# LYMPHOSTROMAL INTERACTIONS IN THYMIC DEVELOPMENT AND FUNCTION

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The generation of a peripheral T-cell pool is essential for normal immune system function. $CD4^+$ and $CD8^+$ T cells are produced most efficiently in the thymus, which provides a complexity of discrete cellular microenvironments. Specialized stromal cells, that make up such microenvironments, influence each stage in the maturation programme of immature T-cell precursors. Progress has recently been made in elucidating events that regulate the development of intrathymic microenvironments, as well as mechanisms of thymocyte differentiation. It is becoming increasingly clear that the generation and maintenance of thymic environments that are capable of supporting efficient T-cell development, requires complex interplay between lymphoid and stromal compartments of the thymus.

PHARYNGEAL POUCH
A lateral diverticulum of the pharynx that meets a corresponding groove in the ectoderm.

ECTODERM
The uppermost layer of the three primary germ layers in the embryonic disc; it develops into the epidermis and epidermal tissues, the nervous system, the external sense organs and the mucous membranes that line the mouth and anus.

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T cells that bear the αβ form of the T-cell-receptor complex (αβTCR) develop in the thymus from migrant, blood-borne precursors which are recruited through a poorly defined chemotactic mechanism[1,2]. On entering the thymus, these immature cells embark on a differentiation programme which can be readily characterized by changes in cell-surface phenotype, proliferation status and functionality. Key events during T-cell development include commitment to the T-cell lineage, although controversy exists as to whether this is an intra- or extrathymic event[3–6], and the choice between αβ versus γδ T-cell lineages[7]. Important events relating specifically to αβ T-cell development include rearrangement and expression of *TCRα* and *TCRβ* genes, and positive and negative selection of the immature TCR repertoire on the basis of reactivity to self-peptide/major histocompatibility complex (MHC) complexes[8]. Recent research has led to a better understanding of the signalling cascades and transcriptional regulators within thymocytes that control intrathymic maturation. However, in general, thymocyte development is not a cell-autonomous process, and transition to the next stage in development depends on interactions with a complexity of thymic stromal cells, which make up thymic microenvironments (FIG. 1). This review specifically discusses recent advances relating to the development and functioning of stromal cell microenvironments within the thymus. For the sake of brevity, a number of aspects of thymus biology, such as precursor migration and colonization, are not covered here, and the reader is directed to recent data indicating a role for CD44 and integrins in thymus colonization, and reviews covering these areas[9–11].

## Mechanisms of thymus organogenesis

***Molecular regulators of thymic epithelial development.*** Thymic epithelial cells are derived from epithelial tissues that originate from the third PHARYNGEAL POUCH and cleft complex. Whether all thymic epithelial cells in the adult thymus share the same developmental origin remains controversial[12,13]. In particular, there is debate over the possible contribution of ECTODERMALLY derived cells in the generation of thymic epithelium, with apparently ectoderm-free ENDODERMAL thymic rudiments generating a normal thymic architecture in birds[14].

Once formed, and following invagination by mesenchymal cells, the epithelial bud of the thymus develops further into discrete cortical and medullary areas

ENDODERM
The innermost of the three embryonic germ layers; it develops into the epithelium of the pharynx, respiratory tract, digestive tract, bladder and urethra.

STEM CELLS
A subset of cells which has a self-renewing capacity, and under appropriate conditions can give rise to a number of mature cell lineages.

containing increasingly defined subsets of epithelial cells. Relatively few molecules have been identified which have subsequently been shown to be important regulators of thymic epithelium and, consequently, the mechanisms of thymic epithelial cell development and function are unclear (FIG. 2). Of these molecules, the product of the *nude* gene locus, which was identified as Winged-helix nude (Whn)[15,16], has long been known to have an important role in thymus development in rodents[12]. Deficiency in Whn, recently renamed Foxn1 (a transcription factor produced by the *nude* gene locus), results in a cystic thymic rudiment in adults and, consequently, causes a marked reduction of T cells, with some cells still being generated extrathymically. Interestingly, Whn does not seem to be necessary for the initial development of the thymic epithelial primordium, since both *nude* and Whn knockout mice display relatively normal morphology of the thymus anlagen at stages prior to lymphocyte colonization[17]. However, subsequent development of thymic epithelium in the absence of Whn is severely perturbed, with no discernable differentiation into normal thymic architecture occurring. In chimeric mice generated by *nude* and wild-type mouse embryo fusion, nude-derived cells give rise to only a small subset of thymic medullary epithelium[18]. Although the mechanism of Whn function is uncertain, these data indicate that Whn might operate through a cell-autonomous process[18] early in thymic development to influence differentiation of the earliest thymic epithelial cells, by a mechanism that might be dosage dependent[19].

A number of transcription factors, Hoxa3, Pax-1 and Pax-9, previously shown to be expressed by thymic epithelial cells, have recently been implicated in their development and function[20]. Although both Hoxa3 deficiency[21] and Pax-9 deficiency[22] lead to athymia, Pax-1 deficiency, as seen in *undulated* mice, results in a less severe phenotype with a two to fivefold reduction in thymocyte numbers[23]. Interestingly, thymic epithelial cells from *Hoxa3*$^{+/-}$/*Pax-1*$^{-/-}$ compound mutants develop abnormally, showing lower levels of MHC class II expression and a decreased ability to support thymocyte maturation[24]. This indicates a direct role for these molecules in epithelial cell development and function.

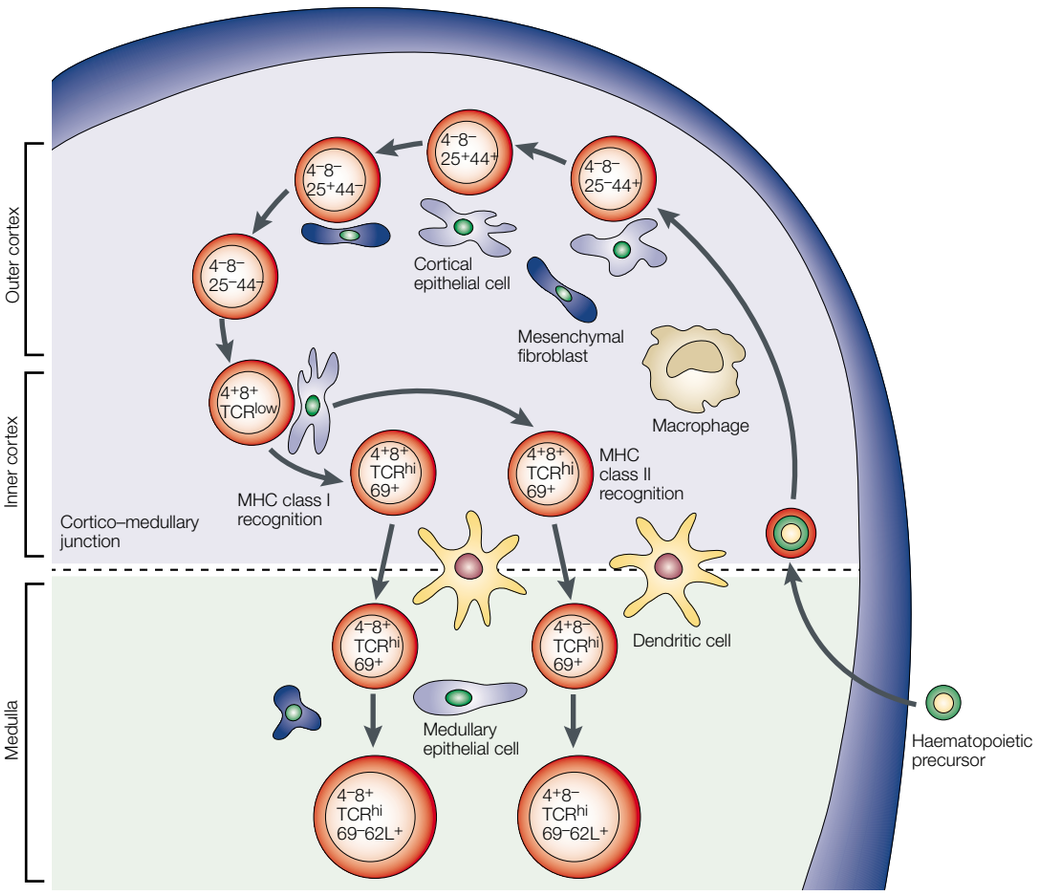


Figure 1 | **Anatomical microenvironments in the adult thymus.** The thymus is a lobed organ divided by mesenchymal septae. Lobes are organized into discrete cortical and medullary areas, each of which is characterized by the presence of particular stromal cell types, as well as thymocyte precursors at defined maturational stages. Thymocyte differentiation can be followed phenotypically by the expression of cell-surface markers, including CD4, CD8, CD44, CD25, CD69 and CD62L (Mel-14), as well as the status of the T-cell receptor (TCR). Interactions between thymocytes and thymic stromal cells are known to be important in driving a complex programme of T-cell maturation in the thymus, which ultimately results in the generation of self-tolerant CD4$^+$ helper and CD8$^+$ cytotoxic T cells, which emigrate from the thymus to establish the peripheral T-cell pool. (4, CD4; 8, CD8; 44, CD44; 25, CD25; 69, CD69; TCR$^{low}$, expressing the TCR at low levels; TCR$^{hi}$, expressing the TCR at high levels.)

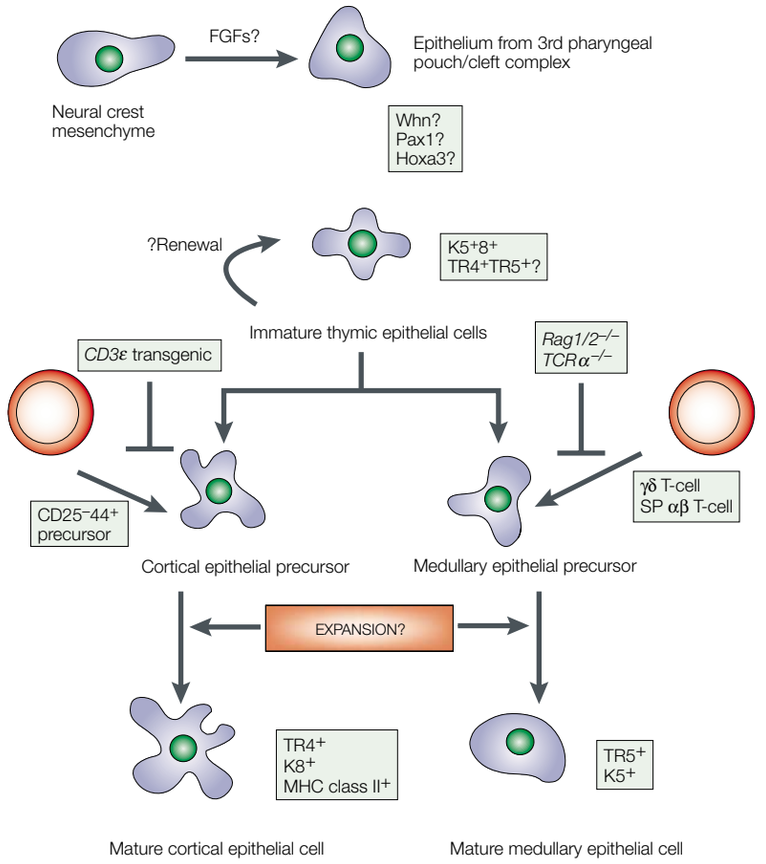


Figure 2 | **Model of molecular regulation during development of thymic epithelium.** Thymic epithelial cells represent a crucial cell type in the functioning of the thymus. The developmental origins of these cells are controversial, and it is unclear whether or not all thymic epithelial cells are derived from a common stem cell. However, subpopulations of thymic epithelial cells co-expressing markers that are restricted to cortical or medullary epithelium — including TR4 and TR5 or Keratin (K)5 and K8 — have been identified. Several studies of naturally occurring, or experimentally induced, genetic mutations have highlighted a number of genes which seem to directly influence the maturational programme of thymic epithelium. These include genes encoding the transcription factors Whn, Pax-1 and Hoxa3. In addition, study of genetic mutations in immature thymocytes has highlighted mechanisms by which immature thymocytes can regulate the growth and development of thymic epithelial cells. (FGF, fibroblast growth factor.)

***A common epithelial stem cell?*** A common feature of many epithelial tissues is that they contain STEM CELLS, which, throughout life, can give rise to committed descendents, providing the potential for further growth and organ regeneration. Whether the thymus contains an epithelial stem-cell pool capable of generating all cortical and medullary epithelial subsets in the adult is not known (FIG. 2). Moreover, given the controversy over a possible dual embryological origin for thymic epithelium[25], the existence of two distinct stem-cell pools — one of ectodermal origin and one of endodermal origin — for the generation of cortical and medullary epithelial cells has not been addressed. However, one study identified a rare thymic epithelial subpopulation in the murine thymus which co-expresses TR4 and TR5 markers which are usually limited to either the cortical or medullary, epithelium[26]. These 'double-positive' epithelial precursors might represent an immature intermediate in the development of thymic epithelium, in the same way that double-positive $CD4^+CD8^+$ thymocytes represent the precursor pool of $CD4^+$ and $CD8^+$ T cells. It is important to note, however, that clonal assays have yet to be performed, either on the origins of thymic epithelium or on possible precursor–product relationships between epithelial subsets.

By analysing patterns of thymic keratin expression, Klug and colleagues[27] detected thymic epithelial cells which simultaneously expressed keratin-5 (normally expressed by most medullary epithelium) and keratin-8 (normally expressed by most cortical epithelium). This double-positive keratin-$5^+$/keratin-$8^+$ ($K5^+K8^+$) epithelial subset is positioned at the corticomedullary junction in normal mice[27], and makes up a large proportion of the total thymic epithelium in line 26 of the *CD3ε* transgenic mouse strain (FIG. 2), in which thymocyte development is blocked at the $CD4^-CD8^-CD25^-CD44^+$ stage[28]. It might therefore be the case that this $K5^+K8^+$ subset represents an immature thymic epithelial intermediate, the further development of which — perhaps into mature $K5^-K8^+$ cortical epithelium[27] — is regulated by immature thymocytes. Whether this $K5^+K8^+$ subset of cells can give rise to medullary epithelial cells is not clear. This mechanism of thymocyte regulation of thymic epithelial cell development will be discussed further in relation to the maturation of cortical and medullary thymic microenvironments.

***Functional importance of thymic mesenchyme.*** In addition to cortical and medullary epithelial cells, an important stromal cell type which influences thymocyte development is MESENCHYME, which is derived from the NEURAL CREST[29]. Since very few markers are available that label only mesenchymal cells, and since heterogeneity within mesenchymal cells is likely to be complex, we have used the term 'mesenchyme' as a global term to cover several cell types of the same developmental origin. In the adult thymus, such cells contribute to the thymic capsule and septae, and can also be located within the thymic cortex where they interact with immature thymocytes[30–32]. The first functional demonstration of a role for mesenchyme in thymus development came from Auerbach in 1960 (REF. 33), who showed that removal of the surrounding mesenchyme from 12-day-old embryonic thymus lobes prevented normal thymus development under fetal thymus organ culture conditions (FTOC) *in vitro*. Shinohara and Honjo[34,35] further confirmed these observations and showed that the mesenchymal production of soluble growth factors, such as epidermal growth factor, might be needed for normal epithelial development. The importance of mesenchyme was emphasized in studies in which extirpation of the neural crest in birds — resulting in a lack of mesenchymal contribution to the developing thymus — disrupted thymic formation and function[36]. Although the precise mechanism by which mesenchymal cells influence development of the thymus is unclear, on the basis of current experimental data we propose a two-stage mechanism for mesenchymal

MESENCHYME
Loosely organized, undifferentiated mesodermal cells.

NEURAL CREST
A group of embryonic cells that separate from the embryonic neural plate and migrate, giving rise to the spinal and autonomic ganglia, peripheral glia, chromaffin cells, melanocytes, some haematopoietic cells and mesenchyme.

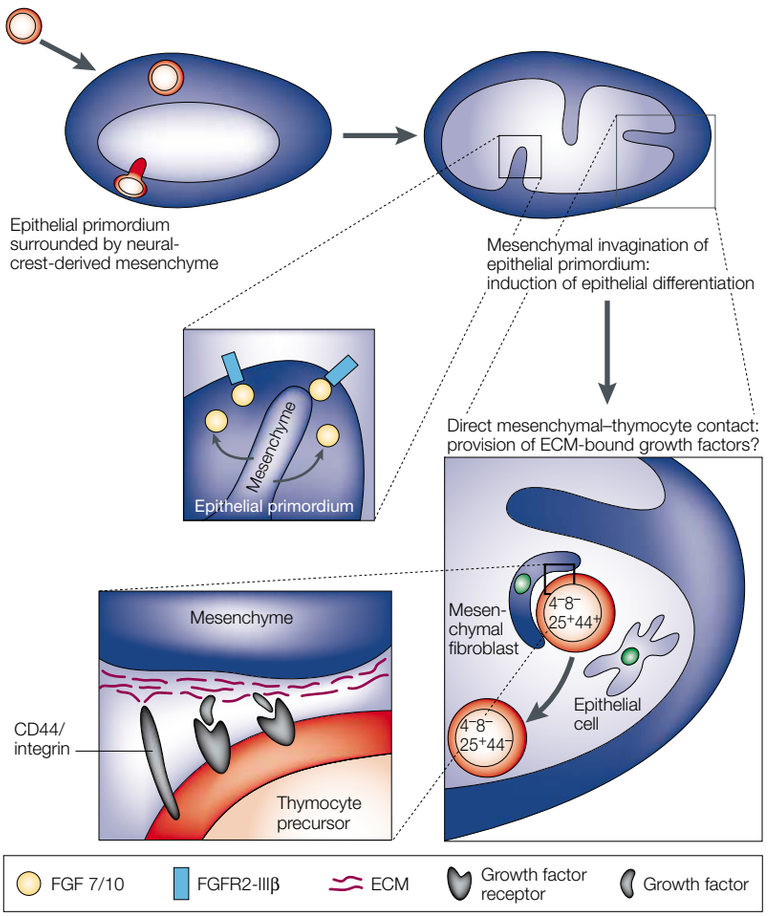


Figure 3 | **Model of mesenchymal involvement in thymus development and function.** Neural-crest-derived mesenchymal cells play an important role in the formation of the early thymic rudiment, by surrounding and eventually invaginating the epithelial promordium. The precise mechanisms by which mesenchymal cells exert their effects on the thymus are not clear. We propose that mesenchymal cells have a dual role in the thymus. First, mesenchyme might directly influence the growth and development of the early thymic epithelium through the production of fibroblast growth factors (FGFs), which have been shown to have a role in the formation of many other epithelio–mesenchymal tissues. Second, mesenchymal cells present in cortical regions of the thymus might directly influence the survival, proliferation or differentiation of immature $CD4^-CD8^-$ T-cell precursors by providing an extracellular framework to present and concentrate essential soluble growth factors. (ECM, extracellular matrix.)

involvement in thymopoiesis (FIG. 3). One requirement for mesenchyme in thymus development might be in the initial stages of thymic formation, when neural-crest-derived mesenchymal cells surround and eventually migrate into the thymic epithelial rudiment. Distinct epithelial–mesenchymal interactions, possibly involving the production of fibroblast growth factors (FGFs) by mesenchyme[37], might then directly regulate the differentiation and/or proliferation of the thymic epithelial primordium. Such interactions have been shown to have an important role in the formation of many other epithelial–mesenchymal tissues, including the limb bud, in which mesenchymal production of FGFs stimulates the growth and differentiation of FGF-receptor-bearing epithelial cells[37]. Interestingly, in mice lacking either Fgf10 (REF. 38) or FgfR2-IIIb (REF. 39), a receptor for FGF10, the thymus is much reduced in size, perhaps indicating a role for these molecules during epithelial–mesenchymal interactions during thymus development (see note added in proof).

A further role for mesenchymal cells in the thymus has also been shown in experiments involving the generation of reaggregate thymus organ cultures (RTOC) from defined stromal components[31,40] (FIG. 4). In addition to mature thymic epithelial cells from 2-deoxyguanosine-treated thymus lobes, mesenchymal fibroblasts were found to be necessary for the further maturation of $CD4^-CD8^-CD25^+CD44^+$ precursors. Rather than influencing epithelial cell function as described above, this further requirement for mesenchyme might be indicative of a direct effect of mesenchyme on immature thymocytes, with cells of a $CD4^-CD8^-CD25^+CD44^+$ phenotype still requiring mesenchymal support in the presence of mature thymic epithelium[40]. How mesenchymal cells influence thymocyte development is still uncertain, although one possibility is that they might be required to present, through components of the EXTRACELLULAR MATRIX (ECM), important soluble growth factors and cytokines to immature T-cell precursors[41] (FIG. 3). Such a mechanism would be analogous with the presentation of cytokines, such as granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin-7 (IL-7), to haematopoietic stem cells and B-cell precursors by bone-marrow stromal cells[42,43].

## Cortical microenvironments

***Crosstalk and organization.*** The thymic cortex represents an important microenvironment in which immature T-cell precursors are held in a complex three-dimensional reticular network of MHC class $II^+$ cortical epithelial cells. These cells seem to be highly specialized in their ability to promote a series of key developmental events. They first lead to the generation of a large pool of $CD4^+CD8^+$ precursors bearing αβTCRs with a wide repertoire of specificities, and then to positive selection of those cells expressing αβTCRs that are capable of recognizing self-peptide/MHC complexes[44]. Recent studies have shown that the generation of intact cortical environments that are capable of supporting these events involves interplay with, and feedback from, immature thymocytes, such that these cells directly influence the formation of environments required for their own development. This process has been termed 'thymic crosstalk'[45–47].

Although the involvement of thymic crosstalk in the development and functioning of the thymic cortex is well accepted, relatively little is known about how developing thymocytes regulate cortical epithelial differentiation (FIG. 2). *CD3ε* transgenic mice (line 26) display a profound block in T-cell development at the $CD4^-CD8^-CD25^-CD44^+$ stage and have severe abnormalities in thymic cortex development and organization[28]. A similar distortion of thymic epithelium is also seen in $CD117^{-/-}/CD132^{-/-}$ double-mutant mice, in which the thymus is alymphoid[48]. The thymus of *CD3ε* transgenic mice lacks the normal three-dimensional

EXTRACELLULAR MATRIX
Secreted products of many cell types which form an organized scaffold for cell support.

reticular epithelial network and, instead, contains numerous cystic structures with poor demarcation of cortical and medullary areas[49]. Restoration of a three-dimensional cortical architecture can, however, be achieved in these animals by introduction of recombination-activating gene 2 (Rag-2)-deficient bone marrow[49], which becomes blocked at the later $CD4^-CD8^-CD25^+CD44^-$ stage of thymocyte development. Transition of immature T-cell precursors, therefore, from the $CD4^-CD8^-CD25^-CD44^+$ to the $CD4^-CD8^-CD25^+CD44^-$ stage marks an important checkpoint in the development of thymic cortical epithelium. Interestingly, such rescue of cortical epithelial cell development in *CD3ε* transgenic mice can only be achieved up to the neonatal period — introduction of wild-type stem cells into *CD3ε* transgenic adult mice fails to reorganize the thymic cortex[27]. Such a finding indicates that there might either be limited plasticity in the ability of immature cortical epithelial cells to respond to signals that are derived from immature thymocytes, or a gradual disappearance of epithelial stem cells in the absence of thymocyte support. A key area in the understanding of cortical epithelium development is to identify the molecules produced by thymocytes, whether cell-surface molecules or soluble factors, which influence the differentiation and organization of the thymic cortex, and to determine to what extent epithelial precursor populations persist into adult life.

***Cortical epithelium and early thymopoiesis.*** In the adult, blood-borne migrant precursors are recruited to the thymus by unknown chemotactic factors, and enter the organ through blood vessels at the corticomedullary junction (FIG. 1). In the embryo, however, thymus colonization occurs prior to vascularization, and precursors have to enter the early thymus rudiment directly through the mesenchymal capsule. Recent data indicates that migration of thymocytes within the thymus is an ordered process[50], regulated, at least in part, by chemokines. Moreover, elegant studies[51] in the neonatal thymus indicate that once newly colonized precursors have entered the thymus at the corticomedullary junction, they undergo development through successive $CD4^-CD8^-$ stages on the way to outer cortical regions, so that the thymic cortex can be divided into zones of thymocyte development.

At present, it is still unclear whether the precursors that colonize the thymus and localize to outer cortical regions represent a group of cells which are already pre-committed to the T-cell lineage, or whether T-lineage restriction occurs under the influence of thymic cortical epithelium. On the one hand, data from a multilineage progenitor (MLP) assay designed by Katsura and colleagues showed that individual cells present in haemoatpoietic sites, such as day 10 aorta-gonad-mesonephros or day 12 fetal liver, can give rise to T cells, but not $B220^+$ cells or $Mac\text{-}1^+/Gr\text{-}1^+$ cells, when introduced into a lymphoid thymi *in vitro*[52–54]. This data is indicative of a prethymic precursor population, which is T-lineage restricted and unable to generate B cells or myeloid cells. However, generation of both αβ T cells and natural killer cells has also been shown from early intrathymic precursors[4,54], indicating that although some degree of commitment can occur prethymically, restriction of immature precursors to the αβ T-cell lineage occurs intrathymically.

How cortical epithelial cells influence T-cell commitment is not clear[55]. Recently, several studies have indicated a role for the transmembrane molecule Notch-1 in T-lineage restriction[56,57]. Notch molecules are good candidates since they are expressed in the thymus and have been shown to have important roles in cellular commitment mechanisms in many other developmental systems[58,59]. Indeed, the Notch family of receptors and its ligands have been implicated in a number of stages of thymocyte differentiation, including αβ/γδ lineage determination and positive selection of $CD4^+CD8^+$ thymocytes[60–73] (TABLE 1). Evidence for a role for Notch-1 in commitment to the T-cell lineage include a conditional knockout of Notch-1 in haemoatpoietic cells, in which thymocyte development was blocked at the earliest stage[61], and experiments involving overexpression of a constitutively active form of Notch-1 (IC-Notch), which caused suppression of B-cell development and promoted extrathymic generation of $CD4^+CD8^+$ cells[62]. As Notch signalling is dependent on interactions with cell-surface ligands[57], understanding expression of Notch ligands in the thymus is central to understanding how thymic microenvironments might regulate Notch activation. To date, although Notch ligands have been detected in thymus, analysis of expression by thymic epithelial cells has either been limited to cell lines[63,71] or performed using *in situ* hybridization[71], in which precise localization of expression can be difficult.

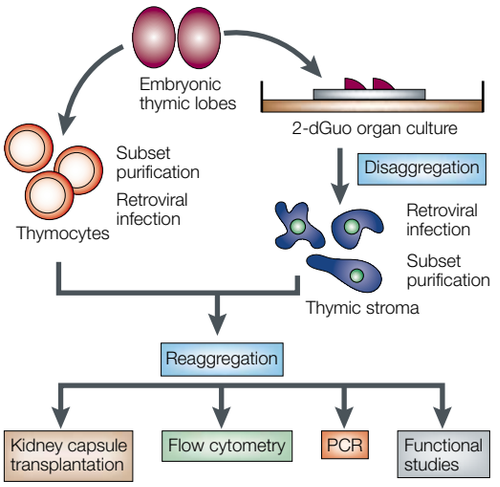


Figure 4 | **Experimental manipulation of the thymus *in vitro*.** Culture systems, such as reaggregate thymus organ cultures, have proved to be useful tools to dissect the stromal cell requirements for individual stages of T-cell development. Such systems can now be readily manipulated to introduce defined genes into specific thymic cellular compartments in order to investigate the molecular mechanisms that regulate thymic stromal cell function and thymocyte development under defined *in vitro* conditions. (2-dGuo, 2-deoxyguanosine; PCR, polymerase chain reaction.)

***Cortical epithelial cells and positive selection.*** As well as Notch being implicated in early stages of thymocyte development, several studies have provided evidence that Notch signalling occurs during positive selection. Generation of $CD4^+$ and $CD8^+$ cells from $CD4^+CD8^+$ precursors, therefore, is accompanied by expression of downstream regulators of Notch signalling, such as Hes-1 and Deltex[65], and in some cases expression of IC-Notch was shown to affect CD4/CD8 lineage choice[67]. Recently, however, elimination of *Notch-1* expression in the T-cell lineage from the $CD4^-CD8^-CD25^+CD44^-$ stage onwards was shown to have no obvious effect on thymocyte development, with the generation and positive selection of $CD4^+CD8^+$ thymocytes occuring normally[60]. So, these findings indicate that Notch-1 signalling is dispensable for generation of $CD4^+$ and $CD8^+$ T cells, although the repertoire of T cells that are generated in the presence or absence of Notch-1 was not compared. Although this might reflect a redundancy in the requirement for Notch-1 molecules in thymocyte development, with other Notch molecules having a role, it might be the case that Notch-1 has a more subtle role in thymic selection processes. It is possible that Notch signalling, perhaps by modulating αβTCR signalling[68], influences the shaping of the αβTCR repertoire of positively selected T cells.

How cortical epithelial cells are unique in their capacity to drive efficient positive selection is unknown. Although a key role of thymic epithelial cells in positive selection is to provide peptide–MHC ligands for the αβTCR, several studies now indicate that the ability of cortical epithelium to promote positive selection is neither due to the provision of a specialized repertoire of MHC-bound peptides[8,74], nor merely to the maintance of thymocyte viability by the production of survival-promoting signals[75]. It is likely, therefore, that thymic cortical epithelial cells express unique, or a unique combination of, cell-surface molecules[76] that act to provide co-stimulatory or accessory signals during positive selection. Indeed, a recent study indicates that thymic cortical epithelial cells, perhaps by way of their specialization for mediating positive selection, when engineered to express an agonistic peptide, lack the ability to promote negative selection of thymocytes that bear a transgenic TCR that is specific for the introduced peptide[77]. Instead of inducing clonal deletion, cortical epithelial cells under these circumstances could induce a 'receptor-editing' mechanism that involves internalization of the transgenic TCRα chain. This promotes expression of an endogenous TCRα chain, allowing cells to escape death by apoptosis[77]. The nature of the accessory molecules expressed by cortical epithelial cells — which seem to be crucial in promoting positive, rather than negative, selection — remains a vital, unresolved issue in thymus development. However, it is clear that these molecules are not the same as those required for activation of mature T cells, as under normal circumstances thymic epithelial cells lack the ability to provide co-stimulation for peripheral T cells[78].

Table 1 | **Notch–Notch ligand interactions in the thymus**

| Molecule | Reported thymic expression | Relevance to T-cell development | References |
|---|---|---|---|
| Notch-1 | Expression detected in all thymocyte subsets | Notch-1 is not essential for maturation of $CD4^-CD8^-CD25^+CD44^-$ precursors | 60 |
| | Thymic epithelial cell lines | Involved in T-cell commitment ? | 61–63 |
| | | Involved in αβ versus γδ T-cell commitment ? | 64 |
| | | Activated on positive selection | 65 |
| | | Overexpression may mediate resistance to apoptosis | 65,66 |
| | | Overexpression interferes with generation $CD4^+$ and $CD8^+$ single-positive cells | 67,68 |
| Notch-2 | Expression detected in all thymocyte subsets<br>Thymic epithelial cell lines | Unknown | 63,69 |
| Notch-3 | Expression detected in all thymocyte subsets<br>Thymic epithelial cell lines | Expression downregulated by pre-TCR signalling | 63 |
| | | Notch-3 IC (intracellular domain) transgenic mice show increased numbers of $CD4^-CD8^-$ thymocytes with sustained NF-κB activity, leading to lymphomagenesis | 70 |
| | | Involved in the regulation of proliferation and survival following successful pre-TCR selection ? | |
| Notch-4 | Low levels detected in fetal, neonatal and adult rat thymus<br>Expression detected in all thymocyte subsets | Unknown | 69,71 |
| Jagged-1 | High in fetal rat thymus, with expression decreasing after birth?<br>Thymic epithelial cell lines | Involved in regulating the CD4/CD8 ratio of mature thymocytes during thymus development? | 63,69,70 |
| Jagged-2 | Thymic epithelial cell lines<br>Embryonic rat thymus<br>Thymocytes | Jagged-2-deficient embryos show normal αβ T-cell development, but reduced γδ T-cell development | 63,69,72,73 |
| Delta-like-1 | High levels in fetal, neonatal and adult rat thymus<br>Thymic epithelial cell lines | Unknown | 69,70 |
| Delta-like-3 | Low levels in fetal, neonatal and adult rat thymus<br>Thymic epithelial cell lines | Unknown | 69,70 |

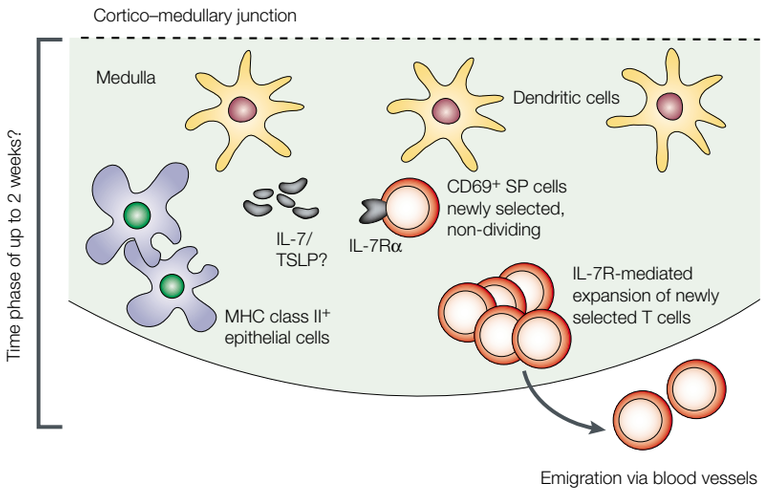


Figure 5 | **Post-positive-selection expansion in the thymic medulla.** Following their screening for reactivity to self-peptide/major histocompatibility complex (MHC) complexes, newly generated CD4+ and CD8+ thymocytes are thought to reside in thymic medullary compartments for extended time periods, indicating that some post-selection events occur prior to their emigration from the thymus. One such event, particularly in the neonatal thymus, seems to be the expansion of newly selected cells, which is dependent on expression of interleukin-7 receptor-α (IL-7Rα), but independent of T-cell receptor (TCR)–MHC interactions. This antigen-independent phase of proliferation might act to increase numbers of positively selected T cells to aid the establishment of the peripheral T-cell pool. (TSLP, thymic stromal lymphopoietin.)

## Medullary microenvironments

***Crosstalk and organization.*** The thymic medulla provides a microenvironment for newly generated single-positive CD4+ and CD8+ thymocytes, which have undergone positive selection in the thymic cortex. As with cortical epithelial cells, medullary epithelium is phenotypically heterogeneous, and any precursor–product relationships existing between medullary epithelial subsets are unclear. Perhaps the first demonstration that organization of the thymic medulla can be regulated by the thymocyte subsets it normally contains, namely CD4+ and CD8+ cells, came from studies in which either TCR transgenic thymocytes[79] or mature peripheral T cells[80] were introduced into severe combined immunodeficient (SCID) mice. In both cases, medullary epithelial cells became organized into discrete regions, indicative of interplay between single-positive T cells and medullary epithelium. Such findings are in agreement with medullary disorganization observed in TCRα$^{-/-}$ mice, in which thymocyte development is blocked at the CD4+CD8+ stage[81]. The nature of the crosstalk in medullary organization is unknown, although analysis of cell kinetics following introduction of wild-type bone marrow into SCID and *Rag2*$^{-/-}$ mice indicate that the regeneration of the thymic medulla observed following the introduction of mature thymocytes does not involve expansion of pre-existing medullary epithelium[82]. Finally, it is interesting to note that the requirement for TCR-bearing T cells in the organization of the thymic medulla might extend to cells bearing the γδTCR, as in some cases γδTCR transgenic mice show an enlarged thymic medulla[83].

Thymic medullary abnormalities also exist in mice lacking RelB, a member of the NF-κB subunit family. In these mice, positive selection of thymocytes seems normal[84], perhaps indicating a second mechanism that regulates medullary organization that is independent of mature thymocytes. The thymi of *RelB*$^{-/-}$ mice have been shown to be lacking some medullary epithelial cells subsets[85,86]. Moreover, some of these RelB-expressing medullary epithelial cells might normally express the product of the autoimmune regulator (*AIRE*) gene, deficiency in which causes multiple-organ autoimmunity[87]. In addition to epithelial defects, *RelB*$^{-/-}$ mice also show perturbation of subsets of thymic dendritic cells (DCs). The thymi of *RelB*$^{-/-}$ mice contain myeloid-derived CD8$^{-}$ DCs, but lack lymphoid-derived CD8+ DCs[88]. Perhaps surprisingly, *RelB*$^{-/-}$ bone marrow gives rise to both lymphoid- and myeloid-derived DCs afterintroduction into irradiated wild-type mice[88]. This raises the intriguing possibility that development and maintenance of subsets of DCs within the thymus are regulated by a subset of *RelB*-expressing medullary epithelium.

***The thymic medulla and tolerance induction.*** As mentioned above, thymic cortical epithelial cells seem to be the most efficient mediators of positive selection, with recent evidence supporting the notion that these cells lack the ability to mediate efficient negative selection[77]. It is generally accepted that the most efficient mediators of negative selection in the thymus are bone-marrow-derived MHC class II+ DCs, which reside at the corticomedullary junction[89,90] (FIG. 1). However, several lines of evidence now point towards a role for medullary epithelium in achieving self-tolerance. Firstly, medullary epithelial cells have been shown to express many proteins that were previously thought to be tissue- or organ-specific, perhaps indicating a role for these cells in tolerance to a variety of tissues[91–93]. Although it is important to note that thymic epithelial expression of these proteins might not necessarily correlate with a role in tolerance induction, for some of these proteins, good evidence exists which indicates that medullary epithelial expression of self-proteins can induce tolerance. Thus, when Klein and colleagues[94] introduced the supposedly liver-specific human C-reactive protein (*hCRP*) gene — and its own regulatory control regions — into mice, expression was detected in thymic medullary epithelium. Moreover, two murine liver acute-phase proteins, CRP and serum amyloid P component (SAP), were also detected in medullary epithelium. Interestingly, when *hCRP* transgenic mice were crossed to *TCR* transgenic mice that were specific for CRP, tolerance was achieved, by means of a mechanism involving deletion of immature thymocytes[94]. Smith and colleagues[95] also have data indicating that medullary epithelial cell expression of supposedly pancreatic-specific proteins can achieve tolerance in the T-cell compartment. Interestingly, this study also showed that the proportion of thymic cells that express these proteins is very low – for example, it was estimated that between 50–500 cells per thymus express the pancreatic protein somatostatin[95]. As already stated[91], this finding has important implications in how

Box 1 | **Experimental systems to study T-cell development *in vitro***

**Fetal thymus organ culture (FTOC)**
Involves microdissection of fetal mouse thymus lobes at day 12–16 of gestation. Isolated lobes are explanted into culture, either on the surface of 0.8-μm filters or in submersion cultures under high oxygen concentrations. A useful system to study phases of thymocyte development in the presence of heterogeneous thymic stromal cells[32,72].

**2-Deoxyguanosine-treated FTOC**
Addition of 2-deoxyguanosine at the outset of the organ-culture period results in a selective elimination of T-cell precursors and dendritic cells, producing alymphoid thymus lobes. Such lobes can then be used as a source of thymic stromal cells, or colonized either *in vitro* by defined precursors or *in vivo* following engraftment under the kidney capsule[108].

**Reaggregate thymus organ cultures**
Purified stromal cell subsets prepared from 2-deoxyguanosine-treated thymus lobes are mixed with suspensions of purified thymocytes and centrifuged to form a cell pellet. Following transfer of the cell pellet to organ culture, these cell mixtures rapidly reform into intact three-dimensional thymus lobes. This system has proved useful to identify the stromal cell requirements of defined stages of thymocyte development[31,41,75,76,110].

**Multilineage progenitor assay**
Single haematopoietic precursor cells from various embryonic sites are allowed to migrate into alymphoid 2-deoxyguanosine-treated thymus lobes in the presence or absence of cytokine cocktails. The progeny of the introduced precursors can then be analysed by flow cytometric and functional assays to help identify haematopoietic lineage relationships[3,52,54].

**FTOC involving retroviral/adenoviral targeting**
In this approach, retroviral or adenoviral constructs can be used to introduce genes of interest into defined T-cell precursors and thymic stromal cells under controlled *in vitro* conditions. Such a system has numerous advantages over conventional transgenesis approaches, including efficiency of targeting of cells and rapidity of introducing defined genes into target cell populations[105–107].

**Gene gun delivery in FTOC**
As well as viral delivery, genes of interest can be introduced into thymocytes using accelerated DNA/particle bombardment, or gene gun technology. Such an approach has recently been modified to analyse gene expression in target cell populations using reporter gene constructs, allowing analysis of gene expression in a biological time frame[109].

these cells might be involved in tolerance induction, as it is difficult to see how such a scarcity of cells would be able to screen large numbers of thymocytes for potential autoreactivity. Finally, it is worth noting that whereas tolerance induction by means of thymic deletion might be a function which medullary epithelium shares with thymic DC, medullary epithelial cells have also been shown to achieve tolerance by non-deletional mechanisms[96], perhaps indicating multiple mechanisms during the establishment of self-tolerance which might be influenced by stromal cell type.

***The thymic medulla and end-stage differentiation.*** As well as having a role in tolerance induction in the thymus, it is likely that medullary epithelial cells are involved in regulating post-selection differentiation events at the level of single-positive thymocytes. Indeed, single-positive thymocytes have been shown to reside in medullary areas for up to two weeks, during which they undergo changes in expression of a variety of cell-surface molecules, such as CD24 (HSA), Qa-2, CD62L (Mel-14), 3G11 and CD69, before they are exported to the periphery[97,98]. However, phenotypically and functionally mature T cells can be generated in the absence of at least some medullary epithelium[84], perhaps indicating that differentiation events occurring at the level of single-positive thymocytes might either be autonomous or capable of being mediated by other thymic stromal cells.

In addition to its possible differentiation-inducing functions, a subtly important role for the thymic medulla has recently emerged, which indicates that this thymic compartment might regulate expansion of newly selected thymocytes prior to their export to the periphery (FIG. 5). So, whereas positive selection of $CD4^+CD8^+$ precursors seems to occur without cell division, a number of studies have shown that, once generated, $CD4^+$ and $CD8^+$ thymocytes undergo an intrathymic phase of cell division[99–101]. This phase has been shown to involve at least six rounds of cell division, and is particularly noticeable in the neonatal period[101]. A hypothesis therefore emerges that this wave of developmentally regulated intramedullary expansion acts to expand the newly selected repertoire of T cells which might help establish the peripheral T-cell pool. This proliferative phase, which occurs independently of TCR–MHC interactions[102] and is, at least in part, dependent on thymocyte expression of interleukin-7 receptor-α (IL-7Rα)[103], might go some way to explaining the retention of single-positive thymocytes in the medulla for prolonged periods[104].

## Conclusions

It is clear that the generation of T cells in the thymus is under strict control of thymic microenvironments. These microenvironments are largely made up of either cortical or medullary epithelial cells, although mesenchymal cells are also now known to have an important role in thymus development and thymocyte differentiation. For many years, thymic epithelial cells have been phenotypically and morphologically characterized, with relatively little data relating to the molecular mechanisms by which these cells influence the development of immature precursors. Recent use of a variety of *in vitro* systems (BOX 1) has enabled a clearer understanding of events occurring within the thymus. Further refinement of these methods, and the generation of new ones[105–110], including retroviral infection of defined thymocyte and stromal cell subsets, will provide powerful tools with which to study mechanisms of thymic stromal cell function and thymocyte development (FIG. 4). The scene is now set to identify the molecular mediators expressed by thymic stromal cells which are involved in key stages of intrathymic development. Most notable, perhaps, is the underlying mechanism of the specialization of cortical epithelial cells for positive selection — a key issue which remains to be resolved. In turn, the role of thymocytes in thymus development and organization will benefit from studies that are aimed at elucidating the molecular basis of thymus crosstalk. Finally, strategies aimed at determining the developmental origins of thymic

epithelial cells, and analysis of putative epithelial cell precursors, will undoubtedly help our broader understanding of how thymic epithelial cells become specialized in their ability to support the multiple stages of thymocyte differentiation.

## Note added in proof

Two recent papers have studied mechanisms of development and functioning of thymic epithelium. In Fgfr2-IIIb-deficient mice, proliferation of immature thymic epithelial cells appears compromised[111]. However, their thymi still support thymocyte development, indicating some epithelial maturation can occur in the absense of Fgfr2-IIIb signalling. Second, ablating thymic epithelial expression of STAT3, an anti-apoptotic regulator linked to growth-factor signalling, leads to severe hypoplasia and cortical disorganization[112]. Moreover, althoug thymocyte differentiaton is not affected, levels of thymocyte apoptois *in vivo* are increased. Thus, STAT3 expression by thymic epithelium has a role in regulating thymocyte survival and organization of epithelial environments.


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*AIRE* | CD24 | CD44 | CD62L | CD69 | Fgf10 | FgfR2-IIIb | Foxn1 | GM-CSF | *hCRP* | Hoxa3 | IL-7 | IL-7Rα | Notch-1 | Pax-1 | Pax-9 | Qa-2 | *Rag-2* | RelB | SAP | somatostatin | TR4 | *undulated*

**FURTHER INFORMATION**

**Graham Anderson's lab:** http://medweb.bham.ac.uk/http/depts/anat/research/graham.html