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Generation of diversity in thymic epithelial cells

Yousuke Takahama[1], Izumi Ohigashi[1], Song Baik[2] and Graham Anderson[2]

[1]Division of Experimental Immunology, Institute of Advanced Medical Sciences, University of Tokushima, Tokushima, Japan.

[2]Medical Research Council Centre for Immune Regulation, Institute for Immunology and Immunotherapy, Medical School, University of Birmingham, Birmingham, United Kingdom.

Correspondence to Y.T. e-mail: takahama@genome.tokushima-u.ac.jp

In the thymus, diverse populations of thymic epithelial cells (TECs), including cortical and medullary TECs and their subpopulations, have distinct roles in coordinating the development and repertoire selection of functionally competent and self-tolerant T cells. Here, we review the expanding diversity in TEC subpopulations, in relation to their functions in T cell development and selection as well as their origins and development.

Some are dead and some are living,
In my life, I've loved them all.

- In My Life, The Beatles

The generation of diversity is a key feature of the immune system, and such importance was highlighted in the 1968 illustration by Richard Gershon, who described it as a conductor of the "immunological orchestra"[1,2]. Initially, generation

of diversity referred to the diversity in antigen recognition specificities by antibodies and T cell receptors (TCRs). Subsequently, it soon expanded to include the diversity in B and T cell subsets and their functional heterogeneity, and further the diversity in macrophages, dendritic cells (DCs), innate lymphoid cells and other haematopoietic cells. Now, this diversity incudes non-haematopoietic cells of the immune system, both in the primary and secondary lymphoid organs. The more we learn about these non-haematopoietic cells within immune organs, the more we realize that they are not just bystander neighbours or resting stroma. They are non-haematopoietic but are diverse and dynamic cells with important roles in the immune system, organizing the development and function of haematopoietic immune cells. Thymic epithelial cells (TECs) are among those diverse and dynamic non-haematopoietic cells.

The organization of adult thymic tissue into cortical and medullary areas, and the presence of an epithelial cell component within each area has for many years provided a way to describe and examine TEC heterogeneity. For example, epithelial cells in the cortex and medulla have differing morphological features, and this is due at least in part to the density of neighbouring thymocytes that they are surrounded by and interacting with[3]. Indeed, it is these differing interactions between cortical TECs (cTECs) and medullary TECs (mTECs) and thymocytes of differing developmental stages that lends support to the idea that the primary reason for TEC diversity is the requirement for their stepwise provision of signals essential for thymocyte development[4,5] (Fig. 1 and Box 1). While the existence of cTEC and mTEC compartments has long been known, recent advances in understanding the nature of the signals they provide, and the ways in which they influence thymocyte development and selection, have provided opportunities to dissect TEC populations further. For example, both cTECs and mTECs can now be defined by their distinct

expression patterns of chemokines, cytokines, costimulatory molecules, antigen processing machinery and transcription factors, observations that have significantly expanded our knowledge of TEC diversity. Moreover, comparable analysis of the expression of at least some of these functional attributes during thymus ontogeny[6-8] has provided important information regarding the developmental programmes that result in the generation of distinct cTEC and mTEC lineages.

Heterogeneity in cTECs

Developmental heterogeneity in cTECs. cTECs are heterogeneous in surface expression of various molecules, many of which are developmentally regulated as previously summarized in REFS [9,10]. Most cTECs in the postnatal thymus, which are defined by expression of epithelial cell adhesion molecule (EPCAM) and CD205, express high levels of MHC class II molecules and CD40. By contrast, the expression levels of MHC class II and CD40 are lower during embryogenesis, and increase during the ontogeny[6]. The expression level of atypical chemokine receptor 4 (ACKR4; also known as CCRL1), which is a non-signalling decoy receptor for chemokines CC-chemokine ligand 19 (CCL19), CCL21 and CCL25, in $CD205^{+}$ cTECs, is also elevated during embryogenesis, but stays broadly heterogeneous in the postnatal thymus[11] (Fig. 2a).

The development of neighbouring and co-developing thymocytes affects the developmental increase in MHC class II, CD40 and ACKR4 expression by cTECs[12-14]. The expression of MHC class II and CD40 by postnatal cTECs remains low in a human CD3ε transgenic mouse strain (tgε26), in which T cell development is arrested at the early double negative 1 stage (which is defined as $CD44^{+}CD25^{-}CD4^{-}CD8^{-}$)[6],

whereas the appearance of ACKR4^{hi} cTECs is diminished in mice deficient in both recombination-activating gene 2 (RAG2) and interleukin-2 receptor γ-subunit (IL-2Rγ), in which thymocyte development is also blocked at an early stage[11].

Functional heterogeneity in cTECs. The thymic cortex provides the microenvironment that supports *de novo* generation of TCR-expressing CD4$^+$CD8$^+$ thymocytes (referred to as double-positive (DP) thymocytes) and positive selection of newly generated DP thymocytes. cTECs express various molecules that are responsible for these processes, as previously summarized in REFS [3,15,16]. Namely, cTECs express the chemokines CCL25 and CXC-chemokine ligand 12 (CXCL12) and NOTCH ligand Delta-like protein 4 (DLL4), which promote thymus seeding and T-cell-lineage specification of lymphoid progenitors. The cytokines interleukin-7 (IL-7) and stem cell factor (SCF) produced by cTECs support the proliferation of developing thymocytes. cTECs also express the thymoproteasome component β5t[7] (also known as PSMB11) and endosomal-lysosomal proteases cathepsin L (CTSL) and thymus-specific serine protease (TSSP; also known as PRSS16), which contribute to the generation of MHC-associated self-peptides that induce positive selection[17,18]. Recently, and relevant to their role in positive selection, cTECs also express CD83, which influences CD4$^+$ SP thymocyte development by regulating their surface expression turnover of MHC class II molecules[19,20]. In addition, cTECs express DLL4 and IL-7[21-24], with evidence indicating that levels of their expression by cTECs may be broad and highly heterogeneous (Fig. 2b). For example, DLL4 expression by cTECs occurs at either low or high levels, which at least in part is regulated by signals from developing thymocytes[21,22]. Moreover, at least two

independently generated IL-7-reporter mouse lines[23,24] identify cTEC heterogeneity with regard to IL-7 expression, and also show that the frequency of IL-7$^+$ cTECs is age dependent. Thus, cTECs are functionally heterogeneous, although T-lineage-specifying and positive-selection-inducing functions appear to overlap between individual cTECs **[Au:OK?]** .

Thymic nurse cells. Thymic nurse cells, which internalize and envelop many thymocytes, are found in the thymic cortex, and constitute approximately 10% of β5t$^+$ cTECs[25-27]. Thymocytes enclosed within thymic nurse cells are enriched with long-lived DP thymocytes that undergo secondary TCR α-chain rearrangement, whereas thymic nurse cells are underdeveloped in many positive-selecting or negative-selecting TCR-transgenic mice, in which transgenic TCRs are capable of inducing positive or negative selection efficiently This suggests that thymic nurse cells provide a microenvironment for optimal TCR repertoire selection by nurturing DP thymocytes to express the secondary rearranged TCR, thereby enabling a second chance of positive selection[27]. The physical and functional properties that underpin the ability of some cTECs to form thymic nurse cell complexes are not fully understood. However, thymic nurse cells have a unique gene expression signature, including high levels of CXCL12 and VCAM1 mRNAs[27]. Given that immature DP thymocytes express high levels of both CXCR4 and integrin α4β1 (also known as VLA4)[28,29], which are receptors for CXCL12 and vascular cell adhesion protein 1 (VCAM1), respectively, it may be the case that thymic nurse cell formation is aided by both chemokine-mediated attraction and integrin-mediated adhesion of DP thymocytes to cTECs. Thus, thymic nurse cells are a subpopulation of cTECs, which are

morphologically and functionally specialized for optimizing positive selection of thymocytes (Fig. 2c).

Heterogeneity in mTECs

mTEC^{hi} and mTEC^{low} populations. Postnatal mTECs contain two major subpopulations that are defined according to their levels of cell surface MHC class II and CD80 molecules; MHC class II^{low}CD80^{low} (mTEC^{low}) cells and MHC class II^{hi}CD80^{hi} (mTEC^{hi}) cells[30]. Embryonic mTEC^{low} subpopulations contain the ability to give rise to mTEC^{hi} subpopulations in reaggregation thymus organ culture (RTOC; Table 1)[31], leading to the widely appreciated notion that mTEC^{low} subpopulations are immature precursors for functionally mature mTEC^{hi} subpopulations. However, it was known from early studies that postnatal mTEC^{low} accumulate during ontogeny[30]. Indeed, it was later shown that cells of postnatal mTEC^{low} populations have heterogeneous expression profiles, including the expression of involucrin and CCL21[32,33]. The involucrin-expressing mTEC^{low} subset resembles terminally differentiated mTECs, possibly representing mTECs that constitute Hassall's corpuscles that are detectable in human thymi[32], whereas the CCL21-expressing mTEC^{low} subset are essential for attracting positively selected thymocytes from the thymic cortex and thereby for the establishment of self-tolerance in T cells[33]. Thus, mTEC^{low} populations are heterogeneous, containing not only immature precursors for mTEC^{hi} populations but also terminally differentiated mTECs and functionally relevant CCL21-expressing mTECs (Fig. 3).

mTEC^{hi} are also heterogeneous with regard to their expression of autoimmune regulator (AIRE)[34], the nuclear protein that critically regulates promiscuous gene

expression for the establishment of self-tolerance in medullary thymocytes[35,36]. AIRE^{+} mTEC^{hi} subsets are further subdivided into osteoprotegerin (OPG)$^{+}$ and OPG^{-} subpopulations[37]. OPG regulates the cellularity of mTECs and the size of the medullary region in the thymus, by attenuating proliferation of mTECs mediated by RANKL (also known as TNFSF11)[38]. Thus, both mTEC^{low} and mTEC^{hi} populations are heterogeneous, and both mTEC^{low} and mTEC^{hi} populations contain functionally relevant mature mTEC subpopulations (Fig. 3).

Heterogeneity in promiscuous gene expression. Promiscuous gene expression is a characteristic that is unique to mTECs, in which 75-90% of all genes, including the tissue-restricted antigens, are expressed[39,40]. Promiscuous gene expression contributes to the establishment of T cell self-tolerance to entire components encoded in the genome[41]. AIRE deficiency causes a failure in optimal promiscuous gene expression and thereby in the establishment of self-tolerance in T cells, leading to the onset of autoimmune diseases in humans and mice[35,42,43]. AIRE binds to hypomethylated lysine residue 4 of histone 3, which is associated with the recruitment of RNA polymerase[44,45]. AIRE also interacts with many other proteins that contribute to the promotion of gene transcription, mRNA stabilization and protein translation[46]. Several layers of heterogeneity in mTECs should be discussed with regard to promiscuous gene expression, as follows.

AIRE-dependency for promiscuous gene expression. AIRE dependency is heterogeneous among genes that are promiscuously expressed by mTECs. Many promiscuously expressed genes are highly dependent on AIRE and are detectable in

AIRE^{+} mTEC^{hi} subpopulations, whereas the expression of a considerable fraction of these genes is unaltered in AIRE-deficient mice and expressed in mTEC^{low} and AIRE^{-} mTEC^{hi} subpopulations[47-49]. A recent study reported that the transcription factor Fez family zinc finger protein 2 (FEZF2) contributes to selective promotion of the transcription of AIRE-independent promiscuous gene expression in mTECs and thereby the establishment of self-tolerance in T cells[50]. Interestingly, whereas AIRE expression by mTECs maps only to the mTEC^{hi} subset, FEZF2 expression was detectable in both mTEC^{low} and mTEC^{hi} populations. In mTEC^{hi} populations, both AIRE^{-}FEZF2^{+} and AIRE^{+}FEZF2^{+} populations were evident, findings that reveal further heterogeneity within the mTEC compartment. Moreover, evidence was provided that mTEC expression of FEZF2 was controlled by lymphotoxin-β receptor (LTβR; also known as TNFRSF3) signalling[50]. This is important as it differs from the requirement for RANK-mediated and/or CD40-mediated signalling in AIRE^{+} mTEC development, and it also further highlights the importance of LTβR in mTEC development[31,33,38,51,52]. However, it remains unclear whether LTβR-mediated regulation of FEZF2 expression fully explains the failure of tolerance induction in LTβR-deficient mice, or whether additional LTβR-mediated effects contribute to thymic T cell tolerance mechanisms.

Heterogeneity at the single-cell level. Single-cell gene expression analysis showed that individual promiscuously expressed genes are transcribed in 2-15% of mTECs[53], and the expression of individual proteins encoded by these genes is detectable in 1-3% of mTECs[54]. A single mTEC co-expresses multiple functionally unrelated promiscuously expressed genes[55], and a single AIRE^{+} mTEC co-expresses both

AIRE-dependent and AIRE-independent genes[53]. Recent studies showed that AIRE-dependent genes tend to be expressed at a lower frequency than AIRE-independent genes[56,57] and that the number of promiscuously expressed gene transcripts in a single mTEC correlates with the expression level of AIRE[39]. In addition, the cluster analysis of single-cell transcriptome data showed that AIRE-dependent promiscuously expressed genes exhibit noticeable co-expression patterns[56,57]. Thus, promiscuous gene expression in individual mTECs is highly heterogeneous, and mosaic expression comprises a sizeable pool of the promiscuously expressed genes in total mTECs.

Heterogeneity during developmental progression. Promiscuous gene expression is also heterogeneous through developmental progression. Promiscuous gene expression is detectable in embryonic mTECs even before the generation of TCRαβ-expressing thymocytes[58,59], and co-expression pattern of promiscuously expressed genes in single mTECs increases in complexity during the ontogeny[59]. Use of a LacZ reporter to trace past and current expression of a particular gene revealed that the promiscuous expression of a particular gene is transient in single mTECs[59]. *In vitro* short-term cultures of human mTECs also showed that mTECs shift from one co-expression pattern to another[55]. The profile of promiscuously expressed self-antigens, including embryonic α-fetoprotein, is different between mTECs derived from embryonic and postnatal progenitors[60]. Thus, during development mTECs shift through heterogeneous patterns of promiscuous gene expression to eventually cover a diverse set of self-genomic components.

Post-AIRE promiscuous gene expression. AIRE-expressing mTEC^{hi} subsets are generally viewed as functionally mature mTECs in terms of their promiscuous gene expression but they contain the developmental potential to further give rise to AIRE^{-} mTEC^{low} subsets, including involucrin-expressing terminally differentiated mTECs[32]. The development of post-AIRE mTECs is predominantly promoted by lymphotoxin-α (LTα)–LTβR signals provided from positively selected thymocytes[32], in contrast to the RANK–RANKL signals provided by positively selected thymocytes that promote the development of AIRE-expressing mTEC^{hi} subpopulation (ref. 31,38). AIRE is also crucial for the development of post-AIRE mTECs[61,62]. Post-AIRE mTECs highly express keratinocyte-related self-antigens, including desmoglin-1 and desmoglin-3, whereas the expression of AIRE-dependent genes is reduced in post-AIRE mTECs[63,64]. Thus, profiles of promiscuous gene expression are different between post-AIRE mTECs and AIRE-expressing mTEC^{hi} subpopulations.

Heterogeneity in chemokine expression. In addition to their provision of self-antigens by promiscuous gene expression, mTECs also produce multiple chemokines that attract positively selected thymocytes and DCs, to optimize the establishment of self-tolerance in T cells[3,15,16]. Ligands for CCR7 produced by mTEC subpopulations guide positively selected thymocytes from the thymic cortex, whereas XCL1 produced by mTECs promotes the accumulation of thymic DCs in the medullary region. mTECs are heterogeneous in the expression of these chemokines.

CCL21, one of CCR7 ligand chemokines, contributes to the recruitment of positively selected thymocytes from the thymic cortex to the medullary region, so that T cells undergo medullary selection and establish self-tolerance[65,66]. The majority of

CCL21-expressing mTECs are distinct from AIRE-expressing mTECs, although both CCL21 and AIRE are detectable in a small subpopulation of mTECs[33]. CCL21-expressing mTECs accumulate during postnatal ontogeny, unlike AIRE-expressing mTECs that dominate during the perinatal period[33]. Similar to post-AIRE mTECs, CCL21-expressing mTECs are enriched in the mTEC^{low} subset, which is regulated by LTβR signals, and reduced in AIRE-deficient mice[33]. Thus, CCL21-expressing mTECs represent a functionally mature mTEC subpopulation, which resemble post-AIRE mTECs.

Positive selection of thymocytes induces the upregulation of expression of multiple chemokine receptors, including CCR4. Unlike CCR7, CCR4 expression during thymic selection is transient, being predominantly expressed by CD69^{+} DP thymocytes and CD69^{+} SP thymocytes. Both CCL17 and CCL22, which are the two known ligands for CCR4, are expressed in the thymic medulla. Within mTECs, the highest levels of CCL17 and CCL22 are seen in mTEC^{hi} subsets compared with mTEC^{low} subsets, although thymic DCs also express high levels of CCL17 and CCL22 mRNAs[67,68]. Whereas T cell development is grossly normal in CCR4-deficient mice, as well as in mice lacking both CCR4 and CCR7, *in vitro* imaging of thymic slices suggested a role for CCR4 in cortex-to-medulla migration of thymocytes, and in supporting thymocyte–DC interactions. Interestingly, the numbers of thymic regulatory T (Treg) cells are slightly increased in the absence of CCR4[67,68], but as yet it is unclear whether this is linked to altered Treg cell selection or increased thymic recirculation of peripheral Treg cells, as seen in the absence of CCR7[69].

The interplay between mTECs and thymic DCs coordinates the establishment of T cell self-tolerance in the thymic medulla[70-72]. Thymic DCs are predominantly localized in the medullary region, which is at least in part controlled by the interaction between the chemokine XCL1 produced by mTECs and its receptor XCR1 expressed by thymic DCs[73]. The expression of XCL1 mRNA is higher in mTEC^{hi} subpopulations than mTEC^{low} populations and is highly AIRE-dependent[73]. XCL1-deficient mice are defective in the optimal accumulation of thymic DCs in the medullary region and in the thymic generation of Treg cells, which depends on the thymus medulla[73]. Thus, XCL1 expression identifies another functionally relevant mTEC subpopulation.

Origin and development of TEC subpopulations

Although some studies suggested a dual germ layer origin of TECs[74,75], cell transplantation experiments in both birds and mice definitively demonstrated that both cTECs and mTECs share a common endodermal origin[76,77]. Moreover, direct clonal analysis of embryonic TECs demonstrated that cTECs and mTECs share a common progenitor[78]. Given the essential requirement for both TEC subsets during T cell development, such findings are significant as they indicate that future cell-based TEC therapies aiming to restore or improve thymic function may focus on manipulating the differentiation of a single germ layer. Subsequently, extensive analysis of both embryonic and adult TEC development has been performed to understand the developmental pathways that lead to the formation of functional cortical and medullary microenvironments.

TEC specification and differentiation in the embryonic thymus. A thymic rudiment is evident in mouse gestation by embryonic day (E) 10-11. At this stage, it is a simple structure that is surrounded by neural crest-derived mesenchyme and contains a single endodermal epithelial layer that arises from the third pharyngeal pouch (3PP). As development proceeds, haematopoietic progenitors colonise the thymus, triggering the formation of increasingly complex three-dimensional epithelial networks[79]. Although the molecular regulators that impose thymus fate within the endodermal epithelium are still unclear, specification of the 3PP endoderm towards a TEC fate is influenced by both sonice hedgehog protein (SHH) and paired box protein PAX3[80]. The presence of a thymus rudiment in forkhead box protein N1 (FOXN1)-deficient *nude* mouse **[G]** embryos demonstrates that specification of the 3PP endoderm towards thymic epithelium occurs independently of the transcription factor FOXN1. However, subsequent TEC differentiation, including the formation of three-dimensional TEC networks and the efficient recruitment and differentiation of T cell progenitors, is critically dependent on FOXN1 expression[81-83]. Indeed, this timing of expression of FOXN1 fits well with the roles of some of its target genes (for example, *CCL25*, *CXCL12* and *DLL4*) that control the recruitment and early differentiation of T cell progenitors[84,85]. Significantly, the use of flow cytometry grade FOXN1-specific antibodies demonstrates that essentially all $EPCAM^+$ TECs within the embryonic mouse thymus are $FOXN1^+$ (ref. 86). Such observations are consistent with the findings that bipotent TEC progenitors residing in the embryonic thymus express FOXN1[82] and that the number of progenitors within the thymus anlage is low in wild-type/*nude* tetraparental chimeric animals[87]. **[Au:OK?]** However, embryonic bipotent TEC progenitors remain poorly defined and further work is required to

examine their phenotypic properties and developmental requirements. Nevertheless, there is now some insight into the developmental stages downstream of these cells that are responsible for the initial emergence of the first cohorts of cTECs and mTECs in the developing embryonic thymus.

Serial progression in embryonic cTEC and mTEC lineages. cTEC and mTEC populations in the adult thymus can be separated on the basis of their differential expression of a panel of intracellular and cell surface markers[10,88]. In the embryonic thymus however, TECs are less well defined by this approach, making analysis of cTEC and mTEC lineage emergence difficult. For example, a keratin5^{+}keratin8^{+} TEC subset dominates in the early embryonic thymus, becoming increasingly rare as thymus development progresses[89,90]. More recently, the cell surface markers CD205 and CD40 that typically identify adult cTECs and mTECs, respectively, were used to assess embryonic TEC development[6]. Interestingly, CD205^{+}CD40^{-} TECs were enriched around E12-13, followed by the progressive appearance of CD205^{+}CD40^{+} and then CD205^{-}CD40^{+} subsets. Surprisingly, RTOC transplantation experiments showed that purified CD205^{+}CD40^{-} TECs could give rise to both cTEC and mTEC lineages that were capable of supporting a complete programme of αβ T cell development[91]. Thus, mTEC potential was evident in embryonic TECs that expressed markers of the cTEC lineage. This 'cTEC-like' phenotype of mTEC progenitors was also revealed by assessing the developmental potential of isolated TECs expressing high levels of a transgene encoding IL-7–yellow fluorescent protein[8]. Significantly, Cre-based fate mapping studies showed that essentially all TECs present in the adult thymus were derived from progenitors that had expressed the cTEC marker β5t during

the embryonic period[92]. The phenotypic identification of mTEC progenitors fits well with the initial description of TEC progenitors that have the ability to form clonal islands specifically within thymic medullary areas[93]. Collectively, such findings are important as they indicate that during initial thymus formation, TEC progenitors acquire hallmarks of the cTEC lineage and then the mTEC lineage in a step-wise manner during initial thymus cortex and medulla formation, findings that support a 'serial progression model' of embryonic TEC development[94] (Fig. 4). Importantly however, given that the only clonal evidence for the existence of embryonic bipotent TECs involved the isolation of cells from the thymus at stages prior to the appearance of such TEC 'co-expressers' (ref. 78), how this progressive acquisition of cTEC and then mTEC markers relates to TEC progenitors with cTEC/mTEC bipotency remains unclear. Given that TEC co-expressers are readily observable by flow cytometry at E15 (ref. 6,91), a time point that correlates with the appearance in E15-17 thymic tissue sections of defined thymic medullary areas, it is likely that these cells represent lineage-restricted mTEC progenitors. If this is the case, then it is perhaps also likely that embryonic TECs that at these stages express cTEC but not mTEC markers are a mixture of bipotent progenitors, together with cells that have committed to the cTEC lineage. The nature of these embryonic cTEC-restricted progenitors remains elusive. Although this has hindered analysis of the mechanisms that control initial thymus cortex formation, further assessment of the developmental properties of TEC subsets using variety of experimental approaches (Table 1) should aid in understanding how formation of the thymic cortex takes place to support the first waves of T cell development.

mTEC stem and progenitor cells. Important advances have been made in identifying the TEC progenitors that initiate thymus medulla formation in the embryonic and postnatal thymus. Understanding of these processes is significant in relation to the mechanisms by which the thymus medulla controls T cell development. For example, the embryonic medulla fosters the generation of distinct waves of tissue-specific γδ T cells[95,96] whereas AIRE$^+$ mTEC availability in the neonatal period has been shown to be particularly important for αβ T cell tolerance induction[97]. Significantly, Sekai, et al. defined a self-renewing subset of embryonic TECs — referred to as mTEC stem cells — that express high levels of Claudin 3 or 4 and SSEA1, and that were capable of long term and specific generation of mTECs[98]. **[Au:OK?]** Interestingly, cells of a similar phenotype were detected in the thymi of *nude* mouse embryos [99], which may be consistent with earlier studies indicating that FOXN1 is dispensable for cTEC/mTEC lineage choice[100]. However, given the importance of FOXN1 in TEC development[83,101,102], it will be difficult to directly assess whether the SSEA1$^+$ TECs generated in the absence of FOXN1 are true mTEC stem cells **[Au:OK?]** . To examine the lineage relationships in early mTEC development, we examined the ontogenetic appearance of mTEC stem cells with TEC progenitors expressing RANK, a key regulator of thymus medulla formation[31,38,51]. Interestingly, SSEA1$^+$ mTEC stem cells were uniformly RANK$^-$ and were detectable earlier in ontogeny than RANK$^+$ TEC progenitors[99]. As with SSEA1$^+$ mTEC stem cells, RANK$^+$SSEA1$^-$ embryonic TECs were restricted to the mTEC lineage. Direct analysis of a precursor–product relationship between these populations remains to be performed. However, it is interesting to note that SSEA1$^+$ mTEC stem cells are detectable in the embryonic thymus of *Relb*$^{-/-}$ mice, which have a profound block in mTEC development, whereas

RANK$^+$ mTEC progenitors are absent[99]. Collectively, these findings are consistent with the idea that specification (through an unknown mechanism) of bipotent TECs to the mTEC lineage is evident by E12. This process then results in the RELB-independent generation of a pool of mTEC stem cells that can generate mTEC progeny in the long term. Downstream of mTEC stem cells, RELB is then required for the generation of RANK$^+$ progenitors, which may then act as a transient source of mTECs (Fig. 4). Additional embryonic mTEC progenitor populations have been described that were also identified on their basis of RANK expression. Termed 'precursors of AIRE$^+$ mTECs' (pMECs), these RANK$^+$ cells were shown to be generated from an earlier 'pro-pMEC' population, and were capable of generating mature AIRE$^+$ mTECs via a TRAF6-dependent mechanism[103]. How pro-pMECs and pMECs relate to SSEA1$^+$ mTEC stem cells is unclear, although both pMECs and mTEC stem cells appear to have the ability to sustain the thymus medulla over the long term. Given the key role of RANK in thymus medulla formation, examination of the mechanisms regulating its expression is perhaps of particular importance. Interestingly, LTβR stimulation was found to increase levels of RANK expression in mTEC progenitors in a RELB-dependent manner[103,104]. Such findings are compatible with the lack of detectable levels of RANK expression in one RANK reporter mouse line when RELB is absent[99], and also the expression of LTβR by early mTEC progenitors, including mTEC stem cells[98]. Although further analysis is required to provide a clearer picture of the pathways and relationships described here, the finding that multiple tumour necrosis factor receptor (TNFR) superfamily members operate in a sequential manner to control the mTEC lineage at least in part explains the importance of these receptors in initial thymus medulla formation.

Maintenance and persistence of adult TECs. The cellular populations that continue to give rise to mature cTEC and mTEC subsets in the postnatal and adult thymus are important to identify, as they are required to maintain thymocyte development for the continued production of naive αβ T cells. The requirement for TEC progenitor populations in the adult thymus is suggested by studies indicating that mature $CD80^{+}$ mTEC turnover is 2-3 weeks[30,58] and that the thymic cortex regenerates following cTEC-specific lineage ablation[105], and by precursor–product studies describing the presence of cTEC-restricted progenitors[106] **[Au:OK?]** . The issue of whether bipotent TEC progenitors exist in the adult thymus has been examined in several studies. Using varying experimental approaches, TEC populations have been identified that would indicate that continued cTEC/mTEC production stems from a bipotent progenitor[106-109]. However, although two of these studies assayed the developmental potential of defined adult TECs using RTOC or thymus transplant assays[106,107], differing phenotypes were ascribed to bipotent adult TECs. Wong et al.[107] described bipotent cells as $EPCAM^{+}$MHC class II^{low} that were further defined as integrin $\alpha6^{hi}$ $SCA1^{hi}$, whereas Ulyanchenko et al. [106] reported bipotency in a $EPCAM^{+}$MHC class II^{hi} subset that also co-expressed Placenta-expressed transcript 1 protein (PLET1) and LY51. In marked contrast, results from thymosphere assays[108,109] indicated that the ability to form these structures was limited to cells that lacked the expression of the pan-epithelial marker EPCAM. This finding is perhaps particularly important, as the starting point for most studies on TEC progenitors involves defining TECs on the basis of an $EPCAM^{+}CD45^{-}$ phenotype. It will be important to determine whether the TEC progenitor properties attributed to

EPCAM$^-$ thymic cells using the thymosphere assay can also be revealed using additional experimental approaches. As such, collective interpretation of these results is difficult. For example, a clear consensus on the overall definition of cells in the adult thymus with cTEC and mTEC potential is awaited. However, it is noteworthy that SCA1 is described as a marker of these cells to two reports[106,107], which may indicate its potential as an adult TEC progenitor marker. In addition, it remains unclear where in the adult thymus such bipotent progenitors reside, although the cortico-medullary junction has been suggested as an attractive site for downstream progeny to replenish both cortical and medullary areas[107], Interestingly, the same site has also been suggested as a site of mTEC-restricted progenitors[110,111]. Relevant to this, inducible cell fate mapping studies showed adult mTECs were derived from progenitors marked by embryonic and neonatal expression of β5t, a finding that is consistent with the serial emergence of initial mTEC populations from progenitors with cTEC markers. By contrast, β5t$^+$ progenitors in the adult thymus contributed little to mTEC generation, even during thymic regeneration following injury[60]. Several explanations for this discrepancy are possible. First, if adult bipotent TECs reside within the adult thymus, then unlike their embryonic counterparts, they may lack expression of the cTEC marker β5t. Second, adult mTEC maintenance may not occur as a result of lineage specification of bipotent cells. Rather, it may take place via pool of mTEC-committed progenitors. This second scenario is perhaps consistent of the presence of SSEA1$^+$ TECs in the adult thymus[98], the embryonic counterparts of which demonstrate mTEC stem cell properties. Interestingly, there is evidence that ongoing T cell development has a negative impact on the availability of TEC progenitor populations, and that blockade of thymocyte differentiation at early stages

prevents this erosion[98]. Although this finding further emphasises the complex thymocyte–TEC crosstalk that underpins thymus function, the possible benefits of controlling TEC progenitor availability via the efficacy of T cell production is not clear. The loss of TEC populations that occurs during age-dependent thymic atrophy **[G]** may have relevance. Here, waves of thymocyte development that establish a peripheral T cell pool early in life may also act to reduce further thymopoietic activity by reducing TEC progenitor frequency, and so limit the energy-consuming process of sustained thymus function. Further work is required to examine how thymocyte development impinges on TEC progenitors and their descendants, and how this may act as a cellular mechanism to regulate thymus function.

TEC therapy and thymus regeneration

An increased understanding of the pathways regulating of TEC development has significance in understanding how the production of functionally capable and self-tolerant T cells is controlled. In addition, it also provides a potential opportunity whereby harnessing TEC progenitor populations might be used as therapeutic means to improve thymus function. For example, several studies have shown that targeting FOXN1, a key transcriptional regulator of thymus development and function, may be a means to either regenerate or create functional thymic tissue. For example, upregulating FOXN1 expression by TECs present in the thymus of aged mice led to improvements in thymopoiesis[112]. Significantly, these changes were notable in mice up to two years of age, indicating that enhancing FOXN1 expression effectively reversed the severe decline in T cell production caused by age-related thymic atrophy. Strikingly, induction of FOXN1 overexpression in mouse embryonic fibroblasts was also shown to be sufficient to promote reprogramming of cells towards the TEC

lineage[113], perhaps providing a complementary approach with the potential to therapeutically restore and/or replace thymus function. However, whereas manipulation of FOXN1 expression increased both recent thymic emigrants and naive T cells[112], the functional properties of these cells was not determined in an immune response, such as following infectious challenge. In terms of potential therapeutic benefit, it will be of interest to examine whether the recovery in thymopoiesis that follows manipulation of FOXN1 expression is also accompanied by improved T cell mediated immune responses.

Finally, several studies have also explored the possibility of generating functional thymic tissue from pluripotent stem cells, an approach that may provide a readily available source of TECs and avoid unwanted issues with HLA-mismatching. Significantly, culture conditions have been reported for both human and mouse embryonic stem cells (ESCs) in which some molecular TEC properties are acquired[114-118]. In some cases when transplanted into mice, cells were also shown to create cortical and medullary thymic tissues and that supported T cell development to varying extents. As discussed in detail elsewhere[119,120], while such approaches hold much promise, the relative efficiency of ESCs to TEC differentiation appears to remain rather low, and it is unclear whether ESC-derived TEC progenitors can give rise to a full spectrum of TEC subsets, including $CCL21^{+}$ and $FEZF2^{+}$ mTEC populations. Moreover, the ability of ESC-derived TECs to maintain T cell development over long periods has not been fully examined. Nevertheless, the adaptation of basic mechanisms of TEC development towards TEC therapies continues to represent an important means to boost immune system function.

Conclusions and perspectives

The intrathymic microenvironments that control the production of functionally competent and self-tolerant αβ T cells are formed by heterogeneous epithelial cell populations that guide immature thymocytes through key phases in their development. Increasingly, TEC heterogeneity is now being revealed at both phenotypic and functional levels, the latter mapping onto the ability of cTEC and mTEC subpopulations to support particular stages of thymocyte development. As such, the importance of TEC diversity for T cell development is reinforced. In addition, our knowledge on TEC diversity has stimulated investigation of the precursor-product relationships that give rise to functionally distinct cTEC and mTEC lineages. In this way, several experimental approaches have resulted in the identification of various TEC progenitors, including cells with cTEC and mTEC bipotency, as well as lineage-restricted TEC progenitors. In the embryonic thymus, a consensus pathway seems to be emerging in which bipotent TEC progenitors initially acquire cTEC features during their development, which is then followed by specification towards the mTEC lineage and the production of $SSEA1^{+}$ mTEC stem cells. Downstream, such cells may then produce mTEC progenitors, including those expressing RANK. Whether this pathway fully explains mTEC heterogeneity within a single mTEC lineage, or whether multiple mTEC 'sub-lineages' exist that can be defined by their expression of functionally distinct molecular profiles, remains to be seen. In contrast to TEC pathways in the embryonic and postnatal thymus, there is still uncertainty regarding how cTEC and mTEC lineages specify and diverge, and how they are maintained in the adult thymus. Whether similar processes regulate both the homeostatic turnover of cTECs and mTECs and thymus recovery following injury, is also not fully clear. A better understanding of the pathways and molecular signals that mediate the recovery of cTEC and mTEC subpopulations in the adult thymus is relevant for future therapies

aiming to restore or improve thymic function in various clinical situations, including post-chemotherapy immune cell reconstitution. The continued identification and understanding of TEC heterogeneity will likely provide significant clues and tools with which to define a clearer picture of the pathways that lead to the continuation of cTEC and mTEC diversity.

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Figure legends

Figure 1 | Diverse TECs constitute multiple thymic microenvironments.

a) The cortex-medulla architecture of the adult mouse thymus. Cortical thymic epithelial cells (cTECs) during the perinatal period are marked by expression of enhanced green fluorescent protein (EGFP) under the control of the thymoproteasome component $\beta5t^{60}$, and medullary thymic epithelial cells (mTECs) are marked by expression of autoimmune regulator (AIRE; red) and keratin 5 (blue).

b) cTEC and mTEC subpopulations coordinate the development and repertoire selection of T cells. Heterogeneous functions of cTECs include, although not limited to, the specification of early thymic progenitors (ETPs) to the T cell lineage and the promotion of positive selection of newly generated $CD4^+CD8^+$ (double-positive, DP) thymocytes. Different mTEC subpopulations attract the migration of positively selected thymocytes to the medullary region and establish self-tolerance in T cells by inducing negative selection of self-reactive $CD4^+CD8^-$ or $CD4^-CD8^+$ (single-positive, SP) thymocytes and by promoting the generation of regulatory T (Treg) cells.

Figure 2 | cTEC heterogeneity.

a) Developmental progression of cortical thymic epithelial cells (cTECs). cTEC expression of cell-surface molecules including MHC class II, CD40 and atypical chemokine receptor 4 (ACKR4) increases with ontogeny. The development of cTECs is regulated by signals provided by co-developing thymocytes.

b) cTECs in adult mice are heterogeneous in the expression of functionally relevant molecules, including delta-like ligand 4 (DLL4) and interleukin-7 (IL-7).

c) Adult mouse cTECs contain thymic nurse cells, which envelop many thymocytes and provide a microenvironment for the optimal T cell receptor (TCR) repertoire selection of $CD4^{+}CD8^{+}$ thymocytes through the secondary TCRα rearrangement.

Figure 3 | mTEC heterogeneity.

In the adult thymus, medullary thymic epithelial cells (mTECs) are typically defined by expression of epithelial cell adhesion molecule $(EPCAM)^{+}$ and lack of expression of CD45, and either reactivity to the lectin UEA1 or lack expression of the cTEC markers LY51 or CD205. The mTEC population can be subdivided on the basis of MHC class II and CD80 expression levels, to identify an MHC class $II^{low}CD80^{low}$ ($mTEC^{low}$) subset and an MHC class $II^{hi}CD80^{hi}$ ($mTEC^{hi}$) subset. While direct precursor-product analysis shows that the $mTEC^{low}$ subset contains $mTEC^{hi}$ progenitors, the $mTEC^{low}$ subset has significant heterogeneity, and probably contains both immature and mature subsets. The mature subsets may include a mature CC-chemokine ligand 21 (CCL21)-expressing $mTEC^{low}$ subset and a $mTEC^{low}$ subset that is generated post-AIRE expression from a $mTEC^{hi}$ subset. The $mTEC^{hi}$ subset is also heterogeneous, with subpopulations defined by their expression of key regulators of tissue restricted self-antigen expression (AIRE and Fez family zinc finger protein 2 (FEZF2)), as well as regulators of mTEC homeostasis (osteoprotegerin (OPG)).

Figure 4 | Origin and development of TECs: lessons from the embryonic thymus.

The diagram summarizes our current knowledge regarding the development of cortical and medullary thymic epithelial cell (cTEC and mTEC) lineages, and it is largely based on studies analysing the embryonic thymus. Bipotent TEC progenitors

differentiate by expressing a panel of markers that are typical of the cTEC lineage. Specification towards the mTEC lineage via an unknown mechanism results in the generation of SSEA1^{+} mTEC stem cells, the subsequent development is RELB dependent and involves expression the mTEC regulator RANK. Crosstalk between RANK^{+} mTEC progenitors then triggers differentiation towards AIRE^{+} mTECs.

Box 1 | Thymocyte traffic during their development in the thymus

The thymus attracts haematopoietic stem cell-derived T lymphoid progenitors and induces their differentiation into T cell receptor (TCR)-expressing $CD4^{+}CD8^{+}$ double-positive (DP) thymocytes in the microenvironment of the thymic cortex. The recruitment of T lymphoid progenitors to the thymus, and their entry to the thymic microenvironment depends on multiple chemokine receptors including CC-chemokine receptor 7 (CCR7), CCR9 and CXC-chemokine receptor 4 (CXCR4). DP thymocytes that interact at low affinity with self-peptide–MHC complexes presented by cortical thymic epithelial cells (cTECs) are induced to survive and differentiate into $CD4^{+}CD8$ or $CD4^{-}CD8^{+}$ single-positive (SP) thymocytes. This process termed positive selection contributes to the enrichment of a self-MHC-restricted and potentially useful T cell repertoire. Positively selected thymocytes are induced to express chemokine receptor CCR7 and are attracted to migrate into the thymic medulla, where CCR7 ligands are abundant. In the thymic medulla, a variety of antigen presenting cells (APCs), including medullary thymic epithelial cells (mTECs) and dendritic cells, present a wide range of self-antigens, including tissue-restricted self-antigens produced by mTECs through the mechanism of promiscuous gene expression. High affinity TCR interactions with those self-antigens displayed in the thymic medulla induce the deletion of positively selected thymocytes or the differentiation into regulatory T cells, contributing to the establishment of self-tolerance in T cells. Mature SP thymocytes express S1P1, a receptor for sphingosine-1-phosphate (S1P), which governs their egress out of the thymus. (Refer to Refs 3, 10, 15 for more detail)

Table 1 | Experimental approaches to study thymic epithelial cell lineage development

Approach	Definition	Advantages	Disadvantages	Refs
Ontogenetic analysis	Phenotypic characterization of embryonic thymic epithelial cell (TEC) populations at defined developmental stages using flow cytometry and/or confocal microscopy.	• Simple to perform. • Ex vivo analysis. • Provides detailed multiparameter characterization of embryonic TECs using well-defined cTEC and mTEC markers.	• Provides static analysis, a 'snapshot' of TEC populations at any given developmental stage. • Does not allow for direct analysis of precursor-product relationships	6, 7, 8, 22, 86
Germline or inducible Cre-mediated cell fate mapping	Expression of a fluorescent protein is driven by a TEC expressed gene promoter in a constitutive or inducible manner.	• *In vivo* analysis of stages in TEC development using defined genetic markers. • Inducible fate mapping allows for examination of stages in TEC development at both embryonic and adult stages.	• Limited availability of cTEC and mTEC lineage-specific Cre-based models, particularly inducible Cre systems.	60, 61, 63, 111
Clonal thymus microinjection	The introduction of a genetically marked single TEC into a wild-type 'foster' thymus, followed by thymus transplantation.	• Provides direct analysis of precursor-product relationships at the single cell level. • Lineage potential is assessed within an intact thymic microenvironment *in vivo*.	• Labour intensive • Relatively low clonal success rate	78
Reaggregate thymus organ culture	The *in vitro* generation of intact three-dimensional thymus structures from purified TEC subsets.	• Can be combined with *in vivo* thymus transplantation to assess lineage relationships. • Simple to perform	• Relies on population analysis and high cell purity • Requires the isolation of candidate TEC populations using a limited array of cell surface markers and/or fluorescent protein tags.	8, 31, 91, 96, 98, 99, 103, 106, 107
Thymospheres	The *in vitro* generation of spheroid structures generated from single cells present within dispersed thymic stromal preparations.	• Enables clonal analysis of thymic stromal cells • Thymosphere-derived cells can be mixed with foster thymic tissue in RTOC and combined with thymus transplantation for *in vivo* analysis.	• Requires low-attachment *in vitro* culture conditions that do not provide the typical reticular organization of TECs.	108, 109

Glossary

Positive selection: The process in that newly generated DP thymocytes that interact at low affinity with self-peptide–MHC complexes presented by cTECs are induced to survive and differentiate into SP thymocytes.

Hassall's corpuscles: A fraction of mTECs that form a concentric epithelial structure, which is apparent in the thymus of several limited species including human.

Autoimmune regulator: A nuclear protein expressed by a subpopulation of mTECs and essential for the establishment of self-tolerance in T cells.

Promiscuous gene expression: A characteristic unique to mTECs in that virtually all genes, including the tissue-restricted self-antigens, are expressed.

***nude* mouse:** A naturally occurring mouse strain in which congenital loss of the transcription factor Foxn1 causes defective hair growth and defective TEC development, resulting in defective T cell production and severe immunodeficiency.

Thymic atrophy: A reduction in size of the thymus, caused by ageing, viral infection, irradiation, and many other stresses, and associated with the decline in T cell production.

Biographies

Yousuke Takahama is Professor of Experimental Immunology and Director of Institute of Advanced Medical Sciences at University of Tokushima, Japan. His main interests are the development and function of the thymus and thymic epithelial cells, especially with regard to repertoire selection of T lymphocytes.

Izumi Ohigashi is Associate Professor of Experimental Immunology at Institute of Advanced Medical Sciences, University of Tokushima, Japan. She is interested in molecular and cellular mechanisms for the development of diverse thymic epithelial cell subpopulations.

Song Baik is a postdoctoral research fellow in the Institute of Immunology and Immunotherapy at the University of Birmingham. Her current research interests focus on thymic epithelial progenitors and their developmental potential.

Graham Anderson is Professor of Experimental Immunology in the Institute for Immunology and Immunotherapy at The University of Birmingham. His research focuses on the role of the thymus in T-cell development. A particular interest is how distinct thymic stromal populations develop to control aspects of thymocyte development that include T-cell progenitor migration and selection.

Online summary

cTECs are functionally heterogeneous, although T-lineage-specifying and positive-selection-inducing functions appear to overlap between individual cTECs.

Thymic nurse cells represent a subpopulation of cTECs, which are morphologically and functionally specialized for optimizing positive selection of thymocytes.

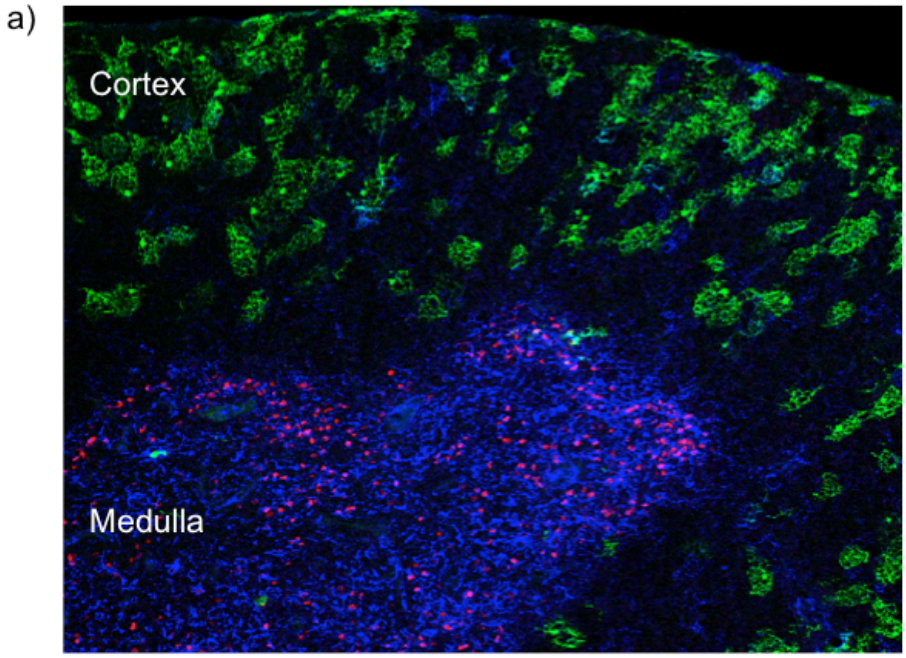
Promiscuous gene expression in individual mTECs is heterogeneous, and mosaic expression comprises a pool of the promiscuously expressed genes in total mTECs.

CCL21-expressing mTECs represent a functionally mature mTEC^{low} subpopulation, which resemble post-AIRE mTECs.

Embryonic TEC progenitors acquire hallmarks of the cTEC lineage and then the mTEC lineage in a step-wise manner during initial thymus cortex and medulla formation.

A self-renewing subset of embryonic TECs, referred to as mTEC stem cells, capable of long term and specific generation of mTECs has been identified.

Figure 1.


a)
Cortex
Medulla

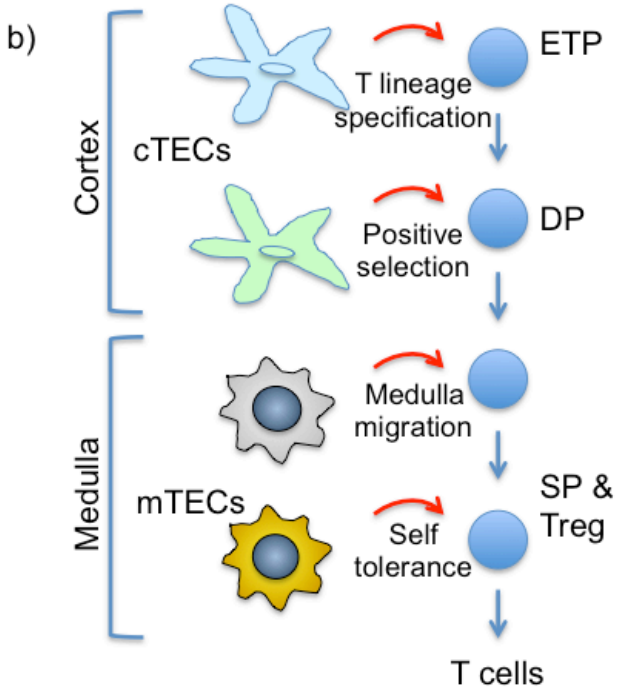

b)
Cortex
cTECs
T lineage specification
ETP
Positive selection
DP
Medulla
mTECs
Medulla migration
Self tolerance
SP & Treg
T cells

Figure 2.

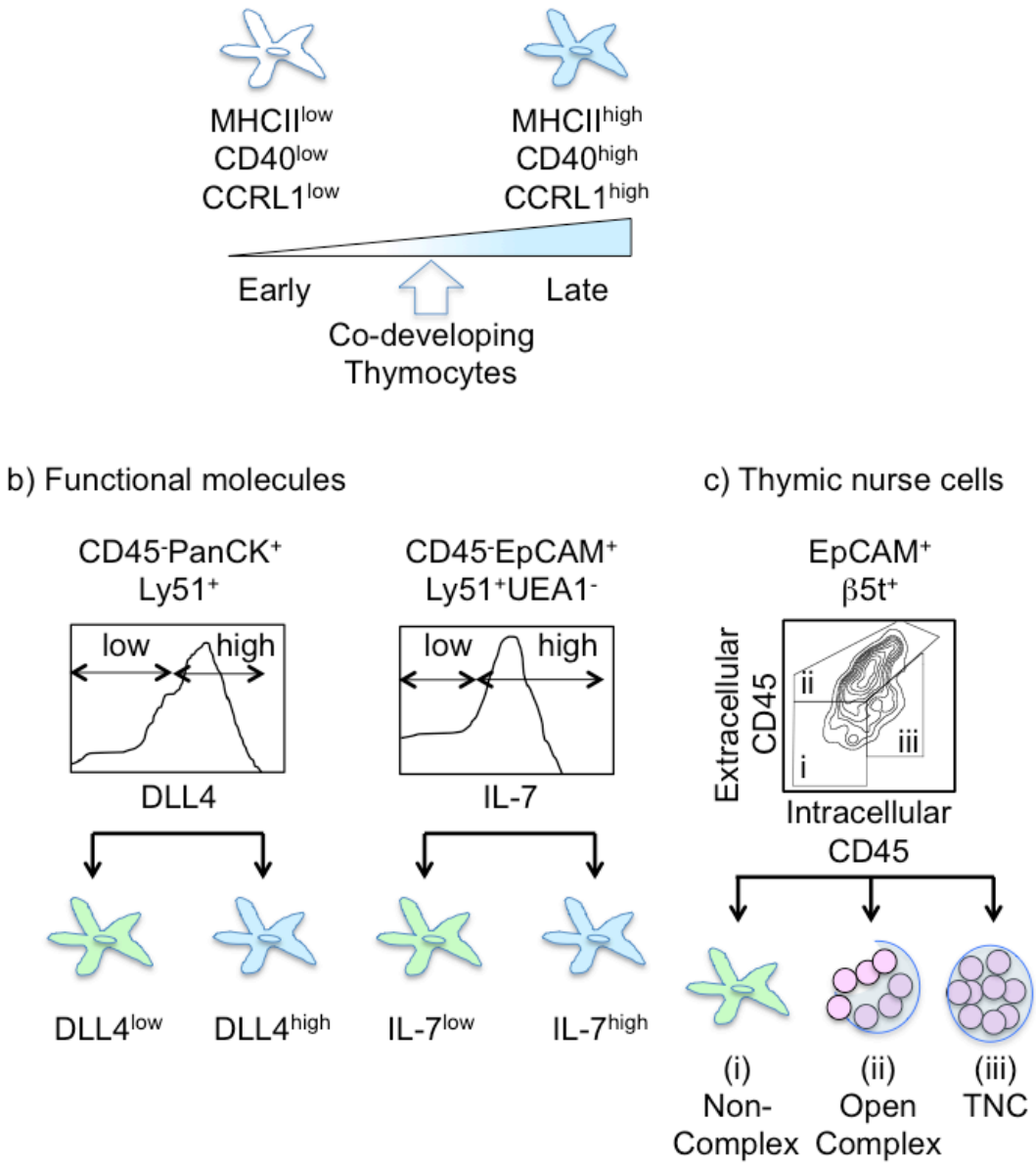

a) Developmental progression
MHCII^low
CD40^low
CCRL1^low
MHCII^high
CD40^high
CCRL1^high
Early
Late
Co-developing
Thymocytes
b) Functional molecules
CD45-PanCK+
Ly51+
low
high
DLL4
DLL4^low
DLL4^high
CD45-EpCAM+
Ly51+UEA1-
low
high
IL-7
IL-7^low
IL-7^high
c) Thymic nurse cells
EpCAM+
β5t+
Extracellular
CD45
ii
iii
i
Intracellular
CD45
(i)
Non-
Complex
(ii)
Open
Complex
(iii)
TNC

Figure 3.

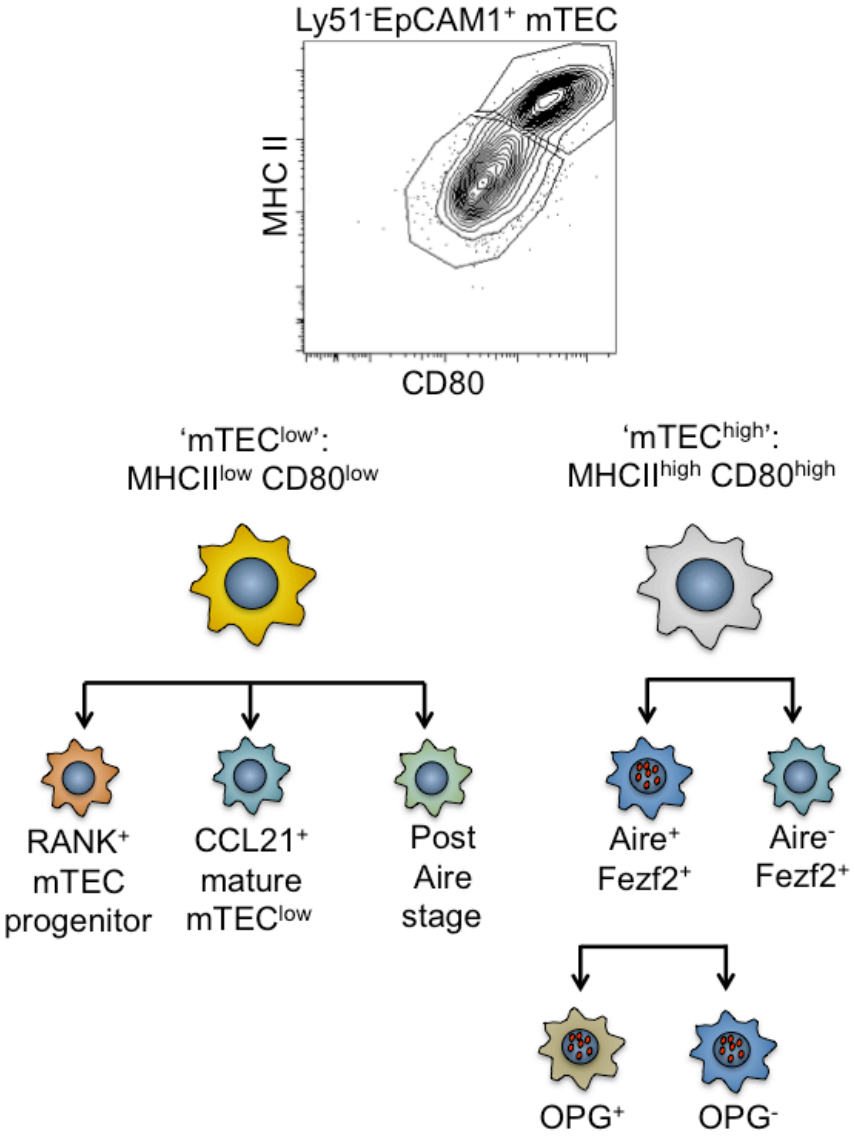
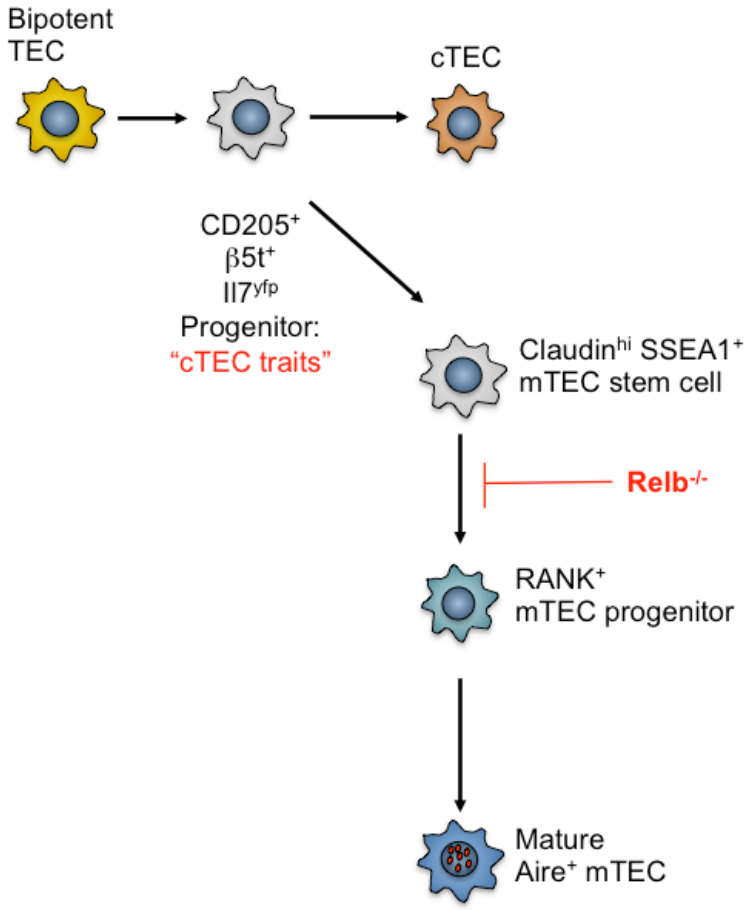

Ly51-EpCAM1+ mTEC
MHC II
CD80
'mTEClow':
MHCIIlow CD80low
'mTEChigh':
MHCIIhigh CD80high
RANK+ mTEC progenitor
CCL21+ mature mTEClow
Post Aire stage
Aire+ Fezf2+
Aire- Fezf2+
OPG+
OPG-

Figure 4.


Bipotent
TEC
cTEC
CD205+
β5t+
Il7yfp
Progenitor:
"cTEC traits"
Claudinhi SSEA1+
mTEC stem cell
Relb-/-
RANK+
mTEC progenitor
Mature
Aire+ mTEC