial cell

TEPC –Thymic epithelial progenitor cell

TEPOC – Thymic epithelial progenitor-based organ culture

TN – Triple negative

TNC – Thymic nurse cell

TRA – Tissue restricted antigen

Treg – Regulatory T cell

TSLP – Thymic stromal lymphopoietin

TSP – Thymus seeding progenitor

Chapter 1: Introduction

1.1 The thymus

The thymus is a bilobed organ situated at the midline above the heart in humans and rodents. It has a complex structure consisting of a highly organised epithelial network, which is tightly packed with thymocytes and interspersed with non-epithelial stromal elements (Boyd et al., 1993; Kendall, 1991). Broadly, the thymus can be subdivided histologically into three main areas, the medulla, the cortex, and the subcapsular region as shown in Figure 1.1.

The thymus is encapsulated by connective tissue layers that penetrate the structure, as trabeculae, at intervals to create lobulations (Boyd et al., 1993). Trabeculae reach through the cortex to the cortico-medullary junction (CMJ) providing a link between the outermost regions and the inner medulla (Figure 1.1)(Boyd et al., 1993). Trabeculae are well innervated and harbour the vessels that form the basis of the extensive thymic vascular network (Boyd et al., 1993). The stromal compartment constitutes a minor fraction of the total cellularity of the thymus, but its complexity is indicative of the array of functions that it performs in order to support the differentiation of thymocytes (Boyd et al., 1993; Gray et al., 2002). The stroma consists of various cell types including epithelial cells, mesenchymal fibroblasts, macrophages, dendritic cells (DC) as well as components of the vascular network (Figure 1.1) (Boyd et al., 1993).

1.1.1 The Thymic Stroma

1.1.1.1 Thymic Epithelial Cells

The epithelial component of the stroma is estimated to be less than 3% of total thymus cellularity (Gray et al., 2002). Thymic epithelial cells (Montecino-Rodriguez and Dorshkind) are morphologically atypical and, unlike in most epithelial structures, form a three-dimensional lattice (van Ewijk et al., 1999). Tonofilaments and desmosomes

provide stability and anchorage throughout the reticular network (Farr and Anderson, 1985). Initial studies into the heterogeneity of the epithelium revealed that the human thymic epithelium could be divided ultra-structurally into six subtypes based on differences in morphology and electron lucency summarised in (van de Wijngaert et al., 1983). Later studies elaborated on these findings with the use of monoclonal antibody panels against unknown determinants and Keratin species (Godfrey et al., 1990; Klug et al., 1998; Nicolas et al., 1985; Van Vliet et al., 1985). A standard nomenclature known as Clusters of Thymic Epithelial Staining (CTES) was adopted to amalgamate these studies, a summary of which is shown in Table 1.2 (Kampinga et al., 1989). Together, these studies map morphologically distinct epithelial subsets to specific areas within the thymus.

The outermost subcapsular and sub-trabecular epithelium consists of a layer of flattened simple Type I epithelium that stains with CTES type II mAbs and is supported by a basal lamina (Kampinga et al., 1989). Although morphologically and phenotypically distinct subtypes exist within the Type I epithelium, it is generally major histocompatibility complex (MHC) Class II^- (Boyd et al., 1993; Godfrey et al., 1990). Underlying the Type I epithelium are the Type II epithelial cells of the outer cortex, which have a pale appearance in electron micrographs (Kampinga et al., 1989). Typically, cortical epithelial cells (cTEC) have long cytoplasmic processes that have been shown to interact with the densely packed cortical thymocytes (Pereira and Clermont, 1971) and are radially orientated with respect to the thymic capsule (van Ewijk, 1988). Type II TEC have been shown to be metabolically active, indicating a possible function in cytokine production (van de Wijngaert et al., 1984). Beneath these cells is the Type III epithelium, which shows even greater electron lucency (Kampinga et al., 1989). Deeper into the thymus, type III epithelium gradually gives way to Type IV epithelium (Kampinga et al., 1989). TEC Types II to IV form an area known as the cortex proper, identifiable with CTES III staining pattern, which is MHC Class II^+ and appears to express MHC Class I at lower levels (Kampinga et al., 1989; Rouse et al., 1985). The orientation of the cTEC, so that cells generally extend in directions

perpendicular to the capsule, is believed to facilitate the directed migration of thymocytes within the thymus (van Ewijk et al., 1999).

The innermost regions of the thymus are populated with heterogeneous medullary epithelial cells (mTEC) designated as Type III, Type V and Type VI, which mainly stain with CTES II and IV mAbs (Kampinga et al., 1989). Among these cell types MHC Class II staining is not uniform although MHC Class I is expressed by most, if not all, mTEC (Rouse et al., 1985; Surh et al., 1992b). In addition, some TEC express non-polymorphic MHC Class II molecules that are typically found on cells of the bone marrow (Surh et al., 1992b). Small clusters of Type V and VI epithelium exist at the CMJ. The epithelial cells of the medulla are proportionally more numerous and are generally rounder with shorter processes (Bearman et al., 1978; Chan and Sainte-Marie, 1968; Lundin and Schelin, 1965). In addition, multicellular structures known as Hassall's corpuscles are also present in the medullary region (Bearman et al., 1978; Chan and Sainte-Marie, 1968).

Epithelial cells can be identified by a characteristic cytoskeletal network of cytokeratin filaments (Sun et al., 1979). The different epithelial cells of the thymus contain a number of cytokeratin heterodimer species, with specific anti-cytokeratin antibodies being able to identify thymic subpopulations (Nicolas et al., 1985; Savino and Dardenne, 1988). Cytokeratins can be grouped into two groups based on their acidic or basic nature. Type I molecules are acidic and have molecular weights in the range 40 to 56 KDa. Type II cytokeratins are basic and have higher molecular weights between 53 and 68 KDa. Broadly, cTEC express type I cytokeratins, which are otherwise generally expressed by simple epithelial structures. Medullary epithelial cells express keratin species with higher molecular weights, typical of more structurally complex epithelia (Savino and Dardenne, 1988; Sun et al., 1979).

One study combined the use of cytokeratin antibodies with other markers of thymic epithelia to characterise the epithelia and to investigate their interrelationships and origin (Klug et al., 1998). The findings of the study indicated that two populations

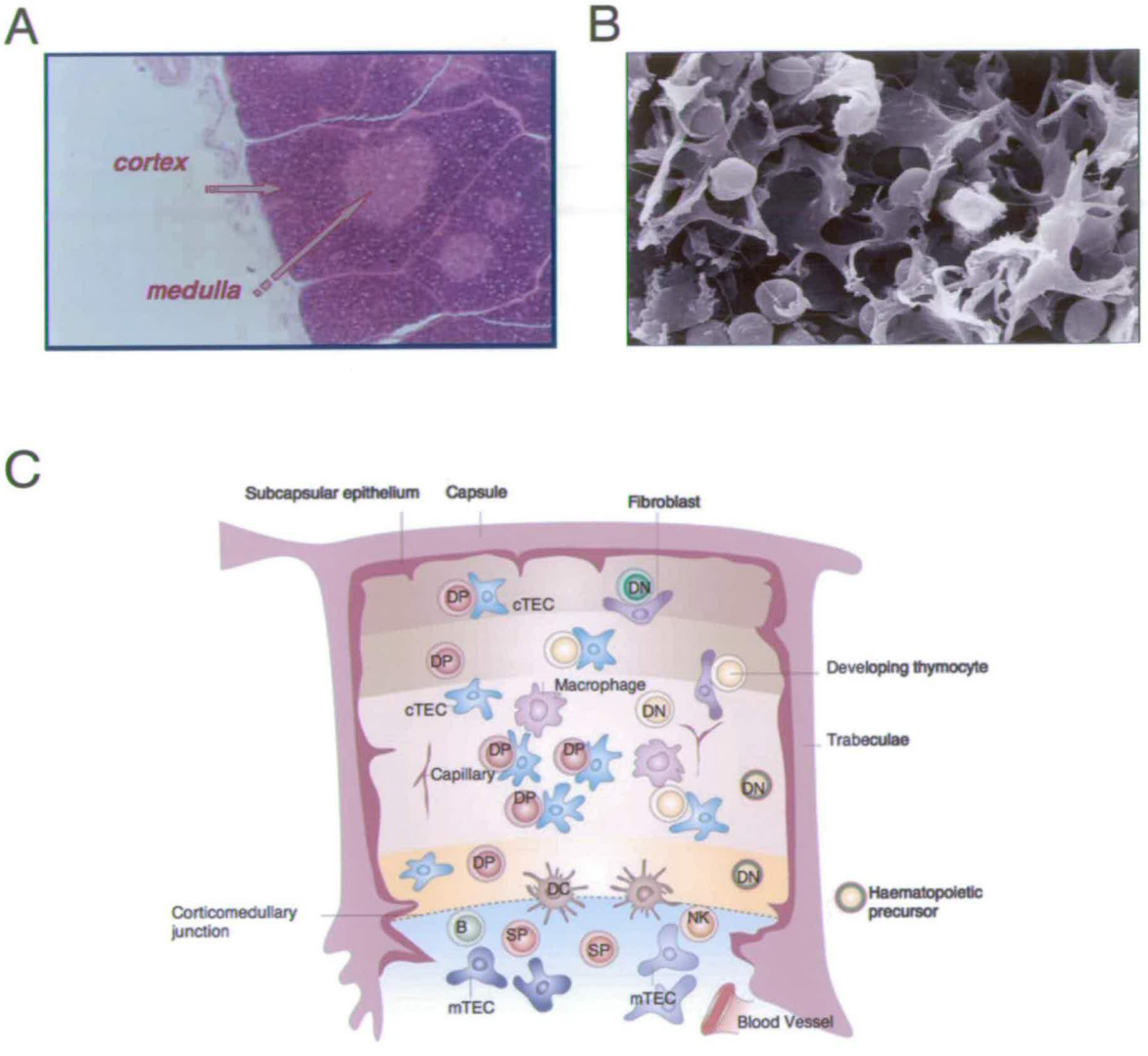

Figure 1.1 Morphology of the postnatal thymus

A, Organisation of the thymus into lobules containing outer cortical and inner medullary areas (M. Ritter, Imperial College, London).

B, Electron micrograph of the thymus showing the epithelial network and developing thymocytes (W. van Ewijk, University of Leiden).

C, Composition of the postnatal thymus (Picture adapted from Nature Reviews Immunology).

Table 1.1 Summary of epithelial subtypes (van de Wijngaert et al., 1984)

DESIGNATION	LOCATION
Type 1	Subcapsular/Perivascular (believed to form the blood-thymus barrier)
Type 2	Outer cortical
Types 3 and 4	Inner cortical (active cytokine producers)
Type 5	Medullary (small foci)
Type 6	Hassall's corpuscles

Table 1.2 Summary of the CTES Classification (Kampinga et al., 1989)

CTES GROUP	SPECIFICITY
I	Pan epithelial
II	Subcapsular/perivascular/medullary
III	Cortical
IV	Medullary/Hassall's Corpuscles
V	Hassall's Corpuscles
VI	Type 1 Epithelial
XX	Miscellaneous

were consistently found in the cortex: a majority population of K (keratin) $8^+K18^+K5^-$ $K14^-$ cells were found in the cortex and the subcapsular cortex and a minority $K8^+K18^+K5^+K14^-$ population was found as scattered cells throughout the cortex but with a concentrated band at the CMJ (Klug et al., 1998). When analysed with keratins and several other markers including the lectin, UEA-1, and CTES II type antibody, MTS10, the medullary epithelium proved similarly heterogeneous. In agreement with earlier studies, UEA-1 and MTS10 were found to be almost entirely restricted to the medullary region although UEA-1 was also found to extend to the CMJ (Farr and Anderson, 1985; Godfrey et al., 1990; Klug et al., 1998).

1.1.1.2 Multicellular complexes

Hassall's corpuscles are small clusters of keratinised medullary epithelial cells, which have a characteristic whorled appearance (Hassall, 1849). Although these are common in the human thymus, they are infrequently found in the medulla of the murine thymus (Farr et al., 2002). Cells within Hassall's corpuscles are highly metabolically active and produce a variety of factors associated with signalling between different cell types including IL-7 (He and Kabelitz, 1995), thymic stromal lymphopoietin (TSLP) (Watanabe et al., 2005), stromal cell-derived factor 1 (SDF-1) (Zaitseva et al., 2002) and macrophage-derived chemokine (MDC) (Annunziato et al., 2000), suggesting that these structures act as communication centres. Several roles have been proposed for these structures, including a site of advanced thymocyte death (Blau, 1973; Senelar et al., 1976). Recently work has shown that human Hassall's corpuscles are able to activate $CD11c^+$ thymic DCs in a TSLP-mediated manner. The resulting activated DCs are then able to recruit $CD25^-CD4^+$ thymocytes into the $CD25^+CD4^+FoxP3^+$ regulatory T cell lineage (Watanabe et al., 2005).

Thymic nurse cells (TNC) were originally identified in stromal cell suspensions as single epithelial cells containing up to 200 thymocytes within their cytoplasm (Ritter et al., 1981; Wekerle et al., 1980). Although there has been some debate whether these are

an artefact of the stromal dissociation process, analogous structures have been found *in situ* throughout the subcapsular cortex and in close association with capillaries in the deep cortex (Kyewski and Kaplan, 1982; van de Wijngaert et al., 1983). The identification of the thymocytes within TNCs as immature $CD3^-CD4^-CD8^-$ triple negative (TN) or $CD4^+CD8^+$ double positive (DP) thymocytes has lead to the hypothesis that these may play a role in the maturation of thymocyte populations (Andrews and Boyd, 1985; Kyewski and Kaplan, 1982; Shortman et al., 1986).

Thymic epithelial cysts are well documented features of the thymus of wild-type mice (Farr et al., 2002; Khosla and Ovalle, 1986). Encapsulating a lumen and laterally joined by intercellular junctions, these were believed to form an integral part of the thymic lymphatic system (Khosla and Ovalle, 1986). Some evidence however suggests that these structures result from inappropriate epithelial differentiation into non-thymic epithelial lineages. Arising in the fetal thymus, and persisting through adult hood, epithelial cysts clearly show features of lung, thyroid and gut tissue, including the presence of cilia and luminal microvilli (Chan, 1986; Khosla and Ovalle, 1986) (Farr et al., 2002). In addition, genes expressed by other endodermally-derived tissues have been observed in the thymus, and antibody staining for proteins such as respiratory surfactants and thyroglobulin show their presence in the cells of the cyst (Dooley et al., 2005b; Farr et al., 2002). There is considerable phenotypic and structural similarity of cysts found in wild-type animals with the prominent cysts of mutant mice showing aberrant epithelial development such as in the nude mouse (Blackburn et al., 1996; Farr et al., 2002). Interestingly, widespread co-expression of cortical and medullary markers (Bennett et al., 2002; Farr et al., 2002; Gill et al., 2002; Klug et al., 2002) in cells of TEC cysts was detected in addition to MTS24 reactivity (Blackburn et al., 1996; Farr et al., 2002).

1.1.1.3 Non-epithelial stromal elements

Many non-epithelial cells contribute to the architecture of the thymus. These include cells derived from the neural crest as well as bone marrow, which are present in all

areas of the thymus (Boyd et al., 1993). Macrophages are found throughout the thymus but are more numerous in the cortex than in the medulla (Boyd et al., 1993). These show great heterogeneity in morphology and MHC expression patterns, although cortical macrophages can be characterised by the presence of numerous lysosomes containing remnants of thymocytes indicating a role in the maintenance of the thymic microenvironment through the removal of apoptosed thymocytes and cellular debris (Duijvestijn and Hoefsmit, 1981; Milicevic and Milicevic, 1988). It is also possible that they play a role in cytokine secretion and antigen presentation, which is important during intrathymic selection events (Kendall, 1991). Similarly, dendritic cells are present throughout the stroma but are concentrated at the CMJ and in the medulla and typically show uniformly high MHC Class II expression (Boyd et al., 1993; Nabarra and Papiernik, 1988). DCs play an important role in imposing negative selection through the presentation of antigens to maturing thymocytes (Ardavin, 1997).

The thymic stroma contains mesenchymally derived fibroblastic cells which, along with cTEC, have been shown to secrete extracellular matrix (ECM) components including collagens, laminin and fibronectin, which form an integrated network throughout the medulla, the subcapsular region, and less extensively, the cortex (Boyd et al., 1993). ECM receptors are widely expressed by both thymocytes and TECs and have been shown to have effects upon thymocyte differentiation and migration as well as TEC differentiation (Boyd and Hugo, 1991; Cardarelli et al., 1988; Savino et al., 1993(Cardarelli, 1988 #329; Schreiber et al., 1991).

1.1.2 Thymic microcirculation

The thymus is a heavily vascularised organ, with a blood supply provided by one or more thymic arteries via the thymic capsule and trabeculae (Kato and Schoefl, 1989). Entering the parenchyma at the CMJ, these branch into arterioles, which in turn support an extensive capillary network. The capillaries reach outwards through the cortex, subcapsular, and capsular region before returning deep into the parenchyma and draining into postcapillary venules in the medulla and at the CMJ (Boyd et al., 1993;

Kato and Schoefl, 1989; Sainte-Marie et al., 1986; Ushiki, 1986). Blood exits the thymus through a one of a few thymic veins (Kato and Schoefl, 1989). There is considerable evidence for a partial blood-thymus barrier, having been demonstrated that inter-endothelial cell junctions, and delineation by Type I epithelium, forms a double-walled barrier to blood-borne antigen (Clark, 1963; Kato, 1997; Marshall and White, 1961; Ushiki, 1986; Weiss, 1963). The use of electron opaque tracers functionally confirmed that the blood-thymus barrier is limited to the cortex and that the medulla shows no such barrier (Raviola and Karnovsky, 1972). Vascular vessels of the thymus are accompanied by lymphatic vessels in many mammals (Odaka et al., 2006). Both the vasculature and lymphatic system appear to constitute a possible route of efflux of lymphocytes into the microcirculation (Ernstrom, 1965; Kato, 1997; Kato and Schoefl, 1989; Miyasaka et al., 1990). In addition to the typical role of vascularisation in an organ, it has been suggested that the elements of the vascular system play a role in the organisation of the thymic medulla. It was found that vessels of intermediate diameter spatially colocalise to areas of medulla (Anderson et al., 2000). This was more clear in recombinase activating gene (RAG) null mice, which have small areas of medulla appearing as perivascular cuffs (Anderson et al., 2000). An example of the organisation of lymphoid structures around vascular structures is in the spleen, where central arteries are surrounded by the white pulp . Vascular elements also interact with epithelial cells to orchestrate the development of other endodermally derived organs such as the liver and pancreas development (Cordier and Haumont, 1980; Lammert et al., 2001; Matsumoto et al., 2001; Nikolova and Lammert, 2003).

1.2 Thymus Organogenesis

1.2.1 Formation of the thymic rudiment

The pharyngeal arches are transient structures comprising ectoderm covered bulges of mesenchymal cells formed in a rostral to caudal manner during embryogenesis (Hogan 1994). The arches are separated by out-pocketings of pharyngeal endoderm known as pharyngeal pouches, and opposing ectodermal invaginations known as pharyngeal clefts

(Cordier and Haumont, 1980). Both the endodermal pouches and the ectodermal clefts initially form as a single layer of simple epithelium.

At around embryonic day (E)9.5 of development in the mouse, the endoderm of the third pharyngeal pouches undergoes a proliferative burst that establishes the outgrowth of common primordia that will give rise to both the thymus and the parathyroid (Cordier and Haumont, 1980; Smith, 1965). Evidence for the patterning of the common primordia into respective thymus and parathyroid domains can be found early in development, with the parathyroid domain being delineated by the transcription factor, *Gcm2* in the dorsal aspect of the third pharyngeal pouch by E9.5 (Gordon et al., 2001). By E11.25, the transcription factor, *Foxn1* is expressed in a complementary manner to *Gcm2*, defining the ventral aspect of the pouch that is destined to become the thymus (Gordon et al., 2001). Evidence that the cells of the third pharyngeal pouch are specified to a thymic fate prior to this time point comes from experiments demonstrating that upon transplantation, isolated E8.5-E9.0 foregut endoderm generates functional thymus-like structures (Gordon et al., 2004). This also shows that *Foxn1* is not the specifying factor.

Each of the common primordia are surrounded by a condensing neural crest-derived mesenchymal layer that persists to form the thymic capsule (Jiang et al., 2000; Le Lievre and Le Douarin, 1975; Schreier, 1952). The encapsulated anlagen continue to proliferate, and segregate from the pharynx so that by E12.5 they have formed discrete structures (Smith, 1965). By E13.5, the thymic and parathyroid primordia have separated and the thymus lobes have begun their migration towards their final anatomical location at the midline (Hammond, 1954; Manley, 2000; Schreier, 1952).

The seeding of extrinsic haematopoietic cells into the thymus takes place in cyclical waves throughout embryonic life (Jotereau and Le Douarin, 1982; Moore and Owen, 1967). Initial colonisation of the thymic anlage begins several days before vascularisation can be seen, with haematopoietic cells migrating into the mesenchymal capsule at E11 (Itoi et al., 2001). By E12.5, colonising cells have entered the epithelial

rudiment and begun to proliferate (Itoi et al., 2001; Jotereau et al., 1987; Suniara et al., 1999). This first wave of precursor influx peaks between E11.5 and E12.5, with successive waves of seeding following. Phenotypic and functional differences have been observed between thymocytes of E12 thymi and those of E14 and E15 thymi, with those from older thymi displaying greater T cell potential when transferred *in vivo* or maintained *in vitro* (Douagi et al., 2000).

1.2.2 Cellular origins of the thymus

1.2.2.1 The thymic epithelium

The origin of the thymic epithelium remained, until recently, a source of controversy with conflicting hypotheses suggesting that the thymus had either a dual endodermal/ectodermal origin or that origin of the epithelium was purely endodermal. Resolution was provided by a study that addressed both lineage and potency of the ectoderm and endoderm (Gordon et al., 2004). Failure to see contribution of labelled early ectoderm to the forming thymic rudiment in cultured embryos strongly suggested that the ectoderm did not contribute to thymic lineage (Gordon et al., 2004). Further investigations into the potency of E8.5 and E9.5 endoderm grafted under the kidney capsule of recipient mice found that both cortical and medullary epithelium could differentiate from endoderm, analogous to results observed in earlier quail-chick chimaera studies (Gordon et al., 2004; Le Douarin and Jotereau, 1973; Le Lievre and Le Douarin, 1975). Taken together, these studies establish that the endoderm can give rise to both cortical and medullary epithelial cells thus supporting the single origin hypothesis.

1.2.2.2 Thymic epithelial progenitor cells

Development of the complex mature thymic epithelial network requires the presence, proliferation and differentiation of an immature progenitor cell population in the developing thymus. Once formed, the maintenance of the thymic structure may require the presence of a persistent postnatal epithelial progenitor or stem cell population. In this way, the cells within the thymic epithelial network could be replenished during

adulthood or following thymic injury in an analogous manner to the replenishment and recovery of other epithelial organs such as the gut and the skin, which is mediated by epithelial stem cells.

Evidence suggestive of the existence of a postnatal thymic progenitor cell has come from a number of epithelial marker studies. A study on human thymic epithelial cancer cells showed that some cells, able to give rise to cells with mTEC and cTEC identity, co-expressed medullary and cortical markers (Schluep et al., 1988). Further analysis showed that a cell type displaying the same staining pattern exists as an epithelial subset in both fetal and postnatal human thymus, supporting the view that a bipotent cortical and medullary precursor exists in human (Schluep et al., 1988). Studies of thymi from mice with developmental blocks have also proved useful in the search for an epithelial progenitor population. One such study comparing the thymus of wild-type and CD3ε26tg mice identified an epithelial sub-population that similarly co-expressed the medullary marker, K5 and the cTEC marker, K8, indicating that an analogous population may also exist in wild-type mice (Klug et al., 1998).

An earlier study of contributions in nude/wild-type thymic chimaeras showed that although nude cells did not contribute significantly to the chimaeric thymus, a few nude-derived cells were able to persist amongst the wild-type cells (Blackburn et al., 1996). Interestingly, the nude-derived cells expressed MTS20 and MTS24, a phenotype shared by some TEC within the nude thymic rudiment, but did not express markers of TEC differentiation such as MHC Class II (Blackburn et al., 1996). These experiments proved that the *nude* gene product Foxn1 is cell-autonomously required for epithelial differentiation and suggested that in its absence, cells undergo maturational arrest and remain in a functionally immature state (Blackburn et al., 1996).

The markers that identify these cells have been analysed further with ontogenic analysis showing that MTS24 and MTS20 are expressed throughout the pharyngeal endoderm by E10.5. At E12.5, a major population of epithelial cells within the thymus primordium expresses MTS20 and MTS24 (Bennett et al., 2002). The proportion of

$MTS24^+$ TECs subsequently decreases so that the MTS20 and MTS24 expression is restricted to only a small fraction of postnatal medullary epithelial cells (Bennett et al., 2002; Gill et al., 2002). In both the embryo and the adult, immunofluorescence analysis showed that there was widespread co-expression of K5 and K8 in the $MTS24^+$ compartment (Bennett et al., 2002; Gill et al., 2002).

Functional data regarding the identity of the fetal TEPC was gained through ectopic grafting of fetal $MTS24^+$ TEC. $MTS24^+$ TEC had the capacity to differentiate into all major TEC subtypes and to recruit and mature T cells precursors (Bennett et al., 2002; Gill et al., 2002). Conversely, the $MTS24^-$ compartment exhibited no thymus function and did not persist under similar conditions, suggesting that progenitor capacity was limited to the $MTS24^+$ population. A recent study using direct functional analysis of sorted E12 TECs concluded that a bipotent progenitor exists in the E12 thymus, with both cortical and medullary cells being shown to be clonally derived from E12 $MTS24^+$ cells (Rossi et al., 2006). The existence of a common progenitor of both cTEC and mTEC has also recently been reported in experiments using conditionally reactivatable *Foxn1* null mice (Bleul et al., 2006). Rare recombination events occurring in these mice led to the apparently clonal reactivation of *Foxn1* in cells which were subsequently able to generate mini thymi containing cortical and medullary regions (Bleul et al., 2006). These data indicate that in the absence of *Foxn1*, bipotent progenitors remain.

1.2.3 Cellular interactions in thymus organogenesis

1.2.3.1 Epithelial-mesenchymal interactions in thymus development

An essential role for neural crest cells (NCC) in the development of fetal thymic epithelial rudiments beyond E12.5 has been demonstrated using tissue culture and grafting experiments, which showed that culture of the thymic rudiment lacking the mesenchymal capsule (Auerbach, 1960) and experimental disturbance of the migrating NCCs (Bockman and Kirby, 1984) results in a failure of normal thymus development and thymic hypoplasia. Chick-quail chimaeras revealed that the early pharyngeal endoderm could induce non-pharyngeal mesenchymal cells to contribute to thymic

structure (Le Douarin and Jotereau, 1973) Furthermore; the perturbed development of the thymic epithelium observed when cultured in the absence of mesenchyme could be corrected by co-culture with mesenchyme from a variety of sources including thymus, kidney and lung, indicating that the ability to support thymic epithelium is not limited to thymus-derived mesenchyme (Auerbach, 1960; Shinohara and Honjo, 1997). Although specific data indicating a role for NCCs prior to E12 of mouse thymus development is lacking, collectively these data suggest that NCCs may respond to pharyngeal endoderm-derived signals in order to establish identity, before playing a role in thymus organogenesis.

Later in development, the role of NCC-derived mesenchyme is less clear. Analysis of the fate of labelled NCCs in *Wnt1-cre* reporter mice (Jiang et al., 2000) and *myelin protein O-cre* reporter mice (Yamazaki et al., 2005) has demonstrated that despite early heavy incorporation of NCCs into the thymus capsule and stroma, beyond E16.5, few labelled cells remain in the thymus although large numbers of mesenchymal cells can be found in the capsule, trabeculae and vascular structures as well as intermittently among epithelial cells of the thymus. This decrease in labelled cells could be due to the gradual replacement of NCC-derived cells with those from other sources. However, the caveat still remains that the loss of labelling may be due to promoter silencing and does not accurately reflect a diminishing contribution of NCCs.

The molecular mechanisms through which NCC influences thymus development are poorly understood, although soluble growth factors expressed by the thymic mesenchyme have been shown to play an important role. Fibroblast growth factor (FGF) 7 and FGF10 are expressed by the mesenchymal component of the thymus (Jenkinson et al., 2003; Revest et al., 2001) whilst their common receptor, FGF receptor (FGFR)2-IIIb is reciprocally expressed by the thymic epithelium. FGFR2-IIIb null mice exhibit thymic hypoplasticity beyond E12, indicating a role for FGFR2-IIIb signalling in normal thymus development (Revest et al., 2001). *In vitro* studies demonstrated that the loss of proliferation caused by the removal of mesenchyme from FTOC and RTOCs

could be corrected by the provision of exogenous FGF 7 and FGF10, indicating that the epithelial-mesenchymal interaction required to maintain epithelial proliferation may be mediated by FGF signalling (Jenkinson et al., 2003). Expression of Insulin-like growth factor (IGF)-I and IGF-II, but not FGF7 and FGF10 has also been reported in the E12 thymic mesenchyme, with the IGF-1 receptor reciprocally expressed in the E12 thymic epithelium (Jenkinson et al., 2007). *In vitro* experiments have indicated a role for IGF-1 and IGF-II and epidermal growth factor (EGF) in the development of the early thymic epithelium with the addition of these factors to cultures leading to upregulation of MHC Class II on the developing epithelium (Shinohara and Honjo, 1997). Recent experiments have additionally implicated the IGFs as mediators of early TEC proliferation with differential expression between mesenchymal cells from thymus and other sources as a possible explanation for the effective but impaired recovery of epithelial proliferation upon grafting under the kidney capsule or in combination with other mesenchymal cells (Jenkinson et al., 2007).

1.2.3.2 Lympho-epithelial interactions in thymus development

The mechanisms that promote the development of mature thymic epithelial cells from immature TEPCs are poorly understood. Several mutant mouse strains, which have defects in thymocyte development, have secondary thymus stromal defects resulting in missing epithelial subsets and/or disorganisation (Naquet et al., 1999). Thus thymic cross-talk between developing thymocytes and the epithelium contributes positively to the establishment and maintenance of microenvironmental niches within the thymus (reviewed in van Ewijk, 1994). Reconstitution experiments using haematopoietic progenitor cells (HPC) from various strains of mutant mice showed that the development of mature thymic epithelial compartments is regulated by distinct thymocyte subsets in a stepwise manner (van Ewijk et al., 2000). In support of this model, $CD4^-CD8^-$ double negative (DN) subsets have been shown to regulate the expansion and organisation of cTEC (Hollander et al., 1995; van Ewijk et al., 2000) with more mature $CD4^+CD8^-$ or $CD4^-CD8^+$ single positive (SP) cells promoting the expansion and organisation of medullary subsets (Palmer et al., 1993; Shores et al.,

1991; Surh et al., 1992a; van Ewijk et al., 2000). Although the adult thymic epithelium is co-dependent upon developing thymocytes for their maturation and maintenance, it is clear that the initial patterning of the thymus is thymocyte-independent. This was demonstrated in both CD3ε26tg and RAG2/γc-deficient mice, which have profound and early blocks in thymocyte development yet show normal TEC development prior to E15.5 (Klug et al., 2002). Despite the apparent lack of further epithelial development beyond E15.5 in mice with thymocyte defects, it is possible to rescue normal thymic organisation by the transfer of wild-type HPCs.

Although the mechanisms involved in lympho-epithelial cross-talk remain unclear, the role of lymphotoxin β receptor (LTβR) signalling in the development of mature mTEC is established (Boehm et al., 2003). Several mouse models have shown that interruption of this pathway, by interfering with the LTβR expressed on the epithelial cells, LTβR ligands reciprocally expressed on thymocytes, or downstream signalling molecules such as NF-κB-inducing kinase (NIK) or the transcription factor, RelB, results in a failure to generate a normal mature medullary compartment and leads to an autoimmune phenotype (Boehm et al., 2003; Burkly et al., 1995). In these mice, although cTEC appear unaffected, the medulla appears disorganised with particular abnormalities among the UEA-1^+ mTEC cells, a phenotype that cannot be corrected by the presence of wild-type haematopoietic cells (Boehm et al., 2003). Furthermore, an LTβR-Fc fusion protein designed to interrupt LTβR signalling was sufficient to qualitatively and quantitatively negatively affect the mTEC compartment in wild-type mice (Boehm et al., 2003). Taken together, this data indicates that sustained interaction of LTβR mediates lympho-epithelial cross-talk is required for the development and maintenance of mature mTECs and thus central tolerance.

1.2.4 Genetic control of early thymus development

1.2.4.1 Tbx1

The heterozygous deletion of contiguous genes on human chromosome 22q11.2 results in complex pathologies typified by DiGeorge's syndrome (DGS), with craniofacial

defects, cardiovascular pathologies and thymic and parathyroid hypoplasia or aplasia (Kirkpatrick and DiGeorge, 1968). This region contains the *Tbx1* locus, which has been demonstrated in mouse mutants as the causative gene for DGS. *Tbx1* is expressed in both the third pharyngeal endoderm and the adjacent mesenchyme but not in the pharyngeal ectoderm or the NCCs of the pharyngeal arches (Garg et al., 2001). *Tbx1*$^{-/-}$ mice have thymic hypoplasia and defects in other derivatives of the third and fourth pharyngeal arch regions, a phenotype similar to that resulting from ablation of NCC prior to migration (Bockman and Kirby, 1984; Jerome and Papaioannou, 2001). Timed deletion of *Tbx1* in conditional mutant mice indicates that it may function in several ways (Xu et al., 2005). Loss of functional *Tbx1* between E9 and E9.5 results in a failure of the formation of a normal third pouch as well as structures located caudally, and a consequent failure in the development of pharyngeal structures (Xu et al., 2005). The loss of *Tbx1* from E10.5 results in thymic hypoplasia although the precise nature of the thymic defect has not been analysed, while later deletion of *Tbx1* from E11.5 has no appreciable effect upon thymus morphogenesis (Xu et al., 2005). In timed fate mapping studies, cells that express *Tbx1* at E8.5 were shown to incorporate heavily into the thymus whereas those form later stages only demonstrated minor incorporation suggesting that the mild hypoplasia seen in the mice with *Tbx1* deletions post E9.5, are likely to result from an indirect effect on thymic organogenesis such as poor factor provision (Xu et al., 2005). Collectively, these experiments indicate that Tbx1 has two main roles: an early role in development of the pharyngeal endoderm, and a later role in the correct development of pharyngeal derivatives that is likely to be indirect (Hollander et al., 2006; Xu et al., 2005).

1.2.4.2 Hoxa3

The Hox family of transcription factors function to regulate embryonic development along the anterior-posterior axis and tissue remodelling in adults. *Hoxa3* is expressed in third pharyngeal pouch endoderm and neural crest-derived mesenchyme of the third and fourth arches (Manley and Capecchi, 1995). Targeted disruption of *Hoxa3* indicated that Hoxa3 directly affects the development of organs such as the thymus and

parathyroid, which derive from this region (Manley and Capecchi, 1995). As a result, homozygous mutants die soon after birth, have neither thymus nor parathyroids and have several other abnormalities including heart defects (Chisaka and Capecchi, 1991; Manley and Capecchi, 1995). Prior to E11.5, thymus organogenesis is normal in *Hoxa3*$^{-/-}$ mutant mice but by E12, although mutant mice retain a discernible third pharyngeal arch, they fail to form a *Foxn1*-expressing thymic anlage (Manley, 2000; Manley and Capecchi, 1995). These observations parallel those seen in DGS, although it has been shown that the defects in *Hoxa3* mutant mice are not attributable to an abnormal number or distribution of NCC cells (Kirkpatrick and DiGeorge, 1968; Manley and Capecchi, 1995). Reduced proliferation among epithelial cells is also apparent, despite the presence of NCCs, suggesting that Hoxa3 plays a role in the intrinsic ability of NCC to induce the normal proliferation and differentiation of epithelial cells (Chisaka and Capecchi, 1991; Manley and Capecchi, 1995). Analysis of compound mutants of *Hox* paralogs, *Hoxb3* and *Hoxd3*, showed that although thymus development in *Hoxb3*$^{-/-}$ *Hoxd3*$^{-/-}$ mice was normal, *Hoxa3*$^{-/+}$*Hoxb3*$^{-/-}$*Hoxd3*$^{-/-}$ thymi failed to migrate normally thus *Hox* genes also play a role in thymus migration (Manley and Capecchi, 1998).

1.2.4.3 Pax1 and Pax 9

Following the onset of pharyngeal pouch development, several transcription factors control the continued development of the third pharyngeal pouch. The paired box (Pax) family of transcription factors plays important roles in embryonic development and organogenesis. Both *Pax1* and *Pax9* are expressed in an overlapping pattern in proliferating regions of the pharyngeal endoderm by E9.5 (Muller et al., 1996; Wallin et al., 1996). *Pax1* is expressed throughout the pharyngeal pouch endoderm at E10.5 before becoming distributed throughout the developing lobe by E12.5. Expression becomes further restricted to cortical epithelium by E14, and *Pax1* is detected in only a small cTEC subpopulation in the adult thymus (Wallin et al., 1996). *Pax1* is required for correct spinal column development and for normal thymus development. *Pax1*$^{-/-}$ mice exhibit mild to moderate thymus hypoplasia (Wallin et al., 1996). *Pax9*$^{-/-}$ mutants show a more severe phenotype having many developmental defects and lacking some

structures deriving from the third and fourth branchial pouches (Peters et al., 1998). The thymus of these mice fails to migrate caudoventrally and are ectopically located in the larynx (Hetzer-Egger et al., 2002). Despite an ability to recruit HPCs, the thymus is severely hypoplastic and does not support γδ T cell development (Hetzer-Egger et al., 2002). Taken together, these findings indicate that *Pax9* is able to instruct TEC in a Foxn1-independent manner to maintain thymic epithelial cell functionality. Both Pax1 and Pax9 are downregulated from E10.5 in *Hoxa3*$^{-/-}$ mice indicating that both operate downstream of Hoxa3.

1.2.4.4 Eya1 and Six1

Eya1 is a murine homologue of the Drosophila Eyes Absent gene (Bonini et al., 1993). The *Eya* genes are frequently involved in the regulation of genes involved in tissue growth. Six1 is a transcriptional regulator that is co-expressed with *Eya1* during organogenesis of a number of embryonic structures. Analysis of the mutant *Eya1* phenotype revealed that, in addition to general hypoplasia of the pharyngeal pouch endoderm, thymus organogenesis was terminated and no thymus was present at E12.5 (Xu et al., 2002). *Eya1* is expressed in pharyngeal endoderm, ectoderm and NCC between E9.5 and E10.5 and therefore interpretation and explanation of the phenotype is difficult (Xu et al., 2002). In *Eya1* null mice, thymic primordia are not formed and thymus-specific genes are not expressed. However, expression of *Hoxa3, Pax1* and *Pax9* in these mice is unchanged in the endoderm and NCC prior to outgrowth of the thymus indicating that Eya1 operates downstream or independently of these genes (Xu et al., 2002; Zou et al., 2006). Interestingly, *Six1* expression is markedly lower in the endoderm and ectoderm of the pharyngeal region in *Eya1* mutant mice, indicating that Six1 operates downstream of Eya1 (Xu et al., 2002). Although the specification of the third pharyngeal pouch is initiated in *Six1*$^{-/-}$ mice, failure to maintain previous *Gcm2* or *Foxn1* expression is followed by increased apoptosis and the resultant loss of the thymus and parathyroid primordia by E12.5 (Zou et al., 2006). Consistent with this observation, upon interaction with Eya1, Six1 is converted from a repressor to an

activator of transcription and is believed to play a role in the proliferation and survival of epithelia at early stages of development of other organs.

1.2.4.5 BMP4 and Shh

There is strong evidence to suggest that opposing gradients of Bone Morphogenetic Proteins (BMP) and Sonic Hedgehog (Shh) play a role in the specification of the thymus and parathyroid domains of the third pharyngeal pouch. *BMP4* is expressed by the ventral aspect of the third pharyngeal pouch at E10.5 (Moore-Scott and Manley, 2005). While *Shh* is expressed at the opposing dorsal end of the pouch. In the absence of Shh, the *BMP4* expression domain has been shown to extend dorsally and the *Foxn1* expression domain also extended in a similar manner (Moore-Scott and Manley, 2005), consistent with a role for *BMP4* as a positive regulator of *Foxn1* expression (Moore-Scott and Manley, 2005; Tsai et al., 2003). Noggin, a BMP inhibitor, is expressed reciprocally to *BMP4* in the prospective parathyroid domain at E10.5, suggesting a role in constraining the prospective thymus domain (Patel et al., 2006).

1.2.4.6 Foxn1

Foxn1 is a gene mutated in the classical mouse mutant nude, characterised by hair loss and athymia (Blackburn et al., 1996; Flanagan, 1966; Nehls et al., 1996; Nehls et al., 1994). Nude mice retain small cystic thymic rudiments, which are devoid of T cell precursors (Blackburn et al., 1996; Scheiff et al., 1978). Foxn1 is a transcription factor that plays a crucial role in the development of the thymic epithelium (Blackburn et al., 1996; Nehls et al., 1996). High levels of *Foxn1* expression can be detected from E11 by *in situ* hybridisation and in LacZ reporter strain analyses (Manley and Blackburn, 2003), although expression from E9.5 can be detected by RT-PCR (Balciunaite et al., 2002). By E17.5, *Foxn1* expression is restricted to specific tissues including the skin and the thymic primordia (Nehls et al., 1994). *Foxn1* expression within TEC becomes gradually restricted so that postnatal expression of Foxn1 is restricted to a subset of TEC (Itoi et al., 2007). In the skin, *Foxn1* expression is found in the epidermis and hair follicle, where it affects hair growth (Flanagan, 1966).

Foxn1 is not required for the initiation of thymus organogenesis (Nehls et al., 1996) but is subsequently absolutely required cell autonomously for the differentiation of all thymic epithelial subtypes after E12.5 (Blackburn et al., 1996; Itoi et al., 2001). The phenotype of nude cells within the thymic rudiment is heterogeneous with cells having morphological and expression profiles of various non-thymic tissues including respiratory and glandular epithelium and some retain markers of immature thymic epithelial cells (Dooley et al., 2005a; Dooley et al., 2005b). As a result, it has been suggested that in the thymus, Foxn1 plays a role in the maintenance of the TEC differentiation programme and limiting alternative fate choices. Recent confirmation of this came from experiments utilising a conditional *Foxn1* null mouse. When *Foxn1* was reactivated, individual TECs in the thymus of postnatal mutant mice were able to differentiate to produce both cortical and medullary TEC indicating that in the absence of Foxn1, TEC are maintained in a progenitor state (Bleul et al., 2006).

1.2.4.7 Wnts

The Wnt gene family members are secreted glycoproteins that have roles in intercellular signalling (Nusse and Varmus, 1992). Wnt glycoproteins are highly conserved between vertebrate species and are known to have important roles in development and organogenesis. Wnts are pivotal in lymphocyte development (van de Wetering et al., 2002) with Wnts 1 and 4 providing thymocyte proliferation and survival signals (Staal and Clevers, 2001; Staal et al., 2001). *Wnt* expression is observed in thymic tissue and Wnt receptors are specifically expressed in TEC of both the nude and wild-type fetal thymi and wild-type adult thymi (Balciunaite et al., 2002). Balciunate *et al* correlated the spatial and temporal patterns of embryonic *Wnt4* and *Wnt5b* expression with the onset of *Foxn1* expression and hypothesised a role for Wnt signalling in the positive regulation of *Foxn1*. This is supported by the observation that thymic epithelial cell lines that overexpress Wnt4 also demonstrate elevated levels of *Foxn1* expression (Balciunaite et al., 2002).

1.3 Intrathymic haematopoietic development

1.3.1 Postnatal T cell development

Intrathymic T cell development is characterised by the ordered progression of haematopoietic progenitor cells through phenotypically defined stages. These stages are supported by a phenotypically and spatially distinct thymic microenvironments, which provide cues that enable the commitment, differentiation and expansion of relatively few HPCs into a diverse T cell repertoire (Figure 1.2).

1.3.1.1 Intrathymic T cell differentiation

The programmed differentiation of bone marrow-derived haematopoietic precursors into mature T cells is a well characterised process that can be conceptually divided into three phases. Thus the most immature stage, characterised as double negative (DN) due to the absence of the co-receptors CD4 and CD8, gives rise to the more mature intermediate $CD4^+CD8^+$ double positive (DP), which then develop into either $CD4^+CD8^-$ or $CD4^-CD8^+$ single positives (SP) (Figure 1.2). Upon colonisation of the thymus, the DN cells have their T cell receptor (TCR) genes in the germline configuration and therefore they express no cell surface TCR molecules (Zuniga-Pflucker and Lenardo, 1996). The DN thymocytes can be further subdivided into 4 distinct subsets based on their expression of CD44 and CD25 as well as a range of other markers (Godfrey et al., 1993; Porritt et al., 2004)

The most immature $CD3^-CD4^-CD8^-$ triple negative (TN) subset, TN1, is characterised by CD44 and c-kit expression without CD25 co-expression (Godfrey et al., 1993; Ogawa et al., 1991). Cells within this compartment have the ability to differentiate into B cells, DCs, macrophages, natural killer (NK) cells as well as T cells (Antica et al., 1993; Ardavin et al., 1993; Balciunaite et al., 2005; Porritt et al., 2004). DN1 have recently been shown to be a heterogeneous population of cells that can be subdivided into 5 subpopulations using CD24 and c-kit (Porritt et al., 2004). These populations show varied expression of Thy-1 and CD127 (IL-7R) (Porritt et al., 2004). The subpopulations have been shown to have differing

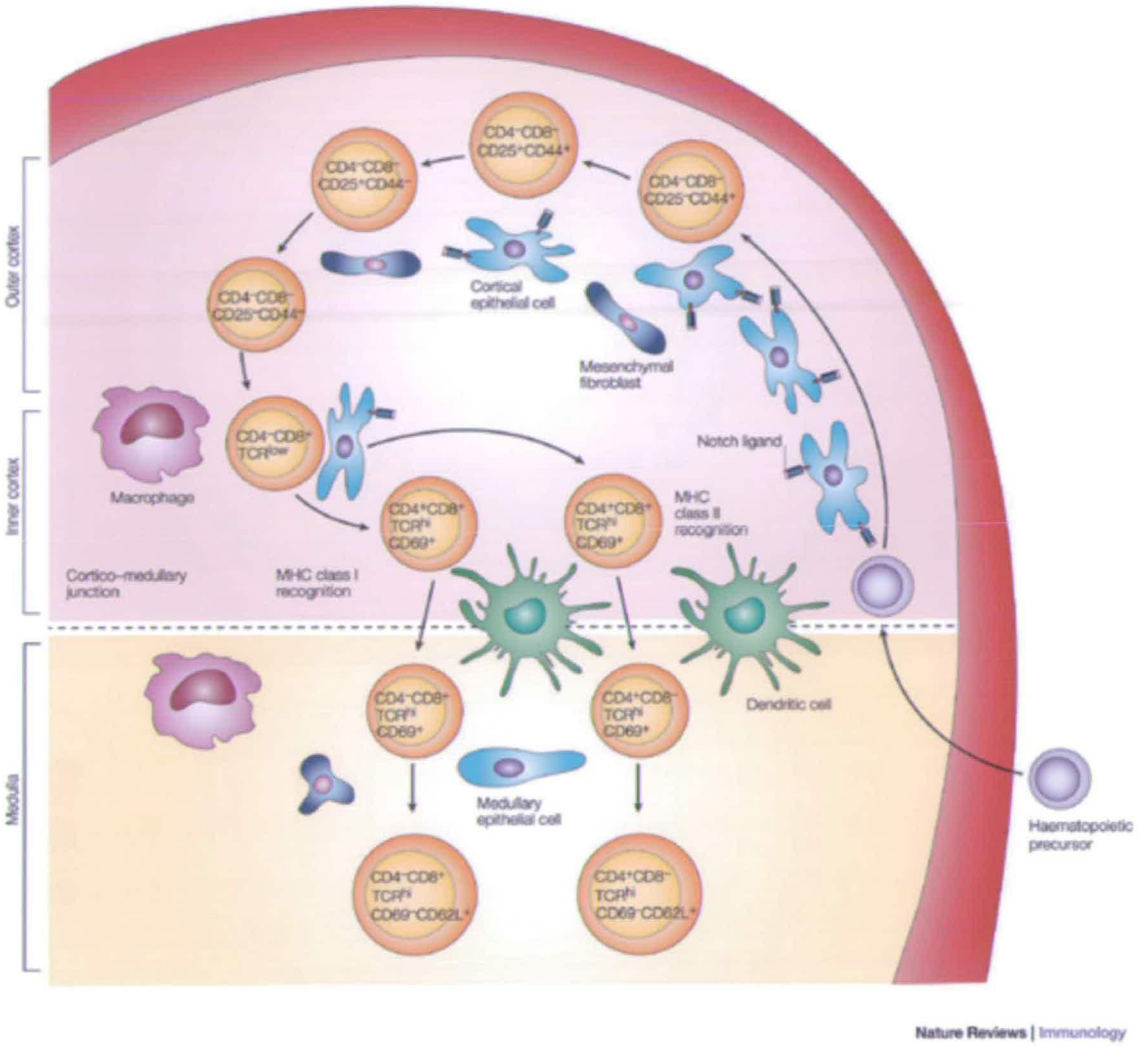

Figure 1.2 Intrathymic T cell differentiation

Thymocytes undergo a well characterised differentiation process that is supported by distinct thymic stromal microenvironments (Adapted from Zuniga-Pflucker, 2004).

lineage potentials as well as proliferative capacities, indicating that each subpopulation may represent a separate precursor (Porritt et al., 2004).

The progression from DN1 to DN2 is marked by the maintenance of CD44 and c-kit and the upregulation of both CD25 and Thy-1 (Godfrey et al., 1993). These phenotypic changes are not accompanied by a limiting of lineage potency, and DN2 cells can give rise to NK cells, DCs, macrophages and T cells (Balciunaite et al., 2005; Lee et al., 2001; Lucas et al., 1998; Schmitt et al., 2004a). At this stage cells undergo IL-7 and SCF-dependent proliferation, thereby increasing the pool of progenitors available for differentiation (Godfrey et al., 1992; Moore and Zlotnik, 1995; Penit et al., 1995).

Subsequent transition to the DN3 stage is accompanied by downregulation of both CD44 and c-kit expression and upregulation of CD24, as well as continued expression of CD25 and Thy-1 (Godfrey et al., 1993). It is during the DN3 stage that widespread rearrangement of TCRβ and TCRγ chains is initiated (Dudley et al., 1994; Godfrey et al., 1993; Hozumi et al., 1994; Mombaerts et al., 1992a; Shinkai et al., 1993). The production of functional TCRβ or TCRγ chain is required for the continued differentiation of thymocytes beyond the DN3 stage, thus thymocytes of recombinase activating gene (RAG)-deficient mice, which are unable to carry out V(D)J rearrangements, show a severe developmental block at DN3 (Kishi et al., 1991; Mombaerts et al., 1992b; Shinkai et al., 1993). Severe combined immunodeficiency (SCID) mice, which are unable to initiate TCRβ rearrangement also show a similar phenotype (Rothenberg et al., 1993). Interestingly, the onset of TCR rearrangements is not immediately initiated by the expression of either RAG1 or RAG2 as these are readily detectable in DN1 and DN2 thymocytes (Wilson et al., 1994).

In αβ T cell precursors, the pre-TCR complex is formed when the product of successful TCRβ rearrangement pairs with the pre-Tα chain, a transmembrane glycoprotein that shares homology with TCR chains (Saint-Ruf et al., 1994) (Groettrup et al., 1993). The formation and expression of the pre-TCR is an important checkpoint in αβ T cell differentiation, termed β selection (Godfrey and Zlotnik, 1993; Groettrup and von

Boehmer, 1993) and acts as a mediator for the allelic exclusion of the second TCRβ allele (Aifantis et al., 1997; Uematsu et al., 1988). While complexed with both CD3 subunits and $p56^{lck}$, the pre-TCR mediates the β-selection process, enabling the transduction of a selective signal that permits the proliferation and further differentiation of the developing thymocyte (Fehling et al., 1995; Godfrey et al., 1993; Levelt et al., 1995; Penit et al., 1995). Thus the disruption of pre-TCR formation via the ablation of either pre-Tα, CD3 or $p56^{lck}$, leads to a developmental arrest between the DN3 and DN4 stages (Groettrup and von Boehmer, 1993; Malissen et al., 1995).

That TCRγδ thymocytes differentiation in the thymi of pre-Tα deficient indicates that the pre-TCR does not play a role in γδ T cell differentiation mice (Fehling et al., 1995). Although the rearrangement of the β locus is critical in the development of αβ T cells, it is clear that the complete rearrangement of the TCRβ locus does not preclude development along the γδ lineage as DN4 cells retain the capacity to generate both αβ and γδ T cells upon intrathymic transfer, despite pre-TCR expression (Petrie et al., 1992). Furthermore, γδ T cells with in-frame TCRβ rearrangements are common (Burtrum et al., 1996). The successful rearrangement of the TCRγ and TCRδ genes ensures that DN cells commit to the γδ lineage (Burtrum et al., 1996).

The progression beyond β-selection to the DN4 stage is accompanied by loss of CD25 expression ($CD44^{-}CD25^{-}$) as well as increased pre-Tα expression and the onset of TCRα gene rearrangement (Godfrey et al., 1993; von Boehmer et al., 1999). The successful rearrangement of the TCRα gene produces a TCR chain that has a greater affinity for the TCR chain already being expressed than the Pre-Tα chain (Trop et al., 2000). This competition permits the formation of TCRαβ heterodimers (Trop et al., 2000). Moreover, expression of the pre-Tα is terminated ensuring that the TCRαβ and pre-TCR complexes are never co-expressed (Trop et al., 2000). In contrast to the allelic exclusion event following TCRβ rearrangement, the successful rearrangement of one TCRα allele does not preclude further TCRα rearrangement (Malissen et al., 1988; Marolleau et al., 1988). Only cells that express a TCRα chain that is able to form a αβ

heterodimer that recognises a self peptide-MHC complex will terminate recombination (Borgulya et al., 1992; Brandle et al., 1992). Cells at this stage begin to express both CD4 and CD8 at the cell surface, marking the transition from the DN stage to the $CD4^+CD8^+$ double positive (DP) stage of development.

The process of positive selection ensures that only thymocytes whose αβ TCRs have a propensity to interact with self peptide-MHC complexes are able to survive. Most DPs either non-productively rearrange their TCR chains or express αβ TCRs that do not react to self-MHC ligands present on the cortical stroma with sufficient affinity to generate the intracellular signals required to sustain viability and it is estimated that greater than 95% of DPs die as a result (Benoist and Mathis, 1989; Egerton et al., 1990). Those cells that are able to interact with self peptide-MHC ligands do not die by neglect and are instead selected for further maturation by the multi-step process of positive selection. As a result of positive selection cells terminate RAG1 and RAG2 activity, thus preventing further rearrangements of the TCR locus (Borgulya et al., 1992; Brandle et al., 1992). In addition, they increase cell-surface TCR expression and elevate Bcl-2 protein levels, protecting the cells from programmed cell death (Linette et al., 1994; Veis et al., 1993). It is at the TCR^{hi} DP stage that early markers of activation such as CD69, CD44 and MHC Class I are expressed or upregulated (Bendelac et al., 1992) (Swat et al., 1993) and CD24 is downregulated (Bendelac et al., 1992; Scollay et al., 1984). $CD4^+CD8^+CD69^+$ cells represent a population of cells that are actively undergoing positive selection, as upon completion of positive selection, cells downregulate either CD4 or CD8 as well as CD69 and no longer require continued MHC-peptide interaction (Hare et al., 1999). CD4 and CD8 bind non-polymorphic regions of MHC Class II and MHC Class I respectively (Doyle and Strominger, 1987; Norment et al., 1988), and cells that bear a MHC Class I restricted αβ TCR retain CD8 expression (Teh et al., 1988), where as those bearing a MHC Class II restricted αβTCR continue to express CD4 (Kaye et al., 1989).

The SP subsets leave the cortex so that cells undergoing negative selection are mostly SPs that reside either at the CMJ or in the medulla (Surh and Sprent, 1994). Negative selection ensures that most cells with strong affinity for self-peptides presented by the thymic stroma are deleted from the emerging repertoire (Bevan, 1997). In this way, autoreactive clones are deleted to leave a T cell repertoire biased towards self-MHC recognition but that shows minimal self-reactivity. To ensure tolerance to self antigens, other mechanisms also limit the activation and proliferation of autoreactive clones that have escaped negative selection, in the periphery. The induction of clonal anergy and the activity of regulatory T cells (T_{reg}) both serve to limit the destructive nature of autoreactive clones that have escaped negative selection (Maloy and Powrie, 2001; Sakaguchi et al., 1995; Schwartz, 1990).

1.3.2 Thymocyte progenitors and lineage commitment

1.3.2.1 Seeding of the postnatal thymus

HPCs enter the postnatal thymus via a process of extravasation at the cortico-medullary junction (Kyewski, 1987; Lind et al., 2001; Ushiki, 1986). This process is likely follow other leukocyte extravasation models and involves three steps: leukocyte rolling, characterised by a loose attachment of the leukocyte to the endothelium; firm adhesion, whereby integrins on the surface of rolling leukocytes form a strong attachment to their receptors expressed on the surface of endothelial cells; and finally transendothelial migration, involving a multitude of cellular adhesion molecules and factors that enable the leukocyte to repeatedly attach and reattach itself to endothelial cell membranes and finally attach itself to the ECM of the thymus (Petrie, 2003).

The ingress of HPC to the thymus is a non-continuous process with the thymus demonstrating periods of receptivity interspersed with longer periods of refractivity (Foss et al., 2001; Jotereau and Le Douarin, 1982). The observed competition for stromal niches required to mediate the DN stages of development is likely to be a limiting factor that moderates the number of imported HPCs and ultimately means that the size of the thymus remains constant and does not fluctuate with the cyclical seeding

(Foss et al., 2001). It has been proposed that the bone marrow periodically releases HPCs that are able to home to the thymus in response to the requirement for renewed thymic seeding (Donskoy et al., 2003). The expression of P-selectin on thymic endothelium, and the reciprocal expression of P-selectin ligands such as PSGL-1 on both intrathymic HPCs and circulating Lineage$^-$Sca-1$^+$c-kit$^+$ (LSK) cells, is consistent with a role in HPC ingress (Rossi et al., 2005). *PSGL-1* deficient mice show reduced numbers of c-kit$^+$ TN1 cells despite the thymus being receptive to wild-type cell influx in transplantation experiments indicative of a homing defect (Rossi et al., 2005). Furthermore, upregulation of P-selectin in thymi with stromal niche availability is consistent with a specific role for P-selectin as a mediator for the gated importation of HPCs (Rossi et al., 2005).

Although the mechanisms of HPC homing to the thymus are poorly understood it is likely that chemokines play a pivotal role. Several chemokines are expressed in the thymus including lymphotactin, CCL12 (SDF-1), CCL17 (TARC), CCL19 (MIP3β), CCL21 (SLC) and CCL25 (TECK) (Takahama, 2006). That the seeding of fetal lobes *in vitro* is mediated by soluble, diffusible factors via a G-protein coupled signal, strongly supports this notion (Jotereau et al., 1980; Wilkinson et al., 1999). Studies using mice deficient for CCR9 (the receptor for CCL25), showed that although CCR9-deficient BM is able to seed the thymus and produce normal T cell subsets in a non-competitive situation (Wurbel et al., 2001), CCR9-deficient BM is out-competed by wildtype BM cells in competitive reconstitution experiments (Uehara et al., 2002). Using anti-CCL25 antibodies in blockade experiments did not prevent cells from seeding irradiated lobes *in vitro* (Wilkinson et al., 1999). These studies establish a likely role for CCL25 in prothymocyte recruitment to the thymus although the lack of a severe phenotype indicates the presence of functional redundancy and suggests that the recruitment process is mediated by multiple chemokines.

1.3.2.2 HPCs

There has been much controversy surrounding the phenotype and functional characteristics of the cells that seed the postnatal thymus (Bhandoola and Sambandam, 2006; Petrie and Kincade, 2005). What is known is that commitment to the T cell lineage requires Notch signalling mediated by Notch ligands, and that these are expressed in both the thymic microenvironment and by the haematopoietic and stromal cells of bone marrow (Felli et al., 1999; Singh et al., 2000; Tsukamoto et al., 2005). Furthermore, there are inherent difficulties in determining the functional capacity of thymus seeding progenitors (TSP) as contact with the thymic microenvironment during the seeding process is likely to have immediate effects on phenotype and function. Thus it remains a source of debate whether TSPs show commitment to the T cell lineage prior to or as a result of exposure to the thymic microenvironment.

There is general agreement that cells within the postnatal TN1 fraction, can give rise to T cells, B cells, NK cells, DCs and myeloid lineage cells (Allman et al., 2003; Wu et al., 1991). As previously discussed, the TN1 compartment is heterogeneous and contains cells that exhibit differentiation and proliferation kinetics expected of a canonical T cell precursor (Bhandoola et al., 2003; Porritt et al., 2004). In addition, cells that can home to the thymus and show both T and B-linage potential do exist in the postnatal DN1 fraction but do not show strong proliferation or normal developmental kinetics (Porritt et al., 2004). Thus, it remains possible that the thymus is seeded by several progenitor cell types that have different phenotypes and functional properties.

The LSK population identified in the bone marrow contains the haematopoietic stem cell (HSC) population and these cells are known to circulate in the bloodstream (Wright et al., 2001). Despite this, few people believe that these cells are the TSP and instead focus on downstream progenitor populations isolated from the bone marrow, blood or thymus, which show skewed potential to generate T cells.

A population of $Lin^{-}IL\text{-}7R^{+}Thy\text{-}1^{low}Sca\text{-}1^{low}c\text{-}kit^{low}$ cells were purified from adult bone marrow and were able to give rise to T cells, B cells, NK cells and DCs but not myeloid

cells upon *in vivo* transfer (Kondo et al., 1997). This population corresponded to a common lymphoid progenitor (CLP) population, indicating that the long-hypothesised bifurcation between the lymphoid and myeloid lineages existed (Kondo et al., 1997). However, despite this increased propensity to give rise to lymphoid cells, these could be induced to differentiate along the myeloid lineage under appropriate conditions (Kondo et al., 2000) highlighting the importance of distinguishing between artificially induced lineage choices and those that are actually made *in vivo*. These results still supported the view that cells exist *in vivo* that preferentially give rise to lymphoid cells and that cells homing to the thymus may already have undergone some lineage restriction or fate-skewing process. Several studies attempting to find analogous cells in the thymus and blood failed to do so, raising the question of whether the CLP homes to the thymus under normal conditions to act as a T cell progenitor (Allman et al., 2003; Bhandoola et al., 2003). The discovery of a population of cells, which was common to both the thymus and the bone marrow and had a propensity to generate T cells and B cells, appeared to satisfy the criteria of a lymphoid-biased progenitor that could originate in the BM and home to the thymus (Gounari et al., 2002; Martin et al., 2003). As it appeared downstream of the CLP, with an IL-7R^{+}B220^{+}c-kit^{low} phenotype, it was termed the CLP2 (Gounari et al., 2002; Martin et al., 2003).

In addition to the BM CLP, other BM populations are also candidates for TSPs. A RAG1^{+}Lin-Sca-1+ c-kit^{+} Flt-3^{+}IL-7Rα^{-} cell with minimal myeloid potential and preferential differentiation towards the lymphoid lineage was identified in BM by Kincade and colleagues using GFP expressed under the RAG1 locus. These cells, known as early lymphoid progenitors (ELP) are considered less differentiated than the CLP population as RAG1 locus is active before IL-7Rα, used to recognise the CLP, is expressed.

1.3.3 Fetal T cell differentiation

As previously discussed, HPCs can first be detected in the mesenchymal capsule of the thymic anlage at E11 (Itoi et al., 2001). Between E11 and E12, these traverse into the

epithelial core of the lobe with the migration coinciding with, but not triggering, phenotypic changes in the epithelial structure so that epithelial cells form a clustered structure (Douagi et al., 2000; Itoi et al., 2001). Currently, the regulation of the influx of precursors into the fetal thymus is poorly understood, although transfilter migration assays have indicated a role for soluble, diffusible chemoattractant factors produced by thymic epithelial cells (Fontaine-Perus et al., 1981; Jotereau et al., 1980; Wilkinson et al., 1999). Several chemokines are expressed in the fetal thymus, including MIP1-γ, RANTES, MCP-1 and SDF-1 (Bleul and Boehm, 2000; Liu et al., 2006; Wilkinson et al., 1999). However, their role as chemoattractants for thymic precursors is uncertain as these are also detectable in non-attracting tissues (Bleul and Boehm, 2000; Liu et al., 2006; Wilkinson et al., 1999). In contrast, the level of CCL25 expressed by fetal TECs is greatly increased over those of non-attracting tissues and CCL25 is able to mediate the chemotaxis of isolated E14 precursors *in vitro* (Bleul and Boehm, 2000; Wilkinson et al., 1999). Interestingly, both CCL25 and SDF-1 expression is lacking in the nude mouse thymus and in these mice prothymocyte entry into the fetal thymus does not occur, although localisation to the CCL21-expressing parathyroid proceeds normally (Bleul and Boehm, 2000). The use of neutralising antibodies to block the action of CCL25 or CCL21 in fetal thymus colonisation experiments showed markedly reduced colonisation efficiency, whereas those against SDF-1 showed no reduction (Liu et al., 2005). Taken together, these studies show that CCL25 is a candidate agent for the chemoattraction of HPC into the fetal thymus prior to vascularisation although some degree of functional redundancy is likely to operate.

Until E16, thymocytes cells appear DN with most cells exhibiting a $CD3^-CD2^-Lin^-c\text{-}kit^+$ phenotype. By E17, most cells have reached the DP stage and these rapidly mature so that both CD4 SP and CD8 SP populations are present prior to birth (Antica et al., 1993). In addition to αβ T cells development, the fetal thymus generates γδ T cells, with the first cells to express γδ appearing by E16 (Pennington et al., 2005). Unlike αβ T cells, γδ T cells are produced in waves that populate specific tissues. The first wave that appears at E15 expresses invariant TCR chains and populates the skin as dendritic

epidermal T cells (DETC). Subsequently, fetally produced γδ T cell cohorts populate the tongue, the reproductive tract, and the intestines (Allison et al., 1991; Havran et al., 1991). Functionally, this represents an inherent difference between fetal and postnatal HPCs as the TCR restriction is cell intrinsic and is not seen in cells developing from postnatal HPCs.

In addition to differences in differentiation potential, fetal and postnatal thymocytes have different requirements for their survival and differentiation. In *Ikaros* null mice, fetal thymocyte development is absent where postnatal development is evident despite some abnormalities (Wang et al., 1996). In contrast, despite the profound block at the DN stage in postnatal *IL-7Rα* null mice, fetal thymocytes in these mice are able to express CD25, express RAG proteins and produce neonatal SPs (Crompton et al., 1998).

1.3.4 The role of the thymic stroma in thymocyte differentiation

The T cell differentiation programme consists of several waves of regulated gene expression. The resultant changes in cell-cell signalling receptivity and cytokine responsiveness sequentially mediate the downregulation of non-T lineage genes, the upregulation of T-lineage associated genes and the commitment to the T cell lineage, as well as later events involved in T cell differentiation. Inherent in this is the survival and proliferation of cells that have successfully passed the checkpoints that mediate the production of a suitable T cell repertoire.

The intrathymic differentiation of T cells from HPCs is critically dependent upon interactions with the thymic stroma. The ability of the thymus to support the differentiation of T cells has been shown *in vitro* to be dependent upon the presence of MHC Class II^+ epithelial cells (Anderson et al., 1993). The thymic epithelium exerts its functional capacity via several mechanisms including cell-cell signalling and the provision of soluble factors.

1.3.4.1 Intrathymic thymocyte migration

The complexity of the heterogeneous thymic epithelium and the compartmentalisation of the thymus structure provides spatially distinct microenvironments that support the various stages of thymocyte development (Figure 1.1; Figure 1.2). The ordered migration of thymocytes within the thymus permits thymocytes at distinct stages of development access to specific niches that are likely to control differentiation events. Chemokines play an important role in the control of intrathymic migration events and cells at different stages of development have been shown by a number of mutant mouse models to differentially respond to several TEC-expressed chemotactic factors. In response to SDF-1, a factor expressed by cortical TEC, early thymocytes expressing the SDF-1 ligand, CXCR4, display a migratory response *in vitro* (Plotkin et al., 2003). In *CXCR4*$^{-/-}$ mice, early thymocytes fail to efficiently colonise cortical areas and demonstrate a block in development at TN1 (Plotkin et al., 2003). In addition, to CXCR4, CCR7 has also been implicated as a factor controlling the intrathymic migration of early thymocytes to the outer cortex (Misslitz et al., 2004). CCR7 expression in TN thymocytes is limited to the TN1-TN2 population and these cells accumulate at the CMJ in mice lacking CCR7 (Misslitz et al., 2004). Paradoxically, CCR7 expressing SP cells and a proportion of DP cells has been implicated in thymocyte migration towards the medullary region (Ueno et al., 2004). Further, overexpression of CCR7 in thymocytes led to the accumulation of DPs in the medulla (Ueno et al., 2004). That CCR7 ligand expression is concentrated in the medulla as well as at the SCZ supports the view that CCR7 plays roles in both the outward migration of immature thymocytes and the inward migration of more mature thymocytes (Misslitz et al., 2004; Ueno et al., 2004).

In the thymus, CCR9 expression is limited to cortical DP and early SP thymocytes and is not found on late medullary SPs, TECK expression is limited to the cortex (Bleul and Boehm, 2000). Consistent with a role in cortical thymocyte migration, the CCR9 ligand, TECK is reciprocally expressed in the cTEC and macrophages of the newborn thymus (Bleul and Boehm, 2000). Post-selection thymocytes expressing CCR4 have been

shown to migrate in response *in vitro* to CCR4 ligands, TARC and MDC. *In vivo*, both TARC and MDC show medullary-restricted expression, indicating that in addition to CCR7 ligands, CCR4 ligands also play a role in cortex to medulla migration (Bleul and Boehm, 2000; Campbell et al., 1999; Suzuki et al., 1999).

The data presented above indicate that differential expression of chemokines expressed by the thymic epithelium ensures that thymocytes expressing chemokine receptors follow an ordered migratory pathway. The complete dissection of the actual role played by each chemokine-ligand pairing in the thymus is made difficult by the functional redundancy that appears to operate in the system. This is reflected in the normal lymphopoiesis and thymocyte migration observed in the thymus of mutant mice that lack CCR9 (Uehara et al., 2002).

1.3.4.2 Major histocompatibility complex

The development of a self-tolerant T cell repertoire is heavily reliant upon interactions with MHC Class I and II complexes in the thymus. The MHC is a polymorphic gene complex, whose products are known to be associated with cell-cell recognition and discrimination. MHC Class I and II molecules are both membrane-bound glycoproteins, which present antigen to cells of the immune system. MHC Class I is expressed on somatic cells at varying levels, and functions to present processed peptides to $CD8^+$ T cells, often referred to as cytotoxic T cells. $CD4^+$ restricted T cells, referred to as helper T cells, are responsive to peptides presented by MHC Class II molecules expressed on specialist antigen presenting cells such as macrophages, dendritic cells and B cells as well as subtypes of the thymic epithelium.

All TECs express MHC I at high density (Ritter and Palmer, 1999). MHC Class II shows differential expression levels between different stromal cell types. Cortical epithelium has high level MHC Class II expression, as does a subpopulation of medullary epithelium. In addition, 50-70% thymic macrophages and all dendritic cells also show strong MHC Class II expression, while all express MHC Class I (Surh et al., 1992b; Van Ewijk et al., 1980).

1.3.4.3 Notch signalling in T cell differentiation

The Notch family of proteins regulates cell fate decisions during fetal and adult development. The thymic epithelium expresses several Notch ligands including Jagged-1, Jagged-2 and delta-like 1 (Dll-1) and delta-like 4 (Dll-4) (Felli et al., 1999; Harman et al., 2003; Tsukamoto et al., 2005). Evidence that Notch-1 plays a critical role in thymopoiesis comes from experiments on mice with induced loss of Notch1 (*iNotch*$^{-/-}$). Deletion of *Notch1* using cre-recombinase under an IFN-α responsive element resulted in a block at the TN1 stage of development with a coincident increase in B cells in the thymus (Radtke et al., 1999; Wilson et al., 2001). Reciprocal experiments utilising an active form of Notch, the intracellular Notch domain (ICN) to act as a signal transducer, demonstrated that Notch can commit Lin$^-$c-kit$^+$ cells to the T cell lineage in conditions that would normally induce B cell differentiation (Hozumi et al., 2003). Similarly, DP cells develop in transplanted BM transduced with ICN, with a simultaneous block in B cell development (Pui et al., 1999).

Deletion of *Notch1* in thymocytes prior to pre-TCR expression using cre-recombinase driven by the *Lck* proximal promoter, severely restricts the development of αβ but not γδ T cells by impairing *VDJβ TCR* rearrangements (Wolfer et al., 2002). These cells, which would ordinarily undergo apoptosis as a result of lack of Pre-TCR signalling, instead bypass this checkpoint, a result that would ordinarily lead to the production of an inappropriate repertoire. Controversially, Notch1 signalling has also been implicated in subsequent lineage decisions such as those deciding whether cells adopt an αβ or γδ fate, and also the CD4 and CD8 fate decision (reviewed in Radtke *et al* 2002).

Collectively, these studies suggest that Notch1 signalling is an essential actor for the T cell versus B cell fate decision with the ability to induce T cell differentiation in uncommitted progenitors. This fact has been exploited in the production of the OP9-Dll-1 and OP9-Dll-4 cell lines, which have proved a useful tool for analysis of T cell development (Hozumi et al., 2004; Porritt et al., 2004; Schmitt and Zuniga-Pflucker, 2002).

1.3.4.4 Soluble factors in T cell development

Several cytokines are involved in the proliferation of early thymocytes with SCF, IL-7 and Flt-3-ligand all being secreted by the thymic epithelium (Moore et al., 1993). The SCF receptor, c-kit is expressed on both DN1 and DN2 thymocytes but is downregulated by DN3 (Godfrey et al., 1993; Lind et al.). C-kit is a tyrosine kinase with an essential role in the proliferation of early thymocytes (Rodewald et al., 1995). IL-7Rα is similarly expressed on subpopulations of DN1 and DN2 thymocytes (Moore et al., 1993; Porritt et al., 2003). Whilst cell culture experiments indicate that many factors are required to maintain TN1 cells, IL-7 signalling is sufficient to maintain TN2 cells (Moore and Zlotnik, 1995). IL-7R signalling promotes rearrangement of TCRγ locus (Durum et al., 1998) (Appasamy, 1992) and in mice lacking the IL-7Rα chain, γδ thymocytes are absent (Maki et al., 1996). *IL-7*$^{-/-}$ mice and *IL-7Rα*$^{-/-}$ mice show severely reduced thymic and peripheral lymphocyte numbers (Peschon et al., 1994; von Freeden-Jeffry et al., 1995). Interestingly, in contrast to the severe developmental arrest seen in the postnatal thymus of *IL-7Rα* null mice, fetal thymopoiesis proceeds normally, although total numbers remain reduced indicating that the IL-7Rα chain is not essential for fetal thymopoiesis (Crompton et al., 1998). IL-7 signalling retains a role in later differentiation and is responsible for post-positive selection thymocyte expansion (Hare et al., 2000). Other factors that have been implicated in the differentiation of the earliest thymocyte subsets such TNFα and IL-1α (Zuniga-Pflucker et al., 1995) are also expressed by TEC (Moore et al., 1993).

1.3.4.5 Cortical epithelial cells

In addition to providing factors for the commitment and early differentiation of thymocytes, cTECs have been implicated in the positive selection of immature thymocytes. Mice in which MHC Class II expression was limited to different stromal compartments illustrated that only when MHC Class II was expressed on the cortical epithelium, were normal numbers of intrathymic and peripheral $CD4^+$ T cell populations found (Benoist and Mathis, 1989; Cosgrove et al., 1992). This is consistent with reports of mice with transgenic restricted TCR expression, which demonstrate that

when the selecting MHC-peptide complex is expressed on cTEC, positive selection is observed (Berg et al., 1989). These data indicate that cTECs can drive positive selection efficiently using MHC-peptide complexes as a ligand for the thymocyte-expressed TCR. It is likely that some of this activity is dependent upon the provision of co-stimulatory accessory signals. Some studies have cast doubt on the unique ability of cTEC to mediate positive selection. The demonstration that intrathymic injection of fibroblasts bearing either MHC Class I or MHC Class II into the thymi of MHC deficient mice could rescue CD8 and CD4 SP compartments, respectively suggests that any cell expressing an MHC-peptide complex can mediate positive selection events (Hugo et al., 1993) (Pawlowski et al., 1993). However, these studies do not exclude the possibility that the endogenous epithelial cells provide accessory co-stimulatory signals to developing thymocytes during positive selection.

The nature of the signals provided by the cTEC are yet to be elucidated. Notch signalling has been suggested to occur during positive selection as the transition from DP to SP phenotype is accompanied by the expression of downstream regulators of Notch including Deltex and Hes-1 (Deftos et al., 1998). Despite this, Notch1 deletion in thymocytes from DN3 onwards showed that the absence of Notch1 signalling during positive selection did not impede the DP generation or selection events (Wolfer et al., 2001).

1.3.4.6 Medullary epithelium

Following positive selection in the cortex, newly generated SP cells migrate across the CMJ to the medullary region. The most efficient mediators of clonal deletion in the thymus are thymic DCs, which are concentrated at the CMJ (Anderson et al., 1998a; Barclay and Mayrhofer, 1981; Barclay and Mayrhofer, 1982). However, several lines of evidence have suggested that the medullary epithelium is essential for the avoidance of autoimmune phenotypes resulting from inefficient negative selection and the breakdown of central tolerance. Several studies have demonstrated that murine and human mTECs express a wide range of tissue specific antigens as well as other proteins

such as α-fetoprotein that are temporally regulated (Gotter et al., 2004; Kyewski et al., 2002). Following the introduction of liver-specific human *C-reactive protein* (*hCRP*) gene and its regulatory elements into mice, *hCRP* was detected in both the liver and mTEC (Klein et al., 1998). When these mice were crossed with others that had TCRs specific for CRP, tolerance was induced, providing strong evidence for the direct involvement in the induction of tolerance to medullary expressed antigens (Klein et al., 1998).

Work investigating the role of the transcription factor, Autoimmune regulator (AIRE), analysed the medullary expression profile of thymic cells taken from mice deficient in AIRE and noted a reduction in the expression of peripheral tissue-restricted genes (Anderson et al., 2002). The *AIRE* knockout animals used in this study exhibited autoimmune diseases, which were attributed to the loss of AIRE activity indicating that that AIRE plays an important role in the promotion of ectopic expression of TSAs. The AIRE-associated disease spectrum parallels with that which is seen in humans known to possess a defective form of AIRE (Anderson et al., 2002; Liston et al., 2003).

In addition to the enforcement of central tolerance, mTECs support post-selection thymocyte maturation. Medullary thymocytes have been shown to reside in the medulla for upto two weeks, during which time several markers of late maturation are upregulated (Gabor et al., 1997; Ge and Chen, 1999). During this time, SPs undergo TEC-dependent proliferation to increase the size of the post-selection pool prior to export to the periphery (Hare et al., 2000; Hare et al., 1998).

1.3.5 Molecular regulation of intrathymic T cell differentiation

During T cell differentiation, several transcriptional regulators have been identified which either regulate T cell specific genes or repress alternative lineage genes, thus mediating commitment. Ikaros is a zinc finger transcription factor that plays an important role in the development of many haematopoietic lineages (Nichogiannopoulou et al., 1998). *Ikaros* null mice lack fetal T cell development although postnatally some T cell development is seen, which generally results in T cell

leukaemia (Wang et al., 1996). A more severe phenotype is seen in mice expressing a dominant negative form of Ikaros, which show a complete early block in all lymphoid differentiation as well as defects in the haematopoietic stem cell compartment (Nichogiannopoulou et al., 1999). Taken together, these studies indicate that Ikaros plays an important role in the regulation of lineage branch points of haematopoietic lineages from the HSC onwards, and exerts an effect on the T cell lineage at various time points. Furthermore, Ikaros plays a vital role as a tumour suppression agent in the postnatally produced T cell compartment.

GATA-3 is another zinc-finger transcription factor expressed in thymocyte and mature T cell populations. In chimaeric experiments, *GATA3*$^{-/-}$ cells are unable to contribute to even the earliest thymocyte populations indicating that it is an essential early regulator of the T cell lineage (Ting et al., 1996). Upregulation of *GATA-3* expression in DP thymocytes is triggered proportionally to the strength of TCR stimulation signal and is ultimately expressed more highly in CD4 SPs than CD8 SPs (Hernandez-Hoyos et al., 2003). Overexpression of *GATA-3* in DP thymocytes inhibits CD8 SP development (Hernandez-Hoyos et al., 2003) whereas interference resulting in reduced GATA-3 expression limits CD4 SP development (Pai et al., 2003) illustrating that in addition to being an early positive regulator, GATA-3 is indispensable for the later stages of $CD4^+$ T cell development.

PU.1 is a haematopoietic cell specific transcription factor expressed in HSCs and the earliest thymocyte compartments. Maintenance of high levels of PU.1 expression drives cells towards the myeloid lineage, with lower levels favouring B cell development (DeKoter and Singh, 2000). PU.1 levels diminish as T cell development progresses and it is possible that this reduction is an important shield against development along inappropriate lineages, which might otherwise be mediated by PU.1 in the thymus (Anderson, 2006; Dionne et al., 2005).

Mediators of thymocyte proliferation and cell cycle status are vital to ensure that only appropriately selected thymocyte populations are able to expand whilst others, which

fail checkpoint controls, are unable to continue cycling and are susceptible to apoptosis. E box binding factors, also known as basic helix-loop-helix (bHLH) factors, such as E2A, HEB and ITF-1 and their inhibitors Id-2 and Id-3 are critical for the control of cell cycle entry and exit that is vital for the controlled differentiation of thymocytes. Generally speaking, interplay between bHLH factors, which inhibit entry into G1 and promote differentiation, and the Id proteins that mediate entry into the cell cycle thus inhibiting further differentiation, accompanies several thymocyte differentiation checkpoints (Bain et al., 2001; Engel et al., 2001; Morrow et al., 1999; Peverali et al., 1994). In this way, the E box binding factors and their Id protein inhibitors exert cell cycle control over many of the processes that govern T cell differentiation and ensure the generation of a large, appropriate, and self-tolerant repertoire.

1.4 In vitro *models of thymocyte development*

The differentiation of cells towards a more restricted cell fate is a complex process involving an interplay of various environments and cell intrinsic factors. The ability to control the environment that differentiating cells occupy permits the dissection of these processes and this requires the dissociation of the system from influences that cannot be regulated or altered. Therefore, development of an *in vitro* organ system, which can be easily controlled and manipulated is an attractive goal.

1.4.1 Organ culture systems

Early attempts to differentiate T cells *in vitro* were made using *ex vivo* fetal thymic lobes (Ceredig et al., 1982; Kamarck and Gottlieb, 1977). As with other *ex vivo* culture systems, fetal thymic organ culture (FTOC) permits study of T cell precursor differentiation and allows macro-environmental conditions to be controlled. Organ cultures are routinely used in the assessment of T cell competency of haematopoietic progenitors in multipotency assays where they have been pre-treated with deoxyguanosine to eliminate haematopoietic cells before being reseeded with haematopoietic progenitors (Kawamoto et al., 1997).

Stromal elements from dissociated lobes have been found to reaggregate in culture to produce an organoid capable of T cell differentiation (Anderson et al., 1993). Reaggregate fetal thymic organ culture (RFTOC) has been effectively used to elucidate the cellular requirements for thymocyte differentiation as well as providing a model for studying positive selection (Anderson et al., 1993; Anderson et al., 1994). However, the reliance upon fresh fetal tissue and the limitations that this places upon the scale of experiments is a drawback with both FTOC and RFTOC.

1.4.2 Scaffold cultures

Biocompatible materials have been successfully used to provide mechanical support and niche availability with the three-dimensional structure acting as an artificial matrix offering stability and spatial organisation to the cells, which they harbour. The belief that the open-meshwork of the thymic reticulum was of great importance in the differentiation of T cells, made the use of scaffolds a good candidate for improving *in vitro* T cell differentiation cultures, with experiments being undertaken to establish the optimal pore size for the maturation of T cell precursors (Poznansky et al., 2000). Experiments in which a carbon coated tantalum matrix supporting a co-culture of a murine thymic cell line with human haematopoietic progenitor cells showed that in principle, artificial matrices may be of used to enhance *the vitro* production of T cells (Poznansky et al., 2000). Recently, another group has used a similar protocol to support the differentiation of HPCs on a matrix seeded with human skin cells and keratinocytes, with resultant cells showing a diverse repertoire with evidence of self-tolerance (Clark et al., 2005). Although potentially useful, it remains unclear whether these matrices form a three-dimensional cell structure or are elaborate monolayer cultures due to imaging limitations.

1.4.3 Monolayer cultures

The *in vitro* development of a simple culture system capable of supporting the differentiation of lymphocytes has proved more difficult than the production of other haematopoietic lineages in simple cultures although a number of BM-derived stromal

cell lines have been shown to support the full differentiation of other non-T lineage lymphocyte populations (Kodama et al., 1994; Williams et al., 1999). One such line, OP-9, is particularly suited for the differentiation of lymphocytes because its derivation from a macrophage colony-stimulating factor (M-CSF) deficient mouse (op/op) means that this cell line does not support the extensive monocyte expansion typically seen on other BM cell lines (Kodama et al., 1994). However, despite this advantage, full T cell differentiation using OP-9 remained difficult (Kodama et al., 1994; Tong et al., 1999).

Stromal cell lines derived from the thymic epithelium also proved to be similarly inefficient at supporting T cell differentiation. The reasons for this remain unclear, but the morphological and functional changes in stromal cells grown in monolayer lines are suggestive of changed in functional capabilities (Anderson et al., 1998b). This led to the suggestion that the microenvironment provided by the three-dimensional lattice of the thymic epithelium was required to support T development (Anderson et al., 1998b). However, recent advances in our understanding of the requirements for T cell commitment and differentiation led to re-evaluation of stromal cell lines as a tool for the differentiation of T cells. Many molecules such as IL-7 and SCF were known to have important roles in the fate and activity of T cells although these were unlikely to be the critical missing factors in T cell differentiation cultures, as they have roles in the differentiation of many other cell types that have been supported by monolayer culture.

The discovery of the critical role for Notch signalling in the B versus T cell decision was of particular interest, suggesting that Notch signalling provides a unique signal that permits the choice of a T cell fate at the expense of other cell fates (Radtke et al., 2004). The finding that delta-like Notch ligands were essentially missing from stromal cultures but present on thymic epithelium provided impetus for further investigations into a potential role as the missing component in stromal cultures. Transduction of the Notch ligand Dll-1 into the OP-9 BM stromal cell line dramatically altered their lymphocyte supporting potential (Schmitt and Zuniga-Pflucker, 2002). While the OP-9 cell line efficiently supported B cell differentiation, the transduced OP9-Dll1 cell line supported T cell differentiation at the expense of B cell lineage (Schmitt and Zuniga-Pflucker, 2002). The simplicity of the culture system and the ease of working with cell lines has

meant that the OP9-Dll-1 has become a commonly used method for studies into the commitment status and differentiative potential of HPCs. However, although the OP-9-Dll-1 line efficiently supports differentiation to the DP stage, the absence of MHC Class II and CD1d precludes efficient positive selection of CD4 SPs (Schmitt and Zuniga-Pflucker, 2002). As yet, efficient positive and negative selection has not been demonstrated on the OP-9-Dll-1 cell line and currently requires transfer of thymocytes FTOC for this to be facilitated (Schmitt et al., 2004b; Schmitt and Zuniga-Pflucker, 2002). Furthermore, the inability of unmanipulated OP-9-Dll-1 cells to express the range of AIRE, or the range of self-antigens that can be found in mTECs, would indicate that negative selection of self-reactive T cells would not be possible without further manipulation of the cell line (Zuniga-Pflucker, 2004).

1.5 Aims

The heterogeneous epithelial cell network of the thymus provides the microenvironments required for intrathymic T cell differentiation and repertoire selection. When grafted *in vivo,* a population of fetal thymic epithelial cells, marked by the monoclonal antibodies MTS20 and MTS24, can generate all major thymic epithelial cell subtypes and form an organised thymus-like structure. Furthermore, the resultant thymic organoid can recruit T cell precursors and support their differentiation into mature $CD4^+$ and $CD8^+$ T cells. This was not achieved in grafts of $MTS20^-MTS24^-$ TEC or fibroblasts alone. Despite efforts to elucidate the mechanisms involved in thymic epithelial cell differentiation, little is known about the processes that govern the differentiation and compartmentalisation of the thymic epithelium.

The overall aim of this thesis was therefore to establish whether $MTS20^+MTS24^+$ fetal TEPC could provide the basis for an *in vitro* thymus-equivalent that could be used as a means of generating haematopoietic populations *in vitro* and also as a manipulatable model for TEC differentiation. Chapter Three describes the characterisation of fetal thymic stroma and also outlines the development of the experimental system, which was to be utilised in subsequent chapters. Chapter Four details the establishment of *in*

vitro conditions permissive for the differentiation of T cells in $MTS20^+$ cell-based cultures and characterises the haematopoietic populations supported by such cultures. Chapter Five describes the *in vitro* differentiative capacity of defined $MTS20^+$ and $MTS20^-$ fetal TEC populations and outlines the potential for these populations to become organised in these cultures.

Chapter 2: Materials and Methods

2.1 Materials and Solutions

Unless otherwise stated, materials were obtained from either BDH laboratory supplies, Gibco, Sigma, or Invitrogen.

RFTOC Medium:

- 1x Dulbecco's modified eagle medium F12:NUT mix
- 10% fetal calf serum (FCS)
- 4mM glutamine
- 2mM sodium pyruvate
- 50U/ml penicillin
- 50µg/ml streptomycin
- 0.1% non-essential amino acids
- 0.1mM 2-mercaptoethanol

Fibroblast Medium:

- 1 x 1x Dulbecco's modified eagle medium
- 10% FCS
- 2mM sodium pyruvate
- 50U/ml penicillin
- 50µg/ml streptomycin

Lobe dissociation mixture:

- 1.4mg/ml hyaluronidase
- 0.7mg/ml collagenase
- 0.05mg/ml deoxyribonuclease
- 1ml PBS

FACS Wash:

- 95ml Ca^{2+}/Mg^{2+}-free PBS
- 5ml FCS

Thymocyte Wash:

- 95ml Hanks balanced salt solution (HBSS)
- 5 ml FCS

Table 2.1 Antibody clones and their source

NAME	CLONE AND SOURCE	SPECIES
MTS20	from R.L. Boyd, Monash University Medical School, Australia	Rat IgM
MTS24	from R.L. Boyd, Monash University Medical School, Australia	Rat IgG2a
αK14	LL002; from B Lane, Dundee	Mouse IgG3
anti-pancytokeratin	polyclonal anti-keratin, Dako Corporation	Rabbit
αK5	polyclonal anti-keratin 5, Covance Research Products	Rabbit
αK8	Troma 1; DSHB	Rat IgG
CDR1	from B Kyewski, German Cancer Research Center, Heidelberg	Rat IgG
UEA1	biotinylated, Vector labs	Lectin
αCD31	MEC13.3, BD PharMingen	Rat IgG2a
αMHC Class II	M5114, BD PharMingen	Rat IgG
αCD3ε	145-2C11, BD PharMingen	Hamster IgG1
αCD4	RM4-5, BD PharMingen	Rat IgG2a
αCD8	53-6.7, BD PharMingen	Rat IgG2a
αCD11c	HL3, BD PharMingen	Hamster
αCD25	7D4, BD PharMingen PC61, BD PharMingen	Rat IgM Rat IgG1
αCD44	IM7, BD PharMingen	Rat IgG2b
αCD11b	M1/70, BD PharMingen	Rat IgG2bk
αCD45	30-F11, BD PharMingen	Rat IgG2b
αTCRβ	H57-597, BD PharMingen	Hamster IgG2
αCD140a (PDGFRα)	APA5, BD PharMingen	Rat IgG2a
TER119	BD PharMingen	Rat IgG2bk
αNK1.1	PK136, BD PharMingen	Mouse IgG2ak
ERTR7	From W van Ewijk, University of Leiden	Rat IgG2a

2.2 Mice

All animals were housed within the Division of Biological Sciences Animal Facility at the University of Edinburgh under conditions outlined in the Animals (Scientific Procedures) Act 1986. Mice were housed in a stabilised environment with a 12 hour light/dark cycle and were supplied with food and water *ad libitum*. Adult mice were sacrificed by the schedule 1 method of cervical dislocation.

2.2.1 Embryo collection

Matings to provide embryos were between C57BL/6 females and CBA males. To this end, male and female mice were together overnight and females were examined the following morning for the presence of a vaginal plug. This was taken as time point E0.5. Pregnant females at the desired stage were sacrificed and the uterine horns were isolated and placed in PBS. Embryos were removed from the uterus and transferred to clean PBS for further dissection.

2.3 Cell Preparations

Embryonic thymic lobes were dissected of excess extrathymic tissue and dissociated into a single cell suspension by incubation at 37°C for 15 minutes in dissociation mixture followed by 5 minutes in 0.025% trypsin at room temperature. Pelleted cells were resuspended in FACS wash for antibody staining. E13.5 foetal livers were mashed between frosted slides into room temperature PBS before being transferred to PharMLyse (BD Bioscience) for RBC depletion as per manufacturers instructions. Following this depletion step, cells were spun and resuspended in FACS wash for further processing. For the isolation of adult thymocytes, thymi from 6-8 week old mice were homogenised by mechanical disaggregation on ice between two frosted glass slides and rinsed with cold thymocyte wash to liberate thymocytes from stromal cells. Cell suspensions were then transferred to 15ml Falcon tubes, spun at 1300rpm for 5 minutes at 4^0C in a bench top centrifuge and resuspended at $1x10^8$ cells/ml in cold thymocyte wash.

2.3.1 Complement depletion

For the enrichment of adult DN cells prior to sorting, complement depletion was used. Adult thymi were collected into cold thymocyte wash from mice aged between 4 and 8 weeks. On ice, thymi were mashed between frosted slides to release thymocytes into a single cell suspension. The cell suspension was spun at 1,300 rpm for 5 minutes at 4 °C. The cell pellet was resuspended in 2ml cold HBSS and 2 ml supernatant from hybridoma RL172.4 and 3.16881 were added. Following a 30 minute incubation on ice, 10ml HBSS was added to the tube and the cells were respun. The supernatant was removed and discarded and the cells were resuspended in 4ml cold HBSS. To this, iml reconstituted LowTox rabbit complement (Cedarlane) was added and incuated at 37°C for 60 minutes with gentle agitation every 20 minutes. Cold HBSS was added to the mix before a further centrifuge spin was used to pellet the cells and debris. The pellet was resuspended in 4 ml HBSS and gently layered above 4ml Lymphoprep M (Cedarlane). The gradient was spun for 20 minutes at 1,400rpm to allow debris to settle at the bottom of the tube. The cells at the interface between the HBSS and the Lymphoprep M were collected and thoroughly mixed with fresh HBSS to remove the contaminatig Lymhoprep M. The cell pellet was then resupended in thymocytes wash and ready to be stained as per the flow cytometry protocols.

2.4 Cell Culture

All cell manipulations were carried out in Class II laminar flow sterile hoods (Heraeus) using aseptic technique. Solutions were routinely sterility tested.

2.4.1 Organ culture

FTOC and RFTOC culture protocol was adapted from protocols published by Anderson *et al* and Dou *et al.* Briefly, fetal thymic lobes were isolated as described previously and RFTOC were generated as described in Chapter Three. These were cultured overnight at the gas-liquid interface, floating on a polycarbonate (Millipore) raft in RFTOC medium. Subsequently, FTOC and RFTOC were submersed in RFTOC medium and cultured under high oxygen submersion (HOS) conditions for defined periods in a gas chamber containing a gas mix of 80% oxygen, 5% carbon dioxide and 15% nitrogen (BOC).

2.4.2 Murine embryonic fibroblast culture

E13.5 embryos were decapitated and stripped of all internal organs and extra-embryonic tissue. The remaining tissue was washed in PBS before being gently mashed between frosted slides and immersed in 5ml trypsin. Following incubation at room temperature for 10 minutes, 10ml fibroblast medium was added and the cell solution was centrifuged for 3 minutes at 200rpm. The cell pellet was resuspended in 5ml fibroblast medium and filtered to removed cell aggregates using a 40μm filter. The resulting cell suspension was added at a range of volumes to 25cm^2 tissue culture treated flasks and incubated with 5 ml fibroblast medium at 37°C for at least 5 hours but typically overnight to allow viable cells to adhere to the flask. The media was completely changed after this time to remove cellular debris. The medium was partially changed every 3 days and MEFs were used at confluence.

2.4.3 Hybridoma supernatant production

Hybridomas were maintained at 37°C in 7% CO_2 in an upright position. Every three days, an equivalent volume of medium was added to the flask so that the total volume in the flask doubled. Once the volume reached the desired level, the cultures were allowed to continue growth without further media addition. When the medium turned yellow and the cultures showed signs of cell death, the cultures were spun to pellet the hybridoma cells then the supernatant was filtered to remove any remaining cells. The hybridoma supernatant was then batch tested for potency using flow cytometry before being aliquoted and stored at 4°C until use.

2.5 Flow cytometry

2.5.1 Sample preparation

Cells in a suitable volume of FACS wash or thymocyte wash were incubated with primary antibody for 20 minutes on ice and then washed and incubated for 20 minutes with secondary antibody if necessary. In some cases, a further 20 minute incubation with a tertiary antibody was also required. In the case of intracellular antigen detection,

cells were fixed and permeabilised using the BrdU Flow Kit (BD Bioscience) prior to staining with antibodies against intracellular determinants. Following staining, cells were washed in appropriate buffer and were resuspended in thymocytes or FACS wash containing 1:25 7AAD (BD Bioscience) or 1:2000 ToPro3 (Molecular Probes) as viability markers if needed. Sorting strategies included the consideration of events with low take-up of viability dyes and appropriate scatter and stainig characteristics.

2.5.2 Flow cytometric cell sorting and analysis

Cells were sorted on either a MoFlo (Dakocytomation) or a FACStar (BD Bioscience) into cold FACS wash using isotonic sheath fluid. Analysis events were acquired on either a FACSCalibur (BD Bioscience) or a Cyan ADP (Dakocytomation) using either CellQuest (BD Bioscience) or Summit (Dakocytomation) software, respectively. Downstream analysis was performed using FlowJo (Treestar Inc).

2.6 Immunofluorescence

2.6.1 Frozen section preparation

Tissues were embedded in OCT compound (Tissue Tek, Miles Inc., USA), snap frozen on dry ice and stored at -80°C. Prior to sectioning, frozen embedded tissues were placed in the cryostat (Leica CM1900) and allowed to equilibrate for 30 minutes at -20°C. Sections were then cut at 10μm thickness and collected onto poly-L-lysine coated glass slides (VWR International). Sections were air dried for 20 minutes before being fixed for two minutes in 100% acetone (-20°C) and air dried for a further 20 minutes. Prepared slides were stored at -80°C prior to use.

2.6.2 Cytospin preparation

Cells suspended in 100μl of PBS were loaded into a cytospin chamber which includes a polysine coated slide (VWR International) and a filter card (Thermo). Preparations were centrifuged at 1,000rpm (Cytospin 3; Shandon) for five minutes at room temperature. Slides were removed from the chamber and allowed to air dry for two minutes and then fixed in 100% acetone (-20°C) for two minutes. Following fixation

slides were left to air-dry for 2 minutes. Prior to staining the area around the cells was marked using a PAP pen (Daido Sangyo).

2.6.3 Staining

Frozen, fixed sections were allowed to air dry for 5 minutes before briefly rinsing in PBS. Slides were subsequently blocked for 15 minutes with PBS containing 5% serum. In order to minimise non-specific antibody binding, the species from which the serum was obtained was the same species in which the secondary antibody was raised. Primary antibody solutions diluted in PBS were then added to the sections and incubated at room temperature for 1 hour. Sections were then washed for 3 x 5 minutes using PBS containing 0.1% Tween 20 (PBS-Tween). Following the washes, the slides were then incubated at room temperature in the dark for 30 minutes with PBS containing fluorescent protein-conjugated secondary antibody combinations at a suitable dilutions, and DAPI (1:2000). Typically, anti-rat IgG Alexa 488 (Molecular Probes), anti-mouse IgG Alexa 568 (Molecular Probes), Streptavidin-conjugated Alexa 568 (Molecular Probes) or anti-rabbit IgG Alexa 647 (Molecular Probes) were used in combination to provide secondary fluorescence. After washing with PBS-Tween for a futher 3 x 5 minutes, slides were rinsed with water and air dried before mounting with Vectashield Hardset mountant (Vector Labs).

2.6.4 Imaging

For detection of immunofluorescence, slides were examined with a Leica AOBS confocal microscope under the appropriate excitation conditions. Images were processed using Adobe Photoshop CS.

Chapter 3: Results: Investigation into the cellular composition of the developing thymus.

3.1 Introduction

The overall aim of this work was to investigate the potential of $MTS20^{+}MTS24^{+}$ fetal TEPCs to form the basis of a functional *in vitro* thymus equivalent. Recent work has identified that MTS20 and MTS24 mAbs recognise the same antigen (M. Depreter and C. Blackburn, manuscript in preparation). It is well established that the developing thymus rudiment contains TECs, fibroblasts, vascular cells and haematopoietic cells (Boyd et al., 1993). At the outset of this project, the relative contributions of these cell types to the developing thymic stroma between E12.5 and E15.5, a period critical to the establishment of stromal organisation, were poorly defined. Although recent publications have added some understanding to this area, the roles that these cell types play during the organisation and maturation of the developing thymic microenvironment remain poorly understood. Therefore, there was a requirement to characterise the populations of cells used during any *in vitro* experiments in order to interpret the outcome of experiments.

The RFTOC technique is a well established method of supporting T cell differentiation *in vitro* and has proved a powerful tool for the identification of cell types required in the thymic microenvironment (Anderson et al., 1993). Standard RFTOCs are cellular reaggregates formed from a cell slurry, which is drawn into a finely-pulled pipette and then pipetted onto filter paper raft floating on medium, where it is allowed to reaggregate at the gas-liquid interface (Anderson et al., 1993). The success of this reaggregation is dependent upon the generation of a cell slurry having great enough viscosity and a small enough volume to enable the creation of a standing drop on the filter. Inherent in the creation and use of a small volume of cell slurry are the cell losses

resulting from the residual cells that remain outside the RFTOC following the reaggregation process, as well as those incurred in the pipetting process. The variability introduced using this method has lead to inconsistency in size and productivity among successful reaggregates, and is a likely contributory factor in the relatively high failure rate of standing drop RFTOC (unpublished observation). This ultimately limits the utility of RFTOC for analysing the functional potential of TEC populations.

In Chapter Three I therefore determine the cellular composition of the developing fetal thymus and the spatial relationship between the different cell types, between E12.5 and E15.5. Particular attention is paid to the thymic epithelial populations present during this time. In addition, I describe the development and optimisation of an improved RFTOC method that minimises cell loss and generates greater intra- and inter-experimental reproducibility than existing techniques.

3.2 Composition of fetal thymic lobes (E12.5-E15.5)

To determine the composition and organisation during the development and maturation of the thymic microenvironment, monoclonal antibodies against markers of stromal elements were used in flow cytometric and immunofluorescence analyses of thymic lobes dissected from E12.5 – E15.5 mouse embryos.

3.2.1 Thymic mesenchyme

PDGFRα identifies NC-derived mesenchymal cells in and around the early thymic primordia (Jenkinson et al., 2007; Morrison-Graham et al., 1992). An anti-PDGFRα antibody was therefore used to identify mesenchymal cells by flow cytometric analysis. As shown in Figure 3.1, PDGFRα$^+$ cells initially make up a significant proportion of stromal cells in the primordium and steadily decline in percentage as the thymus matures. At E12.5, PDGFRα stains 39% of all viable cells, although it is likely that this figure overestimates the true proportion of thymic mesenchyme due to the presence of

extrathymic connective tissue carried through the dissection process (Figure 3.1). At E13.5, PDGFRα$^+$ cells are approximately one fifth of the viable cells, with this figure dropping to 9% by E14.5. This decrease continues so that by E15.5, fewer than 2% of cells express this marker (Figure 3.1). Confirmation that these cells were non-epithelial was provided by cytospin analysis with routinely greater than 95% of purified PDGFRα$^+$ cells not staining with anti-pancytokeratin (Figure 3.2 and (Table 3.1). These observations are consistent with recently published data showing that at E12 PDGFRα is not co-expressed with EpCam, a marker of thymic epithelium, and that beyond E12, PDGFRα$^+$ cells decrease as a proportion of the EpCam$^-$ CD45$^-$ compartment (Jenkinson et al., 2007).

The monoclonal antibody ERTR7 marks an unknown intracellular determinant of thymic fibroblasts. The localisation and arrangement of ERTR7$^+$ fibroblasts was visualised in E13.5 and E15.5 fetal thymic lobes by immunofluorescence (Figure 3.3). In E13.5 thymic lobes, the epithelial core of the primordium is surrounded by a capsule, which stains brightly with ERTR7. Scattered ERTR7$^+$ cells can be seen throughout the epithelium but more commonly the intrathymic fibroblasts are present as clusters (Figure 3.3). Some cells appear to be projections of the capsule that extend into the epithelial areas of the lobes, possibly representing the early stages of trabecularisation (Figure 3.3). By E15.5, the number of ERTR7$^+$ cells appears to be increased proportionally with thymic size, and the staining pattern remains very similar with trabecular fibroblast projections now extending from the capsule deep into the epithelial core of the lobes (Figure 3.3). The relationship between ERTR7$^+$ cells and those that are PDGFRα$^+$ is not clear as studies rarely use both antibodies, and where both are used, they are not compared due to technical difficulties. The loss of PDGFRα$^+$ may represent developmental progression. Despite the proportional reduction in PDGFRα$^+$ cells

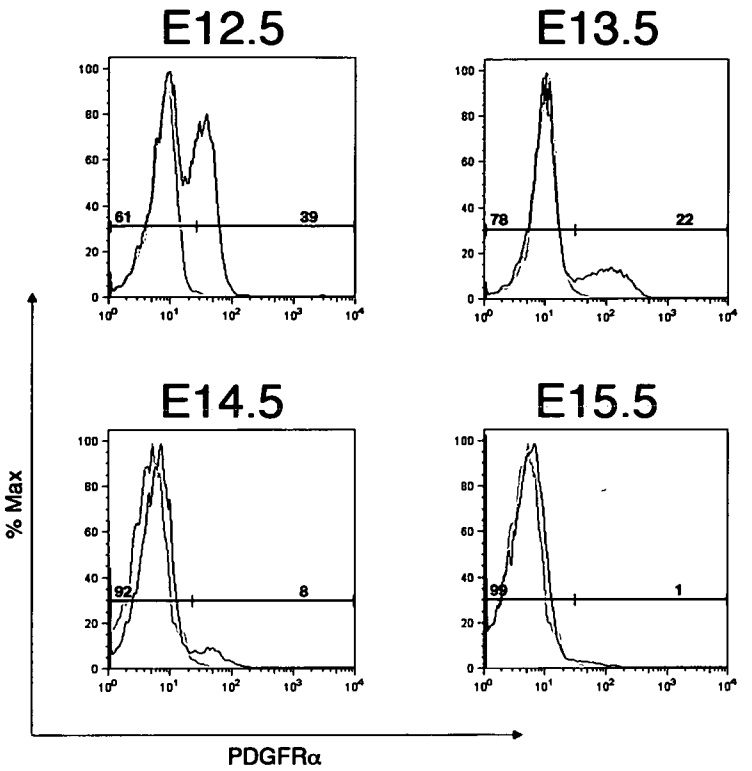

Figure 3.1 PDGFRα staining of fetal thymic lobes

Flow cytometric analysis was performed on cleanly dissected fetal thymic lobes following enzymatic dissociation. Cells were incubated with anti-PDGFRα antibody to identify mesenchymal fibroblasts. A large population of cells in the E12.5 lobes expresses PDGFRα. As the lobes mature and increase in size, this population becomes relatively smaller and constitutes only a small portion of the stromal compartment by E15.5. Plots shown are representative of at least two experiments.

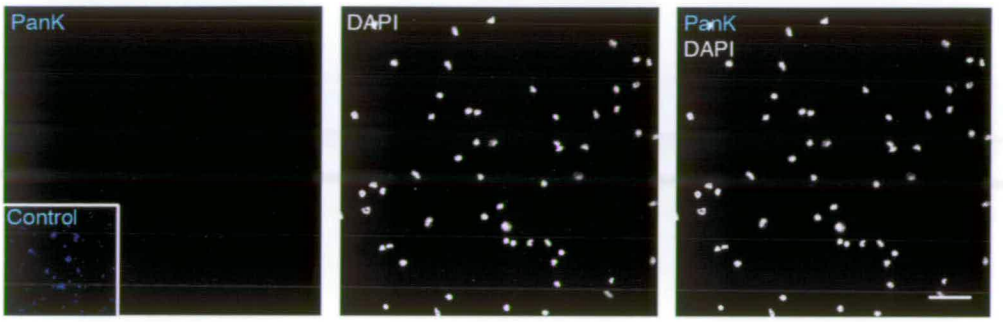

Figure 3.2 PDGFRα$^+$ cells are non-epithelial

Flow cytometrically isolated E13.5 PDGFRα$^+$ cells were cytospun onto glass slides prior to fixation and stained with anti-pancytokeratin (PanK) and DAPI. Three fields of view were scored to establish the total number of DAPI$^+$ cells and PanK$^+$ epithelial cells present following three separate sorting experiments. Only two epithelial cells were identified within the PDGFRα$^+$ population in any of the sorts consistent with the level expected as a result of contamination. The results presented are representative of at least three separate experiments.

Table 3.1 PDGFRα$^+$ cells are non-epithelial

	EXPERIMENT		
	1	**2**	**3**
Cell (DAPI$^+$) count	**56**	**80**	**77**
PanK$^+$ count	**2**	**0**	**0**
% Epithelium	**3.6**	**0**	**0**

Flow cytometrically purified E13.5 PDGFRα$^+$ cells were cytospun onto glass slides prior to fixation and staining with anti-pancytokeratin (PanK) and DAPI. Fields of view were scored to establish the total number of DAPI$^+$ cells and PanK$^+$ epithelial cells. The PDGFRα$^+$ cells exhibit PanK staining in fewer than 5% of cells consistent with their non-epithelial identity. The level of PanK$^+$ staining is equivalent to that expected from contaminating epithelial cells given sort purities of greater than 95%.

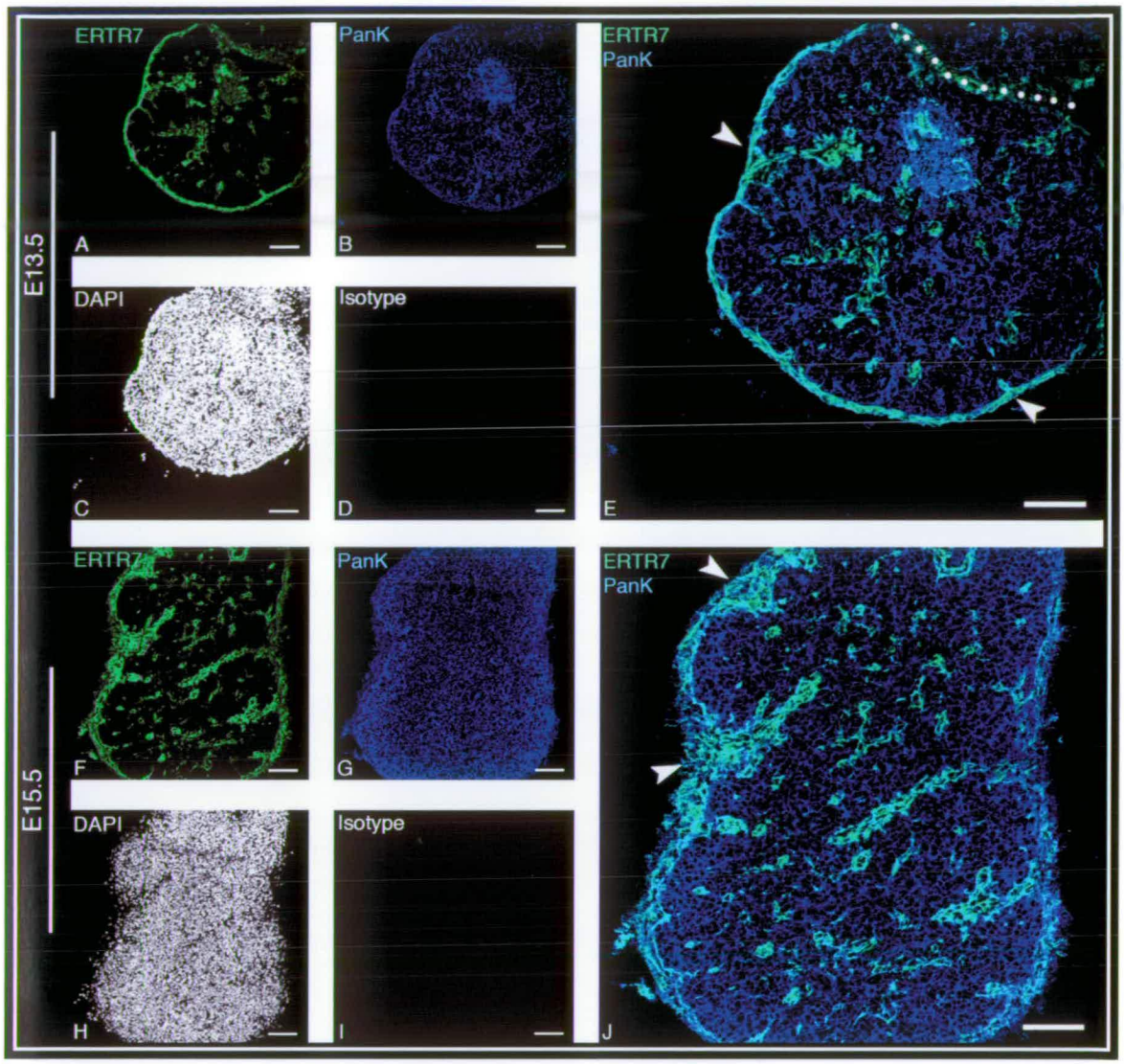

Figure 3.3 Distribution of fibroblasts within fetal lobes

Representative immunofluorescence staining of fetal thymic lobes showing the localisation of ERTR7^{+} fibroblasts in the PanK^{+} epithelial core. At both E13.5 (Panels A-E) and E15.5 (Panels F-J), fibroblasts encapsulate the lobes and are beginning to infiltrate the epithelial core at several points (white arrowheads). Dotted line represents the capsule between two adjacent lobes. Scale bars, 100μm.

beyond E12.5 and the implication in published data that this represents a decrease in actual PDGFRα$^+$ cell number from data analysing the minority non-epithelial, non-haematopoietic population of the embryonic thymus (Jenkinson et al., 2007), it remains unclear whether or not this is the case as other minority populations were not excluded in the analysis. A possible contaminant would be the vascular compartment of the thymus, which makes up an increasing proportion of cells beyond E13.5 (Figure 3.4).

3.2.2 Thymic vasculature

The mature adult thymus is a heavily vascularised organ, with a complex network of vascular elements that form during organogenesis. In other epithelial organs, vascular endothelium plays a critical role in outgrowth or organisation (Nikolova and Lammert, 2003). Vascular elements and their spatial relationship with the developing thymic lobes was investigated using immunofluorescence. Analysis of E13.5 and E15.5 lobes was performed on frozen sections using CD31 as a marker of vasculature and pancytokeratin as a counterstain for TEC (Figure 3.4). Figure 3.4 illustrates that at E13.5, endothelial cells are located throughout the epithelial structure and have begun to cluster into tubular structures. Of note are the endothelial cells that appear to be associated with the capsule and outermost epithelial regions. This staining pattern is more obvious at E15.5 when the epithelium is interspersed with tubular endothelial structures, many of which lie perpendicular to the subcapsule in an arrangement typical of mature cortical vasculature (Kato and Schoefl, 1989) (Figure 3.4).

3.2.3 Phenotypic characterisation of the MTS20$^+$ and MTS20$^-$ populations.

To analyse the remaining compartment of the thymic stroma, a lineage (Lin) cocktail of mAbs against haematopoietic (anti-CD45 and TER-119), vascular (anti-CD31) and fibroblastic (anti-PDGFRα) components of fetal thymic lobes was used to exclude CD45$^+$ and most CD45$^-$ stromal elements. The composition of the MTS20$^-$Lin$^-$ and MTS20$^-$Lin$^-$ populations was then analysed by flow cytometric analysis of thymus lobes and cytospin analysis of populations purified, using the protocol shown in Figure 3.5.

In the latter analysis, total cell numbers were counted in a field using DAPI as a nuclear stain and Photoshop CS to provide an artificial grid across the field to aid with orientation and enumeration.

As shown in Figure 3.6, cells identified as haematopoietic cells, fibroblasts or vascular cells by the use of an antibody lineage cocktail accounted for roughly 45% of total cells at E13.5 and were initially excluded by electronic gating. The relative proportion of $MTS20^+$ and $MTS20^-$ cells are summarised in Table 3.2 and show that at E13.5, 70% (S.D ± 8) were $MTS20^+$ and 30% (S.D ± 8) were $MTS20^-$. Cytospin analysis revealed that 96% (S.D± 0.8) of $MTS20^+$ cells and 86% (S.D± 4) of $MTS20^-$ cells express cytokeratin (Figure 3.7, Table 3.4 and Table 3.5). The expression of two cell-surface markers of epithelial cells, H2-A/H2-E and UEA-1 was then detected by flow cytometric analysis in E13.5 thymic lobes. In the example shown, 18% of E13.5 $MTS20^+$ cells expressed MHC Class II, indicating functional maturation of some cells within the population (Figure 3.6D). A similar proportion of $MTS20^-$ cells also showed MHC Class II staining (16%)(Figure 3.6E). Cytospin analysis showed reasonable concordance with these data with an average of 16% (S.D. ± 0.2) of $MTS20^+$ cells, and 6% (S.D. ± 2) of E13.5 $MTS20^-$ cells showing detectable MHC Class II staining (Figure 3.9, Table 3.8 and Table 3.9). Variability amongst samples and/or increased detection sensitivity in flow cytometric analysis could contribute to observed differences. Furthermore, the cell sorting protocols as shown in Figure 3.5 necessitate a conservative gating strategy to ensure that high purities are obtained for selected populations whereas flow cytometric analysis gating was based on isotype control staining thus cytospin data reflects the phenotype of input populations used in later experiments.

In the example shown in Figure 3.6G, 5% of the $MTS20^+$ cells costained with UEA-1, whereas few or no $MTS20^-$ cells appeared to express UEA-1 (Figure 3.6H). Furthermore, at 13.5 $MTS20^+$ cells were invariably $K14^-$ although occasional $MTS20^-$ $K14^+$ cells were present in cytospins (Figure 3.9, Table 3.8 and Table 3.9). The presence

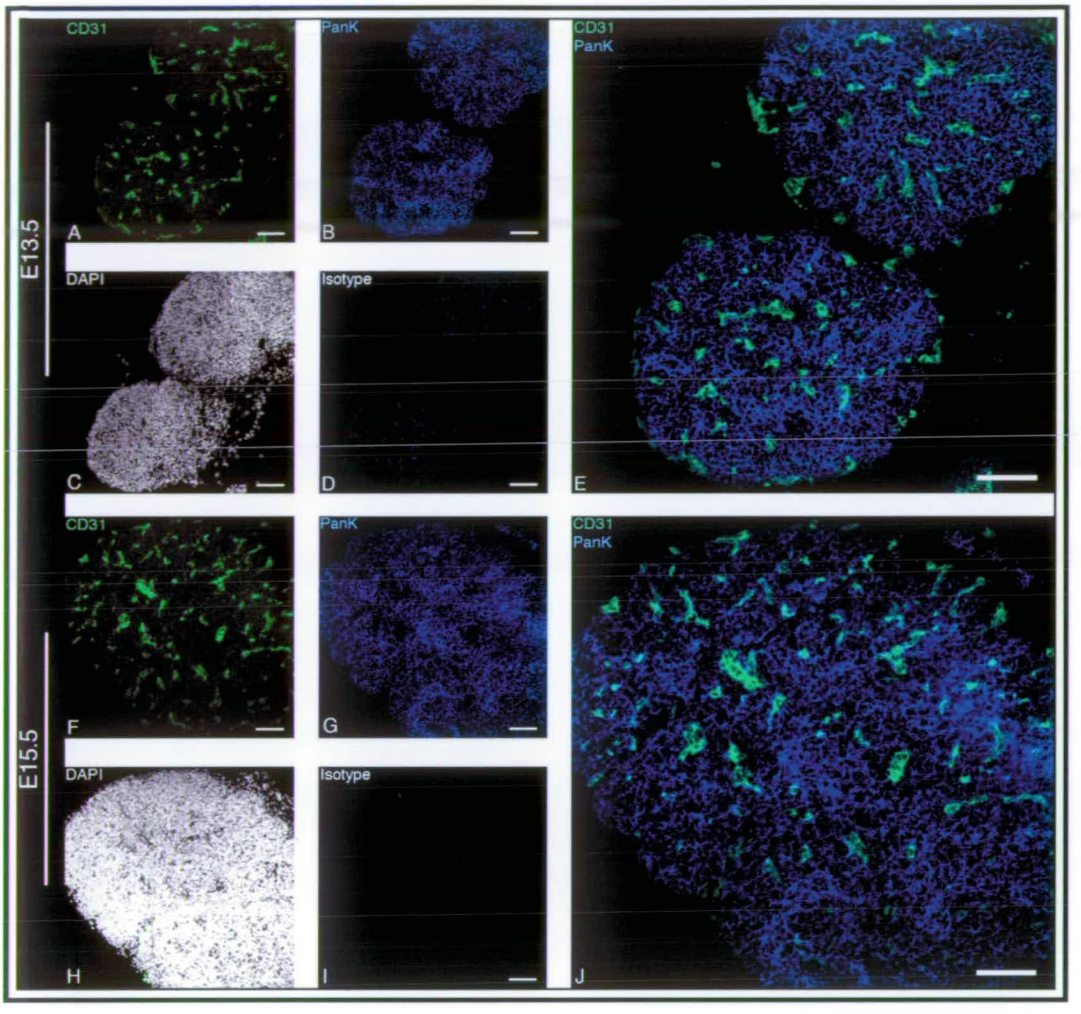

Figure 3.4 Distribution of endothelial cells within fetal thymic lobes

Representative immunofluorescence showing the presence of $CD31^+$ endothelial cells within the $PanK^+$ epithelial core of E13.5 (Panels A-E) and E15.5 (Panels F-J) fetal thymic lobes. Cells of the developing vascular system are scattered throughout the $PanK^+$ epithelial core at E13.5 and E15.5 and have begun to form tubular networks. Scale bars, 100μm.

of UEA-1$^+$ cells in the MTS20$^+$ compartment is interesting as this perhaps reflects a population of cells destined to maintain UEA-1 expression and give rise to mature UEA-1$^+$ cells in the medulla, as demonstrated in a recent publication investigating the lineage of adult UEA1$^+$ mTEC (Hamazaki et al., 2007). The absence of these cells in the MTS20$^-$ population may therefore reflect restricted potential.

A similar data set showing analysis of dissociated and stained E15.5 lobes is presented in Figure 3.8 and Table 3.3. At E15.5 24% cells do not stain with markers of non-epithelial lineages. 25% (S.D. ± 3.4) of these Lin$^-$ cells are MTS20$^+$, whereas the remaining 74% (S.D. ± 3.4) are MTS20$^-$. The representative flow cytometric analysis shown in Figure 3.8D indicates that 26% of the E15.5 MTS20$^+$, and 44% of the MTS20$^-$ population expressed MHC Class II, while few cells showed UEA-1 staining (Figure 3.8G). Cytospin analysis however indicated MHC Class II expression on 63% (S.D. ±4.5) of the MTS20$^+$ and 78% (S.D± 1.9) of the MTS20$^-$ population (Figure 3.9 and Table 3.10) This discrepancy is likely to be attributable to the intracellular detection of MHC Class II with cytospin analysis. No MTS20$^+$ cells were found to co-express K14 by cytospin and only a very small number of MTS20$^-$ K14$^+$ cells were detected (Figure 3.9, Table 3.10 and Table 3.11).

The expression analysis of cytokeratins revealed further differences among the flow cytometrically purified populations. The most common cell phenotype in all populations tested was K8$^+$K5$^-$, a phenotype consistent with cortical identity or early simple epithelium. K8$^-$ K5$^+$ cells, probably representing a developing mTEC population, were present only at E15.5 but not at E13.5, with fewer than 5% of epithelial cells staining with this combination in both the E15.5 MTS20$^+$ and MTS20$^-$ fractions (Figure 3.10, Table 3.12, Table 3.13, Table 3.14 and Table 3.15). Of particular interest were the differing proportions of K8$^+$K5$^+$ cells, since this phenotype is thought to represent more primitive thymic epithelial cells that lack obvious cTEC or mTEC identity. It should

Table 3.2 MTS20 expression in Lin$^-$ cells of E13.5 thymic lobes

	% LIN$^-$ CD45$^-$MTS20$^-$	% LIN$^-$ CD45$^-$MTS20$^+$
Experiment 1	30	70
Experiment 2	19	81
Experiment 3	35	65
Experiment 4	37	63
Mean	30.3	70
Standard Dev.	8.1	8.1

Dissociated E13.5 thymic lobes were stained with anti-CD45 and an antibody lineage cocktail including TER-119, CD31 and PDGFRα as well as MTS20 for flow cytometric analysis.

Table 3.3 MTS20 expression in Lin$^-$ cells of E15.5 thymic lobes

	% LIN$^-$ CD45$^-$MTS20$^-$	% LIN$^-$ CD45$^-$MTS20$^+$
Experiment 1	80	20
Experiment 2	74	26
Experiment 3	72	28
Experiment 4	76	24
Mean	76	25
Standard Dev.	3.4	3.4

Dissociated E15.5 thymic lobes were stained with anti-CD45 and an antibody lineage cocktail including TER-119, CD31 and PDGFRα as well as MTS20 for flow cytometric analysis.

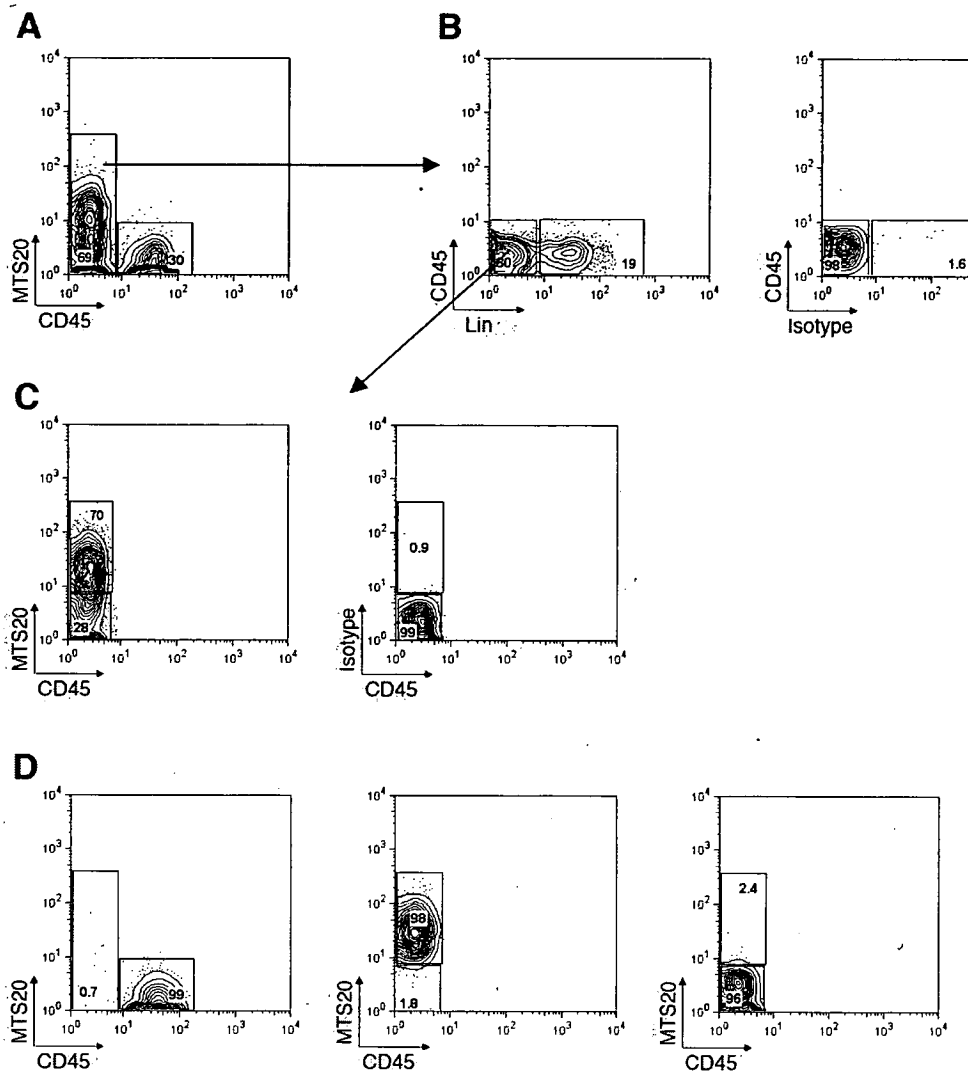

Figure 3.5 Sort criteria for the purification of MTS20$^+$ thymic epithelial cells, fetal thymocytes and MTS20$^-$ thymic epithelial cells from E13.5 lobes

Following enzymatic dissociation and antibody staining, cells were identified for flow cytometric sorting. A, staining for MTS20 and CD45. B, after gating against CD45$^+$ cells, anti-PDGFRα and anti-TER-119 antibodies are used to mark other non-epithelial lineage cells (Lin$^+$). C, after gating against CD45$^+$ and Lin$^+$ cells, remaining cells are further analysed to reveal MTS20+ and MTS20- populations. D, panels show purity checks on flow cytometrically purified CD45$^+$, MTS20$^+$ and MTS20$^-$ populations. Following sorting, population purities are routinely greater than 95%.

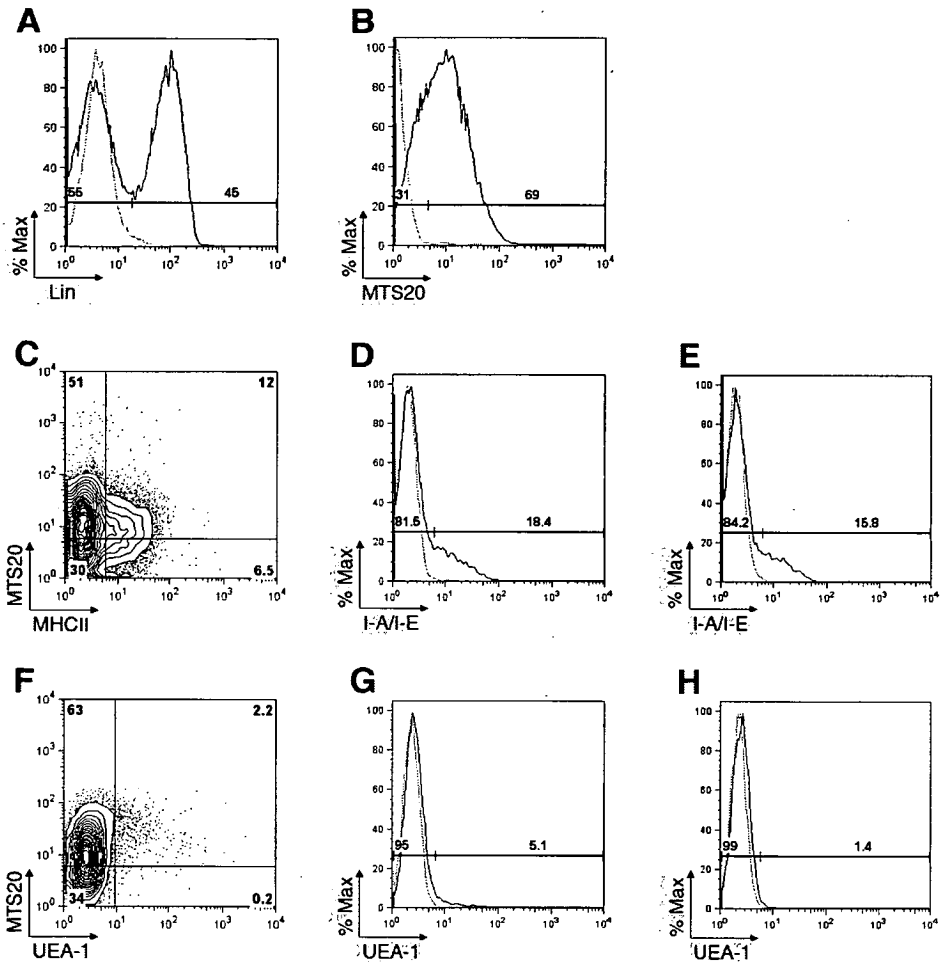

Figure 3.6 Phenotypic analysis of E13.5 thymic epithelial cells

Dissociated thymic lobes were stained with a cocktail of antibodies against haematopoietic cells, vascular cells and fibroblasts. A, at E13.5 approximately 55% of cells do not stain with the lineage cocktail and represent an epithelium-enriched fraction of the lobes (See also Figure 3.7 and Table 3.5). B, most Lin^- cells express MTS20. C-E, co-staining with MTS20 and I-A/I-E reveals MHC Class II is expressed by a significant proportion of both $MTS20^+$ (C and D) and $MTS20^-$ cells (C and E). F-H, co-staining with MTS20 and UEA-1 shows that UEA-1 is expressed by few if any Lin^- cells (F). Expression is mainly restricted to the E13.5 $MTS20^+$ fraction (G). Cells staining brightest with MTS20, express little or no MHC Class II although some express UEA-1 (C and F)

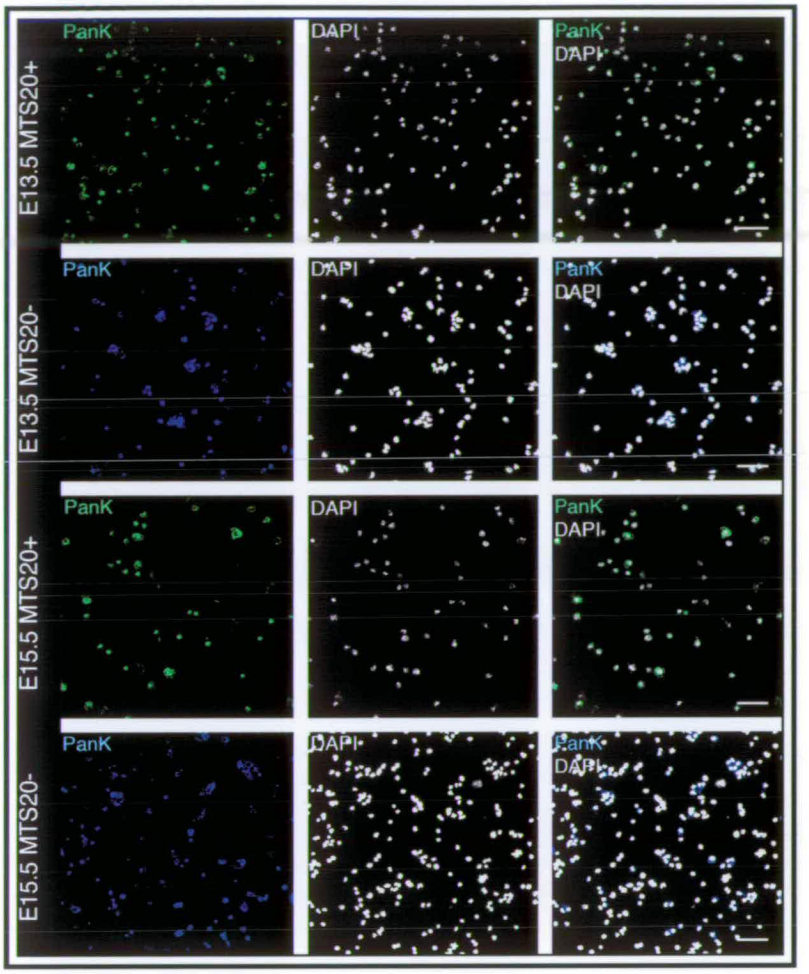

Figure 3.7 Cytospin analysis of purified populations

Lineage negative MTS20^{+} and MTS20^{-} populations were isolated by flow cytometric sorting and cytospun onto glass slides, before being fixed and stained with anti-PanK to confirm the purity of the epithelial preparations. Scale bar, 100μm.

Table 3.4 Determination of the proportion of epithelial cells in the E13.5 MTS20$^+$ population by cytospin analysis

	EXPERIMENT			MEAN±S.D.
	1	2	3	
Cell (DAPI$^+$) count	130	143	110	
PanK$^+$ count	127	137	106	
% epithelium	98	96	96	96±0.8

Flow cytometrically purified E13.5 Lin$^-$MS20$^+$ cells were cytospun and stained for immunofluorescence analysis.

Table 3.5 Determination of the proportion of epithelial cells in the E13.5 MTS20$^-$ population by cytospin analysis

	EXPERIMENT					MEAN±S.D.
	1	2	3	4	5	
Cell (DAPI$^+$) count	106	56	76	31	34	
PanK$^+$ count	93	47	69	28	27	
% epithelium	88	84	91	90	79	86±4.3

Flow cytometrically purified E13.5 Lin$^-$MS20$^-$ cells were cytospun and stained for immunofluorescence analysis.

Table 3.6 Determination of the proportion of epithelial cells in the E15.5 MTS20$^+$ population by cytospin analysis

	EXPERIMENT					MEAN±S.D.
	1	2	3	4	5	
Cell (DAPI$^+$) count	108	108	154	166	112	
PanK$^+$ count	96	103	142	156	102	
% epithelium	89	95	92	94	91	92±2.3

Flow cytometrically purified E15.5 Lin$^-$MTS20$^+$ cells were cytospun and stained for immunofluorescence analysis.

Table 3.7 Determination of the proportion of epithelial cells in the E15.5 MTS20$^-$ population by cytospin analysis

	EXPERIMENT				MEAN±S.D.
	1	2	3	4	
Cell (DAPI$^+$) count	230	331	161	77	
PanK$^+$ count	181	290	135	65	
% epithelium	79	88	84	84	84±3.2

Flow cytometrically purified E15.5 Lin$^-$MTS20$^-$ cells were cytospun and stained for immunofluorescence analysis.

be noted that throughout these experiments, the reactivity of the anti-K8 antibody clone used, was not restricted to the cTEC compartments, as published for K8 expression (Klug et al., 1998), and instead appeared to detect to all cytokeratin expressing cells. This alternate reactivity profile has been observed by several independent research groups (personal communication) and may reflect differing sensitivity of confocal versus conventional fluorescence microscopy. Therefore, the profiling described above does not accurately distinguish between the $K5^{+}K8^{-}$ and $K5^{+}K8^{+}$ populations and will consistently overestimate the size of the $K5^{+}K8^{+}$ compartment.

A caveat of the flow cytometric analysis of fetal thymic lobes is the absence of data on cell losses incurred as a result of the cell processing method. Despite attempts to optimise the dissociation method, some cells are notably under-represented in the analyses. In particular, cells expressing markers of differentiation such as K14 are easily detectable by immunofluorescence analysis of whole fetal thymic lobes, but rarely seen in either the flow cytometric analysis or the in the cytospin preparations (data not shown, Table 3.9 and Table 3.11). The probable reason for this is that some cell types are either difficult to digest or prone to cell damage, and are therefore selectively lost during isolation. It is also possible that some markers may be lost during sample processing and the cells, despite being present, are not identifiable.

An interesting feature of the cytospin analyses is the evidence presented that supports the presence of a cell type that does not express any of the markers tested. In particular, the $MTS20^{-}$ populations, which are sorted on the basis of their non-reactivity to a panel of antibodies, contain a small proportion of non-epithelial cells beyond the boundaries explicable by impurities resulting from the sort procedure (Table 3.5 and Table 3.7). Thus, 14% (S.D± 4.3) and 16% (S.D± 3.2) of $MTS20^{-}$ cells at E13.5 (Table 3.5) and E15.5 (Table 3.7) respectively, do not stain with anti-PanK or the lineage cocktail that includes antibodies against CD45, TER-119, CD31 and PDGFRα. On the basis of

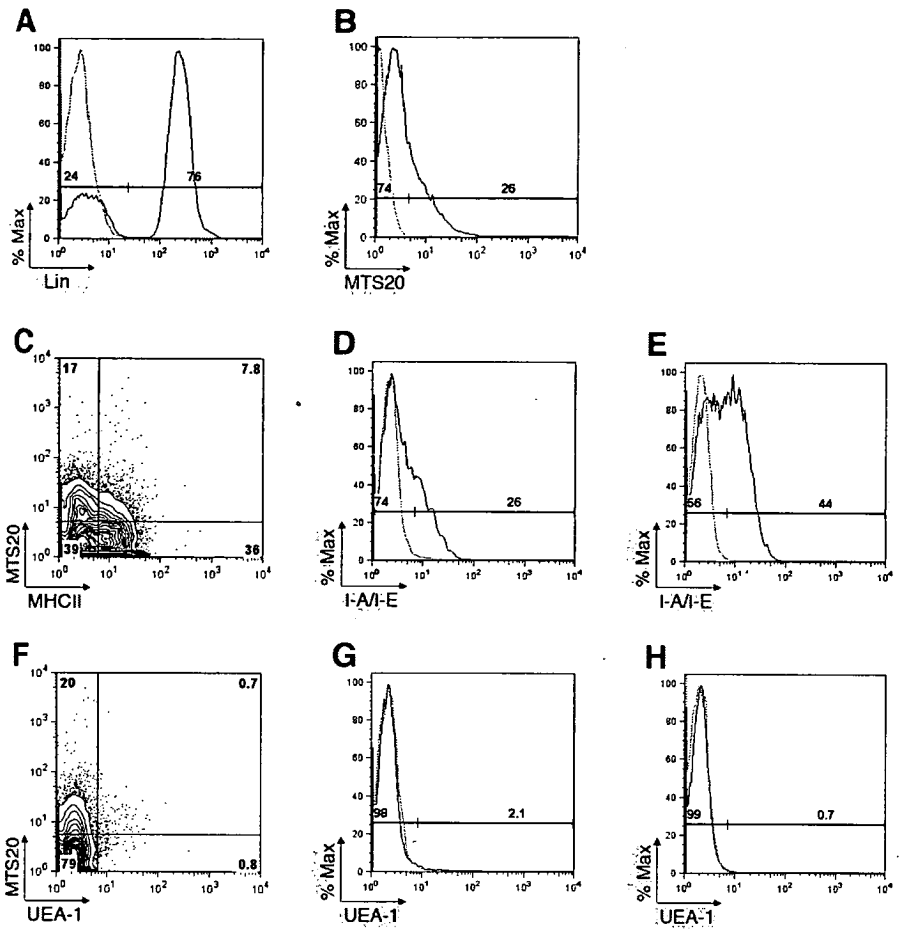

Figure 3.8 Phenotypic analysis of thymic epithelial cells at E15.5

Dissociated thymic lobes were stained with a cocktail of antibodies against haematopoietic cells, vascular cells and fibroblasts. A, at E15.5 24% of cells do not stain with the lineage cocktail and represent an epithelium-enriched fraction of the lobes (See also Figure 3.7 and Table 3.7). B, expression of MTS20 is heterogeneous in Lin$^-$ cells and staining intensity is lower than at E13.5 (see Figure 3.6). C-E, co-staining with MTS20 and I-A/I-E reveals MHC Class II is expressed by both MTS20$^+$ (C and D) and MTS20$^-$ cells (C and E). F-H, UEA-1 is expressed by relatively few cells within the E13.5 Lin- fraction (F) and UEA-1$^+$ cells are mainly restricted to the MTS20$^+$ compartment (G). Similar to E13.5, TECs staining brightest with MTS20 express little or no MHC Class II, although some express UEA-1 (C and F). These profiles are representative of three experiments.

typical 95% sort purity, still greater than 10% of cells remain unidentifiable. Many cell types could comprise some of this remaining compartment. For example, poor flow cytometric resolution of the PDGFRα^{+} and PDGFRα^{-} cell populations means that some PDGFRα^{low} mesenchymal cells are likely to remain in the negative fraction (Figure 3.1). Furthermore, the lineage cocktail would not exclude mesenchymal cells from non-neural crest sources if they do not express PDGFRα. In addition, cell types that comprise the thymic lymphatic system are not all excluded on the basis of either PDGFRα or CD31 staining and will contribute to the non-epithelial fraction particularly at later stages (Odaka et al., 2006).

3.3 Improvement of the RFTOC protocol

Since the analysis of fetal TEC populations requires the ability to utilise small numbers of cells, I initially elected to optimise the reproducibility of the RFTOC technique and to increase its utility for analysis of small but defined cell numbers. This was achieved as follows: in initial experiments the hanging drop method described in Bennett *et al*, 2002, was employed to generate RFTOCs as shown in Figure 3.12. Briefly, cells were mixed and suspended in a small volume of medium before being deposited onto the lid of a tissue culture plate. This was then inverted and incubated for 24-48 hours in humidified conditions to allow reaggregation to occur, prior to subsequent submersion and culture. Although small structures could be retrieved using this method, many problems were apparent. The size of reaggregates that could be made was severely limited as the use of increased cell number resulted in cell death and cell loss probably due to exhaustion of the culture medium constituting the drop. Significantly, many cells remained peripheral to the solid structure of the reaggregate and as these often comprised a great proportion of the total input cells, the eventual size and composition of the reaggregates was difficult to control.

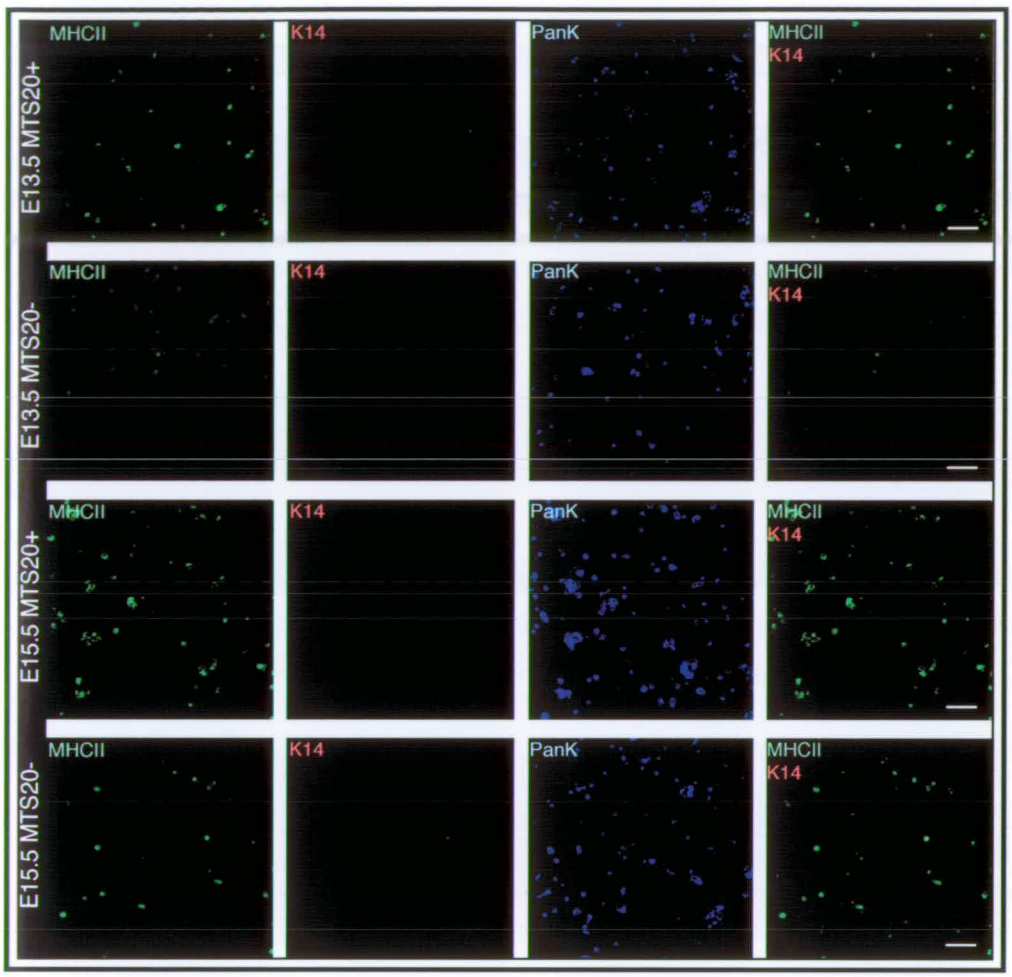

Figure 3.9 Cytospins of purified populations for K14 and MHC Class II

Lineage negative MTS20^{+} and MTS20^{-} populations were sorted and cytospun onto glass slides before fixing and staining, with anti-PanK, anti-K14 and anti-MHC Class II mAbs. Scale bar, 100μm. These cytospins are representative of three experiments.

Table 3.8 Phenotypic analysis of purified E13.5 MTS20$^+$ cells

CELLS	EXPERIMENT			MEAN±S.D.
	1	2	3	
PanK$^+$	127	106	137	
MHC Class II$^+$	20	-	22	
K14$^+$	0	-	0	
% PanK$^+$MHC Class II$^+$	15.8	-	16.1	15.9±0.2
% K14$^+$	0	-	0	-

Flow cytometrically purified E13.5 Lin$^-$MTS20$^+$ cells were cytospun and stained for immunofluorescence analysis.

Table 3.9 Phenotypic analysis of purified E13.5 MTS20$^-$ cells

CELLS	EXPERIMENT			MEAN±S.D.
	1	2	3	
PanK$^+$	93	43	69	
MHC Class II$^+$	8	2	3	
K14$^+$	0	1	0	
% PanK$^+$MHC Class II$^+$	8.6	4.3	4.4	5.7±2.0
% K14$^+$	0	2.3	0	0.8±1.1

Flow cytometrically purified E13.5 Lin$^-$MTS20$^-$ cells were cytospun and stained for immunofluorescence analysis.

Table 3.10 Phenotypic analysis of purified E15.5 MTS20$^+$ cells

CELLS	EXPERIMENT			MEAN±SD
	1	2	3	
PanK$^+$	96	103	142	
MHC Class II$^+$	55	65	97	
K14$^+$	0	0	0	
% PanK$^+$MHC Class II$^+$	57.3	63	68	63±4.5
% K14$^+$	0	0	0	0

Flow cytometrically purified E15.5 Lin$^-$MTS20$^+$ cells were cytospun and stained for immunofluorescence analysis.

Table 3.11 Phenotypic analysis of purified E15.5 MTS20$^-$ cells

CELLS	EXPERIMENT			MEAN±S.D.
	1	2	3	
PanK$^+$	181	290	135	
MHC Class II$^+$	145	219	106	
K14$^+$	0	1	0	
% PanK$^+$MHC Class II$^+$	80.1	76	79	78.1±1.9
% K14$^+$	0	0.4	0	0.1±0.2

Flow cytometrically purified E15.5 Lin$^-$MTS20$^-$ cells were cytospun and stained for immunofluorescence analysis

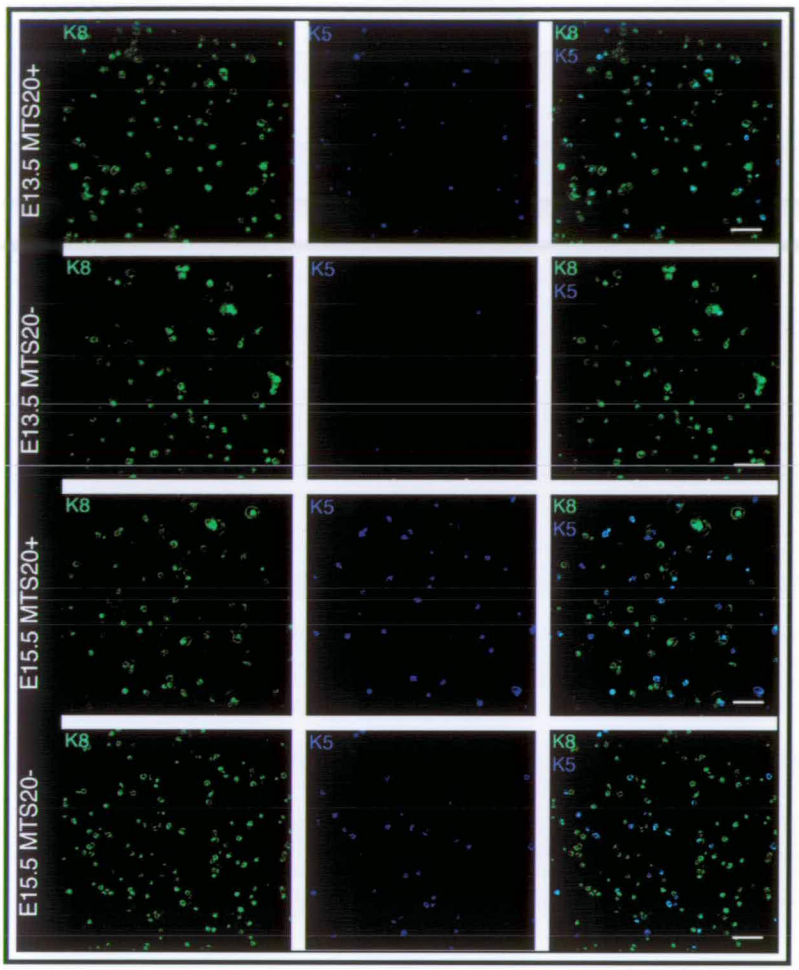

Figure 3.10 Cytospins of sorted populations to reveal K5 and K8 expression

Lineage negative MTS20^{+} and MTS20^{-} TEC populations were sorted and cytospun onto glass slides before fixing and staining, with anti-K5 and anti-K8 mAbs. Scale bar, 100μm. These profiles are representative of three experiments.

Table 3.12 Phenotypic analysis of purified E13.5 MTS20$^+$ cells II

CELLS	EXPERIMENT			MEAN±S.D.
Total cell number (DAPI$^+$)	111	102	74	
K8$^+$	109	100	71	
K5$^+$	42	30	28	
K8$^+$K5$^+$	42	30	28	
% K8$^+$K5$^-$	60.4	68.6	58	62±4.5
% K8$^-$K5$^+$	0	0	0	0
% K8$^+$K5$^+$	37.8	29.4	38	35±4

Flow cytometrically purified E13.5 Lin$^-$MTS20$^+$ cells were cytospun and stained for immunofluorescence analysis.

Table 3.13 Phenotypic analysis of purified E13.5 MTS20$^-$ cells II

CELLS	EXPERIMENT			MEAN±S.D.
Total cell number (DAPI$^+$)	92	49	58	
K8$^+$	82	38	44	
K5$^+$	3	10	8	
K8$^+$K5$^+$	3	10	8	
% K8$^+$K5$^-$	89.1	78	76	81±6
% K8$^-$K5$^+$	0	0	0	0
% K8$^+$K5$^+$	3.3	20	14	12±7

Flow cytometrically purified E13.5 Lin$^-$MTS20$^-$ cells were cytospun and stained for immunofluorescence analysis.

Table 3.14 Phenotypic analysis of purified E15.5 MTS20$^+$ cells II

CELLS	EXPERIMENT			MEAN±S.D.
	1	2	3	
Total cell number (DAPI$^+$)	112	136	116	
K8$^+$	60	118	98	
K5$^+$	34	54	44	
K8$^+$K5$^+$	29	53	41	
% K8$^+$K5$^-$	27.7	48	49	42±9.8
% K8$^-$K5$^+$	4.5	0.7	2.6	3.3±1.5
% K8$^+$K5$^+$	25.9	39	35	33±5.5

Flow cytometrically purified E15.5 Lin$^-$MTS20$^+$ cells were cytospun and stained for immunofluorescence analysis.

Table 3.15 Phenotypic analysis of purified E15.5 MTS20$^-$ cells II

Cells	Experiment			Mean±S.D.
	1	2	3	
Total cell number (DAPI$^+$)	90	94	212	
K8$^+$	65	70	165	
K5$^+$	10	16	33	
K8$^+$K5$^+$	6	16	18	
% K8$^+$K5$^-$	65.6	57	69.3	64.1±5.0
% K8$^-$K5$^+$	4.4	0	7.1	3.8±2.9
% K8$^+$K5$^+$	6.7	17	8.49	10.7±4.5

Flow cytometrically purified E15.5 Lin$^-$MST20$^-$ cells were cytospun and stained for immunofluorescence analysis.

In order to overcome the difficulty of controlling the number and type of cells present in each reaggregate, a cell pellet method was therefore investigated. Cells that were to comprise each reaggregate were mixed to form a cell suspension and added to a well of a 96 well V-bottomed plate. The plate was then centrifuged to pellet the cells at the bottom of each well. After a 24-48 hour incubation period it was clear that although the cells remained as a pellet at the bottom of the well, they did not adhere into a solid structure using this method, and the pellets obtained were easily disturbed and dispersed. It appeared that under these conditions the cells were unable to form or be maintained as a reaggregates.

A possible explanation for these observations was that the location of the cells at the gas-liquid interface is a critical factor in reaggregate formation. Therefore, a method permitting the deposition of pelleted cells on a filter at the gas-liquid interface was devised (Figure 3.13). Thus, a suspension containing the cells to make the reaggregate was drawn into a 200µl pipette tip and the aperture at the apex of the tip blocked using a small piece of folded Parafilm. The blocked pipette tip containing the cell solution was then placed into a 15ml centrifuge tube and centrifuged for 3 minutes at 300xg so that a cell pellet formed at the covered end of the tip. The tip was then removed from the tube and the cell pellet gently pipetted onto a filter paper raft floating on medium in a 6-well plate. This was then cultured overnight to allow reaggregation. Using this method, robust reaggregates formed within 18 hours. These were then transferred to high oxygen submersion cultures (HOS) (Dou et al., 1994) for further incubation.

The pellet-based technique has several advantages over previously published RFTOC protocols. Firstly, the cellularity of RFTOCs produced in this manner can be well controlled, as few cells are lost in the formation of a pellet or remain outside the RFTOC following reaggregation. This allows a consistent and predictable RFTOC size to be achieved. In addition, the system is more amenable to manipulation than the

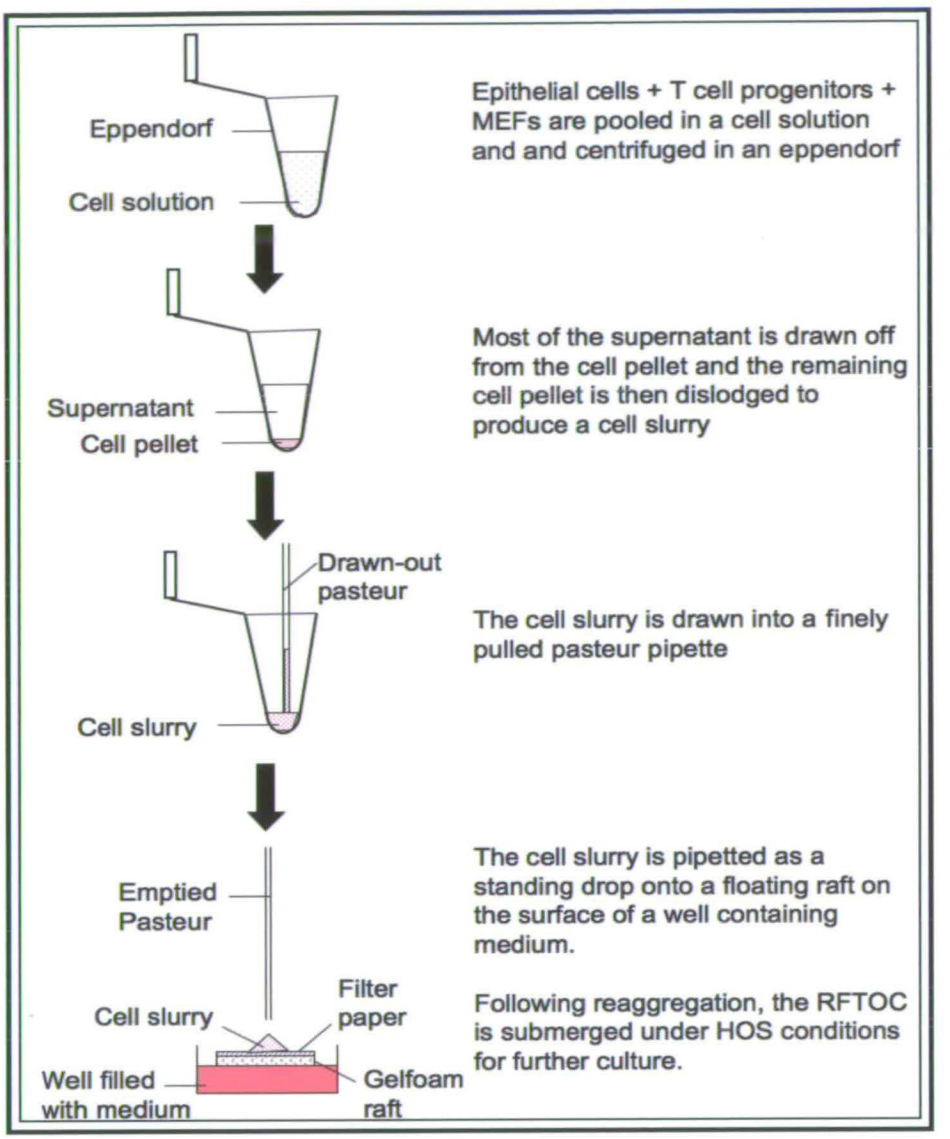

Figure 3.11 Production of a standard standing-drop RFTOC

Cells that will make up the RFTOC are mixed and pelleted. As much of the supernatant as possible is removed and the pellet subsequently disturbed along with the remaining medium, to produce a cell slurry. The cell slurry is pipetted onto a filter paper raft floating on the surface of tissue culture medium using a drawn-out glass pipette (Anderson *et al*, 1993).

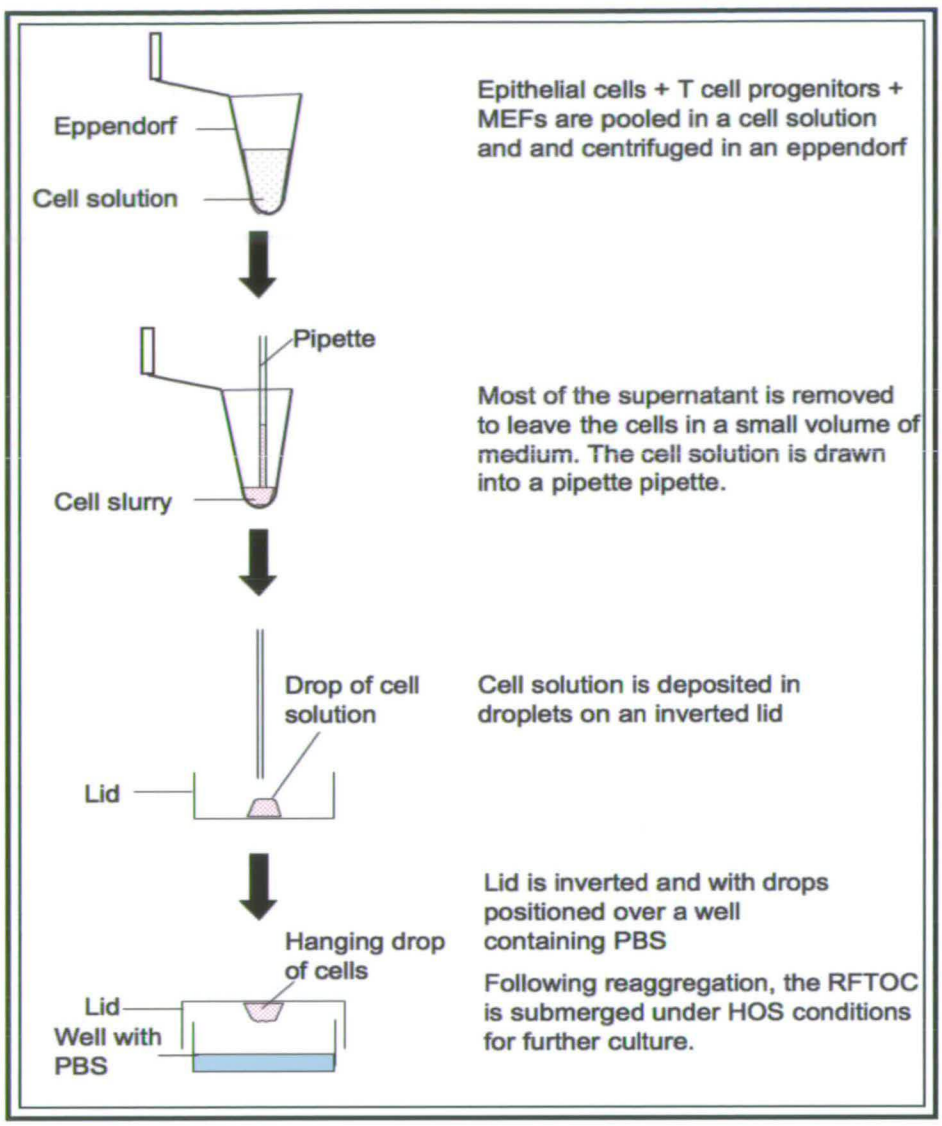

Figure 3.12 Production of a RFTOC by the hanging drop method

Cells that will make up the RFTOC are mixed and centrifuged. As much medium as possible is removed, and the cell pellet resuspended in a small volume of medium. The cell suspension is then pipetted onto the underside of a tissue culture plate lid. The lid is inverted and placed above a well containing PBS so that the cells remain in a hanging drop above the humidified well (Bennett *et al*, 2001).

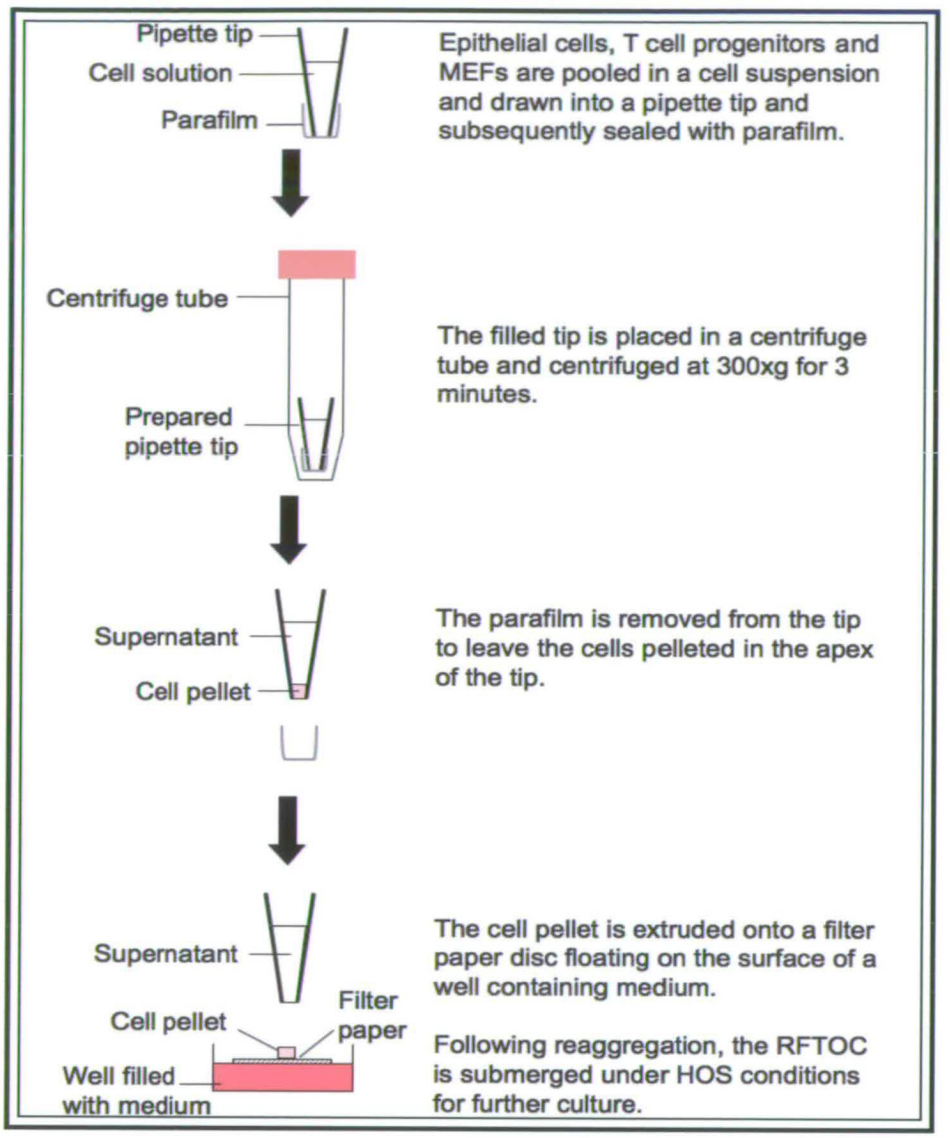

Figure 3.13 Production of a pellet RFTOC

The required cell populations for the RFTOC are mixed and drawn into a pipette tip. The apex of the tip is then sealed with Parafilm before being transferred to a centrifuge tube. Following centrifugation, the tip is removed from the tube and the parafilm is peeled away. The pellet formed during the centrifugation is then deposited onto a filter paper raft floating on the surface of tissue culture medium.

slurry method. Of note is the ability to localise cell types to specific areas within the reaggregate by adding further cells to the pipette tip after the initial centrifugation step, thus creating cell layers. Taken together, the modified RFTOC protocol therefore improved the efficiency and cellular yield of cultured RFTOCs to an extent that allowed individual RFTOCs to be analysed by flow cytometric analsysis, when previously the pooling of several RFTOCs had been required (See Chapter Four).

3.4 Concluding Remarks

Chapter three details the types and location of the various cell types that constitute the fetal thymus. At E13.5, prior to overt thymus organisation, several non-haematopoietic cell types that will eventually complement the epithelial stroma in the adult thymus are already present. Although the roles of observed mesenchymal and vascular cell types in the patterning of the fetal thymus are poorly understood, roles as inducers of epithelial proliferation (Jenkinson et al., 2003; Jenkinson et al., 2007) and possible mediators of epithelial patterning (Anderson et al., 2000) have been reported.

At the stages analysed, the epithelial populations are already heterogeneous and beginning to show MHC Class II expression at E13.5 indicating that some of the epithelial cells are maturing even at this early stage. Although both the $MTS20^+$ and $MTS20^-$ fractions show increasing levels of MHC Class II expression as the thymic lobes mature, the $MTS20^+$ fraction has a consistently smaller MHC Class II^+ population than the respective $MTS20^-$ population, suggestive of a less mature phenotype. There are several other interesting differences between the $MTS20^+$ and $MTS20^-$ populations such as the presence of $UEA1^+$ population restricted to the $MTS20^+$ compartment, and the observed $K14^+$ cells in the $MTS20^-$ fraction. One possible explanation is that these populations represent the divergence of two medullary epithelial cell lineages and therefore could indicate that $MTS20^+$ and $MTS20^-$ cells do not have equivalent differentiation potentials. In summary, the data presented in Chapter Three outlines the

phenotype of cells in the fetal thymus and the composition of populations that will be used for downstream experiments in Chapters Four and Five.

Chapter three provides an overview of the development of an improved RFTOC method, 'the pellet RFTOC', and its uses. Principally adapted from the hanging drop RFTOC method, the use of a pellet to produce the RFTOC structure has several features that make it particularly useful as a means to study T cell development as well as TEC differentiation. The minimisation of cell loss during pellet RFTOC generation enables more accurate input cell counts and improved size consistency between RFTOCs. The formation of a pellet enables cells prior to reaggregation to be tightly packed, which may confer a reaggregation or incorporation advantage over those in RFTOCs formed by a cell slurry method. This may be particularly true when many non-adherent cells such as thymocytes are present as these incorporate poorly and many remain external to the reaggregated structure in standard reaggregation methods. In addition, the greater control afforded by the use of the pellet method could be used to further manipulate the RFTOC structure as the use of repeated cell additions with subsequent centrifugation steps could be used to spatially separate cell types in to cell layers. Taken together, these advantages recommend the pellet method for use in other systems, which currently rely upon the use of the standing drop method.

Taken together, data presented in Chapter Three provides the starting point for detailed analysis of differentiative and functional potential of the $MTS20^{+}$ and $MTS20^{-}$ populations of fetal mouse thymus, which is described in Chapters Four and Five.

Chapter 4: Results: Haematopoietic development within TEPOC

4.1 Introduction

Previous data have shown that E12.5 or E15.5 MTS20$^+$ TEC were able to generate a properly organised functional thymus-like structure upon ectopic transplantation *in vivo* (Bennett et al., 2002; Gill et al., 2002). Surprisingly neither E12.5 nor E15.5 MTS20$^-$ TEC were able to persist in similar grafts and failed to support T cell differentiation *in vivo* (Bennett et al., 2002; Gill et al., 2002). The normal thymic microenvironment is characterised by the presence of several haematopoietic populations including thymocytes, B cells, NK cells and NKT cells, macrophages and DCs as seen in Figure 4.1 panels F, G, H and I, respectively. Recent publications (Bleul et al., 2006; Rossi et al., 2006) have confirmed the hypothesis that a common TEPC gives rise to all TEC subpopulations (Bennett et al., 2002; Blackburn et al., 1996; Gill et al., 2002). This raises the possibility of using TEPC to generate a thymus-equivalent that is able to support T cell differentiation *in vitro*, providing a potentially scaleable system based on a single cell type.

In Chapter Four I optimise conditions permissive for T cell differentiation from adult and fetal HPCs in TEPOC and assess the capacity of TEPOC to support the development of normal intrathymic haematopoietic populations. In addition I investigate the ability of MTS20$^-$ cells to support T cell development *in vitro* using the pellet RFTOC protocol described in Chapter Three.

4.2 In vitro T cell differentiation in the MTS20$^+$cell-based RFTOC system

The optimised RFTOC protocol summarised in Chapter Three was utilised to test the potential of MTS20$^+$ cells harvested from E13.5 thymi to support T cell development *in*

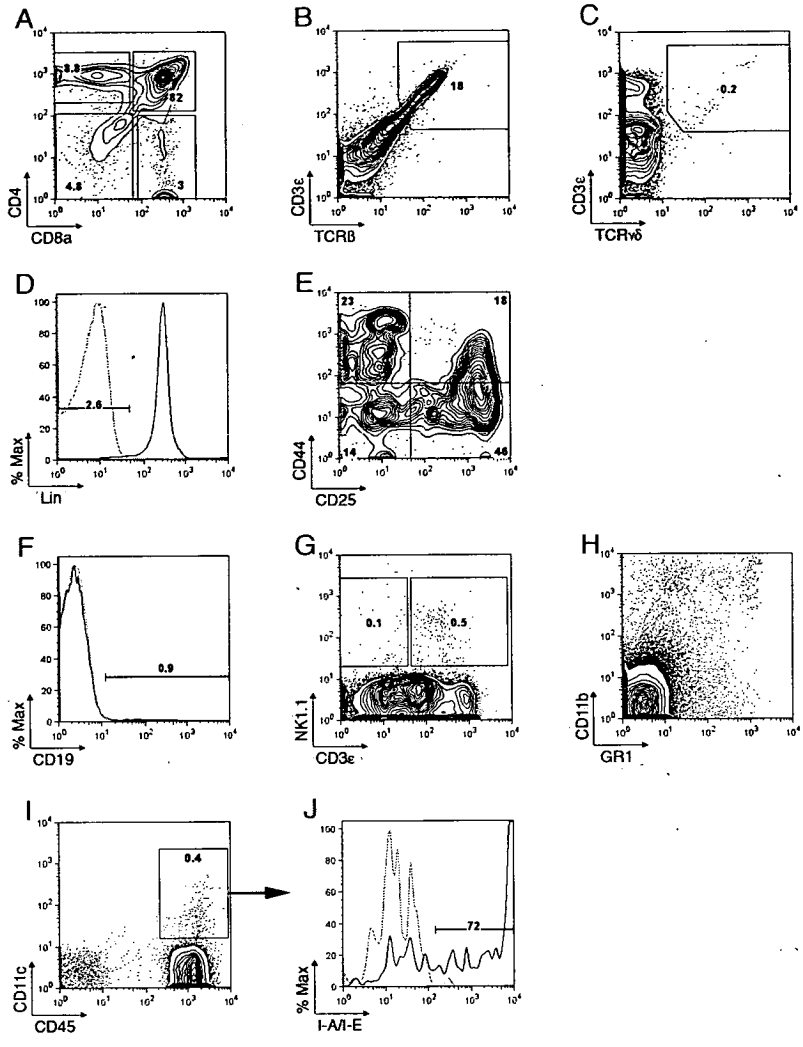

Figure 4.1 Haematopoietic profile of the adult thymus

Cells were mechanically released from a thymus dissected from an 8 week-old mouse. Following appropriate antibody staining, cells were analysed by flow cytometry. Panels A to C, typical antibody staining patterns of developing thymocytes with a large DP population and emerging CD4 and CD8 $TCR\beta^{+}CD3\varepsilon^{+}$ SP thymocytes. Panels D and E, greater than 95% of cells stain with an antibody lineage cocktail that includes CD3ε, CD8a and CD4, and those that remain can be subdivided into four DN populations based on CD44 and CD25 staining. Panels F to J, show the expected minor populations of CD19 $^{+}$ B cells, $NK1.1^{+}CD3\varepsilon^{+}$ NKT cells, cells which stain with Gr-1 and Mac-1 with varying intensity, indicative of non-lymphoid identity as well as $CD11c^{+}$I-A/I-E^{+} dendritic cells.

vitro. Reaggregates established as previously described were routinely analysed after 10 days in culture for the presence of $CD4^+$, $CD8^+$ and $CD3\varepsilon^+$ populations by flow cytometry.

4.2.1 Adult thymocyte development can be supported by TEPC-based RFTOC

In these preliminary experiments, TN thymocytes harvested from 6 to 10 week old mice were used as a source of HPCs. Initially, 50,000 purified $MTS20^+$ cells, 300,000 TN adult thymocytes and 200,000 MEFs were used to form TEPOCs. As shown in Figure 4.2 and Table 4.1, following the 10 day (d10) culture, routinely above 85% of cells harvested from the reaggregates showed light scatter profiles characteristic of lymphocytes (Figure 4.2 and Table 4.1). The presence of DP and $CD4^+$ SP and $CD8^+$ SP indicated maturation of the DN cells had taken place. Some DN cells remained following culture although the exact nature of these remained unconfirmed. The DP and both SP populations expressed low and high levels of CD3ε, respectively, consistent with phenotypes exhibited during intrathymic T cell development. In keeping with previous reports, this T cell differentiation depended on TEC, no DP or SP cells were present in parallel cultures containing 250,000 MEFs and 300,000 TN adult thymocytes. The altered cell input number ensured that the size and therefore set-up of the cultures were approximately the same. Following 10 days of culture, it was often the case that the MEF-only cultures had dissipated or decreased in size. Flow cytometric analysis revealed that most cells within the culture could not exclude 7-AAD and therefore were deemed to be non-viable (Figure 4.2). The small fraction of cells, which appeared viable were invariably $CD4^-$ $CD8^-$ and it was clear that T cell development could not be supported by MEFs alone (Figure 4.2 and Table 4.1). In contrast, the reproducibility of the RFTOC-supported T cell differentiation is demonstrated by Figure 4.3 and Table 4.1. All RFTOCs set up produced viable lymphocytes that contributed up to 96% of total viable cells on the basis of 7AAD exclusion, scatter characteristics and antibody

staining. DP and SP populations were present at varying frequencies, reflecting some variation in maturity level achieved within the RFTOCs during the 10 day culture period. This is best illustrated by the differing percentage of the most mature $CD3\varepsilon^{hi}$ cells among the replicate RFTOCs in Figure 4.3, which varies between 16 and 30%. The robust and reproducible T cell maturation observed in this system is comparable with the widely used fetal thymic organ culture (FTOC), which uses the intact ex-vivo fetal thymic lobes to culture endogenous or exogenous haematopoietic precursors.

4.2.2 Maturation of fetal T cell precursors

4.2.2.1 Differentiation of E13.5 TN1 and 2 thymocytes into T cells.

To test whether TEPOCs were able to support T cell differentiation from fetal HPCs, RFTOCs were established using fetal HPCs. E13.5 thymic lobes can be isolated and maintained in culture and demonstrate robust development of T cells from their resident immature TN thymocytes. Thus, E13.5 thymocytes were purified by FACS from embryonic lobes and added to RFTOCs as a direct replacement for the adult thymocytes. Specifically, 300,000 E13.5 thymocytes were mixed with 50,000 TEPC and 200,000 MEFs and allowed to reaggregate for 18 hours before being transferred to high oxygen submersion for a further 9 days. Unexpectedly, despite the successful reaggregation of these cultures and their normal appearance at early media changes, they did not survive for 10 days (Figure 4.4A). As represented in Table 4.2, repeated experiments indicated that this was a reproducible phenomenon.

In order to address this issue, an attempt was made to better recapitulate the E13.5 thymic environment that is known to be permissive for E13.5 thymocyte maturation. The E13.5 thymus has proportionally far fewer thymocytes than the adult thymus,

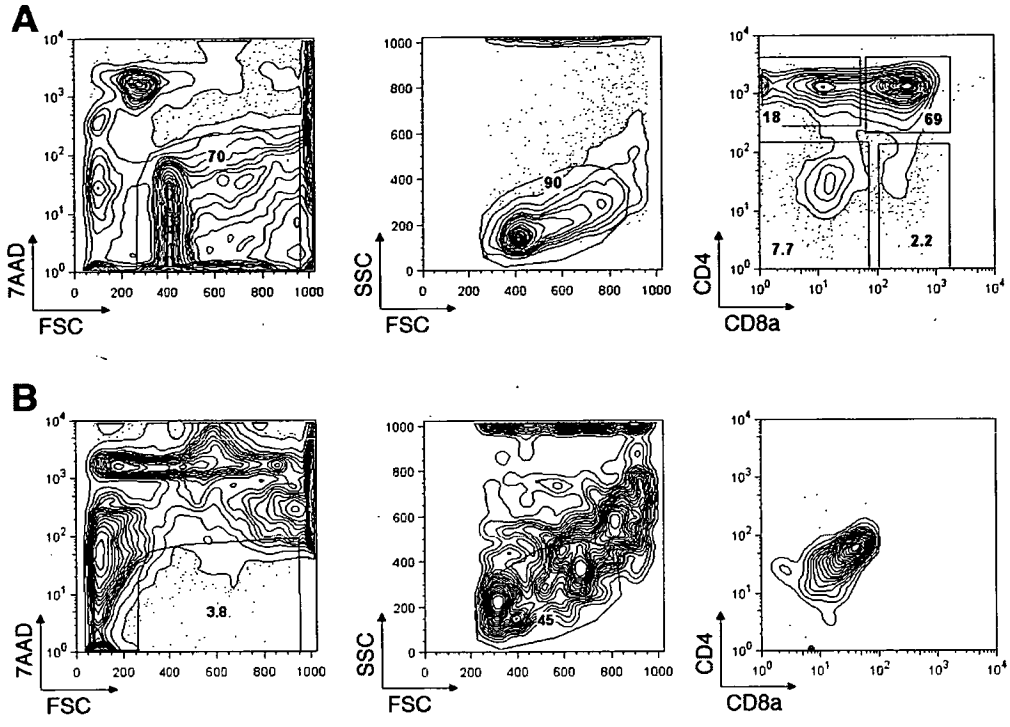

Figure 4.2 TEPCs are responsible for T cell differentiation in TEPC-based RFTOC

TEPOCs were established and allowed to reaggregate before being transferred to HOS. Following a total of 10 days in culture, TEPOCs were mechanically dissociated and stained with appropriate antibodies and 7-AAD for flow cytometric analysis. A, 50,000 TEPCs were reaggregated with 300,000 adult TN thymocytes and 200,000 MEFs. Approximately 90% of viable cells are lymphocytic based on flow cytometric light scatter profiles. Both DP and SP populations are present. B, 250,000 MEFs were reaggregated with 300,000 TN adult thymocytes. Greater than 95% of cells showed uptake of 7-AAD indicating that most if not all cells in the culture had died. The remaining viable cells did not express either CD4 or CD8a.

Table 4.1 Reproducibility of T cell maturation in TEPC-based RFTOC

RFTOC	$MTS20^+$	ADULT TN	MEF	% LYMPHOCYTE	DP PRESENT
RFTOC A	50,000	220,000	150,000	94	Yes
RFTOC B	50,000	240,000	100,000	96	Yes
RFTOC C	50,000	240,000	100,000	94	Yes
RFTOC D	50,000	350,000	100,000	91	Yes
RFTOC E	50,000	300,000	200,000	82	Yes
RFTOC F	50,000	300,000	200,000	62	Yes
RFTOC G	50,000	300,000	200,000	76	Yes
RFTOC H	50,000	300,000	200,000	72	Yes
RFTOC I	50,000	300,000	200,000	84	Yes
RFTOC J	50,000	300,000	200,000	79	Yes
RFTOC K	0	300,000	200,000	2	No
RFTOC L	0	300,000	200,000	-	-
RFTOC M	0	300,000	250,000	-	-

Cell numbers as indicated were mixed and reaggregated for 18 hours before being transferred to HOS for a further 8 or 9 days. T cell production is represented here as the percentage of cells within the RFTOC that are within a lymphocyte gate, as determined by flow cytometric analysis. RFTOC A to RFTOC J all contained TEPCs and show high lymphocyte proportions and contain DP cells, which have matured from the TN precursors. RFTOC K, L and M did not contain TEPCs and following culture, these had either few or no cells within the lymphocyte gate (RFTOC K) or died during culture so that no analysis was possible (RFTOC L and M).

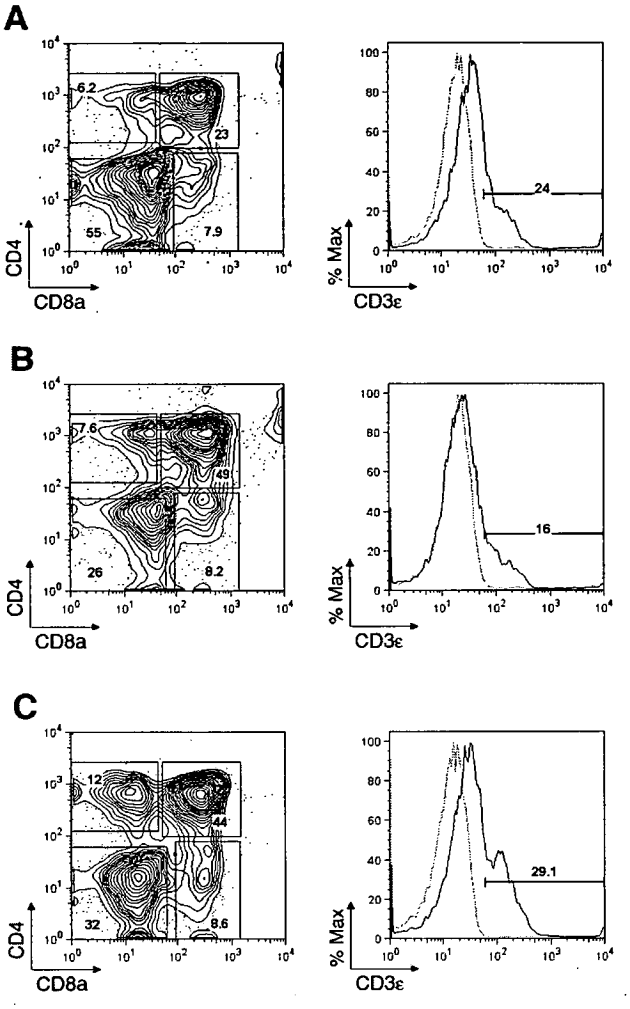

Figure 4.3 Reproducibility of the T cell maturation supported by TEPC-based RFTOC

Flow cytometric analysis of 3 representative RFTOCs established and cultured under identical conditions. In each case, 300,000 TN adult thymocytes were mixed with 50,000 TEPC and 200,000 MEFs and allowed to reaggregate for 18 hours before being transferred to high oxygen submersion for a further 9 days.

giving the E13.5 thymus a high TEC:thymocyte ratio. It was therefore possible that E13.5 thymocytes might require a proportionally higher contribution of epithelial cells thus it would be necessary to provide far more epithelial cells than were present in the failing RFTOCs. Therefore, RFTOCs were established at various HPC:TEC ratios. These experiments demonstrated that RFTOC with HPC:TEC rations close to 1:1 were able to support the differentiation of E13.5 DN cells. These conditions were termed $RFTOC^{high}$, as compared to the $RFTOC^{low}$ conditions established to support adult TN cells.

In the $RFTOC^{high}$, the number of MEFs in the cultures was reduced proportionally to the increased TEC contribution, to compensate for the increase in size afforded by the increased epithelial cell number. This ensured that the cell pellets generated were comparable and thus avoided complications associated with limitations of the tip diameter and dislodging of the pellet during the set up procedure. The reaggregation procedure resulted in a RFTOC of similar size as $RFTOC^{low}$ that were set up in parallel. Flow cytometric analysis following enzymatic dissociation revealed that the $RFTOC^{high}$ could support the development of DN E13.5 thymocytes DP and SP thymocytes, as shown in Figure 4.4B. Virtually all $RFTOC^{high}$ that were set up survived the culture period (Table 4.2). It should be noted that despite the apparent drop in resultant lymphocyte proportions in the RFTOC containing fetal thymocytes compared to those seen in Table 4.1 with adult TN cells, the proportions are not comparable as fewer haematopoietic cells were included in the initial cell pellet. In addition, data presented in Table 4.2 and Figure 4.4 were obtained using an enzymatic harvesting method introduced for the dissociation of the RFTOCs, which released a greater proportion of the non-lymphocytic cell populations than the mechanical method used previously.

4.2.3 Extrathymic fetal T cell precursors mature in RFTOC but require a high contribution of TEPC

To determine whether the TEPOCs were sufficient to support the commitment and differentiation of extrathymic HPC to the T cell lineage, 50,000 TEPC were mixed with 300,000 RBC-depleted E13.5 fetal liver cells (FL) and 200,000 MEFs and cultured as described above. These RFTOC^{low} were unable to support T cell differentiation from FL (Figure 4.5A and Table 4.3). These 'FL-RFTOC^{low}' often died and dispersed during culture, although some remained as a small remnant that could be analysed. Invariably, the cells contained both in the RFTOC remnant and the culture medium were no longer viable, as determined by 7AAD exclusion during flow cytometric analysis. Therefore, the conditions described in 4.2.2.1 as permissive for fetal DN development were utilised. These FL-RFTOC^{high} cultures were viable and gave rise to robust DP populations within 10 days of culture; SP populations were also beginning to develop (Figure 4.5B and Table 4.3). The small number of SPs produced in these cultures likely reflects a differentiation delay, which was expected due to the immature developmental stage of these cells compared to those that are DN1 or DN2 (Figure 4.3, Figure 4.4 and Figure 4.5). As a result, extra culture time would be required to produce mature cell populations of a size comparable with those produced from intrathymic progenitors. As before, the inclusion of TEPCs into the cultures was necessary to enable reaggregate survival and produce DP lymphocytes as determined by 7AAD exclusion, scatter characteristics and antibody staining of cultures containing only MEFs and FL in the absence of MTS20^{+} TEC. (Table 4.3).

Taken together, these data indicate that MTS20^{+} cells can form the basis of an *in vitro* system for supporting T cell differentiation. They further suggest that innate differences between the fetal and adult HPC populations that were used in these experiments exist with respect to the requirements for epithelial support during the differentiation process.

Therefore, it was important to address the developmental stage of the HPCs that were added to RFTOCs.

4.2.4 Adult TN1 &2 thymocytes do not require high-level TEPC contribution to differentiate in RFTOC

Adult DN thymocytes can be subdivided into DN1, DN2, DN3 and DN4 populations based on their expression of CD44 and CD25 (Figure 4.6A). E13.5 thymocytes contain DN1 and DN2 populations only (Figure 4.6B). It was therefore possible that the failure of $RFTOC^{low}$ to support T cell differentiation from E13.5 thymocytes was due to the insufficiency of the $RFTOC^{low}$ conditions to support T cell differentiation from HPCs prior to the DN2 to DN3 transition. To establish whether the ability of $RFTOC^{low}$ to support T cell differentiation was dependent upon the presence of cells beyond DN2, $RFTOC^{high}$ were set up containing 200,000 adult DN1 and 2 thymocytes, 200,000 TEPC and 100,000 MEFs. As expected, following culture, these precursors matured to produce DP and SP populations (Figure 4.7B). However, $RFTOC^{low}$ containing 300,000 adult DN1 and 2 thymocytes, 50,000 TEPC and 200,000 MEFs also produced DP and SP cells, indicating that the absence of DN3 and DN4 in the E13.5 thmocytes could not explain the differences observed between adult and fetal HPCs (Figure 4.7A). The principle difference between these conditions was that with increased TEPC input, the RFTOCs became more robust with a greater proportion of RFTOCs surviving culture. In addition, in those reaggregates that survived culture, far more lymphocytes were seen in $RFTOC^{high}$ than $RFTOC^{low}$ and showed a greater level of maturity (Figure 4.7B and Table 4.4).

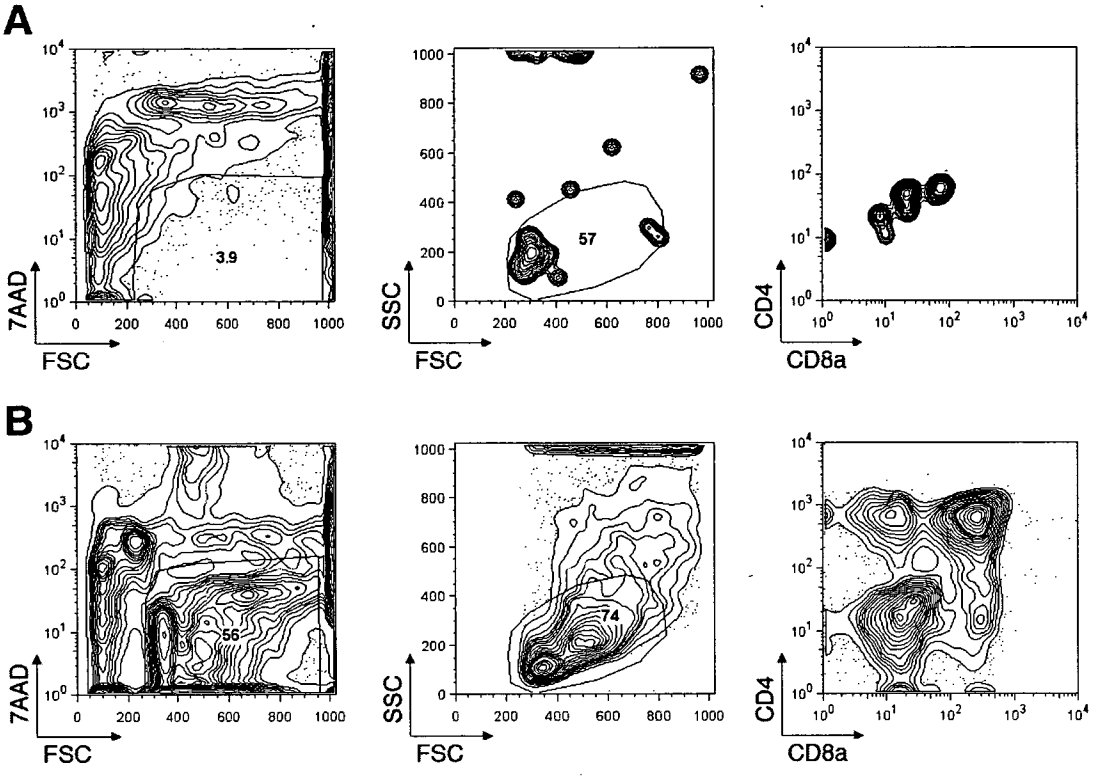

Figure 4.4 Maturation of fetal thymocytes is supported by RFTOC but requires a high proportion of TEPCs

Following reaggregation and a further 9 days HOS culture, TEPOCs were enzymatically dissociated and stained with suitable antibodies for flow cytometric analysis. A, 50,000 TEPCs were reaggregated with 150,000 E13.5 thymocytes and 200,000 MEFs. Following culture, most cells in this TEPOC are inviable (7-AAD+) and any viable cells were invariably CD4-CD8a-. B, 190,000 TEPCs were reaggregated with 200,000 E13.5 thymocytes and 120,000 MEFs. Following culture, most cells had light scatter lymphocytes and both DP and SP populations of thymocytes had developed.

Table 4.2 RFTOCs that have a low ratio of TEPC:HPC are unable to support E13.5 fetal thymocyte development

RFTOC	TEPC	E13.5 THYMOCYTES	MEFS	PERCENTAGE LYMPHOCYTES	DP?
RFTOC A	50,000	150,000	200,000	1	No
RFTOC B	100,000	275,000	150,000	1	No
RFTOC C	75,000	250,000	100,000	2	No
RFTOC D	170,000	100,000	100,000	55	Yes
RFTOC E	170,000	100,000	100,000	50	Yes
RFTOC F	170,000	100,000	100,000	30	Yes
RFTOC G	190,000	200,000	120,000	74	Yes
RFTOC H	200,000	200,000	100,000	65	Yes
RFTOC I	200,000	200,000	100,000	42	Yes
RFTOC J	210,000	200,000	100,000	82	Yes
RFTOC K	220,000	200,000	100,000	78	Yes
RFTOC L	230,000	200,000	100,000	80	Yes
RFTOC M	230,000	200,000	100,000	85	Yes

Cell numbers as indicated were mixed and reaggregated for 18 hours before being transferred to HOS for a further 8 or 9 days. T cell productivity is represented here as the percentage of cells within the RFTOC that are within a lymphocyte gate, as determined by flow cytometric analysis. The presence of DP thymocytes demonstrates T cell differentiation. RFTOCs A to C contain relatively low TEPC: HPC ratios and most cells are non-viable following culture. RFTOC D to M shows that RFOCs with higher TEPC:HPC ratios (approximately 1:1) result in robust T cell development.

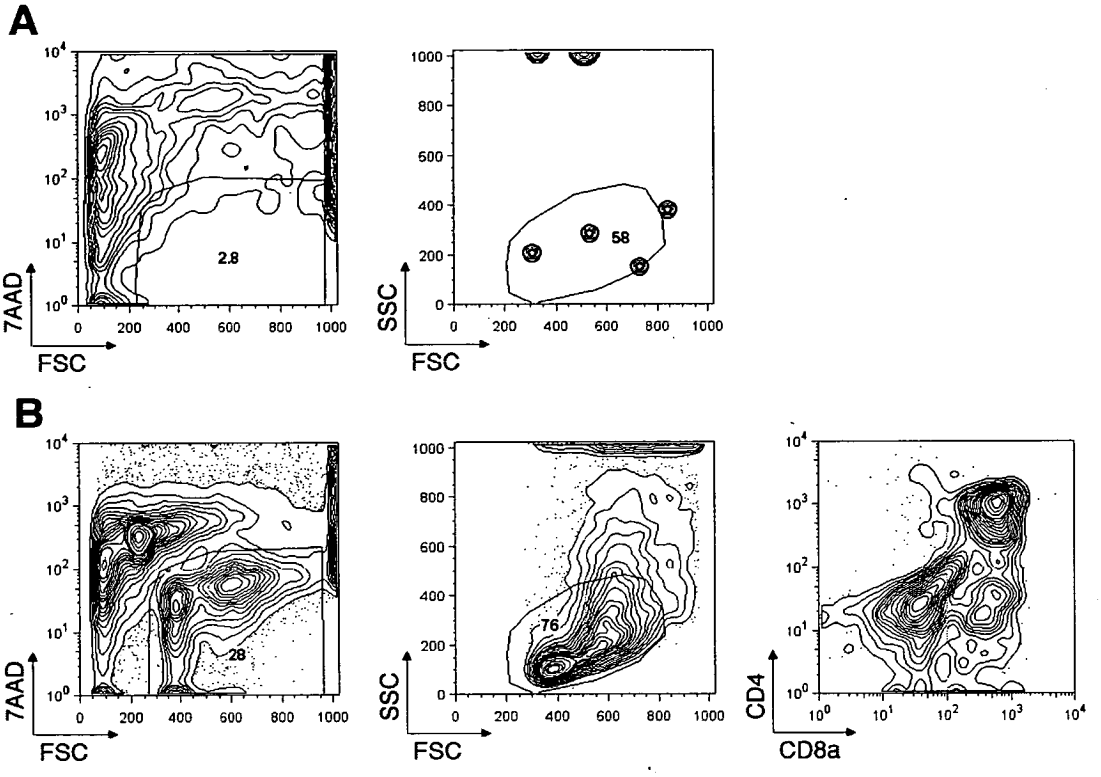

Figure 4.5 Maturation of E13.5 fetal liver cells is supported by RFTOC but requires a high proportion of TEPCs

Flow cytometric analysis of representative examples of TEPOC illustrating the viability, size and staining pattern with anti-CD4 and anti-CD8a mAbs. A, 50,000 TEPCs were added to 300,000 E13.5 (red blood cell-depleted) FL cells and 150,000 MEFs. The cells comprising this RFTOC had died during culture so that very few viable cells were present during analysis. B, 200,000 TEPCs were mixed with 200,000 E13.5 (RBC-depleted) FL cells and 100,000 MEFs. Typical of a RFTOC with high TEPC:HPC ratio, this TEPOC contains a large lymphocyte population, which contains both DP and emerging SP populations.

Table 4.3 RFTOC that have a low TEPC:HPC contribution are unable to support T cell development from E13.5 fetal liver cells

RFTOC	TEPC	FL (RBC-DEPLETED)	MEF	PERCENTAGE LYMPHOCYTES	DP?
RFTOC A	50,000	300,000	150,000	3	No
RFTOC B	50,000	300,000	150,000	1	No
RFTOC C	50,000	300,000	150,000	2	No
RFTOC D	200,000	200,000	100,000	76	Yes
RFTOC E	200,000	200,000	100,000	86	Yes
RFTOC F	200,000	200,000	100,000	88	Yes
RFTOC G	200,000	200,000	100,000	82	Yes
RFTOC H	0	300,000	200,000	2	No
RFTOC I	0	300,000	200,000	1	No
RFTOC J	0	300,000	200,000	6	No

Cell numbers as indicated were mixed and reaggregated for 18 hours before being transferred to HOS for a further 8, 9 or 10 days. T cell productivity is represented by the percentage of cells within a lymphocyte gate as determined by flow cytometric scatter analysis. The presence of DP thymocytes demonstrates T cell differentiation. RFTOCs A to C, RFTOCs with a low TEPC:HPC ratio do not support T cell differentiation from E13.5 FL cells and most cells die during the culture period.RFTOCs D to G, RFTOCs with high TEPC:HPC ratio support robust lymphocyte development. RFTOCs H to J demonstrate that without the addition of TEPCs, the RFTOCs die during culture and do not support T cell differentiation.

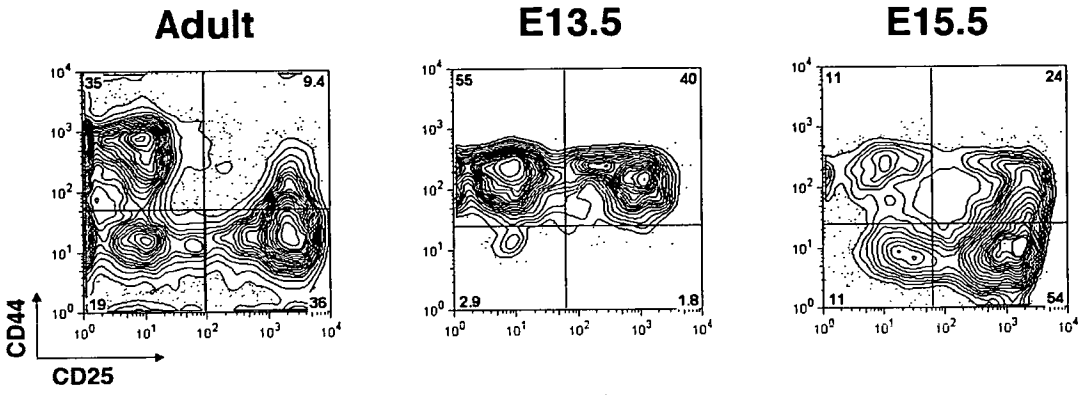

Figure 4.6 Representative flow cytometric analysis of DN thymocytes in adult and embryonic thymi

Thymic cells in single cells suspensions were stained with anti-CD25 and anti-CD44 mAbs to demarcate DN populations. Adult thymocytes gated against lineage positive cells can be seen to have DN1, DN2, DN3 and DN4 subsets. Total E13.5 thymocytes can be seen to be made up of DN1 and DN2 only and lack major DN3 and DN4 compartments. E15.5 thymocytes gated against a lineage cocktail can be seen to contain DN1 to DN4 populations.

4.2.4.1 E15.5 thymocytes do not require high-level TEPC contribution to mature in RFTOC

The above data indicated different requirements for TEC represented an innate difference between the adult and fetal HPCs. To determine when this change occurred, the outcome of culturing E15.5 thymocytes, which contain DN1, DN2, DN3 and DN4 populations as shown in Figure 4.6C, were tested for differentiation potential in the RFTOC. E15.5 thymocytes were co-cultured in RFTOCs of both high and low TEPC contribution. In contrast to E13.5 fetal thymocyte RFTOCs, Figure 4.8 illustrates the robust T cell development that was seen in both $RFTOC^{low}$ and $RFTOC^{high}$, with a very high proportion of cells being $TCR\beta^{hi}CD3\varepsilon^{hi}$, consistent with a mature phenotype. The survival of the E15.5 thymocyte RFTOCs demonstrated that the difference in HPC potential occurred between E13.5 and E15.5. This time frame correlates with the reported end of first wave thymus seeding and the proposed beginning of another. It is thus possible that the conditions of the $RFTOC^{low}$ are not able to support T cell differentiation from the cells that seed the fetal thymus prior to E13.5 as these have requirements that are only met by the $RFTOC^{high}$. Another possibility is that E13.5 thymocytes are less proficient at supporting TEC survival than the other HPCs tested.

4.2.4.2 TEPOC support T cell differentiation from adult thymus-derived DN1 precursors

In order to determine whether thymocyte differentiation in the $RFTOC^{high}$ system followed the normal developmental progression, the earliest stages of thymocyte differentiation were investigated in $MTS20^{+}$ cell-based RFTOC seeded with DN1 cells from postnatal thymus. Thus, 200,000 TEPC were reaggregated with 200,000 DN1 cells conditions, isolated from the thymus of five week-old mice and 100,000 MEFs, under the optimised conditions established in Chapter Four. Following three further days

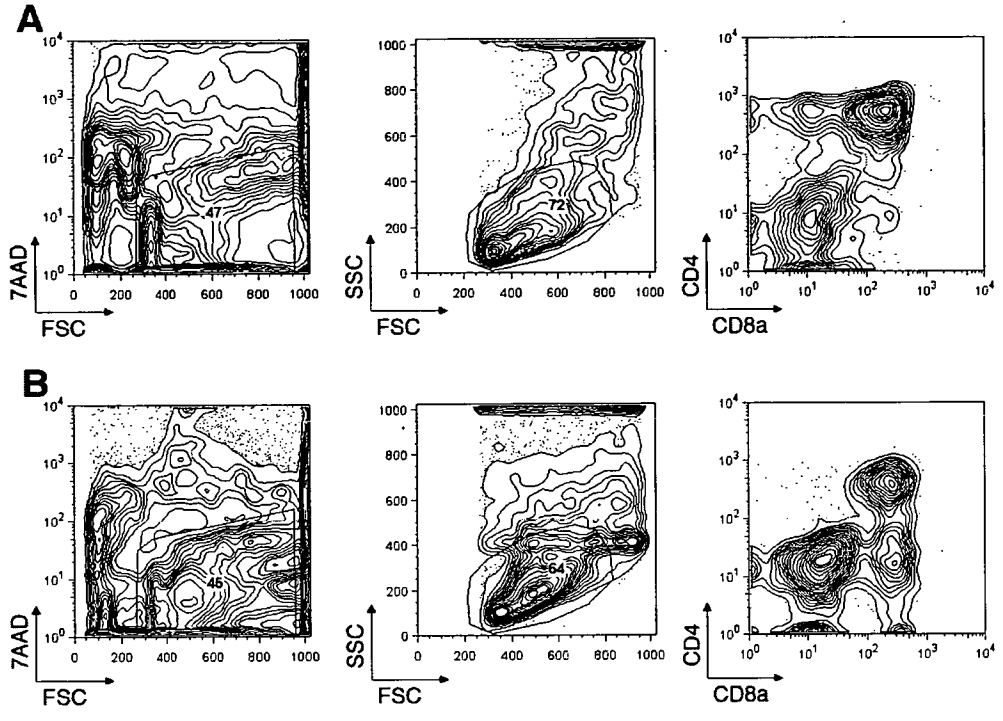

Figure 4.7 Maturation of Adult TN1&2 thymocytes is supported by RFTOC and is not dependent upon a high TEPC input

Flow cytometric analysis of representative examples of TEPOC, illustrating the viability, size and staining pattern with anti-CD4 and anti-CD8a mAbs. A, 200,000 TEPCs were mixed with 200,000 thymocytes and 100,000 MEFs. B, 50,000 TEPCs were added to 300,000 TN1&2 thymocytes and 200,000 MEFs. Both RFTOC^{high} and RFTOC^{low} contain a large lymphocyte population and have developing DP and SP populations although the CD4 SP population is greatly reduced with the lower TEPC:HPC ratio.

Table 4.4 T cell development from TN1&2 thymocytes is supported by the RFTOC system and does not require high-level TEPC contribution

RFTOC	TEPC	TN 1&2	MEFS	VIABLE CELLS	% LYMPHOCYTE	DP
RFTOC A	50,000	300,000	200,000	22	64	Yes
RFTOC B	50,000	300,000	200,000	35	75	Yes
RFTOC C	50,000	300,000	200,000	1	7	No
RFTOC D	50,000	300,000	200,000	59	44	Yes
RFTOC E	180,000	200,000	120,000	64	91	Yes
RFTOC F	190,000	200,000	120,000	0	0	No
RFTOC G	200,000	200,000	100,000	68	87	Yes
RFTOC H	200,000	200,000	100,000	60	88	Yes
RFTOC I	200,000	200,000	100,000	40	74	Yes
RFTOC J	200,000	200,000	100,000	48	84	Yes
RFTOC K	220,000	160,000	100,000	54	68	Yes
RFTOC L	200,000	200,000	100,000	61	81	Yes
RFTOC M	200,000	200,000	100,000	63	75	Yes

Cell numbers as indicated were mixed and reaggregated for 18 hours before being transferred to HOS for a further 8 or 9 days. Cell viability was determined by scatter profiles and the exclusion of 7AAD. T cell productivity is represented here as the percentage of cells within the RFTOC that are within a lymphocyte gate as determined by flow cytometric analysis. The presence of DP thymocytes demonstrates T cell differentiation. RFTOCs A - D contain relatively low numbers of TEPCs and cell viability is generally lower ranging from with a smaller proportion of lymphocytes. RFTOCs E - M demonstrate that the inclusion of higher numbers of TEPCs results in more robust T cell development

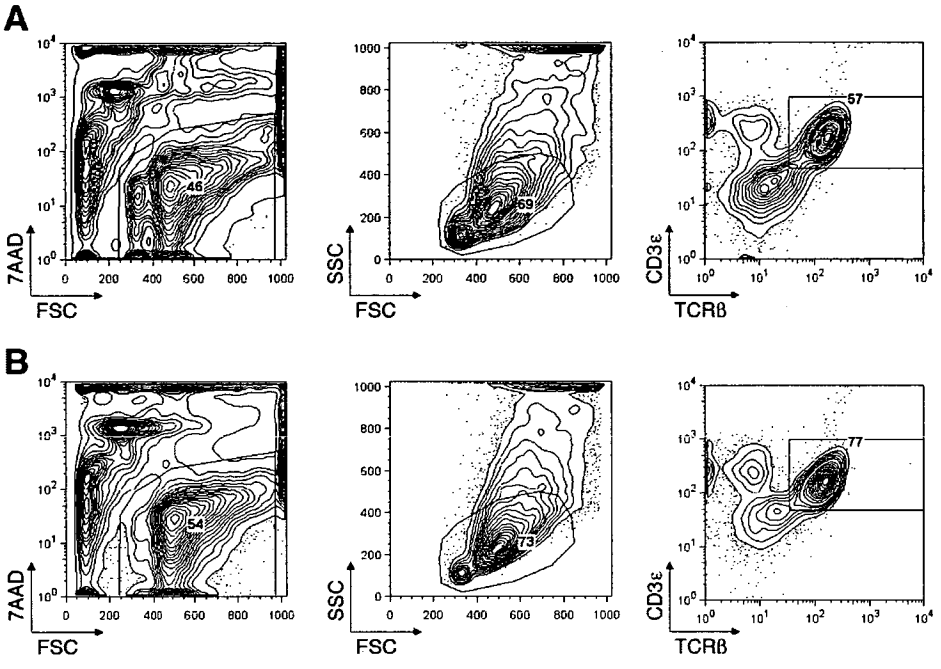

Figure 4.8 Maturation of E15.5 DN fetal thymocytes is supported by RFTOC and is not dependent upon a high TEPC input

Flow cytometric analysis of representative examples of RFTOC illustrating the viability, size and staining pattern with anti-TCRβ and anti-CD3ε mAbs. A, 50,000 TEPCs were added to 300,000 E15.5 DN thymocytes and 200,000 MEFs. B, 200,000 TEPCs were mixed with 200,000 E15.5 DN thymocytes and 100,000 MEFs. Both $RFTOC^{high}$ and $RFTOC^{low}$ contain a large lymphocyte population and have mature $CD3\varepsilon^{hi}$ $TCR\beta^{hi}$ populations indicating successful and comparable T cell differentiation capabilities.

under HOS RFTOCs were enzymatically dissociated and stained with antibodies against CD45 and markers of differentiating thymocytes. Figure 4.9 shows flow cytometric analysis of two representative TEPOCs established under these conditions. At day 4, populations of DN1, DN2, DN3 and DN4 cell populations are present suggesting that T cell differentiation proceeds normally in TEPOC culture. A small DP population was also evident in these cultures, although no SP populations were yet apparent. Notably, many cells remained in the DN1 population at this stage.

4.2.5 Haematopoietic development from adult-derived DN thymocytes

To investigate whether the normal array of haematopoietic cell types were supported by the TEPOCs, 200,000 TEPC were reaggregated with 200,000 Lin^- TN cells isolated from the thymi of postnatal (6-8 weeks old) mice and 100,000 MEFs under the optimised conditions established in Chapter Four.

Large DP populations were seen in all TEPOC at d10 of culture, and in most cases, constituted the majority of cells (Figure 4.10A and B). A $TCR\beta^{hi}CD3\varepsilon^{hi}$ population was always present and both CD4 SP and CD8 SP cells developed during culture (Figure 4.10A, B and C). Typically more $CD8^+$ $CD4^-$ cells than $CD4^+CD8^-$ cells were produced under these $RFTOC^{high}$ conditions, although many $CD4^-CD8^+$ cells were $CD3\varepsilon^{-/low}$ identifying these as immature single positives rather than mature CD8 SP cells. As shown in Figure 4.10C, large immature SP populations are most commonly seen in RFTOC, which have a lower DP frequency and a large DN fraction, consistent with these RFTOCs being developmentally less mature than other RFTOC examples made under identical conditions (see Figure 4.10A and B). In addition, $\gamma\delta^+CD3\varepsilon^+$ cells were present in all RFTOCs, typically at a frequency of less than 6% (Figure 4.10). Although the proportion of γδ T cells produced from DN cells was much higher in TEPOCs (Approximately 5%) (Figure 4.10) than in wild type thymus (Less than 1%) (Figure 4.1C), the number of $\gamma\delta^+$ cells always remained several fold lower than the number of $\alpha\beta^+$ cells (Figure 4.10). In some cultures, substantially elevated numbers of $\gamma\delta^+$ cells

were observed (Figure 4.10C) and in these cases an unusually large DN population were also present. TEPOCs were also analysed for the presence of other haematopoietic populations (Figure 4.11). When compared with wild type adult thymus, relatively high numbers of $NK1.1^{+}CD3\varepsilon^{+}$ cells were observed within TEPOC (Figure 4.1G and Figure 4.11A). As would be expected from an adult precursor population, the expression of TCR chains that are fetally restricted *in vivo*, such as TCR Vγ3 were not found in RFTOCs seeded with adult-derived DN thymocytes and few or no mature $CD19^{+}$ B cells were present (Figure 4.11A). The presence of $CD11c^{+}$ haematopoietic cells, which express high levels of MHC Class II, is indicative of the development or maturation of dendritic cells (Figure 4.11B). These data establish that TEPOC support development of all normal intrathymic populations. However, the reason for the observed development of the unexpectedly low αβ:γδ lineage ratio was affected by the RFTOC conditions. To gain a clearer picture of αβ:γδ lineage ratio attained in the TEPC-RFTOC system, TEPOCs were also seeded with postnatal DN1 and DN2 progenitors, a stage when HPCs have not yet completed TCRβ gene rearrangement and are not committed to the αβ lineage. Thus, RFTOCs containing 200,000 TEPC, 200,000 DN1&2 and 100,000 MEFs were generated, maintained for a further 9 days under standard conditions then analysed for the presence of appropriate haematopoietic cell types by flow cytometric analysis.

Haematoietic differentiation in these cultures was essentially as described above (Figure 4.10 to Figure 4.11), except that proportionally more DN cells were present after 10 days in culture (Figure 4.10 to Figure 4.13). The αβ:γδ lineage ratios obtained in TEPOC seeded with TN thymocytes (17.5±2.5)(Figure 4.10) was several fold greater than that produced in TEPOCs cultured for 10 days with adult-derived DN1-2 thymocytes (6.4±1.7)(Figure 4.12). This difference may be due to either an increase in the number of γδ cells generated from DN1 and 2 cells or may represent reduced

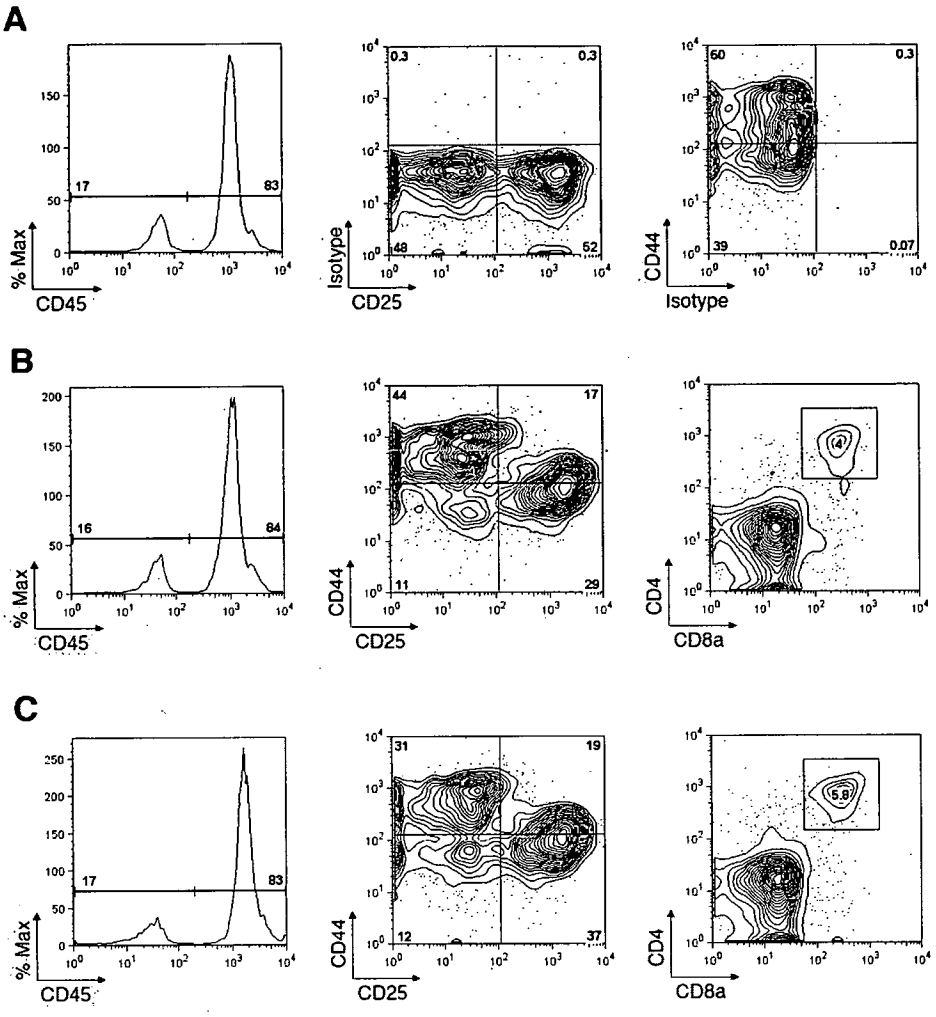

Figure 4.9. DN2, DN3 and DN4 thymocytes develop from DN1 cells in TEPOCs

Purified MTS20^{+} cells, postnatal DN1 thymocytes and MEFs were allowed to reaggregate at the gas-liquid interface for 18 hours before transfer to HOS for a further 72 hours after which they were harvested and processed for flow cytometric analysis. A, staining with anti-CD45 and isotype control mAbs against CD44 and CD25. B and C, examples of TEPOCs showing the development of DN2, DN3, DN4 and DP populations from DN1 thymocytes. Results are representative of at least three experiments.

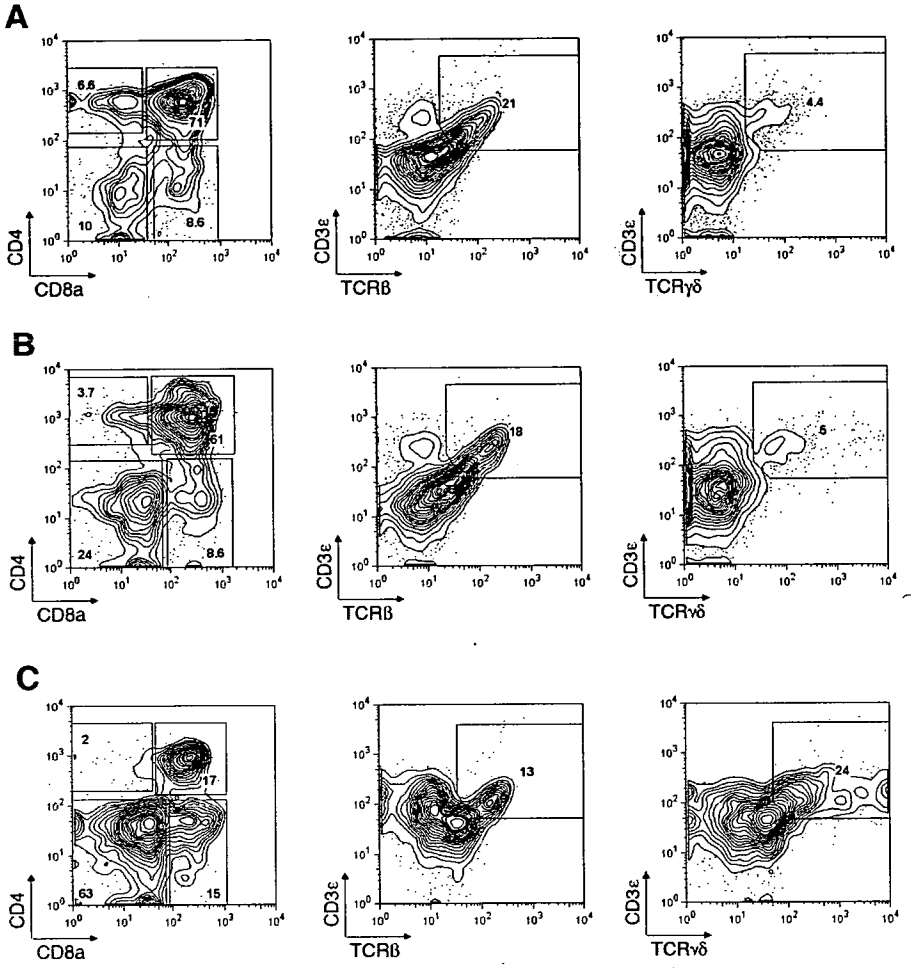

Figure 4.10 TEPOC-support $CD4^+$ and $CD8^+$ αβ as well as γδ T cell differentiation from adult TN thymocytes

200,000 E13.5 TEPCs, 200,000 Lin^- adult TN thymocytes and 100,000 MEFs were reaggregated for 18 hours, then transferred to HOS and maintained for a further 9 days. Following enzymatic dissociation, the cells were stained with appropriate antibodies and analysed by flow cytometry. A-C, replicate RFTOCs showing the development of DP and SP populations from DN progenitors. A large $TCR\beta^+$ $CD3\varepsilon^+$ population illustrates the presence of mature αβ T cells. In addition, a variable number of $CD3\varepsilon^+TCR\gamma\delta^+$ T cells are also produced.

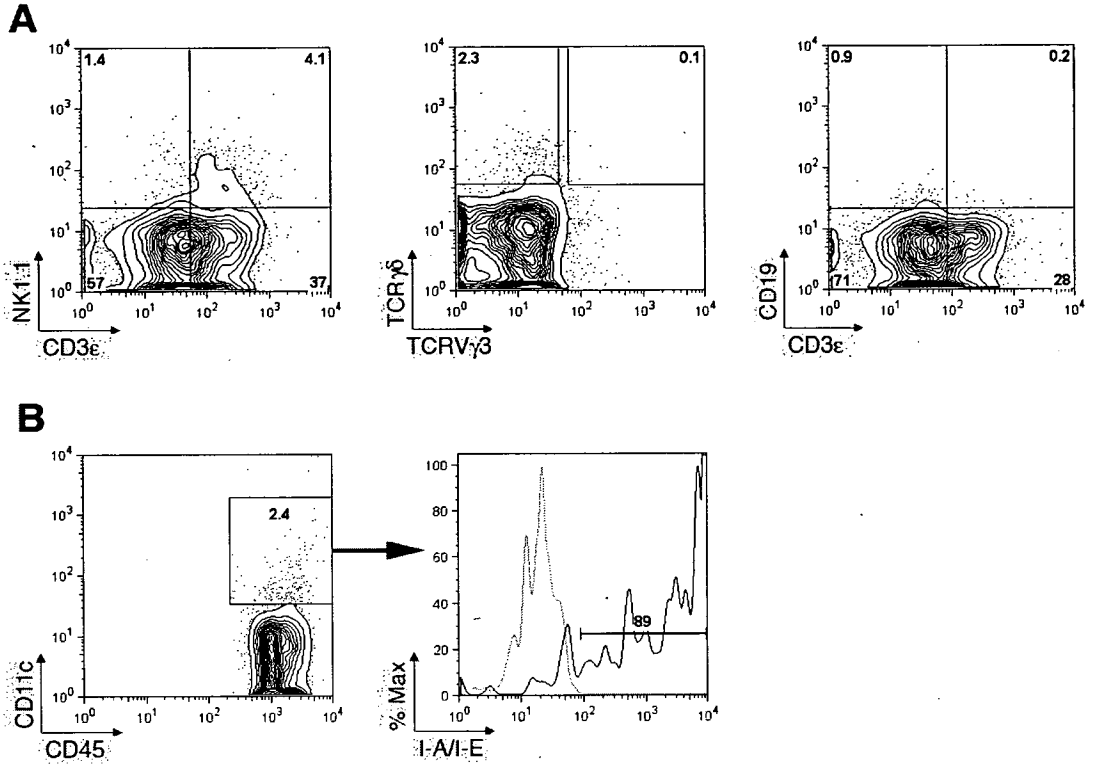

Figure 4.11 TEPOC^{high} support the development of all normal haematopoietic populations of the thymus from adult TN thymocytes

200,000 E13.5 TEPCs, 200,000 Lin$^-$ thymocytes and 100,000 MEFs were reaggregated for approximately 18 hours, then transferred to HOS and maintained for a further 9 days. Following enzymatic dissociation cells were analysed by flow cytometry. A, NK1.1$^+$CD3ε$^+$ NKT cells can be identified along with a small number of NK1.1$^+$CD3ε$^-$ cells. Few, if any, CD19$^+$ B cells are present. Analysis shows populations following electronic gating for lymphocyte populations. B, CD45$^+$ dendritic cells expressing CD11c and high-level MHC Class II are also generated.

numbers of αβ lineage cells being generated due to time constraints upon the system. In either case, the αβ:γδ ratio appears low compared to wildtype adult thymus (188:1)(Figure 4.1).

4.2.6 TEPOCs support the differentiation αβ and γδ T cells from fetal thymocytes

To determine whether similarly appropriate haematopoietic populations, and in particular, fetal-thymus specific T cell populations develop in fetal thymocyte seeded TEPOCs, reaggregates containing 200,000 TEPC, 200,000 E13.5 thymocytes and 100,000 MEFs were generated and analysed for the presence of various haematopoietic cell types after 9 days in culture. As shown by the three examples in Figure 4.14A, B and C, in contrast to RFTOCs seeded with adult-derived thymocytes, the relative proportions of the $CD4^+CD8^-$ and $CD4^-CD8^+$ populations after 10 days of culture varies considerably between individual RFTOCs so that a 'typical' output is difficult to describe. However, a large $CD4^-CD8^+$ population is routinely present although the relatively small $TCR\beta^+CD3\varepsilon^+$ population suggests that these are not mature SPs. A large $TCR\gamma\delta^+CD3\varepsilon^+$ T cell population is always present, which contributes to the large DN population seen in E13.5 thymocyte seeded RFTOCs(Figure 4.14). Figure 4.15 demonstrates that other haematopoietic cell types normally found in the thymus develop from E13.5 thymocytes, including $NK1.1^+$ $CD3\varepsilon^+$ NKT cells and $NK1.1^+CD3\varepsilon^{-/low}$ cells, which are present in appreciable numbers. All RFTOCs seeded with E13.5 thymocytes produced $TCRV\gamma3^+$ γδ T cells (Figure 4.15A), consistent with the TCR repertoire that would be expected to result from the differentiation of fetal thymocytes *in vivo* (Ikuta et al., 1992). $CD11c^+MHCII^{hi}$ DCs were also present in the cultures, and the presence of $CD11b^+$ population possibly corresponds with the presence of a small number of thymic macrophages (Figure 4.15C).

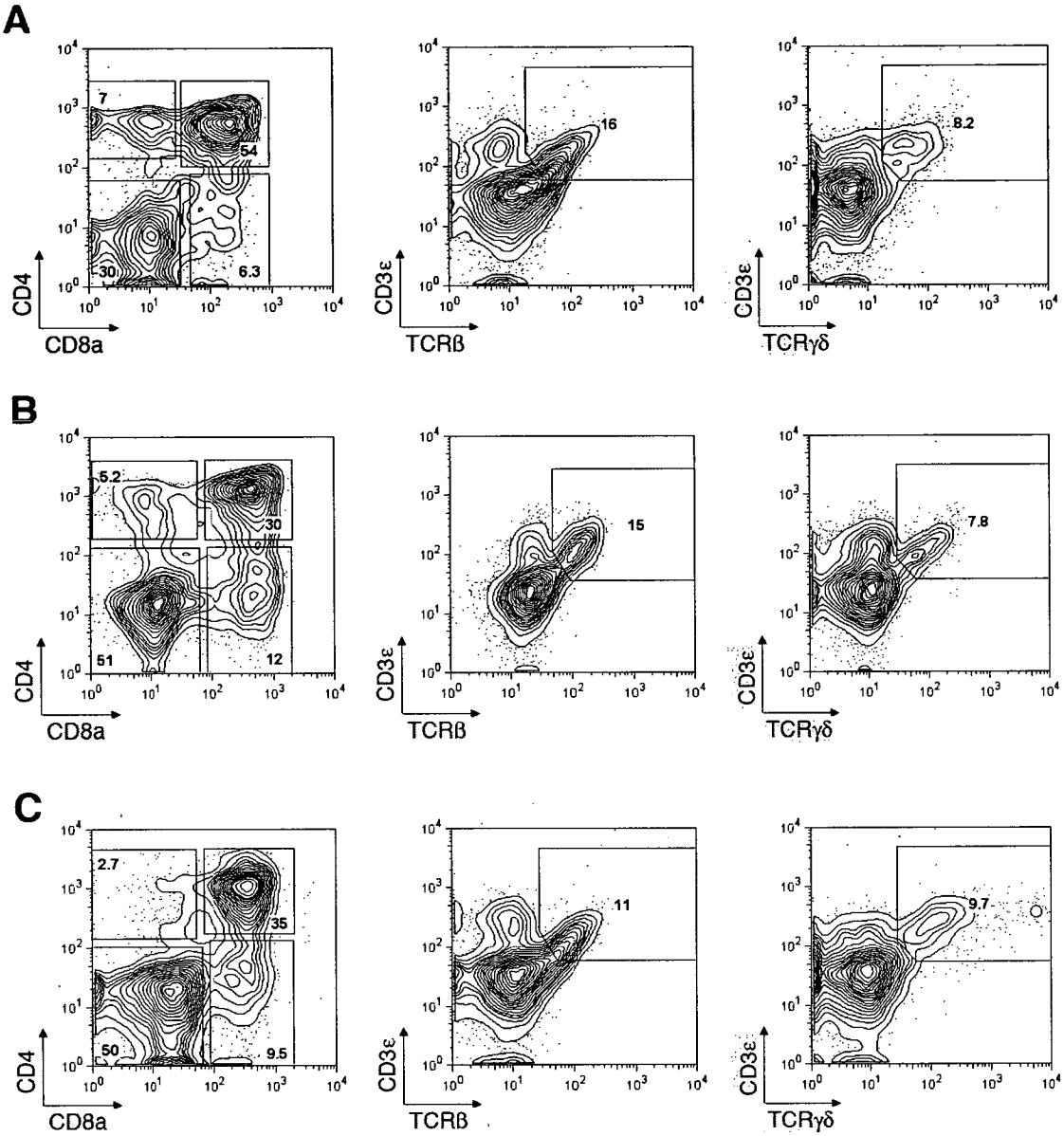

Figure 4.12 TEPOC supports CD4$^+$ and CD8$^+$ αβ as well as γδ T cell differentiation from adult TN1 and 2 thymocytes

200,000 E13.5 TEPCs were mixed with 200,000 Lin$^-$ TN1 and TN2 thymocytes and 100,000 MEFs and reaggregated for 18 hours. TEPOCs were then transferred to high oxygen culture and maintained for a further 9 days. Following enzymatic dissociation cells were analysed by flow cytometry. A-C, replicate RFTOCs showing the development of DP and SP populations from TN1 and TN2 progenitors. A large TCRβ$^+$ CD3ε$^+$ population illustrates presence of mature αβ T cells. A consistently large proportion of TCRγδ$^+$ CD3ε$^+$ population is always present.

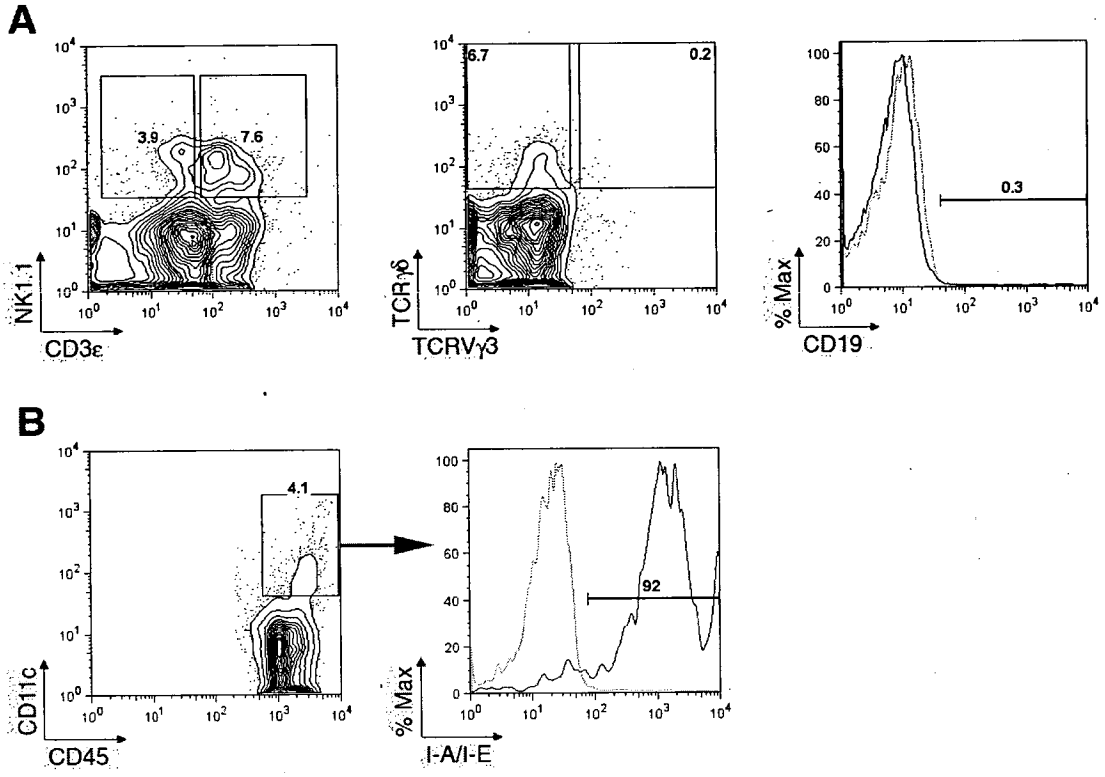

Figure 4.13 TEPOC-supported haematopoietic development from adult-derived TN1 and TN2 thymocytes

200,000 E13.5 TEPCs were mixed with 200,000 Lin^- $CD44^+$ thymocytes and 100,000 MEFs and then reaggregated for 18 hours. TEPOCs were then transferred to HOS conditions and maintained for a further 9 days. Following enzymatic dissociation cells were stained with suitable antibodies and analysed by flow cytometry. A, cells within a lymphocyte scatter gate include NKTs and $NK1.1^+CD3\varepsilon^{lo}$ cells. Few or no B cells or $TCRV\gamma3^+$ cells were identified. B, $CD11c^+MHCII^{hi}$ dendritic cells were present in these TEPOC.

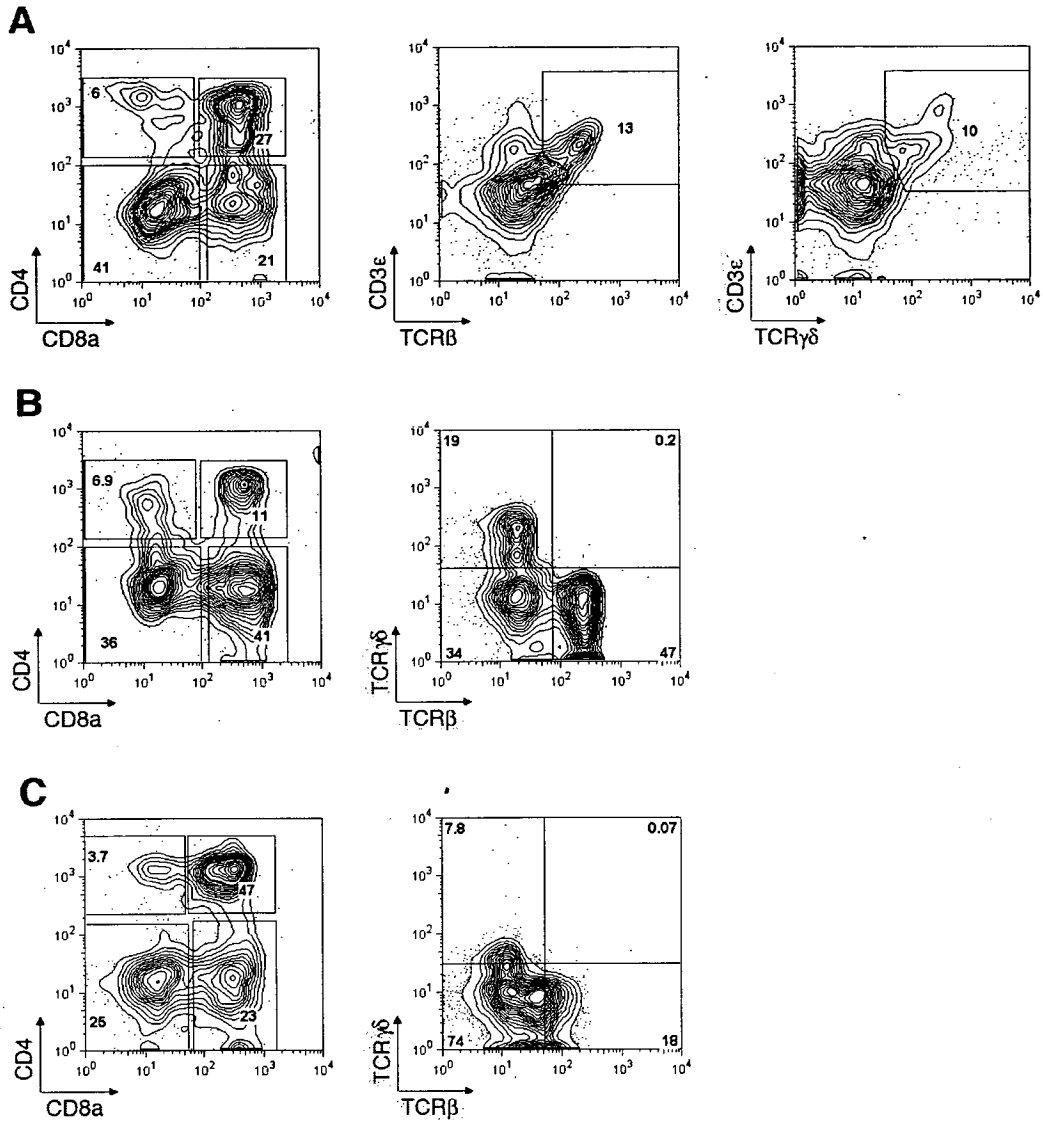

Figure 4.14 TEPOC-support $CD4^+$ and $CD8^+$ αβ as well as γδ T cell differentiation from E13.5 thymocytes

200,000 E13.5 TEPCs were mixed with 200,000 E13.5 thymocytes and 100,000 MEFs and reaggregated for 18 hours. TEPOCs were then transferred to HOS conditions and maintained for a further 9 days. Following enzymatic dissociation cells were stained with appropriate antibodies and analysed by flow cytometry. A, B and C, replicate TEPOCs showing the development of DP and SP populations from DN E13.5 thymocytes. A large $TCR\beta^+$ $CD3\varepsilon^+$ population illustrates presence of mature αβ T cells. A large population of $TCR\gamma\delta^+$ $CD3\varepsilon^+$ T cells is also present in each of the examples.

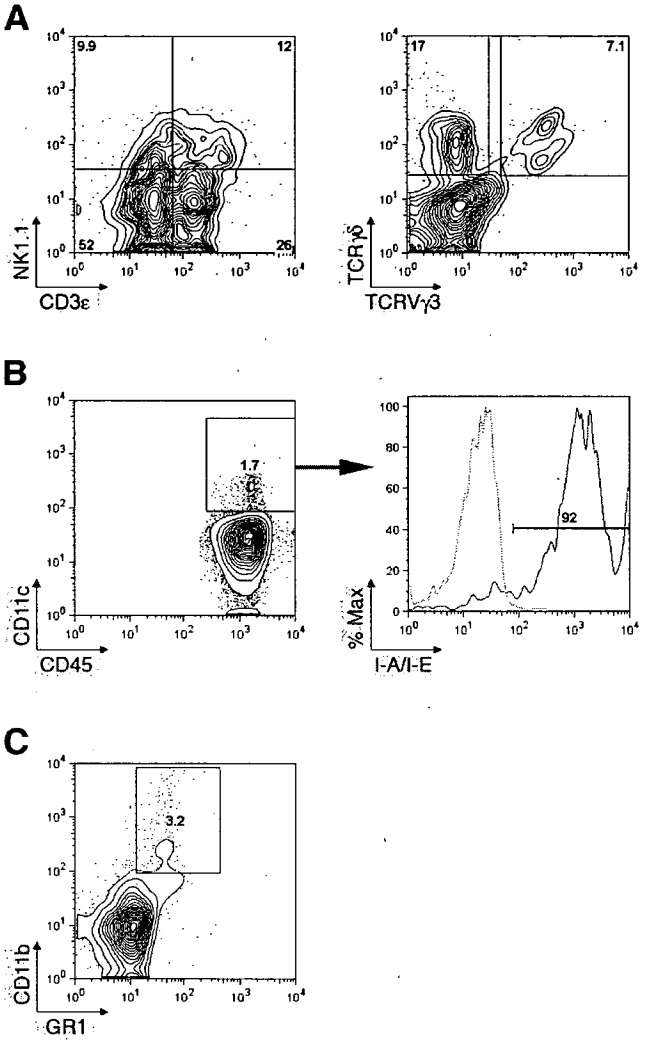

Figure 4.15 TEPOC-supported haematopoietic from E13.5 thymocytes

200,000 E13.5 TEPCs were mixed with 200,000 E13.5 thymocytes and 100,000 MEFs and reaggregated for 18 hours. TEPOCs were then transferred to high oxygen culture and maintained for a further 9 days. Following enzymatic dissociation cells were stained with appropriate antibodies and analysed by flow cytometry. A, Following application of a gate for cells of lymphocytic light scatter properties, $NK1.1^{+}CD3\varepsilon+$ NKT cells and $TCRV\gamma3^{+}$ T cells were present. B, $CD11c^{+}$ $MHCII^{hi}$ DCs were found to have developed in situ. C, $CD11b^{+}$ cells indicative of a macrophage population were also present.

In conclusion, TEPOCs were able to support the differentiation of haematopoietic populations normally found in the thymus from E13.5 fetal thymocytes. Interestingly, the αβ:γδ ration of cells produced in these cultures was variable (range 3.1 to 9.5) but was consistently low when compared to he ratios produced from adult thymocytes in wild-type thymus and TEPOC discussed above, consistent with the skew towards γδ cell generation typically observed from fetal HPCs.

4.2.7 Haematopoietic differentiation in E13.5 MTS20$^-$-based RFTOC

Previous attempts to test the functional potential of the E12.5 and E15.5 MTS20$^-$ fetal thymic compartment using grafting experiments demonstrated the failure of these grafts to persist *in vivo*. However, since these populations clearly contain differentiated TECs, it seemed possible that the failure could be attributed to the experimental assay system used. For example, low cell numbers or increased immunogenicity could have resulted in graft failure. The modified RFTOC protocol was therefore used to address the functional potential of MTS20$^-$Lin$^-$ fetal thymic cells *in vitro*.

Initially, 50,000 MTS20$^-$Lin$^-$ cells, 300,000 adult DN cells and 200,000 MEFs were used to generate RFTOC^{low} under standard conditions then transferred to HOS for a further 9 days. These RFTOCs generally disintegrated during culture, leaving only cell debris remaining following culture. On occasions where remnants of the cultures could be identified and harvested, the cells in these were invariably non-viable based on uptake of 7AAD (Figure 4.16A). These data indicate a clear functional difference between the MTS20$^+$ and MTS20$^-$ epithelial fractions, consistent with data described in Bennett *et al* and Gill, et al.

Therefore, the level of epithelium was raised to a one to one ratio with the HPC such that 200,000 MTS20$^-$ cells, 200,000 adult TN cells and 100,000 MEFs were reaggregated and cultured as RFTOC^{high}. These RFTOCs remained viable and were capable of supporting differentiation of both CD4$^+$ and CD8$^+$ αβ T cells as well as a

small proportion of $\gamma\delta^+$ T cells (Figure 4.16B and Figure 4.17). These data indicate that MTS20$^-$ fetal TEC are functionally competent to support T cell differentiation although a greater number of TEC are required when compared to the MTS20$^+$ population. In addition to differences in functional potency, there is some evidence of qualitative differences, as MTS20$^-$ cell-based RFTOC^{high} also show a higher $\alpha\beta$:$\gamma\delta$ ratio with fewer $\gamma\delta$ T cells (Figure 4.17) than TEPOC^{high} (Figure 4.10).

4.2.8 Haematopoietic differentiation within E15.5 epithelial cell based RFTOC

To determine whether E15.5 MTS20$^-$ cells showed improved capacity to support T cell differentiation over those from E13.5 thymi, RFTOC^{low} containing E15.5 MTS20$^-$ cells, adult T thymocytes and MEFs were set up. In contrast to similar RFTOCs set up with E13.5MTS20$^-$ cells, these RFTOCs were able to support the differentiation of both $\alpha\beta$ T cells and $\gamma\delta$ T cells (Figure 4.16A and Figure 4.18A). To compare the functional capacity of E15.5 MTS20$^+$ and MTS20$^-$ cells, parallel RFTOC^{highs} were set up containing 200,000 TEC, 200,000 adult TN thymocytes and 100,000 MEFs. Following enzymatic dissociation and appropriate antibody staining, flow cytometric analysis revealed that both types of RFTOCs had CD3ε^+ populations sizes that were greater than 50%. Of these cells, approximately 75% were TCRβ^+ and 25% were TCR$\gamma\delta^+$ (Figure 4.18C and D). As shown in Figure 4.18C, E15.5 MTS20$^-$ cells, when mixed with adult TN cells and MEFs, performed as efficiently as the comparable MTS20$^+$ cell-based cultures shown in Figure 4.18B. Interestingly, the apparent differential capacity of MTS20$^+$ cells to support increased $\gamma\delta$ T cell differentiation over E13.5 MTS20$^-$ cells was not retained at E15.5, a stage when both epithelial populations show similar capacity to support $\gamma\delta$ differentiation. The level of SP thymocyte maturity as determined by surface CD3ε staining was greater in these RFTOCs than in those based on E13.5

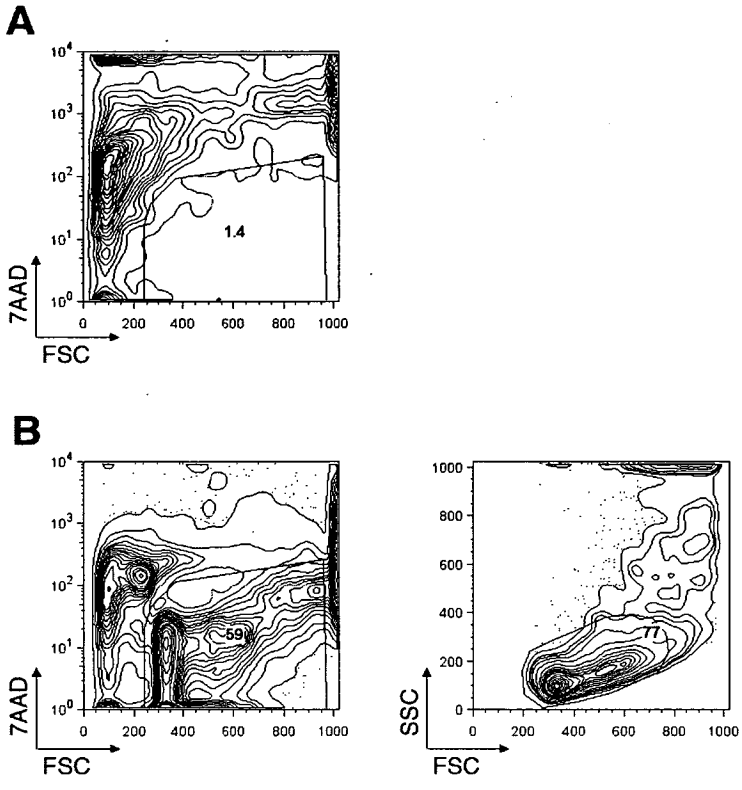

Figure 4.16 E13.5 MTS20$^-$ cells can support T cell development *in vitro* but only at greater TEC:HPC ratios than required for MTS20$^+$ cells

A, 50,000 E13.5 MTS20$^-$ cells were reaggregated with 300,000 TN cells and 200,000 MEFs and cultured for 10 days. B, 200,000 MTS20$^-$ cells were reaggregated with 200,000MEFs and 100,000 MEFs and cultured for 10 days. Following culture, RFTOCs with low-level MTS20$^-$ cell incorporation invariably resulted in cell death as shown by the uptake of 7AAD (A). In contrast, higher level inclusion of MTS20$^-$ cells permitted the survival of the RFTOC cultures and the development of lymphocytes (B).

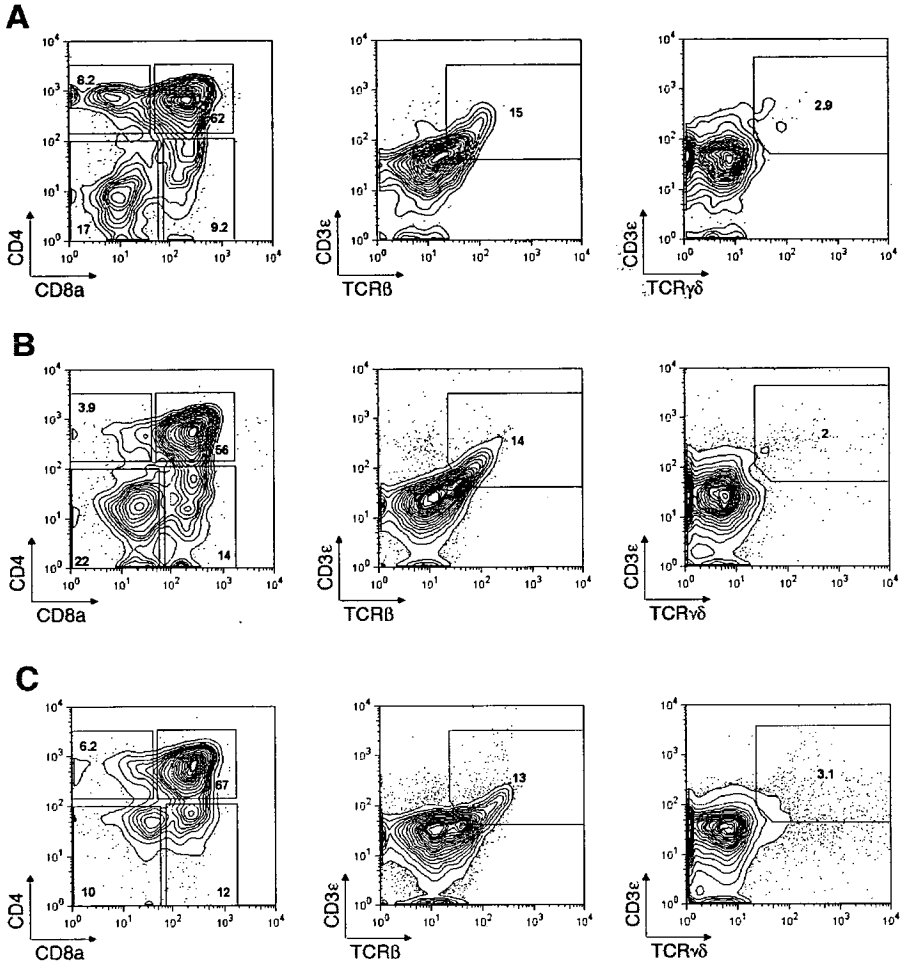

Figure 4.17 αβ and γδ T cell development can be supported by E13.5 MTS20$^-$ cell-based RFTOC

200,000 MTS20$^-$ cells were reaggregated with 200,000 TN and 100,000 MEFs and cultured for 10 days. RFTOCs were then transferred to HOS conditions and maintained for a further 9 days. Following enzymatic dissociation cells were stained with appropriate antibodies and analysed by flow cytometry. A, B and C, Three replicate examples showing the range and reproducibility of such cultures. CD4$^+$ and CD8$^+$ αβ T cells are produced in these cultures as well as a small proportion of γδ T cells.

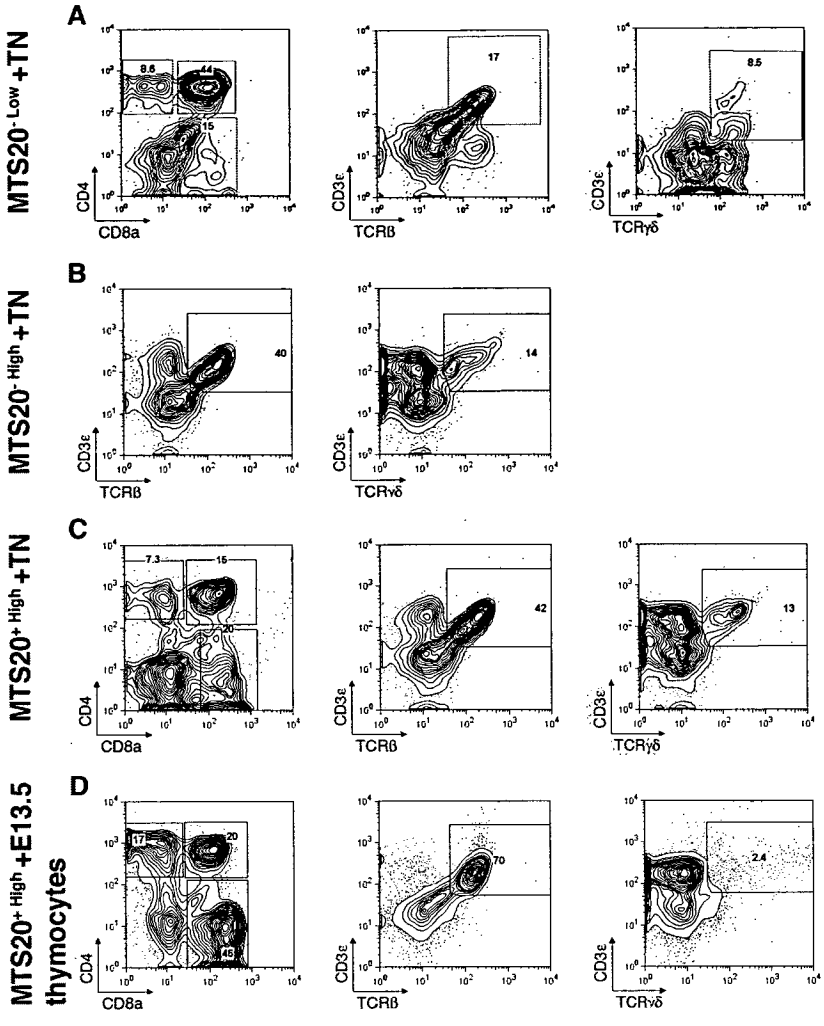

Figure 4.18 E15.5 MTS20$^+$ and MTS20$^-$ cells support T cell differentiation

E15.5 MTS20$^+$ or MTS20$^-$ cells were reaggregated with HPCs and MEFs. Following reaggregation and 10 days of culture RFTOCs were dissociated and stained with antibodies for flow cytometric analysis. A, 50,000 MTS20$^-$ cells were mixed with 300,000 adult TN thymocytes and 200,000 MEFs. Following culture, CD4$^+$ and CD8$^+$ αβ T cells were found as were a small proportion of γδ thymocytes. B, 200,000 MTS20$^-$ cells were mixed with 200,000 adult TN cells and 100,000 MEFs. Following culture, greater than 50% of cells expressed CD3ε with 75% of these coexpressing TCRβ with the remaining 25% being TCRγδ$^+$. C, 200,000 MTS20$^+$ cells were mixed with 200,000 adult TN cells and 100,000 MEFs. The majority of these cells expressed CD3ε with either TCRβ or TCRγδ in a similar manner to MTS20$^-$ cell-based RFTOC shown in B. Few cells remained DP and CD4 SP and CD8 SPs were present. D, 200,000 MTS20$^+$ cells were mixed with 200,000 E13.5 thymocytes and 100,000 MEFs. 70% of cells were TCRβ$^+$CD3ε$^+$, with both CD4 SP and CD8 SPs present in this population. A small population of CD3$^+$TCRγδ$^+$ cells was also observed.

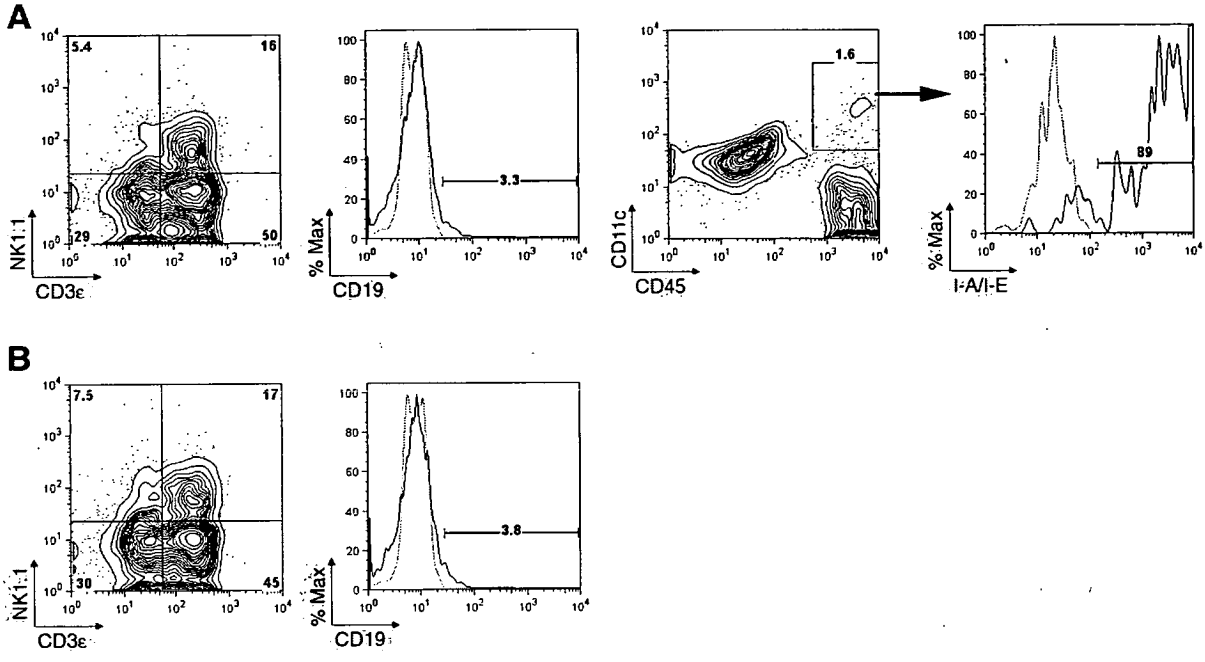

Figure 4.19 Haematopoietic development from adult TN cells in E15.5 TEC based RFTOC

200,000 E15.5 epithelial cells were reaggregated with 200,000 TN cells and 100,000 MEFs and cultured as RFTOC for 10 days. Following dissociation cells were stained with appropriate antibodies for flow cytometric analysis. A, MTS20$^+$ epithelial cells support the development of a large proportion of NK1.1$^+$ CD3ε$^+$ NKT cells. Few or no cells express the B cell marker CD19 at a high level. CD11c$^+$MHCII$^+$ dendritic cells developed *in situ*. B, MTS20$^-$ epithelial cells similarly support the development of NKT cells and CD19$^+$ B cells at a comparable level.

TEC, with large SP populations and a relatively small population of DP cells remaining (Figure 4.10, Figure 4.17 and Figure 4.18). This ability is also evident in E15.5 $TEPOC^{high}$ incorporating E13.5 thymocytes, which, following culture, differentiate to produce a particularly high proportion (70%) of $TCR\beta^{+}CD3\varepsilon^{+}$ cells (Figure 4.18D). $MTS20^{+}$ cells and $MTS20^{-}$ cells also produced similar levels of other haematopoietic cell types, such as B cells and NKT cells (Figure 4.19).

4.3 Discussion

Chapter Four demonstrates that the *in vitro* generation of mature T cell populations from immature intrathymic and extrathymic haematopoietic populations in TEPOC is both efficient and robust. This ability is conferred by the $MTS20^{+}$ TEC component of these RFTOCs, as MEFs alone fail to support this maturation. Differentiation along the $\alpha\beta$ T cell lineage follows the normal DN to DP to SP progression and is able to produce both $CD4^{+}$ and $CD8^{+}$ T cells. Furthermore, $\gamma\delta$ T cell development is also supported and shows the TCRγ chain usage expected from fetal or adult HPCs.

Unexpectedly, $RFTOC^{low}$ did not support the maturation of fetal thymocyte or FLC populations. This could be rectified by increasing the TEC:HPC ratio to approximately 1:1 in the $RFTOC^{high}$ system. Although the reasons for this remain unclear, it is not related to the lack of DN3 and DN4 cells in these cultures, as adult DN1 and DN2 cells survive and differentiate normally in $RFTOC^{low}$. As the development of E15.5 thymocytes proceeds normally in $RFTOC^{low}$, it seems plausible that unknown differential requirements of E13.5 thymocytes versus those from E15.5 and adult thymi are the reason for the observed failure. Fundamental differences between E13.5 thymocytes and those from older mice would best be compared using E13.5 thymocytes that have been cultured for 2 days to the DN3 and DN4 stage of development. Failure of these cultures would support the hypothesis that maturation from early thymocytes cannot be supported by $RFTOC^{low}$ due to the absence of unidentified factors. Successful

maturation would indicate that it is only the very immature E13.5 DN1 and DN2 thymocytes that require additional stimulus, with later thymocytes having no such requirement. It is however difficult to imagine why, when so many E13.5 thymocytes are added to RFTOC^{low}, all cells in these cultures die with no cells appearing to receive the signals required for their survival. This is all the more striking when considered alongside RFTOC^{high}, where such robust fetal T cell maturation is seen. It remains possible that E13.5 thymocytes are unable to support the *in vitro* survival of fetal TEC as efficiently as HPCs from older mice.

Importantly, DCs involved in the education of the thymocytes, are generated alongside T cells populations. These results confirm that TEPOCs are able to support the development of normal intrathymic haematopoietic populations from both fetal and adult progenitors. Although mature cell types arise in the TEPOCs, these often appear at levels inconsistent with those seen in either the adult thymus. This is particularly true of the TCR$\gamma\delta^+$ population and the NK1.1$^+$ CD3ε^+ populations. Many factors may contribute to the qualitative and quantitative differences observed in these cultures. The effect of the contribution of pre-committed progenitors in the DN input populations is one such factor. For example, in RFTOCs seeded with adult DN1-4 cells, the lineage skew of some cells to the TCR$\alpha\beta^+$ lineage fate is established due to the expression of a Pre-TCR complex, a factor known to direct cells towards the $\alpha\beta$ lineage. This is demonstrated by a comparison between the cells stained with anti-CD4 and anti-CD8 in Figure 4.10 and Figure 4.12, where the total number of cells having entered into the $\alpha\beta$ lineage (DP+SP) in the DN1-4 seeded RFTOCs is approximately one and a half fold higher than those in the RFTOCs seeded with DN1 and DN2 cells only, despite both having achieved similar CD3ε staining levels indicating the number of mature $\alpha\beta^+$ and $\gamma\delta^+$ cells.

Interestingly, a relatively high proportion of $\gamma\delta$ thymocytes is evident in all TEPOCs when compared to wild type thymus. This suggests that cells still to make a fate choice

may be influenced by factors in TEPOCs that favour the development of γδ T cells. A similar explanation might be offered for the abundance of NK1.1$^+$ cells that co-express CD3ε, indicative of their identity as NKT cells. Another possible reason for the abundance of ordinarily minor haematopoietic cell populations is possible that the microenvironmental conditions within the RFTOCs orchestrate the proliferation of some populations that would otherwise be dwarfed by more abundant thymic populations. This might be compounded by conditions limiting the proliferation of other cell types. For example, cells entering the αβ T cell lineage may not undergo the proliferative bursts that would normally accompany their maturation. The consequent reduction in the majority DP and SP populations could account for the comparatively large non-αβ lineage cell populations. The acquisition and compilation of data on cellular proliferation rates *in vitro* may begin to elucidate the mechanisms at play in the formation of the observed populations identifiable in the RFTOCs.

Data presented in Chapter Four shows that MTS20$^-$ cells, although apparently unable to support T cell maturation under limiting conditions *in vivo,* are able to persist in culture and support T cell differentiation. This ability is dependent upon the incorporation of large numbers of epithelial cells, although the reasons for this remain unclear. It is possible that these cells die more readily following isolation due to damage sustained during their preparation. Alternatively, these cells may be terminally differentiated and may not be able to persist in the cultures. In either case, the resultant cell loss would mean more cells would be required to provide an equivalently permissive environment as that provided by fewer MTS20$^+$ cells.

The ability of fetal epithelial cells to support T cell differentiation *in vitro* is maintained until at least E15.5, when both the MTS20$^+$ and MTS20$^-$ fractions can efficiently support differentiation of T cell precursors. The large CD3ε$^+$ fractions found in E15.5 TEC-based RFTOCs indicates that by E15.5, the epithelium is more competent to drive

T cell development, possibly reflecting the increasingly mature nature of the epithelium at E15.5 as ontogeny proceeds (Chapter Three).

The data presented above clearly demonstrate that $MTS20^+$ cells are able to support the generation of cell populations that would be expected of the *in vivo* thymic microenvironment, and therefore demonstrate that these cells could form the basis of an *in vitro* thymus-equivalent. Although the $MTS20^-$ compartment is able to support the differentiation of HPCs in RFTOCs, these cells are considerably less efficient than the $MTS20^+$fraction.

Chapter 5: Results: Characterisation of differentiative potential and organisational properties of MTS20$^+$ and MTS20$^-$ fetal thymic epithelial cells.

5.1 Introduction

Upon ectopic transplantation, MTS20$^+$ cells isolated from both E12.5 and E15.5 mouse thymi give rise to a thymus-like structure containing all major thymic epithelial subtypes that is organised into clear medullary and cortical regions comparable to those of the wild-type thymus (Bennett et al., 2002; Gill et al., 2002). Since thymic architecture is believed to be important for the correct functioning of the thymus with respect to the imposition of T cell selection (Naquet et al., 1999), the nature of the organoid generated in RFTOC cultures of MTS20$^+$ cells is of critical importance. The data presented in Chapters Three and Four support the view that MTS20$^+$ fetal TEC could be used to form the basis of an *in vitro* thymus-equivalent, capable of fully supporting T cell development.

In Chapter Five, I therefore investigate and compare the *in vitro* differentiative capacity and organisational properties of E13.5 MTS20$^+$ and MTS20$^-$ fetal TEC.

5.2 Results

5.2.1 RFTOCs contain spatially separated cell types

Although the ability of standard RFTOC (Anderson et al., 1993) to efficiently support T cell development is well described, the organisation of their epithelial structure has been reported to be poor unless grafted (Rodewald, 2000). The outcome of culturing the MTS20$^+$ and MTS20$^-$ populations defined in Chapter Three under the optimised conditions studied in Chapter Four was therefore investigated. Thus, 100,000 and

200,000 epithelial cells were reaggregated with 100,000 to 200,000 MEFs and 200,000 to 300,000 TN thymocytes. Following culture under HOS conditions, these reaggregates were snap-frozen, cryosectioned and fixed briefly. Antibody staining was then used to identify specific TEC subtypes.

The first striking observation that can be made from these studies is the early clustering of cell types. At day (d) 2 of culture, ERTR7$^+$ fibroblasts appear as tight clusters that are already largely segregated from the PanK$^+$ epithelial cells in both MTS20$^+$ and MTS20$^-$ cell-based RFTOCs (Figure 5.1A-E). These epithelial and fibroblastic cells remained spatially segregated at d10 of culture (Figure 5.1F-J and K-O). However, at d10, substantial differences between the MTS20$^+$ cell-based (Figure 5.1F-J) and MTS20$^-$ cell-based (Figure 5.1K-O) RFTOCs were apparent. Epithelial cells within MTS20$^+$-derived RFTOC exhibited a compacted structure at d2, but had adopted a more open meshwork structure by d10 (Figure 5.1F-J). This sparse, networked appearance is reminiscent of the epithelium of the wild type thymus, where the epithelial stroma is tightly packed with developing thymocytes. The images in Figure 5.1K-O show that epithelial cells similarly cluster to one side of the MTS20$^-$ cell-based RFTOC at d10, with fibroblasts making up the remainder of the area in this section. Note that the MTS20$^-$ cell-based RFTOC characteristically contains only a relatively small area of epithelium (Figure 5.1).

The intense DAPI staining in Figure 5.1 indicates the presence of many non-epithelial, non-fibroblastic cells colocalising with the epithelial area. These cells are CD45$^+$ haematopoietic cells (Figure 5.2) and are largely spatially restricted to the epithelial areas of the RFTOC, as expected.

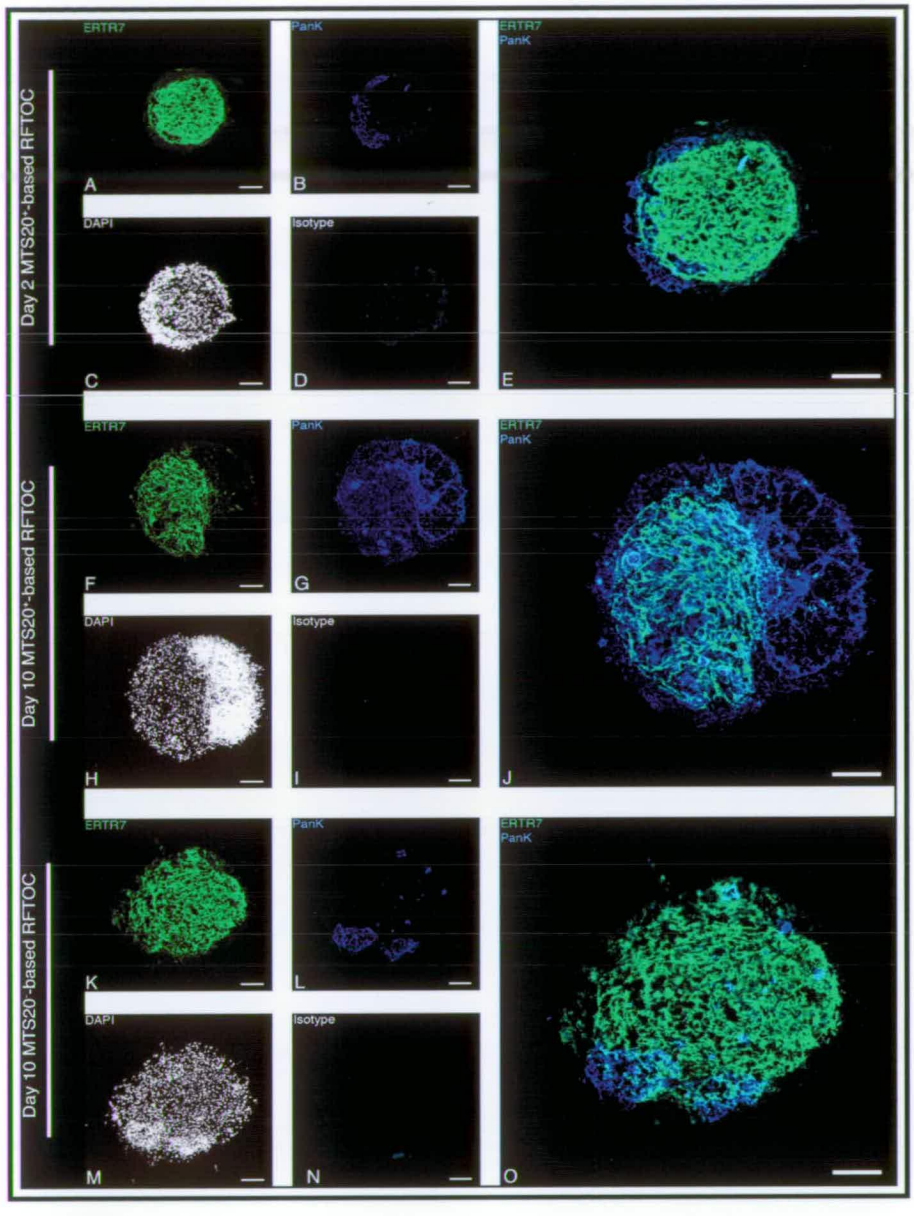

Figure 5.1 Mesenchymal and epithelial cells within the RFTOC are segregated by day two of culture

A-E 120,000 TEC were reaggregated with 120,000 E13.5 thymocytes and 150,000 MEFs. After 2 days in culture, E13.5 $MTS20^{+}$-derived epithelial cells ($PanK^{+}$) were clustered at the edge of the RFTOC adjacent to the $ERTR7^{+}$ fibroblasts. This arrangement persists through culture so that at day 10, $MTS20^{+}$ cell-derived (F-J) and $MTS20^{-}$ cell-derived epithelial cells (K-O) remain clustered. However, $MTS20^{+}$ cell-derived TEC exhibited an open meshwork structure, reminiscent of thymic epithelium. White scale bar , 100μm.

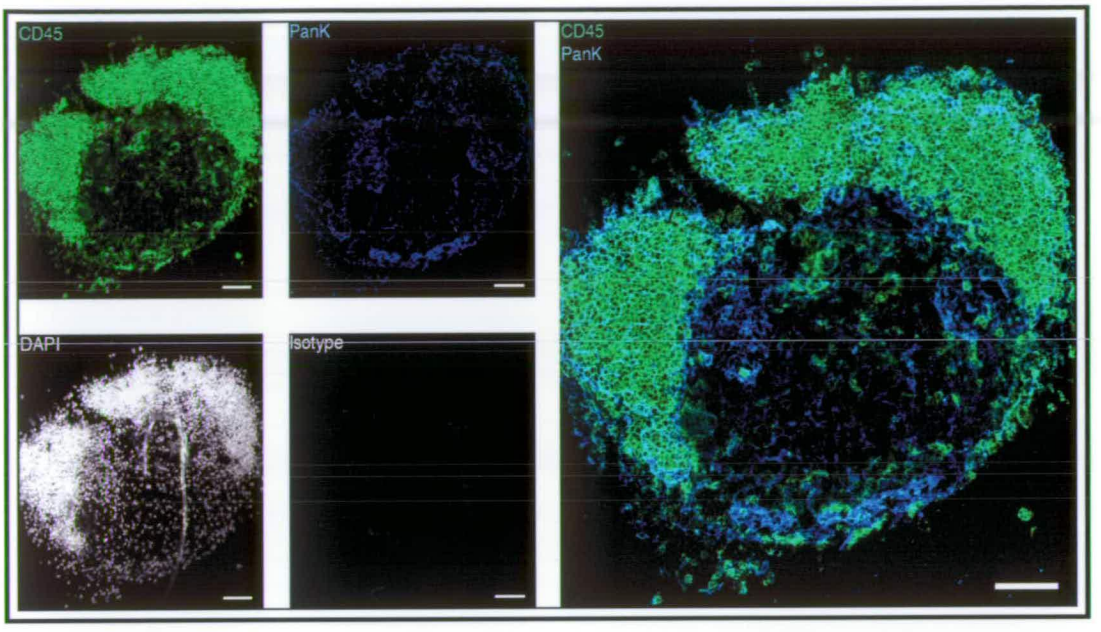

Figure 5.2 Localisation of haematopoietic cells to the epithelial region within the RFTOC

An RFTOC containing 200,000 MTS20^{+} cells, 200,000 adult TN and 100,000 MEFs was generated and cultured for 10 days. After this time, developing thymocytes (CD45^{+}) almost exclusively reside in the network of MTS20^{+} cell-derived epithelium (PanK^{+}). White scale bar, 100μm.

To test the differentiation capacity of E13.5 MTS20$^+$ and MTS20$^-$ cells, reaggregate cultures were set up and cultured for up to 10 days under HOS conditions before being snap frozen and cryosectioned for immunofluorescence analysis. Thus, 100,000 to 200,000 epithelial cells were reaggregated with 100,000 to 200,000 MEFs and 200,000 to 300,000 TN thymocytes. Immunofluorescent detection of MTS24 expression showed that MTS24 antigen expression was downregulated on most cells, such that by d2 of culture a large MTS24$^-$ population was present (Figure 5.3). However, after 10 days in HOS conditions, small areas of intense MTS24 staining remained, either as part of a small cluster of cells or in the cystic structures (Figure 5.3 and Figure 5.4). Higher magnification revealed that in cystic structures, the MTS24 expression was generally strongest along the lumenal side of cystic epithelial cells (Figure 5.4D). Interestingly, in cases where TEPOCs were generated in the absence of HPCs, immunofluorescent analysis showed that similar to nude thymic epithelium (Blackburn et al., 1996), the MTS20$^+$ cell-derived epithelium had become cystic and retention of MTS24 antigen expression persisted for at least 10 days (data not shown). Furthermore, the subsequent addition of HPCs to these RFTOCs showed that they retained the ability to support the differentiation of E13.5 thymocytes but that the epithelium was cystic, and that these cysts showed continued MTS24 expression (data not shown).

5.2.2 Differentiative capacity of MTS20$^-$ epithelial cells in RFTOC

As discussed in Chapter Four, although RFTOC^{low} generated with E13.5 MTS24$^-$ cells do not survive in culture or support T cell development, MTS24$^-$-based RFTOC^{high} survive for 10 days in HOS culture and can support the development of T cells. Due to the failure of MTS20$^-$ cells to persist following grafting under the kidney capsule, a hypothesis was adopted that these cells had very limited or no capacity to function as progenitor cells for either cTEC or mTEC (Bennett et al., 2002; Gill et al., 2002). The data detailed above and in Chapter Four clearly demonstrates that fetal MTS20$^-$ cells are

able to persist and support T cell differentiation under certain conditions, raising questions surrounding their differentiation capacity and the nature of the relationship between $MTS20^+$ cells and their $MTS20^-$ counterparts. The data presented by Bennett *et al*, Gill *et al* and herein shows that $MTS24^+$ cells can give rise to $MTS24^-$ cells. However, the question still remained whether $MTS24^-$ cells can give rise to $MTS24^+$ cells. Figure 5.5 shows a representative RFTOC example, indicating that no MTS24 staining can be detected in the cultured $MTS20^-$ cell-based RFTOCs. This result strongly suggests that there is a lineal relationship between the $MTS20^+$ cells and the $MTS20^-$ cells, with $MTS20^-$ cells of the E13.5 thymus differentiating from an earlier $MTS20^+$ population. The possibility that a lineal relationship does not exist also remains, with the E13.5 $MTS20^+$ and the $MTS20^-$ cells arising separately both with the capacity to give rise to $MTS20^-$ cells but $MTS20^+$ cells being able to give rise to further $MTS20^+$ cells.

In order to address the nature of the E13.5 $MTS20^-$ compartment further, the differentiative potential of this population was therefore assessed using further markers of mature thymic epithelium. Three examples of $MTS20^-$ cell-based RFTOCs containing 200,000 $MTS20^-$ fetal TEC are represented in Figure 5.6. These RFTOCs routinely show broad MHC Class II staining (Panel K) consistent with the functional competence demonstrated in Chapter Four. Panels F and K illustrate the presence of widespread K14 staining, which is consistent with either some differentiation occurring towards mTECs or towards an aberrant cell type, or expansion of the small K14 population already present in the $MTS20^-$ input population, as identified in Chapter Three. Despite staining indicative of mTEC identity, these cells do not occupy distinct areas within the RFTOCs. A large epithelial cyst can be seen in panels G to K, a feature common to epithelium exposed to aberrant differentiation signals or inadequate lymphostromal cross-talk *in vivo*. Another interesting feature of the $MTS20^-$ cell-based

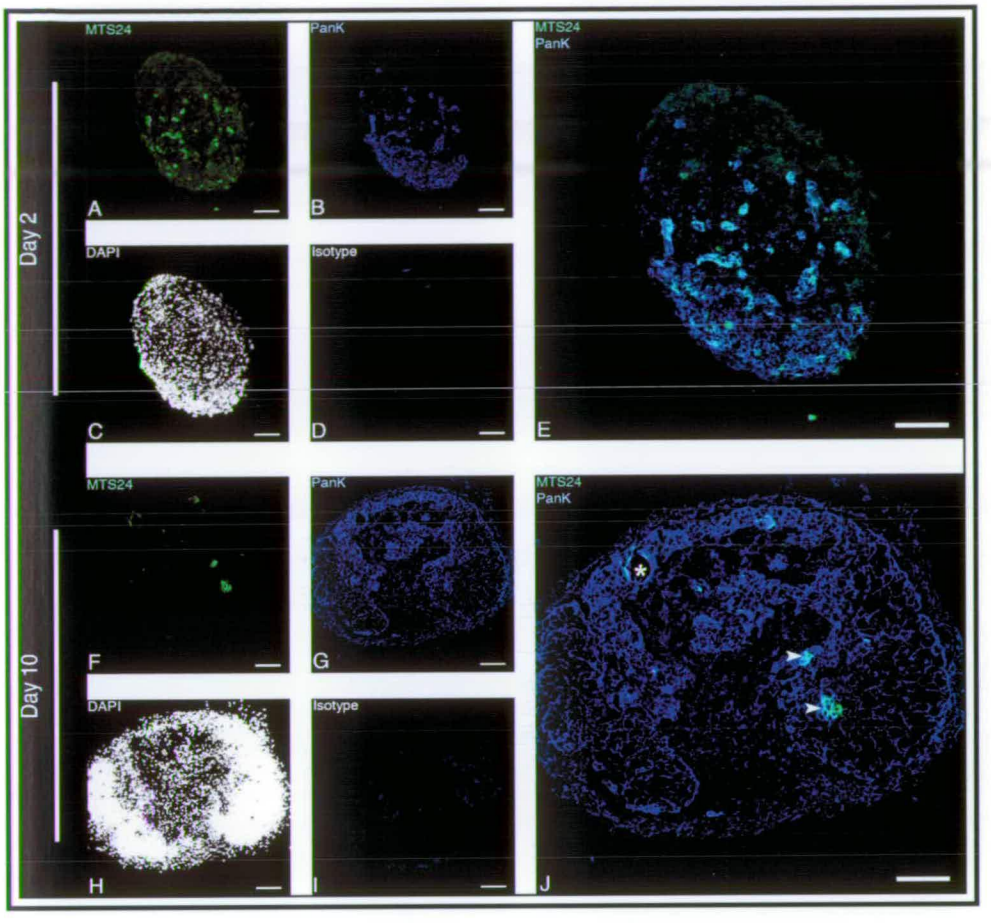

Figure 5.3 Epithelial cells rapidly downregulate MTS24 expression in culture

A-E, 120,000 MTS20$^+$ cells, 120,000 adult TN thymocytes and 150,000 MEFs were reaggregated and cultured for 2 days. Immunofluorescence analysis reveals that MTS24 is rapidly downregulated by most epithelial cells. F-J, RFTOC containing 200,000 MTS20$^+$ cells, 200,000 adult TN thymocytes and 100,000 MEFs cultured for 10 days demonstrating that small MTS24$^+$ areas persist at d10 (same RFTOC pictured in Figure 5.2F-J). Images are representative of greater than three replicate experiments.

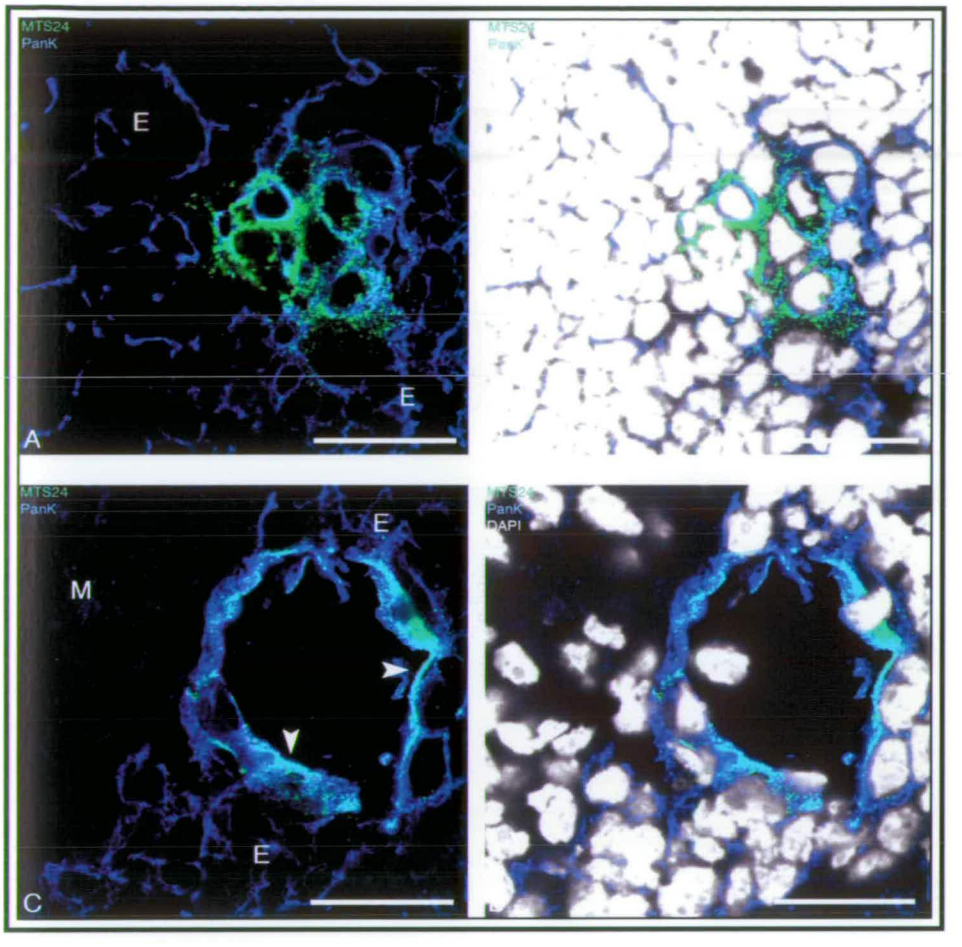

Figure 5.4 Retention of MTS24$^+$ phenotype following culture in TEPOC

Cells that retain MTS24 expression at d10 were found as clusters (Panels A and B) or in cyst-like structures (Panels C and D). White arrowheads indicate MTS24 staining that mostly lines the cyst lumen. Scale bars, 25μm.

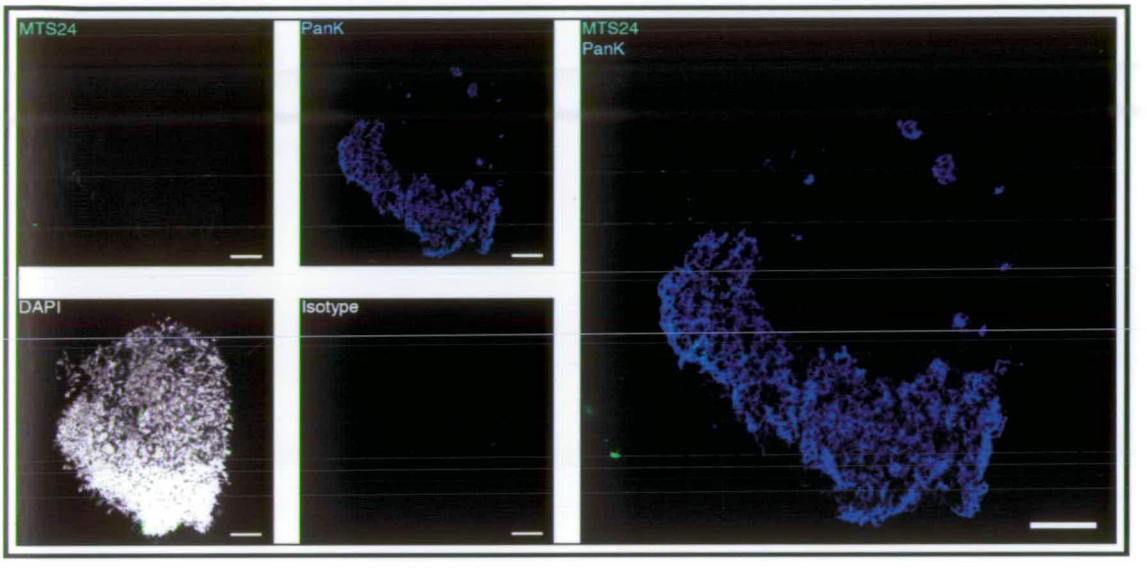

Figure 5.5 MTS20$^-$ cells do not give rise to MTS24$^+$ cells

RFTOCs made with E13.5 MTS20$^-$ epithelial cells were cultured for 10 days and processed for the immunofluorescent detection of MTS24 expression. MTS24$^+$ epithelial cells were never found in these structures. White scale bars, 100μm.

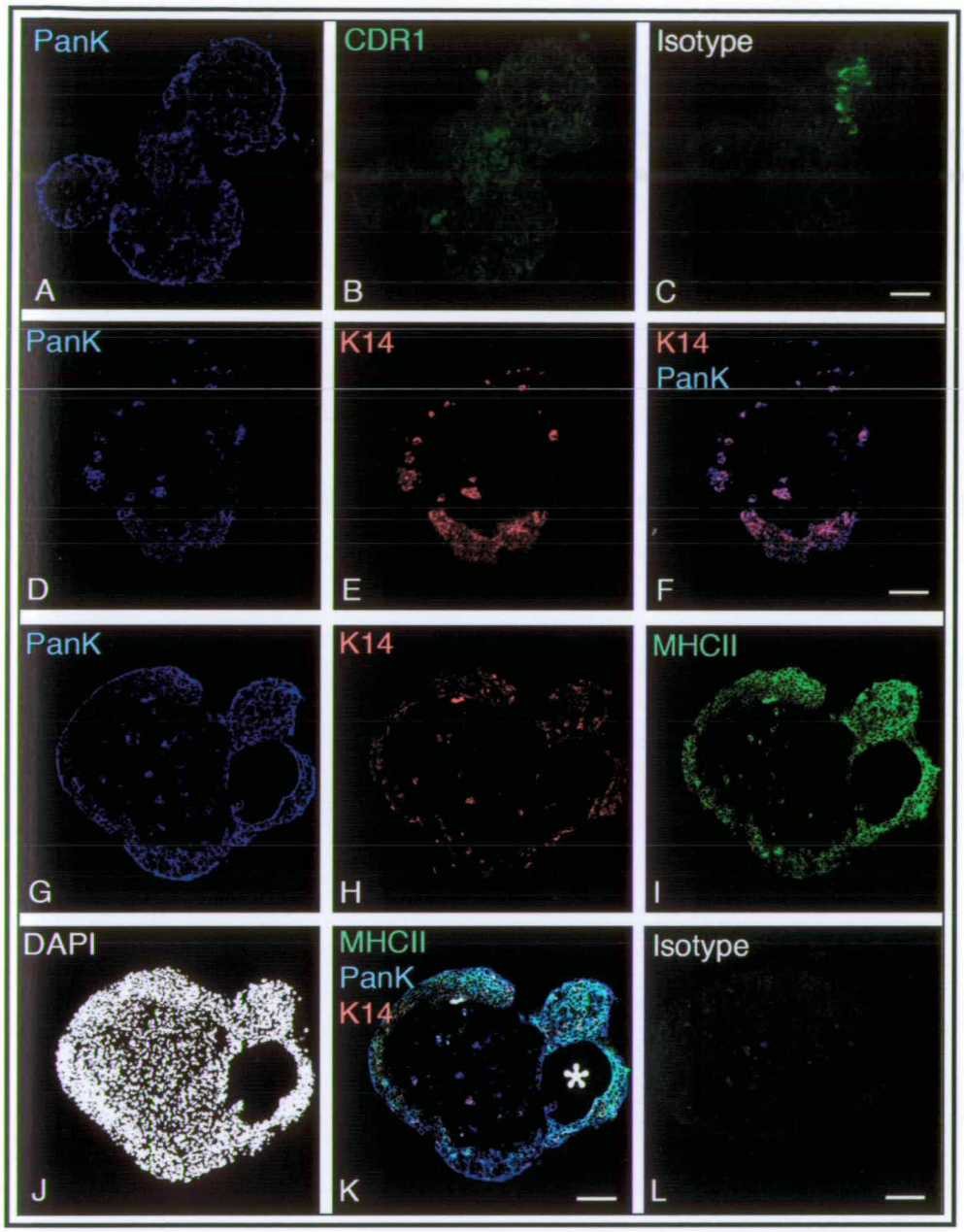

Figure 5.6 MTS20⁻ cells have limited differentiative potential

MTS24⁻ cell-based RFTOCs were cultured for 10 days and processed for immunofluorescence analysis. Staining for markers of mature epithelium in three examples of MTS24⁻-based RFTOCs is represented in panels A-C, D-E, and G-L. MTS24⁻-derived epithelial cells show widespread MHC Class II expression and heterogeneous K14 expression. CDR-1 expression at levels above the isotype control was not detected. MTS20⁻ cells can form epithelial cysts (white asterisk). Scale bar, 100μm

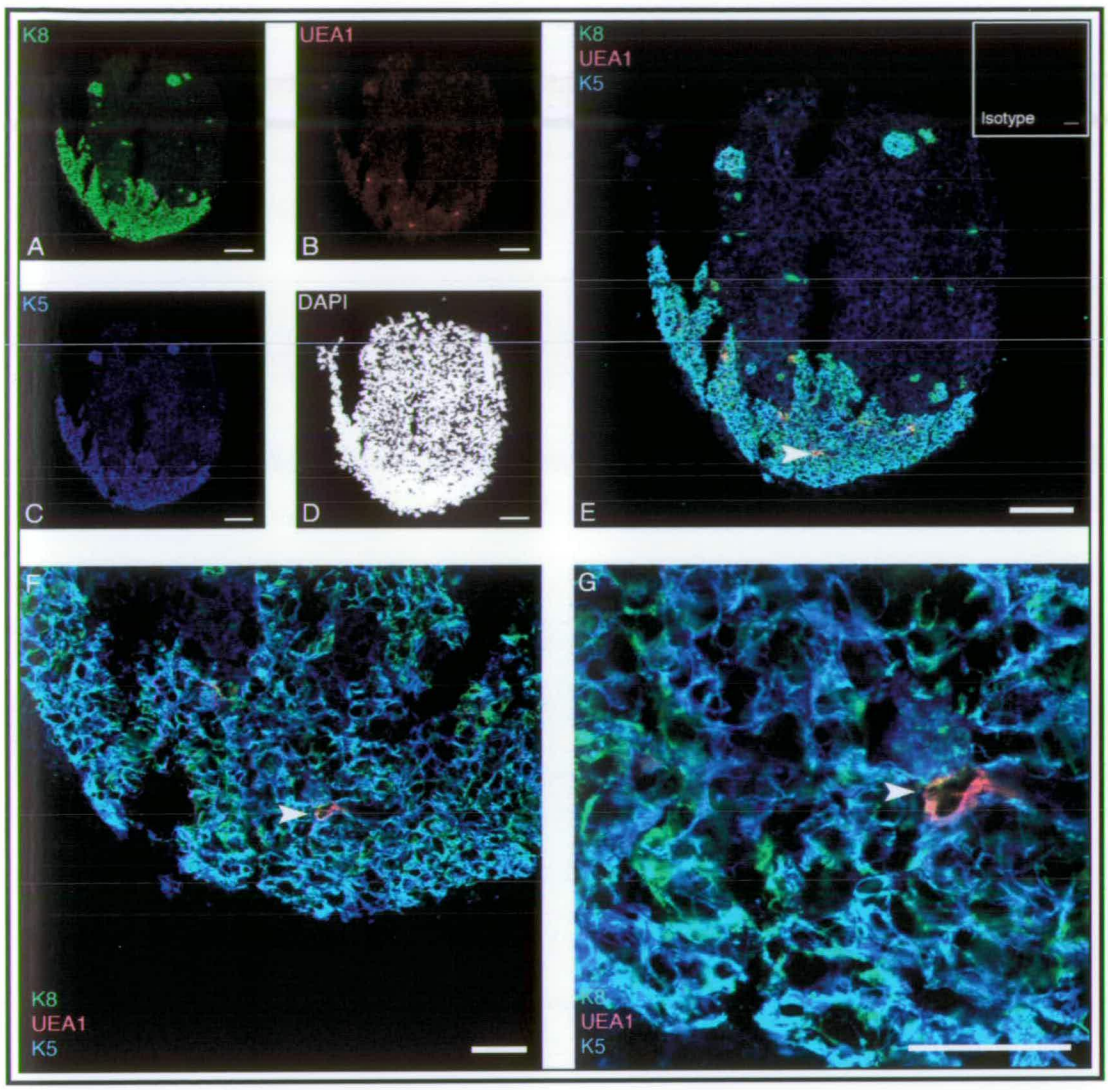

Figure 5.7 The differentiation capacity and organisational ability of MTS20$^-$ cells is limited

MTS20$^-$ cell-based RFTOCs were cultured for 10 days and processed for immunofluorescence analysis. Epithelial areas of the RFTOC delineated with anti-K8 and anti-K5 staining are found within the larger RFTOC structure. Panels F and G highlight the compacted nature of the epithelium and widespread strong K8 and K5 co-staining. Very few UEA1$^+$ cells are found following culture (white arrowhead). White scale bars, 100μm. Grey scale bars, 25μm.

RFTOCs is the almost total lack of UEA1$^+$ cells, with the only UEA-1$^+$ cell observed in several MTS20$^-$ cell-based RFTOCs shown by the arrowhead in Figure 5.7 (Figure 5.6). Figure 5.7 shows another example of a MTS20$^-$ cell-based RFTOC, which was analysed for the presence of cTEC using CDR-1, a marker of cortical cells. Little or no CDR1 staining was observed in the MTS20$^-$ cell-based RFTOC, consistent with the absence of mature cortical lineage cells. Widespread co-expression of K8 and K5 in these RFTOCs could be indicative of failed or improper differentiation but also may correspond to a singular differentiation, expansion, and persistence of the K5$^+$K8$^+$ cell type, found at the CMJ or in the cortex of wild-type thymus. Although some K8$^+$K5$^-$ cells are present, this phenotype preceeds the cortical CDR1$^+$ phenotype *in vivo,* indicating that these may be immature. Moreover, there is no evidence of a morphologically normal K8$^+$K5$^-$ cell type that corresponds to cTEC (Figure 5.7). Further evidence for this is the absence of any morphologically distinct areas within the RFTOCs, which could correspond to the sparse network of cTEC (Figure 5.7). These data establish that MTS20$^-$ cells have only limited ability to differentiate *in vitro*.

5.2.3 Differentiative potential of MTS20$^+$ cells *in vitro*

The functional capacity of the thymic epithelium is dependent upon the expression of MHC Class I and II molecules, and MHC Class II molecules are expressed on the surface of most thymic epithelial cells. As discussed in Chapter Three, prior to culture, only a minority of E13.5 MTS20$^+$ TECs (16%) expressed MHC Class II. Figure 5.8 shows three examples of a TEPOC after 10 days in culture indicating that by this time, MHC Class II expression is widespread with strong reactivity being limited to TEC. Figure 5.8 panels G to I illustrate that the strongest MHC Class II staining often colocalises to areas of epithelium showing K5$^+$ staining, suggestive of mTEC identity, although MHC Class II expression also extends into K5$^-$ areas.

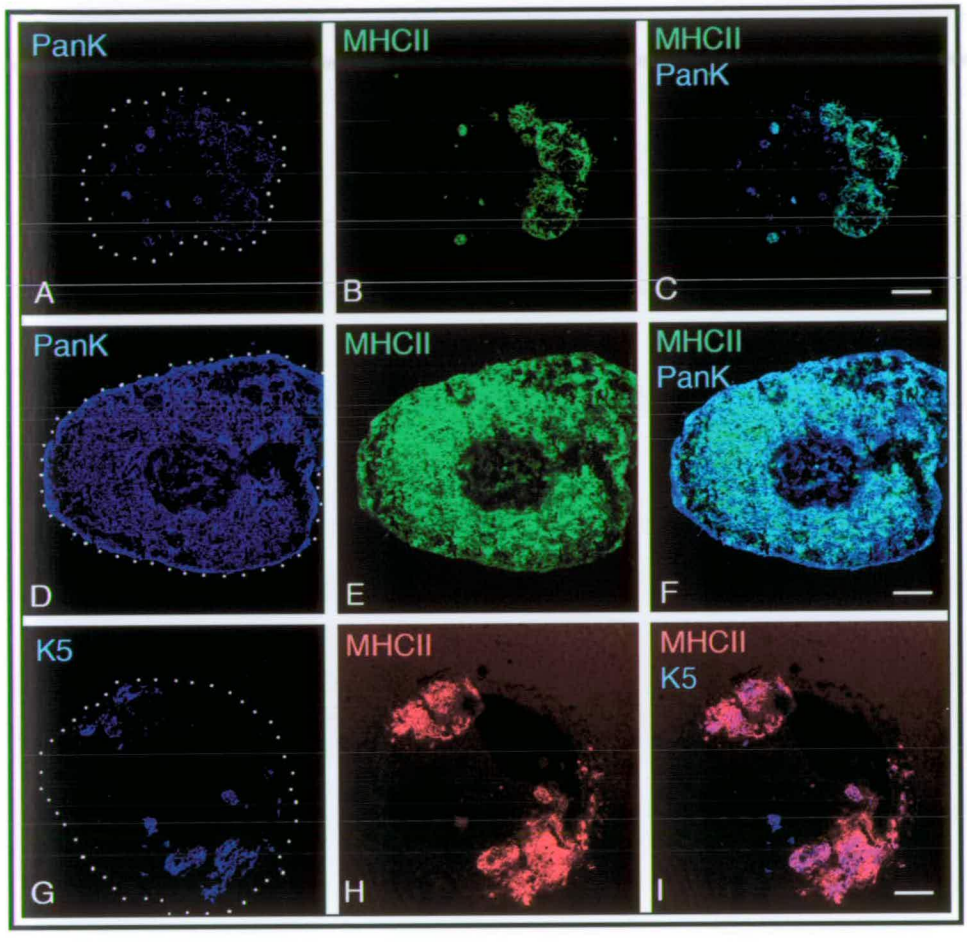

Figure 5.8 Epithelial cells in TEPOC express MHC Class II

Immunofluorescent staining of fixed frozen sections of RFTOC following culture. Using anti-pancytokeratin and anti-keratin 5 antibodies to stain epithelial cells and anti-I-A/I-E to demonstrate MHC Class II expression, it can be seen that the epithelial areas of the RFTOCs show broad MHC Class II expression. Panels A - C, D - F, and G - I represent three examples of MHC Class II staining in RFTOCs. Dotted line indicates the outline of each RFTOC. Scale bars, 100μm

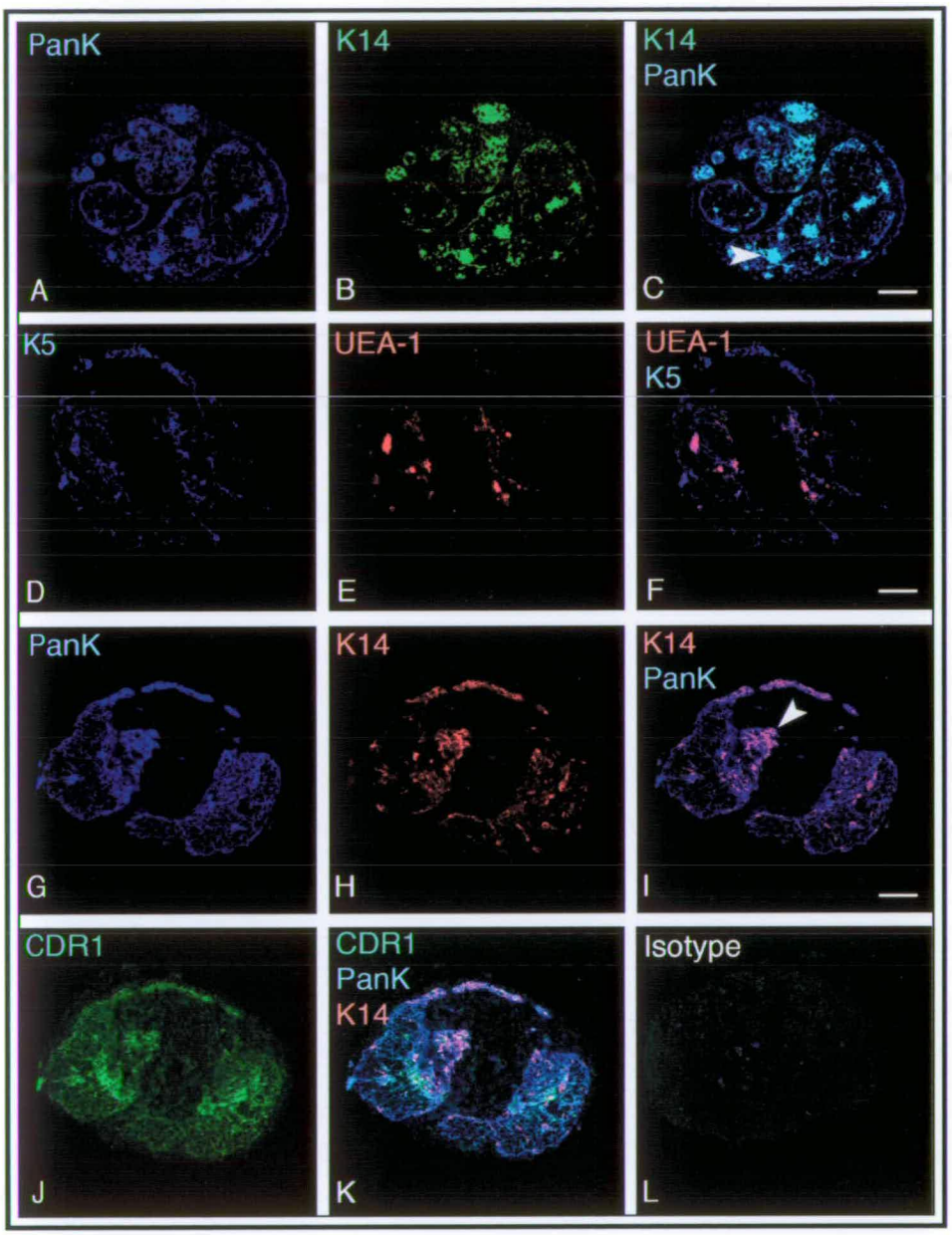

Figure 5.9 Epithelial areas in TEPOC express markers of differentiated epithelium

Immunofluorescent staining of MTS20^{+}cell-based RFTOC following culture. Staining for the mature medullary markers, Keratin 14 and UEA1 demonstrate good medullary development. cTEC are identifiable with CDR1. White arrowheads indicate examples of medullary epithelial areas. Scale bars, 100μm

In order to assess the diversity of the maturing MTS20$^+$ cell-derived epithelium, the presence of other markers of differentiated thymic epithelium was analysed using confocal microscopy. Figure 5.9 shows analysis of markers of medullary epithelium including K5, K14 and UEA-1, and of CDR1, a late marker of cortical epithelial development. Figure 5.9 Panels A to C, D to F and G to L represent three examples of TEPOCs that have been cultured for 10 days prior to processing and analysis. The RFTOCs illustrate that the MTS20$^+$ cells readily differentiate *in vitro* to produce different subsets of mTEC. A common feature is that the K14$^+$ areas appear to stain more brightly with PanK than other TEC, a characteristic typical of mTECs *in vivo* (Figure 5.9). The cells in the areas of presumptive medullary epithelium also appear more compacted and globular, a feature consistent with the morphology expected of mTEC (Figure 5.9). Figure 5.9 panels D to F illustrate heterogeneity within the medullary epithelium established in TEPOCs. A small proportion of the K5$^+$ mTECs co-expresses UEA-1, as would normally be seen in the wild-type thymus (Figure 5.9). Figure 5.9 panels J, K and L demonstrate low-level CDR1 staining in a proportion of epithelium, that is adjacent to the medullary areas identified in panels H and I.

5.2.4 Self-organisation is limited to the MTS24$^+$ cells-based RFTOCs

The presence of segregated epithelial subpopulations within the MTS24$^+$ cell-based RFTOCs is interesting and reveals a self-organising ability inherent that has not previously been demonstrated *in vitro*. This effect is most striking in TEPOCs set up with high numbers of MTS20$^+$ epithelial cells, which demonstrate varying degrees of cortex-medulla segregation. Figure 5.10 shows a representative example; panels A to E show that the MTS20$^+$cell-derived epithelium formed a differentiated network, which in this plane, completely enclosed a non-epithelial area consisting of autofluorescent fibroblasts. Panels F and G reveal that while some cells expressed K14, typical of

mTEC, others expressed CDR1, a marker of postnatal cTEC. Of great interest is the obvious segregation of $CDR1^+$ and $K14^+$ cell types (Figure 5.10).

Figure 5.11 shows a further example of self-organisation within an $MTS24^+$ cell-based RFTOC. Panels A to E demonstrate that several types of epithelial cell have developed within this RFTOC and were segregated in regions suggestive of cortical-medullary organisation. Panel F shows this structure in greater detail. Two main areas are present, one, characterised by a compacted cluster of $K5^+$ cells and the presence of $UEA\text{-}1^+$ cells, represents an area of mTECs (Figure 5.11E, F and G). Adjacent to this is a larger area of K8 staining, with $K8^+$ cells appearing as elongated cells, sparsely spread and more tightly packed with haematopoietic cells (Figure 5.11), indicative of cTEC.

In summary, Figure 5.9 to Figure 5.11, demonstrate that TEPOCs exhibit clearly segregated cortical and medullary areas characterised by the differential expression of markers of TEC subtypes including UEA-1, CDR-1, as well as Keratin 5, and 14. These data indicate that fetal $MTS20^+$ epithelial cells have the capacity to generate an organised thymus in modified RFTOC.

In summary, the data presented in Figure 5.5 to Figure 5.7 demonstrate that in striking contrast to $MTS20^+$ cells, $MTS20^-$ cells do not have the ability to organise into discrete medullary and cortical areas in RFTOC. This may reflect their limited differentiation potential, as they appear unable to produce cells of a cortical lineage based on absence of $CDR1^+$ cells, $K8^+K5^-$ cells, or cells of stellate morphology typical of cTEC. The question still remains whether the $MTS20^-$ population contains a progenitor for the mTEC lineage. Although lack of UEA-1 staining but widespread K14 staining would suggest that the $MTS20^-$ population shows some restricted medullary potential, small numbers of $K14^+$ cells are present prior to culture and thus $K14^+$ cells following culture could be derived from population expansion and not differentiation. Overall, these data

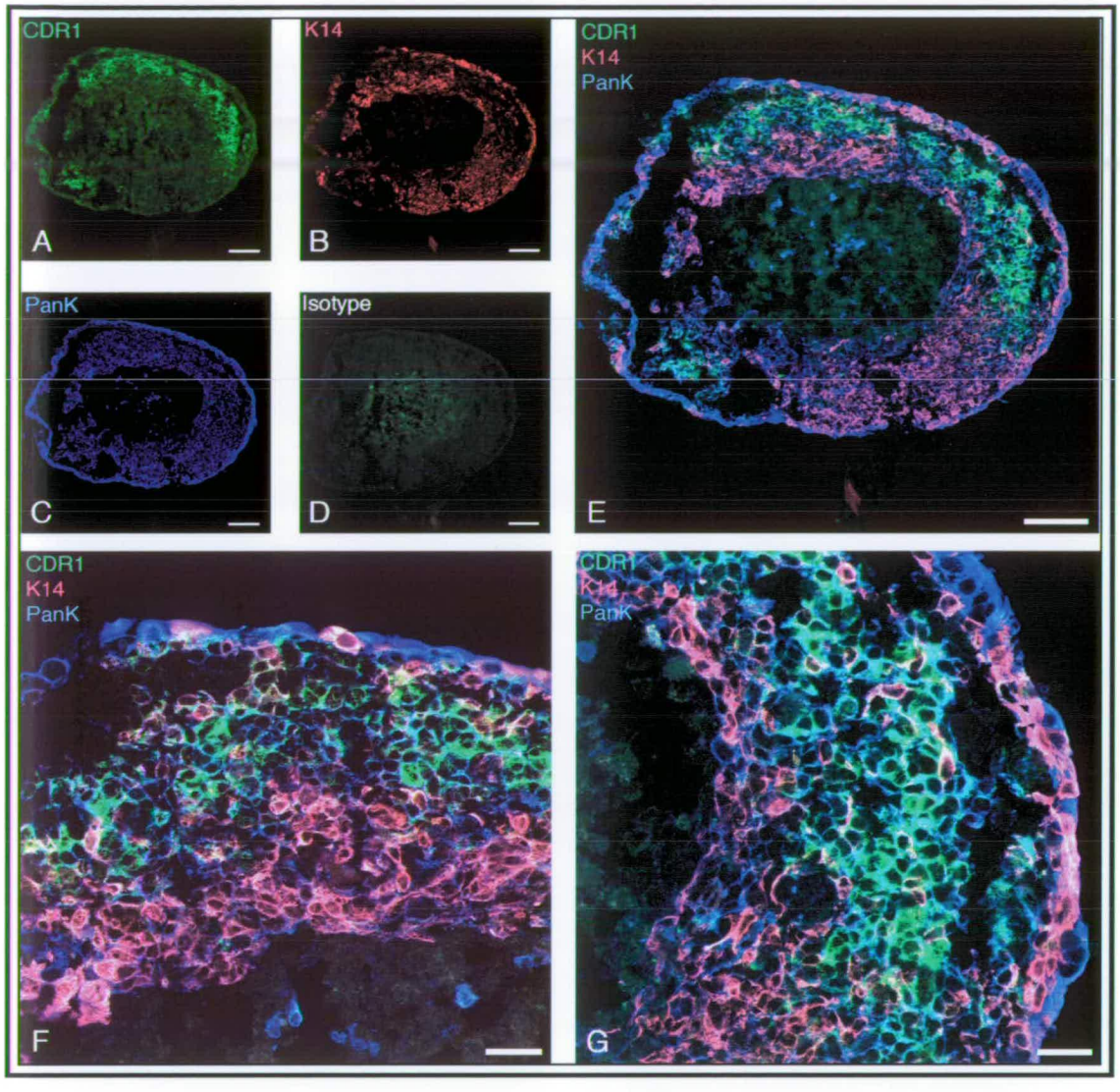

Figure 5.10 Evidence of self-organisation in MTS20$^+$ cell-derived RFTOC I

Immunofluorescent staining of MTS20$^+$ RFTOC with antibodies against cortical and medullary epithelium following 10 days in culture. An enclosed ring of MTS20$^+$ cell-derived TEC is present around a central core of autofluorescent non-epithelial cells. CDR1$^+$ cortical epithelial cells and K14+ medullary epithelial cells are both identifiable in the RFTOC. White scale bars,100μm. Grey scale bars, 25μm.

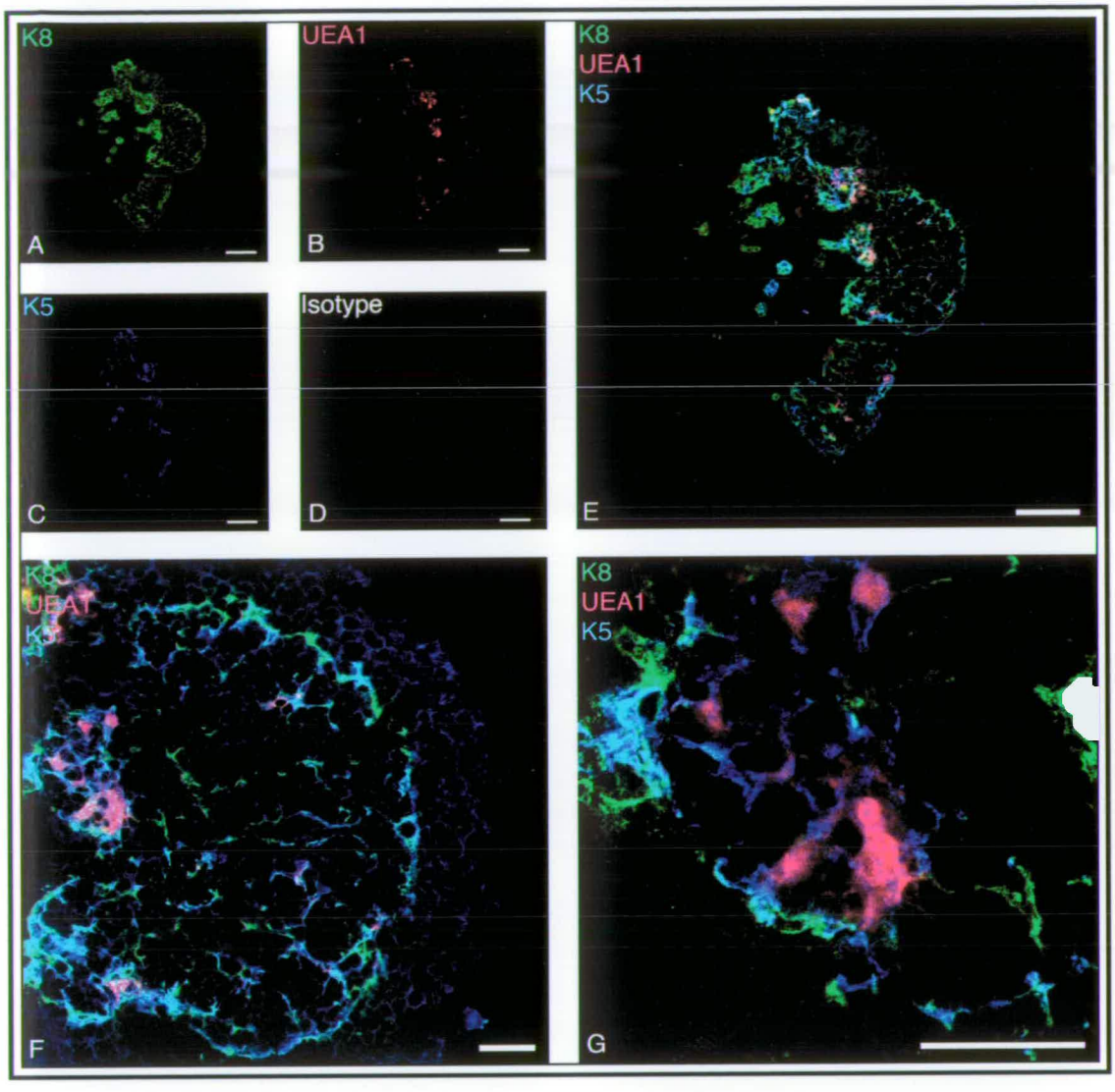

Figure 5.11 Evidence of self-organisation in MTS20$^+$ cell-derived RFTOC II

Staining of the epithelial cells by anti-K8 delineates the epithelial clusters within the larger TEPOC structure. Areas of K5$^+$ UEA1$^+$ cells are present, consistent with a medullary identity. White scale bars, 100μm. Grey scale bars, 25μm.

support the conclusions of Bennett *et al,* which highlighted the progenitor nature of the E12.5 MTS20$^+$ TEC compartment, and conclusively demonstrate that the E13.5 MTS20$^+$ but not the MTS20$^-$ population can generate all major epithelial TEC subtypes.

5.3 Discussion

The data presented in Chapter Five demonstrate that the modified RFTOC system provides a suitable environment for the differentiation of MTS20$^+$ cells into mature TEC subtypes. The MTS20$^+$ cell-derived mTECs and cTECs that developed exhibited staining patterns and morphologies comparable to TEC subtypes found *in vivo*. In contrast, although MTS20$^-$ cells or their progeny persisted in RFTOC, they were unable to generate a range of normal mature TEC subtypes. No K8$^+$K5$^-$ or CDR1$^+$ cells were found, and no cTEC cells were identifiable by morphology, indicating a total lack of cortical development from MTS20$^-$ cells.

Although K14$^+$ cells were found following culture, few or no UEA1$^+$ mTECs developed. A recent report identifies UEA-1 as a marker of the AIRE$^+$ mTEC lineage progenitors at E13.5 (Hamazaki et al., 2007), known to be important for thymic expression of tissue restricted antigens (Anderson et al., 2002; Derbinski et al., 2001). The restriction of UEA-1 to the MTS20$^+$ compartment suggests that only the MTS20$^+$ population would be able to give rise to UEA-1$^+$ mTEC subset consistent with data presented in Chapter Five. Thus, AIRE expression and thymic TRA expression would similarly be limited to the MTS20$^+$ cell-derived progeny indicating that only RFTOC generated using MTS20$^+$, but not MTS20$^-$ cells, could give rise to cells expressing AIRE and thus be fully competent to support negative selection and tolerance to TRAs.

In summary, no markers of mature TEC were present following culture of MTS20$^-$ cell based RFTOC that had not previously been identified in the MTS20$^-$ population prior to culture. However some differentiation markers, such as MHC Class II and K14, became

more prevalent, indicating that limited differentiation or maturation had occurred in the cultures and/or existing minority populations had proliferated or selectively survived to dominate in these cultures. These data are consistent with the hypothesis that the $MTS20^+$ population contains the TEPC population in the E13.5 thymus, with $MTS20^-$ cells representing either terminally differentiating medullary cells or an intermediate medullary progenitor. It is also possible that continued interaction with $MTS20^+$ cells is required to maintain cell identity in $MTS20^-$ TEC. In any case, the ability to autonomously generate cTEC and a diverse medullary compartment is limited to the $MTS20^+$ population.

The striking ability of TEPOCs to organise into spatially segregated cortical and medullary regions, is in contrast to the lack of any epithelial organisation in the $MTS20^-$ cell-based RFTOCs and to a previous reports of standard RFTOCs, which showed no compartmentalisation (Rodewald, 2000). It remains possible that the lack of cTEC and the absence of clustered mTECs within $MTS20^-$ cell-based RFTOCs are causally related to the absence of epithelial organisation and vice-versa. Thus, experiments that assess the ability of $MTS20^-$ cells to contribute to TEC lineages and not just their ability to autonomously differentiate into those lineages would be informative. This could be achieved by incorporating small numbers of labelled $MTS20^-$ cells into self-organising TEPOCs and assessing lineage contribution.

Although Chapter Five demonstrates that TEPOCs become organised, it remains unclear where the organiser capacity lies. It is possible that the $MTS20^+$ population contains cells with organisational capacity or alternatively, organisation observed in $MTS20^+$ cell-based cultures may result from *de novo* organogenesis. RFTOCs incorporating irradiated $MTS20^+$ cells could address whether RFTOCs become organised in the absence of proliferating $MTS20^+$ cells. The observation that areas of mTECs in organised RFTOCs are, for the most part, adjacent to the fibroblast cluster is

perhaps indicative of the involvement of organising factors provided by the fibroblasts. Although this aspect remains to be investigated, cross-talk between the thymic epithelium and LTβR-ligands detected in MEFs is one mechanism that would be able to provide inductive cues for the formation of medullary areas adjacent to the MEFs (Boehm et al., 2003).

Chapter 6: Concluding remarks

Immune deficiency can be caused by many factors including age, genetic disorders, infectious diseases, and as a result of chemotherapy or radiation therapy. Strategies to overcome the slow recovery of T cell compartments in cases of immune deficiency include attempts to enhance T cell reconstitution following HSC transplantation by the provision of exogenous factors, thymic tissue transplantation and techniques based on *ex vivo* culture systems.

When grafted *in vivo,* fetal $MTS20^{+}MTS24^{+}$, but not $MTS20^{-}MTS24^{-}$ fetal thymic epithelial cells, can generate all major thymic epithelial cell subtypes and form an organised thymus-like structure. Furthermore, the resultant thymic organoid can recruit T cell precursors and support their differentiation into mature $CD4^{+}$ and $CD8^{+}$ T cells. The generation of a single cell line corresponding to fetal TEPC would enable the generation and expansion of thymic epithelial cells *in vitro*. These could be used as a source of tissue for thymic epithelial tissue or to support T cell differentiation *in vitro*.

The possibility of supporting T cell differentiation *in vitro* using a TEPC-based thymic-equivalent has several advantages over currently used *in vitro* systems. A thymus-equivalent would necessarily be capable of supporting both T cell differentiation and selection processes thus producing a self-tolerant T cell repertoire. *In vitro*, this has been dependent upon the use of fetal thymic lobes, which not only inherently limits the scale of T cell generation but the intact nature of the lobes means that this is not easily manipulatable, features which ultimately limit the usefulness of FTOC for research or therapeutic purposes. The *in vitro* generation of an organised heterogeneous thymic epithelial structure based on the RFTOC system capable of supporting the differentiation of HPCs into therapeutically useful T cell populations would overcome both of these caveats.

6.1 TEPOC-based T cell repertoire generation

The work presented in this thesis aimed to assess the ability of $MTS20^+$ fetal TEPC to form the basis of an *in vitro* thymus-equivalent. Chapter Three outlines the development of a modified RFTOC method, which has several advantages over the established RFTOC method developed by Anderson and colleagues (Anderson et al., 1993). As demonstrated in Chapter Four, using this method it is possible to reliably and reproducibly support T cell development in $MTS20^+$ fetal TEPC-based TEPOC. Although unable to support T cell differentiation when grafted *in vivo* (Bennett et al., 2002; Gill et al., 2002), fetal $MTS20^-$ TEC are competent to support T cell differentiation using the modified reaggregation protocol, *in vitro,* but remain less efficient than the $MTS20^+$ cells.

The highly organised thymic epithelial stroma provides the developing thymocytes with temporally and spatially separate microenvironments that support distinct stages of thymocyte development. These microenvironments are believed to provide factors that are specifically required for a given stage of thymocyte development. For example, upon production of an appropriate TCRβ chain, and also following positive selection, thymocytes are able to respond to stroma-derived IL-7 that supports the expansion of post-selection thymocytes in a microenvironmentally controlled manner thereby ensuring that only the most useful thymocytes are expanded in number. Similarly, the medullary stromal cell compartment instructs potentially autoreactive T cells to apoptose or become anergic. In this way, the provision of organised specialised microenvironments in the thymus ensures the production of a large self MHC-restricted, self-tolerant repertoire. As such, organisation of the TEPOC structure should aid the *in vitro* production of such a repertoire.

In future experiments the ability of the T cells produced in TEPOC to respond to mitogen stimulation will be tested as an indicator of functional maturity. Such experiments routinely include incubation with phytohemagglutinin or concanavalin A followed by analysis for responses such as a proliferative burst, upregulation of

activation markers, and cytokine production. Once the functional maturity of these cells has been established, the relevance of the observed repertoire should be established. It is envisaged that these experiments will include mixed lymphocyte reaction experiments to establish whether self-tolerance could be established by the organised TEPOC.

6.2 Epithelial differentiation within TEPOC

Under the conditions of the modified RFTOC, E13.5 MTS20$^-$ cells showed limited differentiation capacity and only generated one mTEC subtype and aberrant cell types similar to those seen in the thymus of nude mice and those with thymocyte deficiencies. However, in analogous RFTOC, E13.5 MTS20$^+$ cells showed the capacity to generate all major thymic epithelial subtypes. Furthermore, in contrast to MTS20$^-$cell-based RFTOC, TEPOC demonstrated the ability to self-organise into cortical and medullary regions indicating that MTS20$^+$ cells are unique in their potential to form the basis of an organised in vitro thymus-equivalent. While this is limited to the MTS20$^+$ compartment it remains undetermined whether this potential is retained by the E15.5 MTS20$^+$ population. Similarly, the failure of MTS20$^-$ cell-based grafts to survive precluded any analysis of their potential in previous experiments (Gill et al., 2002) and so it remains possible that these cells can contribute to normal epithelial subtypes in an organised thymus structure. As it is clear from Chapter Four that E15.5 MTS20$^+$ and MTS20$^-$ cells can survive and mature T cells under the conditions of the modified RFTOC cultures, their potentials can now be tested. These experiments may also add to our current understanding of the relationship between the cell populations at E13.5 and those at E15.5.

6.3 TEPOC as a model for thymus organogenesis

It is hypothesised that the method used to make these RFTOC plays a role in establishing the compartmentalisation that is observed, a feature lacking in cell slurry-based RFTOCs (Rodewald, 2000). The ability to separate cell types to produce a desired structural order is desirable due to the potential for possible effects on cell-cell signalling and through the implementation of factor gradients, both features that are

essentially abrogated by the random distribution of cell types produced by the cell slurry-based RFTOC protocol. The early segregation of cell types illustrated in Figure 5.1, is likely achieved through differences associated with the large size of MEFs and the smaller epithelial and haematopoietic cells, meaning that upon centrifugation, larger cells coalesce and become overlaid by smaller cells. In this way, prior to and following the reaggregation process, the epithelial cells are clustered adjacent to the MEFs (data not shown and Figure 5.1). The ability to purposefully layer cells with repeated centrifugation steps during RFTOC generation could be used to make this process more complete or be used to generate more complex RFTOC structures involving several cell layers or placement of a given cell layer in relation to another regardless of cell size.

The observed organisation within RFTOC opens up the possibility for using RFTOC to dissect the pathways that organise and maintain the thymic microenvironment. There are several possible means by which the organisation seen in these RFTOC is established. In one scenario, organisation could be achieved by an initial period of epithelial differentiation in a non-organised or semi-organised manner followed by a phase of restructuring, during which time cells of a given identity coalesce, or proliferate thus creating areas rich in a particular cell type. Post-differentiation reorganisation could be identified in these cultures by analysis of a time-course during the maturation process. In this way, an initial period of epithelial disorganisation would be identifiable if the RFTOCs if organisation was taking place following differentiation. Alternatively, organisation could result from *de novo* organisation, where the *in situ* generation of mTEC and cTEC could result in medullary and cortical areas being generated. Analysis of early time points during TEPOC culture would be able to identify the emergence of cortical and medullary areas as bodies of similar cell types that are spatially distinct.

Pre- or post-differentiation organisation may be driven by an external organising factor or may be a population-intrinsic activity. *In vivo*, although small areas of medulla have been shown to arise as clonal islets (Rodewald et al., 2001), expansion of medullary

areas can be driven by signalling along the LTβR or TRAF6 pathways (Akiyama et al., 2005; Boehm et al., 2003). In this way, the organisation of the medullary compartment is mediated by ligands expressed on non-epithelial cells. Although it remains possible that $MTS20^+$ cells harbor the capacity to self-organise *in vitro*, the observation that medullary compartments within organised TEPOC are typically adjacent to the MEF compartment, would indicate that interplay between developing the epithelium and adjacent MEFs is exerting an organising influence on the epithelium. Although the mechanism of this cross-talk is unclear, preliminary RT-PCR results suggest that the MEFs express several factors including several TNF receptor ligands including the LTα and LTβ (data not shown). Future experiments will aim to establish whether the provision of LTβR ligands by MEFs plays a significant role in the development of medullary areas within TEPOC and whether, in the absence of LTβR signalling, medullary areas persist and/or are abberent. Such experiments will provide be proof of principle for the use of TEPOC as a manipulatable *in vitro* model of thymus patterning.

Although it seems likely that LTβR signalling is playing a role in the expansion of the developing medullary compartment in TEPOC, *in vivo,* some medullary differentiation occurs in the mice deficient for members of the LTβR pathway suggesting that LTβR signalling is not absolutely required for the induction of mTEC differentiation along all mTEC lineages (Boehm et al., 2003; Burkly et al., 1995). This would indicate that a pathway that is not dependent upon LTβR signalling is determining the formation of the medullary compartment although again, the coincident localisation of medullary epithelium and MEFs may suggest that MEF-epithelial cross-talk may play a role in this process in TEPOC.

6.4 Summary

In summary, the data presented in this thesis supports the view that a fetal thymic epithelial population can be used to form the basis of an *in vitro* thymus organoid. Uniquely, the $MTS20^+MTS24^+$ population is able to generate an organised thymic epithelial structure under the conditions of the modified RFTOC. It is believed that this structure will support the development of a self-tolerant repertoire. Future experiments

will address the functional nature of the T cell repertoire produced in these cultures. Further experiments using this system as an *in vitro* model of thymus organogenesis will attempt to elucidate the mechanism of epithelial organisation, which is observed in TEPOC.

List of Figures

List of Tables

Bibliography

Aifantis, I., Buer, J., von Boehmer, H. and Azogui, O. (1997). Essential role of the pre-T cell receptor in allelic exclusion of the T cell receptor beta locus. *Immunity* **7**, 601-7.

Akiyama, T., Maeda, S., Yamane, S., Ogino, K., Kasai, M., Kajiura, F., Matsumoto, M. and Inoue, J. (2005). Dependence of self-tolerance on TRAF6-directed development of thymic stroma. *Science* **308**, 248-51.

Allison, J. P., Asarnow, D. M., Bonyhadi, M., Carbone, A., Havran, W. L., Nandi, D. and Noble, J. (1991). Gamma delta T cells in murine epithelia: origin, repertoire, and function. *Adv Exp Med Biol* **292**, 63-9.

Allman, D., Sambandam, A., Kim, S., Miller, J. P., Pagan, A., Well, D., Meraz, A. and Bhandoola, A. (2003). Thymopoiesis independent of common lymphoid progenitors. *Nat Immunol* **4**, 168-74.

Anderson, G., Jenkinson, E. J., Moore, N. C. and Owen, J. J. (1993). MHC class II-positive epithelium and mesenchyme cells are both required for T-cell development in the thymus. *Nature* **362**, 70-3.

Anderson, G., Owen, J. J., Moore, N. C. and Jenkinson, E. J. (1994). Characteristics of an in vitro system of thymocyte positive selection. *J Immunol* **153**, 1915-20.

Anderson, G., Partington, K. M. and Jenkinson, E. J. (1998a). Differential effects of peptide diversity and stromal cell type in positive and negative selection in the thymus. *J Immunol* **161**, 6599-603.

Anderson, K. L., Moore, N. C., McLoughlin, D. E., Jenkinson, E. J. and Owen, J. J. (1998b). Studies on thymic epithelial cells in vitro. *Dev Comp Immunol* **22**, 367-77.

Anderson, M., Anderson, S. K. and Farr, A. G. (2000). Thymic vasculature: organizer of the medullary epithelial compartment? *Int Immunol* **12**, 1105-10.

Anderson, M. K. (2006). At the crossroads: diverse roles of early thymocyte transcriptional regulators. *Immunol Rev* **209**, 191-211.

Anderson, M. S., Venanzi, E. S., Klein, L., Chen, Z., Berzins, S. P., Turley, S. J., von Boehmer, H., Bronson, R., Dierich, A., Benoist, C. et al. (2002). Projection of an immunological self shadow within the thymus by the aire protein. *Science* **298**, 1395-401.

Andrews, P. and Boyd, R. L. (1985). The murine thymic nurse cell: an isolated thymic microenvironment. *Eur J Immunol* **15**, 36-42.

Annunziato, F., Romagnani, P., Cosmi, L., Beltrame, C., Steiner, B. H., Lazzeri, E., Raport, C. J., Galli, G., Manetti, R., Mavilia, C. et al. (2000). Macrophage-derived chemokine and EBI1-ligand chemokine attract human thymocytes in different stage of development and are produced by distinct subsets of medullary epithelial cells: possible implications for negative selection. *J Immunol* **165**, 238-46.

Antica, M., Wu, L., Shortman, K. and Scollay, R. (1993). Intrathymic lymphoid precursor cells during fetal thymus development. *J Immunol* **151**, 5887-95.

Appasamy, P. M. (1992). IL 7-induced T cell receptor-gamma gene expression by pre-T cells in murine fetal liver cultures. *J Immunol* **149**, 1649-56.

Ardavin, C. (1997). Thymic dendritic cells. *Immunol Today* **18**, 350-61.

Ardavin, C., Wu, L., Li, C. L. and Shortman, K. (1993). Thymic dendritic cells and T cells develop simultaneously in the thymus from a common precursor population. *Nature* **362**, 761-3.

Auerbach, R. (1960). Morphogenetic interactions in the development of the mouse thymus gland. *Dev Biol* **2**, 271-84.

Bain, G., Cravatt, C. B., Loomans, C., Alberola-Ila, J., Hedrick, S. M. and Murre, C. (2001). Regulation of the helix-loop-helix proteins, E2A and Id3, by the Ras-ERK MAPK cascade. *Nat Immunol* **2**, 165-71.

Balciunaite, G., Ceredig, R. and Rolink, A. G. (2005). The earliest subpopulation of mouse thymocytes contains potent T, significant macrophage, and natural killer cell but no B-lymphocyte potential. *Blood* **105**, 1930-6.

Balciunaite, G., Keller, M. P., Balciunaite, E., Piali, L., Zuklys, S., Mathieu, Y. D., Gill, J., Boyd, R., Sussman, D. J. and Hollander, G. A. (2002). Wnt glycoproteins regulate the expression of FoxN1, the gene defective in nude mice. *Nat Immunol* **3**, 1102-8.

Barclay, A. N. and Mayrhofer, G. (1981). Bone marrow origin of Ia-positive cells in the medulla rat thymus. *J Exp Med* **153**, 1666-71.

Barclay, A. N. and Mayrhofer, G. (1982). Ia positive cells in the medulla of rat thymus are bone marrow derived. *Adv Exp Med Biol* **149**, 381-7.

Bearman, R. M., Levine, G. D. and Bensch, K. G. (1978). The ultrastructure of the normal human thymus: a study of 36 cases. *Anat Rec* **190**, 755-81.

Bendelac, A., Matzinger, P., Seder, R. A., Paul, W. E. and Schwartz, R. H. (1992). Activation events during thymic selection. *J Exp Med* **175**, 731-42.

Bennett, A. R., Farley, A., Blair, N. F., Gordon, J., Sharp, L. and Blackburn, C. C. (2002). Identification and characterization of thymic epithelial progenitor cells. *Immunity* **16**, 803-14.

Benoist, C. and Mathis, D. (1989). Positive selection of the T cell repertoire: where and when does it occur? *Cell* **58**, 1027-33.

Berg, L. J., Pullen, A. M., Fazekas de St Groth, B., Mathis, D., Benoist, C. and Davis, M. M. (1989). Antigen/MHC-specific T cells are preferentially exported from the thymus in the presence of their MHC ligand. *Cell* **58**, 1035-46.

Bevan, M. J. (1997). In thymic selection, peptide diversity gives and takes away. *Immunity* **7**, 175-8.

Bhandoola, A. and Sambandam, A. (2006). From stem cell to T cell: one route or many? *Nat Rev Immunol* **6**, 117-26.

Bhandoola, A., Sambandam, A., Allman, D., Meraz, A. and Schwarz, B. (2003). Early T lineage progenitors: new insights, but old questions remain. *J Immunol* **171**, 5653-8.

Blackburn, C. C., Augustine, C. L., Li, R., Harvey, R. P., Malin, M. A., Boyd, R. L., Miller, J. F. and Morahan, G. (1996). The nu gene acts cell-autonomously and is required for differentiation of thymic epithelial progenitors. *Proc Natl Acad Sci U S A* **93**, 5742-6.

Blau, J. N. (1973). Hassall's corpuscles--a site of thymocyte death. *Br J Exp Pathol* **54**, 634-7.

Bleul, C. C. and Boehm, T. (2000). Chemokines define distinct microenvironments in the developing thymus. *Eur J Immunol* **30**, 3371-9.

Bleul, C. C., Corbeaux, T., Reuter, A., Fisch, P., Monting, J. S. and Boehm, T. (2006). Formation of a functional thymus initiated by a postnatal epithelial progenitor cell. *Nature* **441**, 992-6.

Bockman, D. E. and Kirby, M. L. (1984). Dependence of thymus development on derivatives of the neural crest. *Science* **223**, 498-500.

Boehm, T., Scheu, S., Pfeffer, K. and Bleul, C. C. (2003). Thymic medullary epithelial cell differentiation, thymocyte emigration, and the control of autoimmunity require lympho-epithelial cross talk via LTbetaR. *J Exp Med* **198**, 757-69.

Bonini, N. M., Leiserson, W. M. and Benzer, S. (1993). The eyes absent gene: genetic control of cell survival and differentiation in the developing Drosophila eye. *Cell* **72**, 379-95.

Borgulya, P., Kishi, H., Uematsu, Y. and von Boehmer, H. (1992). Exclusion and inclusion of alpha and beta T cell receptor alleles. *Cell* **69**, 529-37.

Boyd, R. L. and Hugo, P. (1991). Towards an integrated view of thymopoiesis. *Immunol Today* **12**, 71-9.

Boyd, R. L., Tucek, C. L., Godfrey, D. I., Izon, D. J., Wilson, T. J., Davidson, N. J., Bean, A. G., Ladyman, H. M., Ritter, M. A. and Hugo, P. (1993). The thymic microenvironment. *Immunol Today* **14**, 445-59.

Brandle, D., Muller, C., Rulicke, T., Hengartner, H. and Pircher, H. (1992). Engagement of the T-cell receptor during positive selection in the thymus down-regulates RAG-1 expression. *Proc Natl Acad Sci U S A* **89**, 9529-33.

Burkly, L., Hession, C., Ogata, L., Reilly, C., Marconi, L. A., Olson, D., Tizard, R., Cate, R. and Lo, D. (1995). Expression of relB is required for the development of thymic medulla and dendritic cells. *Nature* **373**, 531-6.

Burtrum, D. B., Kim, S., Dudley, E. C., Hayday, A. C. and Petrie, H. T. (1996). TCR gene recombination and alpha beta-gamma delta lineage divergence: productive TCR-beta rearrangement is neither exclusive nor preclusive of gamma delta cell development. *J Immunol* **157**, 4293-6.

Campbell, J. J., Pan, J. and Butcher, E. C. (1999). Cutting edge: developmental switches in chemokine responses during T cell maturation. *J Immunol* **163**, 2353-7.

Cardarelli, P. M., Crispe, I. N. and Pierschbacher, M. D. (1988). Preferential expression of fibronectin receptors on immature thymocytes. *J Cell Biol* **106**, 2183-90.

Ceredig, R., Jenkinson, E. J., MacDonald, H. R. and Owen, J. J. (1982). Development of cytolytic T lymphocyte precursors in organ-cultured mouse embryonic thymus rudiments. *J Exp Med* **155**, 617-22.

Chan, A. S. (1986). Ultrastructure of epithelial thymic cysts of the chicks. *Poult Sci* **65**, 177-82.

Chan, C. and Sainte-Marie, G. (1968). Distribution and morphology of the subcapsular and reticular cells of the ten-week-old rat thymus. *J Anat* **102**, 477-91.

Chisaka, O. and Capecchi, M. R. (1991). Regionally restricted developmental defects resulting from targeted disruption of the mouse homeobox gene hox-1.5. *Nature* **350**, 473-9.

Clark, R. A., Yamanaka, K., Bai, M., Dowgiert, R. and Kupper, T. S. (2005). Human skin cells support thymus-independent T cell development. *J Clin Invest* **115**, 3239-49.

Clark, S. L., Jr. (1963). The thymus in mice of strain 129/J, studied with the electron microscope. *Am J Anat* **112**, 1-33.

Cordier, A. C. and Haumont, S. M. (1980). Development of thymus, parathyroids, and ultimo-branchial bodies in NMRI and nude mice. *Am J Anat* **157**, 227-63.

Cosgrove, D., Chan, S. H., Waltzinger, C., Benoist, C. and Mathis, D. (1992). The thymic compartment responsible for positive selection of CD4+ T cells. *Int Immunol* **4**, 707-10.

Crompton, T., Outram, S. V., Buckland, J. and Owen, M. J. (1998). Distinct roles of the interleukin-7 receptor alpha chain in fetal and adult thymocyte development revealed by analysis of interleukin-7 receptor alpha-deficient mice. *Eur J Immunol* **28**, 1859-66.

Deftos, M. L., He, Y. W., Ojala, E. W. and Bevan, M. J. (1998). Correlating notch signaling with thymocyte maturation. *Immunity* **9**, 777-86.

DeKoter, R. P. and Singh, H. (2000). Regulation of B lymphocyte and macrophage development by graded expression of PU.1. *Science* **288**, 1439-41.

Derbinski, J., Schulte, A., Kyewski, B. and Klein, L. (2001). Promiscuous gene expression in medullary thymic epithelial cells mirrors the peripheral self. *Nat Immunol* **2**, 1032-9.

Dionne, C. J., Tse, K. Y., Weiss, A. H., Franco, C. B., Wiest, D. L., Anderson, M. K. and Rothenberg, E. V. (2005). Subversion of T lineage commitment by PU.1 in a clonal cell line system. *Dev Biol* **280**, 448-66.

Donskoy, E., Foss, D. and Goldschneider, I. (2003). Gated importation of prothymocytes by adult mouse thymus is coordinated with their periodic mobilization from bone marrow. *J Immunol* **171**, 3568-75.

Dooley, J., Erickson, M. and Farr, A. G. (2005a). An organized medullary epithelial structure in the normal thymus expresses molecules of respiratory epithelium and resembles the epithelial thymic rudiment of nude mice. *J Immunol* **175**, 4331-7.

Dooley, J., Erickson, M., Roelink, H. and Farr, A. G. (2005b). Nude thymic rudiment lacking functional foxn1 resembles respiratory epithelium. *Dev Dyn* **233**, 1605-12.

Dou, Y. M., Watanabe, Y., Aiba, Y., Wada, K. and Katsura, Y. (1994). A novel culture system for induction of T cell development: modification of fetal thymus organ culture. *Thymus* **23**, 195-207.

Douagi, I., Andre, I., Ferraz, J. C. and Cumano, A. (2000). Characterization of T cell precursor activity in the murine fetal thymus: evidence for an input of T cell precursors between days 12 and 14 of gestation. *Eur J Immunol* **30**, 2201-10.

Doyle, C. and Strominger, J. L. (1987). Interaction between CD4 and class II MHC molecules mediates cell adhesion. *Nature* **330**, 256-9.

Dudley, E. C., Petrie, H. T., Shah, L. M., Owen, M. J. and Hayday, A. C. (1994). T cell receptor beta chain gene rearrangement and selection during thymocyte development in adult mice. *Immunity* **1**, 83-93.

Duijvestijn, A. M. and Hoefsmit, E. C. (1981). Ultrastructure of the rat thymus: the micro-environment of T-lymphocyte maturation. *Cell Tissue Res* **218**, 279-92.

Durum, S. K., Candeias, S., Nakajima, H., Leonard, W. J., Baird, A. M., Berg, L. J. and Muegge, K. (1998). Interleukin 7 receptor control of T cell receptor gamma gene rearrangement: role of receptor-associated chains and locus accessibility. *J Exp Med* **188**, 2233-41.

Egerton, M., Scollay, R. and Shortman, K. (1990). Kinetics of mature T-cell development in the thymus. *Proc Natl Acad Sci U S A* **87**, 2579-82.

Engel, I., Johns, C., Bain, G., Rivera, R. R. and Murre, C. (2001). Early thymocyte development is regulated by modulation of E2A protein activity. *J Exp Med* **194**, 733-45.

Ernstrom, U., Gyllensten, L., Larsson, B. (1965). Venous output of lymphocytes from the thymus. *Nature* **207**.

Farr, A. G. and Anderson, S. K. (1985). Epithelial heterogeneity in the murine thymus: fucose-specific lectins bind medullary epithelial cells. *J Immunol* **134**, 2971-7.

Farr, A. G., Dooley, J. L. and Erickson, M. (2002). Organization of thymic medullary epithelial heterogeneity: implications for mechanisms of epithelial differentiation. *Immunol Rev* **189**, 20-7.

Fehling, H. J., Krotkova, A., Saint-Ruf, C. and von Boehmer, H. (1995). Crucial role of the pre-T-cell receptor alpha gene in development of alpha beta but not gamma delta T cells. *Nature* **375**, 795-8.

Felli, M. P., Maroder, M., Mitsiadis, T. A., Campese, A. F., Bellavia, D., Vacca, A., Mann, R. S., Frati, L., Lendahl, U., Gulino, A. et al. (1999). Expression pattern of notch1, 2 and 3 and Jagged1 and 2 in lymphoid and stromal thymus components: distinct ligand-receptor interactions in intrathymic T cell development. *Int Immunol* **11**, 1017-25.

Flanagan, S. P. (1966). 'Nude', a new hairless gene with pleiotropic effects in the mouse. *Genet Res* **8**, 295-309.

Fontaine-Perus, J. C., Calman, F. M., Kaplan, C. and Le Douarin, N. M. (1981). Seeding of the 10-day mouse embryo thymic rudiment by lymphocyte precursors in vitro. *J Immunol* **126**, 2310-6.

Foss, D. L., Donskoy, E. and Goldschneider, I. (2001). The importation of hematogenous precursors by the thymus is a gated phenomenon in normal adult mice. *J Exp Med* **193**, 365-74.

Gabor, M. J., Godfrey, D. I. and Scollay, R. (1997). Recent thymic emigrants are distinct from most medullary thymocytes. *Eur J Immunol* **27**, 2010-5.

Garg, V., Yamagishi, C., Hu, T., Kathiriya, I. S., Yamagishi, H. and Srivastava, D. (2001). Tbx1, a DiGeorge syndrome candidate gene, is regulated by sonic hedgehog during pharyngeal arch development. *Dev Biol* **235**, 62-73.

Ge, Q. and Chen, W. F. (1999). Phenotypic identification of the subgroups of murine T-cell receptor alphabeta+ CD4+ CD8- thymocytes and its implication in the late stage of thymocyte development. *Immunology* **97**, 665-71.

Gill, J., Malin, M., Hollander, G. A. and Boyd, R. (2002). Generation of a complete thymic microenvironment by MTS24(+) thymic epithelial cells. *Nat Immunol* **3**, 635-42.

Godfrey, D. I., Izon, D. J., Tucek, C. L., Wilson, T. J. and Boyd, R. L. (1990). The phenotypic heterogeneity of mouse thymic stromal cells. *Immunology* **70**, 66-74.

Godfrey, D. I., Kennedy, J., Suda, T. and Zlotnik, A. (1993). A developmental pathway involving four phenotypically and functionally distinct subsets of CD3-CD4-

CD8- triple-negative adult mouse thymocytes defined by CD44 and CD25 expression. *J Immunol* **150**, 4244-52.

Godfrey, D. I. and Zlotnik, A. (1993). Control points in early T-cell development. *Immunol Today* **14**, 547-53.

Godfrey, D. I., Zlotnik, A. and Suda, T. (1992). Phenotypic and functional characterization of c-kit expression during intrathymic T cell development. *J Immunol* **149**, 2281-5.

Gordon, J., Bennett, A. R., Blackburn, C. C. and Manley, N. R. (2001). Gcm2 and Foxn1 mark early parathyroid- and thymus-specific domains in the developing third pharyngeal pouch. *Mech Dev* **103**, 141-3.

Gordon, J., Wilson, V. A., Blair, N. F., Sheridan, J., Farley, A., Wilson, L., Manley, N. R. and Blackburn, C. C. (2004). Functional evidence for a single endodermal origin for the thymic epithelium. *Nat Immunol* **5**, 546-53.

Gotter, J., Brors, B., Hergenhahn, M. and Kyewski, B. (2004). Medullary epithelial cells of the human thymus express a highly diverse selection of tissue-specific genes colocalized in chromosomal clusters. *J Exp Med* **199**, 155-66.

Gounari, F., Aifantis, I., Martin, C., Fehling, H. J., Hoeflinger, S., Leder, P., von Boehmer, H. and Reizis, B. (2002). Tracing lymphopoiesis with the aid of a pTalpha-controlled reporter gene. *Nat Immunol* **3**, 489-96.

Gray, D. H., Chidgey, A. P. and Boyd, R. L. (2002). Analysis of thymic stromal cell populations using flow cytometry. *J Immunol Methods* **260**, 15-28.

Groettrup, M., Ungewiss, K., Azogui, O., Palacios, R., Owen, M. J., Hayday, A. C. and von Boehmer, H. (1993). A novel disulfide-linked heterodimer on pre-T cells consists of the T cell receptor beta chain and a 33 kd glycoprotein. *Cell* **75**, 283-94.

Groettrup, M. and von Boehmer, H. (1993). A role for a pre-T-cell receptor in T-cell development. *Immunol Today* **14**, 610-4.

Guillet, M., Brouard, S., Gagne, K., Sebille, F., Cuturi, M. C., Delsuc, M. A. and Soulillou, J. P. (2002). Different qualitative and quantitative regulation of V beta TCR transcripts during early acute allograft rejection and tolerance induction. *J Immunol* **168**, 5088-95.

Hamazaki, Y., Fujita, H., Kobayashi, T., Choi, Y., Scott, H. S., Matsumoto, M. and Minato, N. (2007). Medullary thymic epithelial cells expressing Aire represent a unique lineage derived from cells expressing claudin. *Nat Immunol* **8**, 304-11.

Hammond, W. (1954). Origin of the thymus in the chick. *J Morphol* **95**, 501-613.

Hare, K. J., Jenkinson, E. J. and Anderson, G. (1999). CD69 expression discriminates MHC-dependent and -independent stages of thymocyte positive selection. *J Immunol* **162**, 3978-83.

Hare, K. J., Jenkinson, E. J. and Anderson, G. (2000). An essential role for the IL-7 receptor during intrathymic expansion of the positively selected neonatal T cell repertoire. *J Immunol* **165**, 2410-4.

Hare, K. J., Wilkinson, R. W., Jenkinson, E. J. and Anderson, G. (1998). Identification of a developmentally regulated phase of postselection expansion driven by thymic epithelium. *J Immunol* **160**, 3666-72.

Harman, B. C., Jenkinson, E. J. and Anderson, G. (2003). Entry into the thymic microenvironment triggers Notch activation in the earliest migrant T cell progenitors. *J Immunol* **170**, 1299-303.

Hassall, A. H. (1849). The microscopic anatomy of the human body, in health and disease. London

Havran, W. L., Carbone, A. and Allison, J. P. (1991). Murine T cells with invariant gamma delta antigen receptors: origin, repertoire, and specificity. *Semin Immunol* **3**, 89-97.

He, W. and Kabelitz, D. (1995). Differential effects of interleukin-2 and interleukin-7 on the induction of CD4 and CD8 expression by double-negative human thymocytes. *Scand J Immunol* **41**, 309-12.

Hernandez-Hoyos, G., Anderson, M. K., Wang, C., Rothenberg, E. V. and Alberola-Ila, J. (2003). GATA-3 expression is controlled by TCR signals and regulates CD4/CD8 differentiation. *Immunity* **19**, 83-94.

Hetzer-Egger, C., Schorpp, M., Haas-Assenbaum, A., Balling, R., Peters, H. and Boehm, T. (2002). Thymopoiesis requires Pax9 function in thymic epithelial cells. *Eur J Immunol* **32**, 1175-81.

Hollander, G., Gill, J., Zuklys, S., Iwanami, N., Liu, C. and Takahama, Y. (2006). Cellular and molecular events during early thymus development. *Immunol Rev* **209**, 28-46.

Hollander, G. A., Wang, B., Nichogiannopoulou, A., Platenburg, P. P., van Ewijk, W., Burakoff, S. J., Gutierrez-Ramos, J. C. and Terhorst, C. (1995). Developmental

control point in induction of thymic cortex regulated by a subpopulation of prothymocytes. *Nature* **373**, 350-3.

Hozumi, K., Abe, N., Chiba, S., Hirai, H. and Habu, S. (2003). Active form of notch members can enforce T lymphopoiesis on lymphoid progenitors in the monolayer culture specific for B cell development. *J Immunol* **170**, 4973-9.

Hozumi, K., Kobori, A., Sato, T., Nozaki, H., Nishikawa, S., Nishimura, T. and Habu, S. (1994). Pro-T cells in fetal thymus express c-kit and RAG-2 but do not rearrange the gene encoding the T cell receptor beta chain. *Eur J Immunol* **24**, 1339-44.

Hozumi, K., Negishi, N., Suzuki, D., Abe, N., Sotomaru, Y., Tamaoki, N., Mailhos, C., Ish-Horowicz, D., Habu, S. and Owen, M. J. (2004). Delta-like 1 is necessary for the generation of marginal zone B cells but not T cells in vivo. *Nat Immunol* **5**, 638-44.

Hugo, P., Kappler, J. W., McCormack, J. E. and Marrack, P. (1993). Fibroblasts can induce thymocyte positive selection in vivo. *Proc Natl Acad Sci U S A* **90**, 10335-9.

Ikuta, K., Kina, T., MacNeil, I., Uchida, N., Peault, B., Chien, Y. H. and Weissman, I. L. (1992). Development of gamma delta T-cell subsets from fetal hematopoietic stem cells. *Ann N Y Acad Sci* **651**, 21-32.

Itoi, M., Kawamoto, H., Katsura, Y. and Amagai, T. (2001). Two distinct steps of immigration of hematopoietic progenitors into the early thymus anlage. *Int Immunol* **13**, 1203-11.

Itoi, M., Tsukamoto, N. and Amagai, T. (2007). Expression of Dll4 and CCL25 in Foxn1-negative epithelial cells in the post-natal thymus. *Int Immunol* **19**, 127-32.

Jenkinson, W. E., Jenkinson, E. J. and Anderson, G. (2003). Differential requirement for mesenchyme in the proliferation and maturation of thymic epithelial progenitors. *J Exp Med* **198**, 325-32.

Jenkinson, W. E., Rossi, S. W., Parnell, S. M., Jenkinson, E. J. and Anderson, G. (2007). PDGFRalpha-expressing mesenchyme regulates thymus growth and the availability of intrathymic niches. *Blood* **109**, 954-60.

Jerome, L. A. and Papaioannou, V. E. (2001). DiGeorge syndrome phenotype in mice mutant for the T-box gene, Tbx1. *Nat Genet* **27**, 286-91.

Jiang, X., Rowitch, D. H., Soriano, P., McMahon, A. P. and Sucov, H. M. (2000). Fate of the mammalian cardiac neural crest. *Development* **127**, 1607-16.

Jotereau, F., Heuze, F., Salomon-Vie, V. and Gascan, H. (1987). Cell kinetics in the fetal mouse thymus: precursor cell input, proliferation, and emigration. *J Immunol* **138**, 1026-30.

Jotereau, F. V., Houssaint, E. and Le Douarin, N. M. (1980). Lymphoid stem cell homing to the early thymic primordium of the avian embryo. *Eur J Immunol* **10**, 620-7.

Jotereau, F. V. and Le Douarin, N. M. (1982). Demonstration of a cyclic renewal of the lymphocyte precursor cells in the quail thymus during embryonic and perinatal life. *J Immunol* **129**, 1869-77.

Kamarck, M. E. and Gottlieb, P. D. (1977). Expression of thymocyte surface alloantigens in the fetal mouse thymus in vivo and in organ culture. *J Immunol* **119**, 407-15.

Kampinga, J., Berges, S., Boyd, R. L., Brekelmans, P., Colic, M., van Ewijk, W., Kendall, M. D., Ladyman, H., Nieuwenhuis, P., Ritter, M. A. et al. (1989). Thymic epithelial antibodies: immunohistological analysis and introduction of nomenclature. *Thymus* **13**, 165-73.

Kato, S. (1997). Thymic microvascular system. *Microsc Res Tech* **38**, 287-99.

Kato, S. and Schoefl, G. I. (1989). Microvasculature of normal and involuted mouse thymus. Light- and electron-microscopic study. *Acta Anat (Basel)* **135**, 1-11.

Kawamoto, H., Ohmura, K. and Katsura, Y. (1997). Direct evidence for the commitment of hematopoietic stem cells to T, B and myeloid lineages in murine fetal liver. *Int Immunol* **9**, 1011-9.

Kaye, J., Hsu, M. L., Sauron, M. E., Jameson, S. C., Gascoigne, N. R. and Hedrick, S. M. (1989). Selective development of CD4+ T cells in transgenic mice expressing a class II MHC-restricted antigen receptor. *Nature* **341**, 746-9.

Kendall, M. D. (1991). Functional anatomy of the thymic microenvironment. *J Anat* **177**, 1-29.

Khosla, S. and Ovalle, W. K. (1986). Morphology and distribution of cystic cavities in the normal murine thymus. *Cell Tissue Res* **246**, 531-42.

Kirkpatrick, J. A., Jr. and DiGeorge, A. M. (1968). Congenital absence of the thymus. *Am J Roentgenol Radium Ther Nucl Med* **103**, 32-7.

Kishi, H., Borgulya, P., Scott, B., Karjalainen, K., Traunecker, A., Kaufman, J. and von Boehmer, H. (1991). Surface expression of the beta T cell receptor (TCR)

chain in the absence of other TCR or CD3 proteins on immature T cells. *Embo J* **10**, 93-100.

Klein, L., Klein, T., Ruther, U. and Kyewski, B. (1998). CD4 T cell tolerance to human C-reactive protein, an inducible serum protein, is mediated by medullary thymic epithelium. *J Exp Med* **188**, 5-16.

Klug, D. B., Carter, C., Crouch, E., Roop, D., Conti, C. J. and Richie, E. R. (1998). Interdependence of cortical thymic epithelial cell differentiation and T-lineage commitment. *Proc Natl Acad Sci U S A* **95**, 11822-7.

Klug, D. B., Carter, C., Gimenez-Conti, I. B. and Richie, E. R. (2002). Cutting edge: thymocyte-independent and thymocyte-dependent phases of epithelial patterning in the fetal thymus. *J Immunol* **169**, 2842-5.

Kodama, H., Nose, M., Niida, S., Nishikawa, S. and Nishikawa, S. (1994). Involvement of the c-kit receptor in the adhesion of hematopoietic stem cells to stromal cells. *Exp Hematol* **22**, 979-84.

Kondo, M., Scherer, D. C., Miyamoto, T., King, A. G., Akashi, K., Sugamura, K. and Weissman, I. L. (2000). Cell-fate conversion of lymphoid-committed progenitors by instructive actions of cytokines. *Nature* **407**, 383-6.

Kondo, M., Weissman, I. L. and Akashi, K. (1997). Identification of clonogenic common lymphoid progenitors in mouse bone marrow. *Cell* **91**, 661-72.

Kyewski, B., Derbinski, J., Gotter, J. and Klein, L. (2002). Promiscuous gene expression and central T-cell tolerance: more than meets the eye. *Trends Immunol* **23**, 364-71.

Kyewski, B. A. (1987). Seeding of thymic microenvironments defined by distinct thymocyte-stromal cell interactions is developmentally controlled. *J Exp Med* **166**, 520-38.

Kyewski, B. A. and Kaplan, H. S. (1982). Lymphoepithelial interactions in the mouse thymus: phenotypic and kinetic studies on thymic nurse cells. *J Immunol* **128**, 2287-94.

Lammert, E., Cleaver, O. and Melton, D. (2001). Induction of pancreatic differentiation by signals from blood vessels. *Science* **294**, 564-7.

Le Douarin, N. and Jotereau, F. (1973). [Embryologic origin of thymus lymphocytes in bird embryos]. *C R Acad Sci Hebd Seances Acad Sci D* **276**, 629-32.

Le Lievre, C. S. and Le Douarin, N. M. (1975). Mesenchymal derivatives of the neural crest: analysis of chimaeric quail and chick embryos. *J Embryol Exp Morphol* **34**, 125-54.

Lee, C. K., Kim, J. K., Kim, Y., Lee, M. K., Kim, K., Kang, J. K., Hofmeister, R., Durum, S. K. and Han, S. S. (2001). Generation of macrophages from early T progenitors in vitro. *J Immunol* **166**, 5964-9.

Levelt, C. N., Mombaerts, P., Wang, B., Kohler, H., Tonegawa, S., Eichmann, K. and Terhorst, C. (1995). Regulation of thymocyte development through CD3: functional dissociation between p56lck and CD3 sigma in early thymic selection. *Immunity* **3**, 215-22.

Lind, E. F., Prockop, S. E., Porritt, H. E. and Petrie, H. T. (2001). Mapping precursor movement through the postnatal thymus reveals specific microenvironments supporting defined stages of early lymphoid development. *J Exp Med* **194**, 127-34.

Linette, G. P., Grusby, M. J., Hedrick, S. M., Hansen, T. H., Glimcher, L. H. and Korsmeyer, S. J. (1994). Bcl-2 is upregulated at the CD4+ CD8+ stage during positive selection and promotes thymocyte differentiation at several control points. *Immunity* **1**, 197-205.

Liston, A., Lesage, S., Wilson, J., Peltonen, L. and Goodnow, C. C. (2003). Aire regulates negative selection of organ-specific T cells. *Nat Immunol* **4**, 350-4.

Liu, C., Saito, F., Liu, Z., Lei, Y., Uehara, S., Love, P., Lipp, M., Kondo, S., Manley, N. and Takahama, Y. (2006). Coordination between CCR7- and CCR9-mediated chemokine signals in prevascular fetal thymus colonization. *Blood* **108**, 2531-9.

Liu, C., Ueno, T., Kuse, S., Saito, F., Nitta, T., Piali, L., Nakano, H., Kakiuchi, T., Lipp, M., Hollander, G. A. et al. (2005). The role of CCL21 in recruitment of T-precursor cells to fetal thymi. *Blood* **105**, 31-9.

Lucas, K., Vremec, D., Wu, L. and Shortman, K. (1998). A linkage between dendritic cell and T-cell development in the mouse thymus: the capacity of sequential T-cell precursors to form dendritic cells in culture. *Dev Comp Immunol* **22**, 339-49.

Lundin, P. M. and Schelin, U. (1965). Ultrastructure of the rat thymus. *Acta Pathol Microbiol Scand* **65**, 379-94.

Maki, K., Sunaga, S., Komagata, Y., Kodaira, Y., Mabuchi, A., Karasuyama, H., Yokomuro, K., Miyazaki, J. I. and Ikuta, K. (1996). Interleukin 7 receptor-deficient mice lack gammadelta T cells. *Proc Natl Acad Sci U S A* **93**, 7172-7.

Malissen, M., Gillet, A., Ardouin, L., Bouvier, G., Trucy, J., Ferrier, P., Vivier, E. and Malissen, B. (1995). Altered T cell development in mice with a targeted mutation of the CD3-epsilon gene. *Embo J* **14**, 4641-53.

Malissen, M., Trucy, J., Letourneur, F., Rebai, N., Dunn, D. E., Fitch, F. W., Hood, L. and Malissen, B. (1988). A T cell clone expresses two T cell receptor alpha genes but uses one alpha beta heterodimer for allorecognition and self MHC-restricted antigen recognition. *Cell* **55**, 49-59.

Maloy, K. J. and Powrie, F. (2001). Regulatory T cells in the control of immune pathology. *Nat Immunol* **2**, 816-22.

Manley, N. R. (2000). Thymus organogenesis and molecular mechanisms of thymic epithelial cell differentiation. *Semin Immunol* **12**, 421-8.

Manley, N. R. and Blackburn, C. C. (2003). A developmental look at thymus organogenesis: where do the non-hematopoietic cells in the thymus come from? *Curr Opin Immunol* **15**, 225-32.

Manley, N. R. and Capecchi, M. R. (1995). The role of Hoxa-3 in mouse thymus and thyroid development. *Development* **121**, 1989-2003.

Manley, N. R. and Capecchi, M. R. (1998). Hox group 3 paralogs regulate the development and migration of the thymus, thyroid, and parathyroid glands. *Dev Biol* **195**, 1-15.

Marolleau, J. P., Fondell, J. D., Malissen, M., Trucy, J., Barbier, E., Marcu, K. B., Cazenave, P. A. and Primi, D. (1988). The joining of germ-line V alpha to J alpha genes replaces the preexisting V alpha-J alpha complexes in a T cell receptor alpha, beta positive T cell line. *Cell* **55**, 291-300.

Marshall, A. H. and White, R. G. (1961). The immunological reactivity of the thymus. *Br J Exp Pathol* **42**, 379-85.

Martin, C. H., Aifantis, I., Scimone, M. L., von Andrian, U. H., Reizis, B., von Boehmer, H. and Gounari, F. (2003). Efficient thymic immigration of B220+ lymphoid-restricted bone marrow cells with T precursor potential. *Nat Immunol* **4**, 866-73.

Matsumoto, K., Yoshitomi, H., Rossant, J. and Zaret, K. S. (2001). Liver organogenesis promoted by endothelial cells prior to vascular function. *Science* **294**, 559-63.

Milicevic, N. M. and Milicevic, Z. (1988). On the nature of the content in the PAS-positive macrophages of the rodent thymus. *Thymus* **11**, 257-9.

Misslitz, A., Pabst, O., Hintzen, G., Ohl, L., Kremmer, E., Petrie, H. T. and Forster, R. (2004). Thymic T cell development and progenitor localization depend on CCR7. *J Exp Med* **200**, 481-91.

Miyasaka, M., Pabst, R., Dudler, L., Cooper, M. and Yamaguchi, K. (1990). Characterization of lymphatic and venous emigrants from the thymus. *Thymus* **16**, 29-43.

Mombaerts, P., Clarke, A. R., Rudnicki, M. A., Iacomini, J., Itohara, S., Lafaille, J. J., Wang, L., Ichikawa, Y., Jaenisch, R., Hooper, M. L. et al. (1992a). Mutations in T-cell antigen receptor genes alpha and beta block thymocyte development at different stages. *Nature* **360**, 225-31.

Mombaerts, P., Iacomini, J., Johnson, R. S., Herrup, K., Tonegawa, S. and Papaioannou, V. E. (1992b). RAG-1-deficient mice have no mature B and T lymphocytes. *Cell* **68**, 869-77.

Montecino-Rodriguez, E. and Dorshkind, K. (2003). To T or not to T: reassessing the common lymphoid progenitor. *Nat Immunol* **4**, 100-1.

Moore, M. A. and Owen, J. J. (1967). Experimental studies on the development of the thymus. *J Exp Med* **126**, 715-26.

Moore, N. C., Anderson, G., Smith, C. A., Owen, J. J. and Jenkinson, E. J. (1993). Analysis of cytokine gene expression in subpopulations of freshly isolated thymocytes and thymic stromal cells using semiquantitative polymerase chain reaction. *Eur J Immunol* **23**, 922-7.

Moore, T. A. and Zlotnik, A. (1995). T-cell lineage commitment and cytokine responses of thymic progenitors. *Blood* **86**, 1850-60.

Moore-Scott, B. A. and Manley, N. R. (2005). Differential expression of Sonic hedgehog along the anterior-posterior axis regulates patterning of pharyngeal pouch endoderm and pharyngeal endoderm-derived organs. *Dev Biol* **278**, 323-35.

Morrison-Graham, K., Schatteman, G. C., Bork, T., Bowen-Pope, D. F. and Weston, J. A. (1992). A PDGF receptor mutation in the mouse (Patch) perturbs the development of a non-neuronal subset of neural crest-derived cells. *Development* **115**, 133-42.

Morrow, M. A., Mayer, E. W., Perez, C. A., Adlam, M. and Siu, G. (1999). Overexpression of the Helix-Loop-Helix protein Id2 blocks T cell development at multiple stages. *Mol Immunol* **36**, 491-503.

Muller, T. S., Ebensperger, C., Neubuser, A., Koseki, H., Balling, R., Christ, B. and Wilting, J. (1996). Expression of avian Pax1 and Pax9 is intrinsically regulated in the pharyngeal endoderm, but depends on environmental influences in the paraxial mesoderm. *Dev Biol* **178**, 403-17.

Nabarra, B. and Papiernik, M. (1988). Phenotype of thymic stromal cells. An immunoelectron microscopic study with anti-IA, anti-MAC-1, and anti-MAC-2 antibodies. *Lab Invest* **58**, 524-31.

Naquet, P., Naspetti, M. and Boyd, R. (1999). Development, organization and function of the thymic medulla in normal, immunodeficient or autoimmune mice. *Semin Immunol* **11**, 47-55.

Nehls, M., Kyewski, B., Messerle, M., Waldschutz, R., Schuddekopf, K., Smith, A. J. and Boehm, T. (1996). Two genetically separable steps in the differentiation of thymic epithelium. *Science* **272**, 886-9.

Nehls, M., Pfeifer, D., Schorpp, M., Hedrich, H. and Boehm, T. (1994). New member of the winged-helix protein family disrupted in mouse and rat nude mutations. *Nature* **372**, 103-7.

Nichogiannopoulou, A., Trevisan, M., Friedrich, C. and Georgopoulos, K. (1998). Ikaros in hemopoietic lineage determination and homeostasis. *Semin Immunol* **10**, 119-25.

Nichogiannopoulou, A., Trevisan, M., Neben, S., Friedrich, C. and Georgopoulos, K. (1999). Defects in hemopoietic stem cell activity in Ikaros mutant mice. *J Exp Med* **190**, 1201-14.

Nicolas, J. F., Savino, W., Reano, A., Viac, J., Brochier, J. and Dardenne, M. (1985). Heterogeneity of thymic epithelial cell (TEC) keratins--immunohistochemical and biochemical evidence for a subset of highly differentiated TEC in the mouse. *J Histochem Cytochem* **33**, 687-94.

Nikolova, G. and Lammert, E. (2003). Interdependent development of blood vessels and organs. *Cell Tissue Res* **314**, 33-42.

Norment, A. M., Salter, R. D., Parham, P., Engelhard, V. H. and Littman, D. R. (1988). Cell-cell adhesion mediated by CD8 and MHC class I molecules. *Nature* **336**, 79-81.

Nusse, R. and Varmus, H. E. (1992). Wnt genes. *Cell* **69**, 1073-87.

Odaka, C., Morisada, T., Oike, Y. and Suda, T. (2006). Distribution of lymphatic vessels in mouse thymus: immunofluorescence analysis. *Cell Tissue Res* **325**, 13-22.

Ogawa, M., Matsuzaki, Y., Nishikawa, S., Hayashi, S., Kunisada, T., Sudo, T., Kina, T., Nakauchi, H. and Nishikawa, S. (1991). Expression and function of c-kit in hemopoietic progenitor cells. *J Exp Med* **174**, 63-71.

Pai, S. Y., Truitt, M. L., Ting, C. N., Leiden, J. M., Glimcher, L. H. and Ho, I. C. (2003). Critical roles for transcription factor GATA-3 in thymocyte development. *Immunity* **19**, 863-75.

Palmer, D. B., Viney, J. L., Ritter, M. A., Hayday, A. C. and Owen, M. J. (1993). Expression of the alpha beta T-cell receptor is necessary for the generation of the thymic medulla. *Dev Immunol* **3**, 175-9.

Patel, S. R., Gordon, J., Mahbub, F., Blackburn, C. C. and Manley, N. R. (2006). Bmp4 and Noggin expression during early thymus and parathyroid organogenesis. *Gene Expr Patterns* **6**, 794-9.

Pawlowski, T., Elliott, J. D., Loh, D. Y. and Staerz, U. D. (1993). Positive selection of T lymphocytes on fibroblasts. *Nature* **364**, 642-5.

Penit, C., Lucas, B. and Vasseur, F. (1995). Cell expansion and growth arrest phases during the transition from precursor (CD4-8-) to immature (CD4+8+) thymocytes in normal and genetically modified mice. *J Immunol* **154**, 5103-13.

Pennington, D. J., Silva-Santos, B. and Hayday, A. C. (2005). Gammadelta T cell development--having the strength to get there. *Curr Opin Immunol* **17**, 108-15.

Pereira, G. and Clermont, Y. (1971). Distribution of cell web-containing epithelial reticular cells in the rat thymus. *Anat Rec* **169**, 613-26.

Peschon, J. J., Morrissey, P. J., Grabstein, K. H., Ramsdell, F. J., Maraskovsky, E., Gliniak, B. C., Park, L. S., Ziegler, S. F., Williams, D. E., Ware, C. B. et al. (1994). Early lymphocyte expansion is severely impaired in interleukin 7 receptor-deficient mice. *J Exp Med* **180**, 1955-60.

Peters, H., Neubuser, A., Kratochwil, K. and Balling, R. (1998). Pax9-deficient mice lack pharyngeal pouch derivatives and teeth and exhibit craniofacial and limb abnormalities. *Genes Dev* **12**, 2735-47.

Petrie, H. T. (2003). Cell migration and the control of post-natal T-cell lymphopoiesis in the thymus. *Nat Rev Immunol* **3**, 859-66.

Petrie, H. T. and Kincade, P. W. (2005). Many roads, one destination for T cell progenitors. *J Exp Med* **202**, 11-3.

Petrie, H. T., Scollay, R. and Shortman, K. (1992). Commitment to the T cell receptor-alpha beta or -gamma delta lineages can occur just prior to the onset of CD4 and CD8 expression among immature thymocytes. *Eur J Immunol* **22**, 2185-8.

Peverali, F. A., Ramqvist, T., Saffrich, R., Pepperkok, R., Barone, M. V. and Philipson, L. (1994). Regulation of G1 progression by E2A and Id helix-loop-helix proteins. *Embo J* **13**, 4291-301.

Plotkin, J., Prockop, S. E., Lepique, A. and Petrie, H. T. (2003). Critical role for CXCR4 signaling in progenitor localization and T cell differentiation in the postnatal thymus. *J Immunol* **171**, 4521-7.

Porritt, H. E., Gordon, K. and Petrie, H. T. (2003). Kinetics of steady-state differentiation and mapping of intrathymic-signaling environments by stem cell transplantation in nonirradiated mice. *J Exp Med* **198**, 957-62.

Porritt, H. E., Rumfelt, L. L., Tabrizifard, S., Schmitt, T. M., Zuniga-Pflucker, J. C. and Petrie, H. T. (2004). Heterogeneity among DN1 prothymocytes reveals multiple progenitors with different capacities to generate T cell and non-T cell lineages. *Immunity* **20**, 735-45.

Poznansky, M. C., Evans, R. H., Foxall, R. B., Olszak, I. T., Piascik, A. H., Hartman, K. E., Brander, C., Meyer, T. H., Pykett, M. J., Chabner, K. T. et al. (2000). Efficient generation of human T cells from a tissue-engineered thymic organoid. *Nat Biotechnol* **18**, 729-34.

Pui, J. C., Allman, D., Xu, L., DeRocco, S., Karnell, F. G., Bakkour, S., Lee, J. Y., Kadesch, T., Hardy, R. R., Aster, J. C. et al. (1999). Notch1 expression in early lymphopoiesis influences B versus T lineage determination. *Immunity* **11**, 299-308.

Radtke, F., Wilson, A., Mancini, S. J. and MacDonald, H. R. (2004). Notch regulation of lymphocyte development and function. *Nat Immunol* **5**, 247-53.

Radtke, F., Wilson, A., Stark, G., Bauer, M., van Meerwijk, J., MacDonald, H. R. and Aguet, M. (1999). Deficient T cell fate specification in mice with an induced inactivation of Notch1. *Immunity* **10**, 547-58.

Raviola, E. and Karnovsky, M. J. (1972). Evidence for a blood-thymus barrier using electron-opaque tracers. *J Exp Med* **136**, 466-98.

Revest, J. M., Suniara, R. K., Kerr, K., Owen, J. J. and Dickson, C. (2001). Development of the thymus requires signaling through the fibroblast growth factor receptor R2-IIIb. *J Immunol* **167**, 1954-61.

Ritter, M. A. and Palmer, D. B. (1999). The human thymic microenvironment: new approaches to functional analysis. *Semin Immunol* **11**, 13-21.

Ritter, M. A., Sauvage, C. A. and Cotmore, S. F. (1981). The human thymus microenvironment: in vivo identification of thymic nurse cells and other antigenically-distinct subpopulations of epithelial cells. *Immunology* **44**, 439-46.

Rodewald, H. R. (2000). Thymus epithelial cell reaggregate grafts. *Curr Top Microbiol Immunol* **251**, 101-8.

Rodewald, H. R., Kretzschmar, K., Swat, W. and Takeda, S. (1995). Intrathymically expressed c-kit ligand (stem cell factor) is a major factor driving expansion of very immature thymocytes in vivo. *Immunity* **3**, 313-9.

Rodewald, H. R., Paul, S., Haller, C., Bluethmann, H. and Blum, C. (2001). Thymus medulla consisting of epithelial islets each derived from a single progenitor. *Nature* **414**, 763-8.

Rossi, F. M., Corbel, S. Y., Merzaban, J. S., Carlow, D. A., Gossens, K., Duenas, J., So, L., Yi, L. and Ziltener, H. J. (2005). Recruitment of adult thymic progenitors is regulated by P-selectin and its ligand PSGL-1. *Nat Immunol* **6**, 626-34.

Rossi, S. W., Jenkinson, W. E., Anderson, G. and Jenkinson, E. J. (2006). Clonal analysis reveals a common progenitor for thymic cortical and medullary epithelium. *Nature* **441**, 988-91.

Rothenberg, E. V., Chen, D. and Diamond, R. A. (1993). Functional and phenotypic analysis of thymocytes in SCID mice. Evidence for functional response transitions before and after the SCID arrest point. *J Immunol* **151**, 3530-46.

Rouse, R. V., Ezine, S. and Weissman, I. L. (1985). Expression of major histocompatibility complex antigens in the thymuses of chimeric mice. *Transplantation* **40**, 422-6.

Saint-Ruf, C., Ungewiss, K., Groettrup, M., Bruno, L., Fehling, H. J. and von Boehmer, H. (1994). Analysis and expression of a cloned pre-T cell receptor gene. *Science* **266**, 1208-12.

Sainte-Marie, G., Peng, F. S. and Marcoux, D. (1986). The stroma of the thymus of the rat: morphology and antigen diffusion, a reconsideration. *Am J Anat* **177**, 333-52.

Sakaguchi, S., Sakaguchi, N., Asano, M., Itoh, M. and Toda, M. (1995). Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *J Immunol* **155**, 1151-64.

Savino, W. and Dardenne, M. (1988). Developmental studies on expression of monoclonal antibody-defined cytokeratins by thymic epithelial cells from normal and autoimmune mice. *J Histochem Cytochem* **36**, 1123-9.

Savino, W., Villa-Verde, D. M. and Lannes-Vieira, J. (1993). Extracellular matrix proteins in intrathymic T-cell migration and differentiation? *Immunol Today* **14**, 158-61.

Scheiff, J. M., Cordier, A. C. and Haumont, S. (1978). The thymus of Nu/+ mice. *Anat Embryol (Berl)* **153**, 115-22.

Schluep, M., Willcox, N., Ritter, M. A., Newsom-Davis, J., Larche, M. and Brown, A. N. (1988). Myasthenia gravis thymus: clinical, histological and culture correlations. *J Autoimmun* **1**, 445-67.

Schmitt, T. M., Ciofani, M., Petrie, H. T. and Zuniga-Pflucker, J. C. (2004a). Maintenance of T cell specification and differentiation requires recurrent notch receptor-ligand interactions. *J Exp Med* **200**, 469-79.

Schmitt, T. M., de Pooter, R. F., Gronski, M. A., Cho, S. K., Ohashi, P. S. and Zuniga-Pflucker, J. C. (2004b). Induction of T cell development and establishment of T cell competence from embryonic stem cells differentiated in vitro. *Nat Immunol* **5**, 410-7.

Schmitt, T. M. and Zuniga-Pflucker, J. C. (2002). Induction of T cell development from hematopoietic progenitor cells by delta-like-1 in vitro. *Immunity* **17**, 749-56.

Schreiber, L., Eshel, I., Meilin, A., Sharabi, Y. and Shoham, J. (1991). Analysis of thymic stromal cell subpopulations grown in vitro on extracellular matrix in defined medium. III. Growth conditions of human thymic epithelial cells and immunomodulatory activities in their culture supernatant. *Immunology* **74**, 621-9.

Schreier, J., Hamilton, JL. (1952). An experimental study of the origin of the parathyroid and thymus glands in the chick. *J Exp Zool* **119**, 165-187.

Schwartz, R. H. (1990). A cell culture model for T lymphocyte clonal anergy. *Science* **248**, 1349-56.

Scollay, R., Bartlett, P. and Shortman, K. (1984). T cell development in the adult murine thymus: changes in the expression of the surface antigens Ly2, L3T4 and B2A2 during development from early precursor cells to emigrants. *Immunol Rev* **82**, 79-103.

Senelar, R., Escola, M. J., Escola, R., Serrou, B. and Serre, A. (1976). Relationship between Hassall's corpuscles and thymocytes fate in guinea-pig foetus. *Biomedicine* **24**, 112-22.

Shinkai, Y., Koyasu, S., Nakayama, K., Murphy, K. M., Loh, D. Y., Reinherz, E. L. and Alt, F. W. (1993). Restoration of T cell development in RAG-2-deficient mice by functional TCR transgenes. *Science* **259**, 822-5.

Shinohara, T. and Honjo, T. (1997). Studies in vitro on the mechanism of the epithelial/mesenchymal interaction in the early fetal thymus. *Eur J Immunol* **27**, 522-9.

Shores, E. W., Van Ewijk, W. and Singer, A. (1991). Disorganization and restoration of thymic medullary epithelial cells in T cell receptor-negative scid mice: evidence that receptor-bearing lymphocytes influence maturation of the thymic microenvironment. *Eur J Immunol* **21**, 1657-61.

Shortman, K., Scollay, R., Andrews, P. and Boyd, R. (1986). Development of T lymphocytes within the thymus and within thymic nurse cells. *Curr Top Microbiol Immunol* **126**, 5-18.

Singh, N., Phillips, R. A., Iscove, N. N. and Egan, S. E. (2000). Expression of notch receptors, notch ligands, and fringe genes in hematopoiesis. *Exp Hematol* **28**, 527-34.

Smith, C. (1965). Studies on the Thymus of the Mammal. Xiv. Histology and Histochemistry of Embryonic and Early Postnatal Thymuses of C57bl-6 and Akr Strain Mice. *Am J Anat* **116**, 611-29.

Staal, F. J. and Clevers, H. C. (2001). Regulation of lineage commitment during lymphocyte development. *Int Rev Immunol* **20**, 45-64.

Staal, F. J., Meeldijk, J., Moerer, P., Jay, P., van de Weerdt, B. C., Vainio, S., Nolan, G. P. and Clevers, H. (2001). Wnt signaling is required for thymocyte development and activates Tcf-1 mediated transcription. *Eur J Immunol* **31**, 285-93.

Sun, T. T., Shih, C. and Green, H. (1979). Keratin cytoskeletons in epithelial cells of internal organs. *Proc Natl Acad Sci U S A* **76**, 2813-7.

Suniara, R. K., Jenkinson, E. J. and Owen, J. J. (1999). Studies on the phenotype of migrant thymic stem cells. *Eur J Immunol* **29**, 75-80.

Surh, C. D., Ernst, B. and Sprent, J. (1992a). Growth of epithelial cells in the thymic medulla is under the control of mature T cells. *J Exp Med* **176**, 611-6.

Surh, C. D., Gao, E. K., Kosaka, H., Lo, D., Ahn, C., Murphy, D. B., Karlsson, L., Peterson, P. and Sprent, J. (1992b). Two subsets of epithelial cells in the thymic medulla. *J Exp Med* **176**, 495-505.

Surh, C. D. and Sprent, J. (1994). T-cell apoptosis detected in situ during positive and negative selection in the thymus. *Nature* **372**, 100-3.

Suzuki, G., Sawa, H., Kobayashi, Y., Nakata, Y., Nakagawa, K., Uzawa, A., Sakiyama, H., Kakinuma, S., Iwabuchi, K. and Nagashima, K. (1999). Pertussis toxin-sensitive signal controls the trafficking of thymocytes across the corticomedullary junction in the thymus. *J Immunol* **162**, 5981-5.

Swat, W., Dessing, M., von Boehmer, H. and Kisielow, P. (1993). CD69 expression during selection and maturation of CD4+8+ thymocytes. *Eur J Immunol* **23**, 739-46.

Takahama, Y. (2006). Journey through the thymus: stromal guides for T-cell development and selection. *Nat Rev Immunol* **6**, 127-35.

Teh, H. S., Kisielow, P., Scott, B., Kishi, H., Uematsu, Y., Bluthmann, H. and von Boehmer, H. (1988). Thymic major histocompatibility complex antigens and the alpha beta T-cell receptor determine the CD4/CD8 phenotype of T cells. *Nature* **335**, 229-33.

Ting, C. N., Olson, M. C., Barton, K. P. and Leiden, J. M. (1996). Transcription factor GATA-3 is required for development of the T-cell lineage. *Nature* **384**, 474-8.

Tong, J., Kishi, H., Matsuda, T. and Muraguchi, A. (1999). A bone marrow-derived stroma cell line, ST2, can support the differentiation of fetal thymocytes from the CD4+ CD8+ double negative to the CD4+ CD8+ double positive differentiation stage in vitro. *Immunology* **97**, 672-8.

Trop, S., Rhodes, M., Wiest, D. L., Hugo, P. and Zuniga-Pflucker, J. C. (2000). Competitive displacement of pT alpha by TCR-alpha during TCR assembly prevents surface coexpression of pre-TCR and alpha beta TCR. *J Immunol* **165**, 5566-72.

Tsai, P. T., Lee, R. A. and Wu, H. (2003). BMP4 acts upstream of FGF in modulating thymic stroma and regulating thymopoiesis. *Blood* **102**, 3947-53.

Tsukamoto, N., Itoi, M., Nishikawa, M. and Amagai, T. (2005). Lack of Delta like 1 and 4 expressions in nude thymus anlages. *Cell Immunol* **234**, 77-80.

Uehara, S., Grinberg, A., Farber, J. M. and Love, P. E. (2002). A role for CCR9 in T lymphocyte development and migration. *J Immunol* **168**, 2811-9.

Uematsu, Y., Ryser, S., Dembic, Z., Borgulya, P., Krimpenfort, P., Berns, A., von Boehmer, H. and Steinmetz, M. (1988). In transgenic mice the introduced functional T cell receptor beta gene prevents expression of endogenous beta genes. *Cell* **52**, 831-41.

Ueno, T., Saito, F., Gray, D. H., Kuse, S., Hieshima, K., Nakano, H., Kakiuchi, T., Lipp, M., Boyd, R. L. and Takahama, Y. (2004). CCR7 signals are essential for cortex-medulla migration of developing thymocytes. *J Exp Med* **200**, 493-505.

Ushiki, T. (1986). A scanning electron-microscopic study of the rat thymus with special reference to cell types and migration of lymphocytes into the general circulation. *Cell Tissue Res* **244**, 285-98.

van de Wetering, M., de Lau, W. and Clevers, H. (2002). WNT signaling and lymphocyte development. *Cell* **109 Suppl**, S13-9.

van de Wijngaert, F. P., Kendall, M. D., Schuurman, H. J., Rademakers, L. H. and Kater, L. (1984). Heterogeneity of epithelial cells in the human thymus. An ultrastructural study. *Cell Tissue Res* **237**, 227-37.

van de Wijngaert, F. P., Rademakers, L. H., Schuurman, H. J., de Weger, R. A. and Kater, L. (1983). Identification and in situ localization of the "thymic nurse cell" in man. *J Immunol* **130**, 2348-51.

van Ewijk, W. (1988). Cell surface topography of thymic microenvironments. *Lab Invest* **59**, 579-90.

van Ewijk, W., Hollander, G., Terhorst, C. and Wang, B. (2000). Stepwise development of thymic microenvironments in vivo is regulated by thymocyte subsets. *Development* **127**, 1583-91.

Van Ewijk, W., Rouse, R. V. and Weissman, I. L. (1980). Distribution of H-2 microenvironments in the mouse thymus. Immunoelectron microscopic identification of I-A and H-2K bearing cells. *J Histochem Cytochem* **28**, 1089-99.

van Ewijk, W., Wang, B., Hollander, G., Kawamoto, H., Spanopoulou, E., Itoi, M., Amagai, T., Jiang, Y. F., Germeraad, W. T., Chen, W. F. et al. (1999). Thymic microenvironments, 3-D versus 2-D? *Semin Immunol* **11**, 57-64.

Van Vliet, E., Jenkinson, E. J., Kingston, R., Owen, J. J. and Van Ewijk, W. (1985). Stromal cell types in the developing thymus of the normal and nude mouse embryo. *Eur J Immunol* **15**, 675-81.

Veis, D. J., Sentman, C. L., Bach, E. A. and Korsmeyer, S. J. (1993). Expression of the Bcl-2 protein in murine and human thymocytes and in peripheral T lymphocytes. *J Immunol* **151**, 2546-54.

von Boehmer, H., Aifantis, I., Feinberg, J., Lechner, O., Saint-Ruf, C., Walter, U., Buer, J. and Azogui, O. (1999). Pleiotropic changes controlled by the pre-T-cell receptor. *Curr Opin Immunol* **11**, 135-42.

von Freeden-Jeffry, U., Vieira, P., Lucian, L. A., McNeil, T., Burdach, S. E. and Murray, R. (1995). Lymphopenia in interleukin (IL)-7 gene-deleted mice identifies IL-7 as a nonredundant cytokine. *J Exp Med* **181**, 1519-26.

Wallin, J., Eibel, H., Neubuser, A., Wilting, J., Koseki, H. and Balling, R. (1996). Pax1 is expressed during development of the thymus epithelium and is required for normal T-cell maturation. *Development* **122**, 23-30.

Wang, J. H., Nichogiannopoulou, A., Wu, L., Sun, L., Sharpe, A. H., Bigby, M. and Georgopoulos, K. (1996). Selective defects in the development of the fetal and adult lymphoid system in mice with an Ikaros null mutation. *Immunity* **5**, 537-49.

Watanabe, N., Wang, Y. H., Lee, H. K., Ito, T., Wang, Y. H., Cao, W. and Liu, Y. J. (2005). Hassall's corpuscles instruct dendritic cells to induce CD4+CD25+ regulatory T cells in human thymus. *Nature* **436**, 1181-5.

Weiss, L. (1963). Electron microscopic, observations on the vascular barrier in the cortex of the thymus of the mouse. *Anat Rec* **145**, 413-37.

Wekerle, H., Ketelsen, U. P. and Ernst, M. (1980). Thymic nurse cells. Lymphoepithelial cell complexes in murine thymuses: morphological and serological characterization. *J Exp Med* **151**, 925-44.

Wilkinson, B., Owen, J. J. and Jenkinson, E. J. (1999). Factors regulating stem cell recruitment to the fetal thymus. *J Immunol* **162**, 3873-81.

Williams, N. S., Klem, J., Puzanov, I. J., Sivakumar, P. V., Bennett, M. and Kumar, V. (1999). Differentiation of NK1.1+, Ly49+ NK cells from flt3+ multipotent marrow progenitor cells. *J Immunol* **163**, 2648-56.

Wilson, A., Held, W. and MacDonald, H. R. (1994). Two waves of recombinase gene expression in developing thymocytes. *J Exp Med* **179**, 1355-60.

Wilson, A., MacDonald, H. R. and Radtke, F. (2001). Notch 1-deficient common lymphoid precursors adopt a B cell fate in the thymus. *J Exp Med* **194**, 1003-12.

Wolfer, A., Bakker, T., Wilson, A., Nicolas, M., Ioannidis, V., Littman, D. R., Lee, P. P., Wilson, C. B., Held, W., MacDonald, H. R. et al. (2001). Inactivation of Notch 1 in immature thymocytes does not perturb CD4 or CD8T cell development. *Nat Immunol* **2**, 235-41.

Wolfer, A., Wilson, A., Nemir, M., MacDonald, H. R. and Radtke, F. (2002). Inactivation of Notch1 impairs VDJbeta rearrangement and allows pre-TCR-independent survival of early alpha beta Lineage Thymocytes. *Immunity* **16**, 869-79.

Wright, D. E., Wagers, A. J., Gulati, A. P., Johnson, F. L. and Weissman, I. L. (2001). Physiological migration of hematopoietic stem and progenitor cells. *Science* **294**, 1933-6.

Wu, L., Antica, M., Johnson, G. R., Scollay, R. and Shortman, K. (1991). Developmental potential of the earliest precursor cells from the adult mouse thymus. *J Exp Med* **174**, 1617-27.

Wurbel, M. A., Malissen, M., Guy-Grand, D., Meffre, E., Nussenzweig, M. C., Richelme, M., Carrier, A. and Malissen, B. (2001). Mice lacking the CCR9 CC-chemokine receptor show a mild impairment of early T- and B-cell development and a reduction in T-cell receptor gammadelta(+) gut intraepithelial lymphocytes. *Blood* **98**, 2626-32.

Xu, H., Cerrato, F. and Baldini, A. (2005). Timed mutation and cell-fate mapping reveal reiterated roles of Tbx1 during embryogenesis, and a crucial function during segmentation of the pharyngeal system via regulation of endoderm expansion. *Development* **132**, 4387-95.

Xu, P. X., Zheng, W., Laclef, C., Maire, P., Maas, R. L., Peters, H. and Xu, X. (2002). Eya1 is required for the morphogenesis of mammalian thymus, parathyroid and thyroid. *Development* **129**, 3033-44.

Yamazaki, H., Sakata, E., Yamane, T., Yanagisawa, A., Abe, K., Yamamura, K., Hayashi, S. and Kunisada, T. (2005). Presence and distribution of neural crest-derived cells in the murine developing thymus and their potential for differentiation. *Int Immunol* **17**, 549-58.

Zaitseva, M., Kawamura, T., Loomis, R., Goldstein, H., Blauvelt, A. and Golding, H. (2002). Stromal-derived factor 1 expression in the human thymus. *J Immunol* **168**, 2609-17.

Zou, D., Silvius, D., Davenport, J., Grifone, R., Maire, P. and Xu, P. X. (2006). Patterning of the third pharyngeal pouch into thymus/parathyroid by Six and Eya1. *Dev Biol* **293**, 499-512.

Zuniga-Pflucker, J. C. (2004). T-cell development made simple. *Nat Rev Immunol* **4**, 67-72.

Zuniga-Pflucker, J. C., Di, J. and Lenardo, M. J. (1995). Requirement for TNF-alpha and IL-1 alpha in fetal thymocyte commitment and differentiation. *Science* **268**, 1906-9.

Zuniga-Pflucker, J. C. and Lenardo, M. J. (1996). Regulation of thymocyte development from immature progenitors. *Curr Opin Immunol* **8**, 215-24.