Thymic epithelial cell heterogeneity

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Thymic epithelial cell heterogeneity: TEC by TEC

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Abstract

The generation of a functional T cell repertoire in the thymus is mainly orchestrated by thymic epithelial cells (TECs), which provide developing T cells with cues for their navigation, proliferation, differentiation and survival. The TEC compartment has been segregated historically into two major populations of medullary TECs and cortical TECs, which differ in their anatomical localization, molecular characteristics and functional roles. However, recent studies have shown that TECs are highly heterogeneous and comprise multiple sub-populations with distinct molecular and functional characteristics, including tuft cell-like or corneocyte-like phenotypes. Here, we review the most recent advances in our understanding of TEC heterogeneity from a molecular, functional and developmental perspective. In particular, we highlight the key insights that were recently provided by single-cell genomic technologies and in vivo fate mapping and discuss them in the context of previously published data.

[H1] Introduction

The thymus is a specialized primary lymphoid organ whose main function is the production of immunologically competent T cells that can recognize and eliminate foreign antigens, but that tolerate the body's own components[1]. T cell 'education' in the thymus is mainly orchestrated by thymic epithelial cells **[G]** (TECs), which provide developing T cells with cues for their navigation, proliferation, differentiation and survival (**BOX 1**).

The TEC compartment has been divided historically into two major subsets, which differ in their anatomical localization and functional roles. Specifically, whereas the early checkpoints of the T cell developmental programme (T cell lineage commitment and positive selection **[G]**) are orchestrated by cortical thymic epithelial cells (cTECs), later steps of T cell development, including negative selection **[G]** of self-reactive thymocytes or their diversion into the $FOXP3^{+}CD25^{+}$ regulatory T (T_{reg}) cell lineage (agonist selection **[G]**), are primarily mediated by medullary thymic epithelial cells (mTECs)[2,3].

In the past several years, however, it has become evident that cTECs and mTECs are not homogeneous compartments, but are rather characterized by a high degree of internal

heterogeneity (**FIG. 1a**). This increasing appreciation of TEC complexity, previously reviewed in REF.[4], is accompanied by growing confusion regarding the molecular and functional characterization of the individual TEC subsets. This may stem from the fact that the characterization and/or isolation of these subsets have been achieved using only a small number of surface markers, hence obscuring the composite picture of the TEC compartment.

Here, we review the most recent advances in our understanding of TEC heterogeneity from a molecular, functional and developmental perspective that have been provided by single-cell genomic technologies and in vivo fate mapping, and discuss them in the context of previously published data. Specifically, we discuss thymic epithelial progenitor cells (TEPCs) and highlight some of the key open questions and controversies regarding their molecular characteristics and progenitor properties at different stages of thymic development. Furthermore, we review in detail our current understanding of cTEC and mTEC heterogeneity and development, with a particular focus on the diverse cell subsets that were recently found to compose the autoimmune regulator **[G]** (AIRE)-negative mTEC compartment, including the mTEC I subset[5], CCL21-expressing mTECs[6,7], podoplanin-expressing (PDPN$^+$) junctional TECs (jTECs)[8,9], corneocyte-like KRT10$^+$ mTECs (also known as post-AIRE mTECs or mTEC III)[5,10–12] and the newly identified DCLK1$^+$ thymic tuft cells (also known as mTEC IV)[5,13–15]. We hope that this Review not only provides the most up-to-date and comprehensive snapshot of the TEC atlas, but also helps in integrating this information with previous knowledge and clarifying some of the outstanding questions from the past.

[H1] Thymic epithelial progenitor cells

The identification of the putative TEPCs has been one of the major challenges in the field. Despite marked progress in this direction in the past two decades, and convincing evidence for the existence of bipotent TEPCs that can concurrently give rise to both mTEC and cTEC lineages in the fetal and early neonatal thymus, a substantial degree of controversy remains regarding the exact identity and molecular characteristics of TEPCs, particularly in the adult thymus. Specifically, while several studies proposed the existence of a bipotent TEPC[16–19], others have argued that mTECs and cTECs are maintained by lineage-specific progenitors[20,21]. Moreover, many of these studies

differ markedly in the molecular characterization of such putative TEPCs, each proposing a different set of markers for their identification.

One of the first pieces of evidence to support the existence of bipotent TEPCs in the embryonic thymus comes from experiments showing that a single EPCAM^{+}CD45^{-} cell isolated from the thymus at embryonic day 12.5 (E12.5) gives rise to both cTEC and mTEC progeny after transplantation **[Au:OK?]**[22]. Similarly, another study using an in vivo fate-mapping approach showed that a single embryonic TEC gives rise to distinct cell islets within the thymus, comprised of both cTECs and mTECs (in 76% of the cases) or of the individual lineages, which suggests that both bipotent and lineage-restricted TEPCs exist in the embryonic thymus[23] (**FIG. 1b**). These findings are further supported by two more recent studies[20,21] that carried out in vivo lineage tracing using a fluorescent reporter activated by a doxycycline-inducible Cre recombinase under control of the *Psmb11* promoter. Importantly, although *Psmb11* is a cTEC-specific gene encoding the β5t subunit of the thymoproteasome **[G]**, it was also previously shown to be active in embryonic bipotent TEPCs[24]. Using this elegant fate-mapping system, both studies[20,21] showed that the *Psmb11*-expressing TEPCs are still present in the neonatal thymus, but that their progenitor capacity disappears ~2 weeks after birth. Although this supports the conclusion that postnatal β5t-expressing cells are not bipotent progenitors in the adult thymus, it does not necessarily show that there is a lack of bipotential within the β5t-negative population, as suggested by REFS[17,19] (**FIG. 1b**).

The molecular characterization of the embryonic TEPCs has been marked by several discrepancies between groups. Specifically, in 2002, two independent groups showed that the embryonic thymus contains bipotent TEPCs that are characterized by expression of the surface marker PLET1, which is recognized by the monoclonal antibody MTS24 (REFS[25,26]). However, a more recent study using larger cell numbers showed that both PLET1^{+} and PLET1^{-} embryonic TECs have a similar capacity to generate functional thymic tissue[27]. As the phenotype of the transplanted PLET1^{-} cells was not determined in the later study[27], it cannot be excluded that PLET1^{-} lineage-restricted TEPCs could arise from PLET1^{+} bipotent TEPCs. Thus, whether PLET1 expression marks bipotent TEPCs remains a point of discussion. Importantly, several subsequent studies have shown that embryonic

TEPCs are characterized by a strong 'cTEC-footprint', including high levels of expression of β5t[24] and CD205 (also known as LY75)[28], which are highly specific to the cTEC lineage in the adult thymus[4]. Based on these findings, a 'serial progression model' of TEC development has been proposed[29], according to which bipotent progenitors commit to the cTEC lineage by default **(FIG. 1b)**, whereas entry into the mTEC lineage requires activation of an mTEC-specific transcriptional programme concomitant with the downregulation of cTEC-specific genes. Importantly, this model is further supported by recent single-cell RNA-sequencing (scRNA-seq) analysis[5], which showed that most embryonic TECs are characterized by a strong cTEC footprint and relatively limited cell heterogeneity. Interestingly, in spite of their marked similarities to mature cTECs, the embryonic TEPCs have several unique characteristics that distinguish them from adult cTECs, including high levels of expression of cell cycle-related genes, fibronectin (*Fn1*) and pyruvate kinase 2 (*Pkm*), and low levels of expression of genes of the MHC class II pathway[5] **(FIG. 1c)**. These data therefore collectively suggest that although the bipotent TEPCs in the embryonic thymus may share many molecular characteristics with mature cTECs, they likely differ markedly in their proliferative, metabolic or antigen-presentation properties, and, most importantly, in their progenitor capacities. Moreover, the proportion and/or progenitor capacity of the putative TEPCs seems to progressively decrease with embryonic age to undetectable levels in the adult thymus, reflecting the gradual emergence of additional TEC subsets corresponding to the cTEC and mTEC lineages[5,20,21].

The existence and molecular characterization of bipotent TEPCs in the adult thymus still remain largely controversial. Several independent studies have shown that the adult thymus does not contain cTEC-like bipotent TEPCs[20,21], whereas other studies have suggested that bipotent TEPCs do exist in the adult thymus, albeit with different molecular characteristics[16,17]. For example, a rare $CD45^{-}EPCAM^{+}PLET1^{+}UEA1^{-}LY51^{+}MHCII^{hi}$ TEC subset was proposed to have bipotent capacity when transferred into reaggregate thymic organ cultures **[G]** (RTOCs)[16]. By contrast, another study suggested that bipotent TEPCs are contained within a small subset of $CD45^{-}EPCAM^{+}LY51^{lo}UEA1^{lo}MHCII^{lo}ITGA6^{hi}SCA1^{hi}$ TECs in RTOC-based experiments[30], and an additional study reported a similar population that is radioresistant and proliferates during thymus regeneration[31]. Moreover, in vitro analysis of TEC subsets using a colony-forming assay showed that $EPCAM^{+}LY51^{lo}UEA1^{lo}MHCII^{lo}ITGA6^{hi}SCA1^{hi}$ TECs have the greatest capacity to form colonies[17] which, upon transplantation under the kidney capsule, gave rise to functionally mature

cTEC and mTEC lineages[17] However, the exact molecular characteristics of such adult bipotent TEPCs are still largely unknown. Recent scRNA-seq analysis of the adult TEC compartment showed that *Itga6* and *Sca1,* which were proposed to mark bipotent TEPCs in the adult thymus[30], are highly enriched in at least two distinct cell clusters, one having a cTEC-specific gene signature and the other having an mTEC-specific gene signature[5]. Thus, ITGA6 and SCA1 may not suffice for the characterization of bipotent TEPCs and the identification of additional and more specific markers is needed. Finally, another study has proposed that bipotent TEPCs in the adult thymus form spheric organoids (termed thymospheres), a feature that is characteristic of stem cells in other tissues, and do not express several key hallmarks of TECs, such as EPCAM and FOXN1[18]. However, these findings were recently challenged by another study showing that the thymosphere-forming cells are mainly mesenchymal cells that can incorporate bystander TECs into the thymosphere[32]. Recent evidence has found that although bipotent TEPCs may exist in the adult thymus, replenishment of the TEC population after puberty relies heavily upon lineage-restricted TEPCs, as the bipotent TEPCs become quiescent[19]. The underlying mechanisms that account for this change in TEPC activity are the result of age-dependent increases in levels of both follistatin (FST) and bone morphogenetic protein 4 (BMP4). FST antagonizes activin A signalling, which is an important factor for TEC differentiation, and BMP4 has been shown to support the maintenance of TEPCs[19,33].

In summary, although there is sufficient evidence to support the existence of bipotent TEPCs in the embryonic and neonatal thymus, the existence of such cells in the adult thymus remains controversial and requires further experimental support based on *in vivo* single-cell fate-mapping experiments. Moreover, future studies should provide detailed molecular characterization of such TEPCs, in particular in the adult thymus.

[H1] Cortical thymic epithelial cells

cTECs are equipped with several key molecules that are crucial for the regulation of different checkpoints of the initial T cell developmental programme (**FIG 2a**)[4,34]. Specifically, mature cTECs express high levels of two key cytokines, CXC-chemokine ligand 12 (CXCL12) and CC-chemokine ligand 25 (CCL25), which promote the homing of blood-borne lymphoid progenitor

cells into the thymus[35–38]. Moreover, cTECs express high levels of the Notch ligand delta like ligand 4 (DLL4), which instructs the recruited lymphoid progenitors to commit to the T cell lineage[39,40]. Mature cTECs also express various cytokines, such as IL-7 and stem cell factor (SCF), that promote the proliferation and survival of the developing thymocytes[41]. Being the exclusive mediators of positive selection of immunocompetent T cells in the thymus[1], cTECs express specific components of the antigen processing and presentation machinery, including the thymoproteasome subunit β5t (encoded by *Psmb11*), and the proteases cathepsin L1 (encoded by *Ctsl*) and thymus-specific serine protease (TSSP; encoded by *Prss16*)[42]. Recently, and relevant to their role in positive selection, cTECs were also found to express CD83 (REF.[43]), which regulates the turnover of surface MHC class II molecules and thereby affects the development of $CD4^+$ single-positive (SP) thymocytes[44,45].

The heterogeneity of cTECs is still poorly understood. In general, most cTECs in the postnatal thymus are defined by high levels of expression of the surface markers LY51 (also known as CD249; encoded by *Enpep*), LY75 (encoded by *Cd205*) and MHC class II. However, a small fraction of $MHCII^{lo}LY51^+LY75^+$ cTECs also exists, likely representing an immature subset[46]. cTECs were also found to have highly heterogeneous levels of expression of DLL4, which depends on the developmental stage of the thymus and decreases with age[47]. Whereas almost all embryonic cTECs express high levels of DLL4, a large fraction of cTECs in the postnatal thymus express lower levels of DLL4 (REF.[47]). Moreover, the existence of two major cTEC subsets distinguished by differential expression of *Dll4* — as well as additional genes, such as *Cd83*, *Ackr4* (also known as *Ccrl1*), *Ccl25, Ly6a* and *Cxcl12*) — was recently validated by scRNA-seq analysis (**FIG. 2b**)[5], but it remains to be investigated whether these $DLL4^{hi}$ and $DLL4^{lo}$ cTEC subsets have different functional roles.

It is also important to stress that the limited cTEC heterogeneity highlighted by scRNA-seq analysis[5] may be largely underestimated, as such single cell-based analyses may have 'filtered out' and thus overlooked a large fraction of cTECs that are tightly associated with thymocytes. In particular, such complex cTEC structures may include thymic nurse cells **[G]** (TNCs) or their precursors (**FIG. 2b**), which are a significant fraction of cTECs in the adult thymus[48,49]. Although

detailed molecular characterization of TNCs is still missing, they were shown to be capable of internalizing between 2 and 200 immature double-positive (DP) thymocytes[50]. They therefore resemble more a complex 'organoid' than a conventional cell (**FIG. 2b**). The engulfment of developing thymocytes by TNCs was shown to be crucial for secondary T cell receptor (TCR) α-chain rearrangement[49]. Furthermore, whereas some of the internalized thymocytes proliferate and mature into TCR^{hi}CD69^{+} DP T cells, which are then released from the TNC, others undergo apoptosis and are degraded within the TNC[50].

The developmental heterogeneity of cTECs is also poorly understood. Although the cTEC compartment can fully regenerate following cTEC-specific ablation in adult mice[51], little is known about the putative cTEC progenitor cells (cTEPCs). One such population that has been briefly described is short-term cTEPCs in the PLET1^{-}LY51^{+} fraction in the adult thymus[16]. More recently, SCA1^{lo}MHCII^{lo} cTECs were shown to be precursors of mature cTECs, and the contribution of these precursors to cTEC maintenance increases with age[19]. Moreover, unlike mTEC development, which is orchestrated by nuclear factor-κB (NF-κB) signals (see below)[52], no specific signalling pathway has yet been identified to regulate cTEC development.

[H1] mTEC progenitors and immature mTECs

The cell precursors that give rise to and/or maintain the mTEC compartment seem to differ greatly between the embryonic thymus and adult thymus. Specifically, whereas the embryonic mTEC progenitors (mTEPCs) are defined by high levels of expression of the tight junction components claudin 3 (CLDN3) and CLDN4, progenitors that maintain the mTEC compartment in the adult thymus do not express CLDN3 or CLDN4[53] and express a different set of molecular markers (discussed below).

Interestingly, a small subset of the CLDN3^{hi}CLDN4^{hi} embryonic mTEPCs was shown to have clonogenic capacity, which suggests that it may comprise unipotent mTEC stem cells (mTECSCs)[54]. Specifically, these CLDN3^{hi}CLDN4^{hi} mTECSCs were found to express stage-specific embryonic antigen 1 (SSEA-1; also known as CD15) and to be present in the embryonic thymus of nude mice **[G]** and RELB-deficient mice, which suggests that they do not require

FOXN1 or NF-κB signalling for their emergence and subsequent maintenance[55]. The NF-κB signalling pathway was, however, crucial for the upregulation of expression of receptor activator of NF-κB (RANK; also known as TNFRSF11A) in these mTECSCs, a step that is necessary for subsequent stages of mTEC development[55,56] **(FIG 1b)**. In addition to RANK, other members of the tumour necrosis factor receptor superfamily[56–60], including CD40 (REFS[56,61–64]) and lymphotoxin-β receptor (LTβR)[58,65,66], were also found to have key roles in controlling different checkpoints of the mTEC developmental programme.

Interestingly, the CLDN3^{hi}CLDN4^{hi} mTECs in the adult thymus do not have any progenitor capacity[53] and are instead restricted to mature AIRE^{+} and terminally differentiated mTECs[53]. Correspondingly, the identification of analogous markers that specifically define mTEC precursors in the postnatal thymus has been more challenging and only partially successful. Several initial studies suggested that the postnatal mTEPCs are contained within the MHCII^{lo}CD80^{lo} mTEC compartment (known as mTEC^{lo} cells), as this population was shown to give rise to mature AIRE^{+}MHCII^{hi}CD80^{hi} cells (known as mTEC^{hi} cells)[27,67]. However, the relatively high abundance and the recently discovered heterogeneity of the mTEC^{lo} compartment suggest that mTEPCs likely constitute only a small fraction of this population[5,13,62,67–70]. More recently, it was suggested that the putative mTEC^{lo} progenitors in the postnatal thymus are characterized by high levels of expression of the surface protein PDPN and several genes encoding various CC-chemokine receptor 7 (CCR7) ligands, including *Ccl21a*, *Ccl21b* and *Ccl21c*[8]. Moreover, the same study showed that these PDPN^{+} mTEC^{lo} cells preferentially localize at the cortico-medullary junction — thus being known as junctional TECs (jTECs) — and accumulate in mice with mTEC-specific deletion of the NF-κB inducing kinase (NIK)[8]. Importantly, the existence of this PDPN^{+} jTEC subset was subsequently supported by data from two independent scRNA-seq studies[5,9]. Specifically, one study identified a distinct mTEC cluster that was characterized by co-expression of *Pdpn, Ccl21a, Ccl21b* and *Lgals1*; based on pseudotime analysis, it was suggested that this cluster is the earliest mTEC population in the adult thymus[9]. Another study highlighted a rather large and heterogeneous MHCII^{lo} cell cluster, referred to as the mTEC I subset, that was characterized by high-level and specific expression of genes encoding various CCR7 ligands (such as *Ccl21a* and *Ccl21c*), surface markers (such as *Itgb4, Itga6* and *Lifr)*, transcription factors (such as *Sox4* and *Ascl1)* and mTEC-

specific cytokeratins (such as *Krt5* and *Krt14*)[5]. Importantly, the mTEC I cluster also contained a small, but distinct, subset characterized by co-expression of *Pdpn, Sca1* and *Lgals1* (REF.[5]), which suggests that the putative PDPN^{+} mTEPCs constitute only a small fraction of the *Ccl21a*- and/or *Ccl21c*-expressing mTEC I cells (**FIG. 3**). It is also important to note that the CCR7 ligand-producing (in other words, CCL21^{+}) mTEC^{lo} cells were previously suggested to be a lymphotoxin signalling-dependent post-AIRE population[6], rather than an early mTEC subset with putative progenitor capacity. This notion was supported by the fact that during mouse ontogeny, the CCL21^{+} mTECs appear only after the emergence of AIRE^{+} cells and they are significantly underrepresented in AIRE-deficient thymi[6]. However, subsequent studies using AIRE reporter mice showed that several key CCR7 ligands (encoded by *Ccl21a* and *Ccl19*) are not expressed in post-AIRE cells, but rather in a separate mTEC subset that developmentally precedes the expression of *Aire* in the adult thymus[12]. Moreover, in vivo cell fate-mapping analysis using *Csn2*$^{Cre+}$*Rosa26*^{tdTomato} reporter mice **[G]** showed that the mTEC I population, which is characterized by specific expression of *Ccl21a* and *Ccl21c*, is the only mTEC subset that is not developmentally derived from mature mTECs, which supports the notion that these cells are at an early rather than late stage of mTEC development. Interestingly, although the mTEC I cells were not derived from mature mTEC^{hi} cells, their cellularity was markedly reduced in AIRE-deficient thymi[5], which suggests that their survival and/or proliferation are regulated by AIRE^{+} mTECs (**FIG. 3**). Although the molecular mechanisms underlying this cellular crosstalk remain elusive, they may possibly involve paracrine signalling from AIRE^{+} mTECs (**FIG. 3**).

Taken together, the currently available experimental evidence suggests that the mTEC I population is likely to be composed of at least two functionally distinct subsets: putative mTEPCs that replenish the mTEC compartment in the adult thymus; and a developmentally mature CCL21-producing population, whose major role is to recruit (and/or retain) positively selected CCR7^{+} thymocytes into the medulla (**FIG. 3**)[6,71]. Additional in vivo studies will be required to further validate such a model and/or to provide additional molecular markers that would enable separation of the putative progenitors from the other CCL21-expressing mTECs. It is also worth mentioning that the individual CCR7 ligands may have diverse and non-redundant roles, as shown by CCL21Ser-deficient mice **[G]**, which have impaired accumulation of positively selected

thymocytes in the thymic medulla and defective establishment of central tolerance[7] or impaired formation of the intrathymic conventional dendritic cell 1 (cDC1) subset[72].

[H1] Mature AIRE-expressing mTECs

The maturation of mTECs is phenotypically defined by the concomitant upregulation of several key genes, including those encoding MHC class II, CD80, AIRE, and AIRE-dependent and AIRE-independent tissue-restricted antigens **[G]** (TRAs)[27,67]. The mature AIRE^{+} mTEC^{hi} population is characterized by a high degree of intrinsic heterogeneity, which is essential for the role of these cells in both negative selection and agonist selection of self-reactive thymocyte clones (reviewed in REF.[73]) (**FIG. 4a**).

In order to carry out these functions, mature mTECs must express and present developing thymocytes with the complete array of self-proteins and peptides that they might encounter once they are released into the periphery, as failure to express even a single peptide during this intricate selection process could result in devastating autoimmunity[74–79]. To this end, the mature mTEC population expresses up to 90% of the entire coding genome, which is a considerably higher proportion than most other cell types[80]. Moreover, to maximize the exposure of thymocytes to diverse protein isoforms, mTECs are equipped with extensive mechanisms for alternative splicing and RNA editing[81,82]. The phenomenon of promiscuous gene expression (PGE) of hundreds of tissue-restricted genes at the population level is an intrinsic property of mTECs[83]. It is readily detected in the mouse embryo as early as E16–E17 with the emergence of mature AIRE^{+} mTECs, and has been shown to increase in complexity until birth[84–87]. Interestingly, when PGE is subtracted from the gene signature of mature mTEC^{hi} cells, their internal heterogeneity almost disappears, which suggests that it primarily stems from the differential expression of TRAs at a single cell level[80]. Indeed, it has been evident for more than two decades that, at the single cell level, not all mature mTECs express all TRAs. Specifically, early studies based on microscopic analysis of the thymus showed that only a few cells in a given thymic section stain positively for TRAs such as insulin, somatostatin or retinol binding protein 3 (REFS[88,89]). In addition, single-cell PCR of mature mTECs of both mouse and human origin showed that each individual mTEC expresses only a limited set of TRAs and an individual TRA is expressed by only 0.4–3% of cells[90–94]. Therefore, the complete expression of all TRAs by the mTEC population must be owing to the summation of

mosaic expression of TRAs by individual mTECs (**FIG. 4b**). Moreover, the expression of TRAs in these studies was suggested to be stochastic, as the TRAs expressed in individual mTECs did not show any discernible pattern, even within a defined locus. It also differed significantly from the expression pattern found in peripheral cell lineages that normally express the TRAs, as seen by discrepancies in terms of monoallelic versus bi-allelic transcription, as well as the use of different transcription start sites and transcription factors[91,92]. The stochastic nature of this process makes intuitive sense as it functions as the initial and most substantial system of checks and balances of the TCR repertoire, which has itself been generated in a semi-random manner through VDJ recombination. Furthermore, the restricted expression of a limited number of TRAs per single mTEC is essential to increase the density of the derived epitopes that are presented to maturing thymocytes on the limited number of MHC class II molecules, such that effective selection may be achieved[92,94,95].

The advent of high-throughput single-cell technologies has enabled a detailed examination of the expression pattern of TRAs per single mTEC at high resolution. This has provided important insights into the molecular intricacies of the PGE process and has helped to correct some inaccurate interpretations based on gene expression profiling of the bulk and heterogeneous mature mTEC population. Deep RNA-seq of ~200 individual mature mTECs showed that up to 95% of TRAs are represented in such a small sample size, which suggests that the number of mature mTECs needed to interact with a developing thymocyte in order to cover the entire TRA library is rather small[80,81,95]. When the repertoire of TRAs per cell was examined, a given AIRE-dependent gene **[G]** was expressed in only ~1% of mature mTECs, compared with ~2% of mTECs for AIRE-enhanced genes **[G]** and 9% of mTECs for AIRE-independent genes, whereas all other genes were expressed in ~33% of all mature mTECs[80]. Interestingly, although a given AIRE-dependent TRA is expressed in only a very small fraction of mTECs, its expression level in the corresponding single cell is very high, although significantly lower than in the peripheral tissue in which it is normally expressed[80,81,91,95,96]. Despite the apparent stochastic nature of TRA expression highlighted by the scRNA-seq analyses[80,81], there do seem to be some rules guiding this process. For example, it was shown that many TRA genes preferentially fall into co-expression clusters, which suggests that PGE in specific individuals follows rules of ordered stochasticity **[G]** (**FIG. 4b**) but has high inter-individual variance[81]. In a parallel study, another group found evidence of overlap between co-

expression clusters and suggested that they could potentially be placed on a continuum of TRA expression, with mTECs transitioning between different co-expression clusters throughout their lifespan and perhaps covering the entire TRA repertoire over time[95]. Experimental evidence to validate that a single mTEC is capable of expressing most of the TRA gene repertoire during its lifetime has yet to be provided and we still lack spatial information that could potentially tie together seemingly unrelated cells.

The mechanism by which mature mTECs bring about this unique gene expression programme has been only partly elucidated and marks another level of heterogeneity within this population, as two factors — namely, AIRE[75] and Fez family zinc finger protein 2 (FEZF2)[97] — have been implicated in inducing PGE. AIRE, which is the sole gene responsible for autoimmune polyendocrine syndrome type 1 **[G]** (APS1)[98,99], has been the focus of many studies over the past two decades and has been extensively reviewed elsewhere[4,73,100]. Importantly, AIRE is expressed by approximately half of the mature mTEC^{hi} population, in which it has been shown to regulate the expression of ~4,000 genes, most of which are TRAs[75,80,81,92]. FEZF2 has been reported to upregulate the expression of ~400 AIRE-independent genes and thereby to complement the role of AIRE in PGE and the subsequent negative selection and/or agonist selection of self-reactive thymocytes[97]. However, it is important to stress that many of the FEZF2-induced TRA genes that were reported in the original study to be AIRE independent (such as *Krt10, Klk1b16, Apoc2, Cyp24a1* and *Muc3*)[97] have been identified by other groups as AIRE-dependent genes[65,80–82]. Therefore, although FEZF2 seems to be an important factor with the potential to shape the mTEC-specific gene signature, the number of AIRE-independent TRA genes that are induced by FEZF2 is still a matter of debate and may be markedly lower than that proposed by REF.[97].

[H1] Terminally differentiated mTECs

Until relatively recently, the AIRE^{+} mTEC^{hi} population was regarded as the final stage of mTEC development[67]. However, over the past several years, a growing body of evidence has shown that the AIRE^{+} mTEC^{hi} cells continue to differentiate and consequently give rise to additional mTEC subsets[6,10–12,68,69,101], including corneocyte-like mTECs and thymic tuft cells[5,9,13] (**FIG 3**).

[H2] ***Corneocyte-like mTECs.*** Probably the first experimental evidence to suggest that AIRE promotes mTEC differentiation was provided by a study showing that AIRE-deficient mice have reduced numbers of KRT10^{+} mTECs and impaired formation of Hassall's corpuscles **[G]** in their thymi[10]. Moreover, a subsequent study showed that the development of these KRT10^{+} mTECs and the subsequent formation of Hassall's corpuscles comes after the development of AIRE^{+} mTECs during ontogeny and is regulated by the lymphotoxin signaling pathway[11]. These findings were further supported by several additional studies based on in vivo fate-mapping experiments, which showed that AIRE^{+} mTEC^{hi} cells continue their differentiation programme by downregulation of AIRE and MHC class II expression and concomitant upregulation of expression of various genes, such as those encoding keratin type II cytoskeletal 1 (KRT1), keratin type I cytoskeletal 10 (KRT10), involucrin, desmogleins and serine protease inhibitor Kazal-type 5 (*Spink5*)[12,69,70,101–103]. Interestingly, all of these genes are also highly specific to terminally differentiated keratinocytes (corneocytes) that compose the outermost layer of the epidermis (stratum corneum) (**FIG. 5a**). To function as an effective physical barrier against the external environment, corneocytes lose their nuclei and cytosolic organelles and acquire a tough outer cell envelope composed of aggregated keratins. Similarly to corneocytes, the post-AIRE KRT10^{+} mTEC^{lo} cells also lose their nuclei as they become Hassall's corpuscles[12] (**FIG. 5a**). Moreover, their striking similarity to corneocytes is further supported by several recent transcriptomic studies showing that, in addition to keratins (such as *Krt1, Krt7, Krt10* and *Krt77*), desmogleins and involucrin, the corneocyte-like mTECs (also known as mTEC III cells)[5] also express clusterin, dermokine, retroviral-like aspartic protease 1 (*Asprv1*), cystatin A and other corneocyte-specific genes[5,13]. Therefore, these data collectively suggest that the terminal development of mTECs highly resembles that of epidermal keratinocytes, where cornification functions as an alternative route to cell death[68] (**FIG. 5a**).

The specific surface markers that would enable the physical isolation and detailed characterization of these post-AIRE corneocyte-like mTECs remained unknown for a long time. Recently, it was shown that these cells can be separated from other mTEC subsets based on staining with *Tetragonolobus purpureus* agglutinin (TPA)[68], a lectin that was previously shown to specifically mark Hassall's corpuscles[104]. More recently, the corneocyte-like mTECs were found to express high levels of LY6D and/or of the gene encoding polymeric immunoglobulin receptor (*Pigr*) (**FIG.**

5a), whereas they did not express ITGB4 (or *Itga6*), which are characteristic of mTEC I cells[5]. Therefore, the combination of these markers now enables effective separation of corneocyte-like mTECs from the other mTEC^{lo} subsets. Interestingly, scRNA-seq analysis of TECs throughout ontogeny showed that the corneocyte-like mTECs cannot be detected in mouse embryonic thymus and appear only later after birth[5]. This is in line with previous studies showing that post-AIRE mTECs appear only after the emergence of their AIRE^{+} precursors[11,68] and that they still express many AIRE-dependent genes, despite the down-regulation of AIRE itself.

The exact functional role(s) of the corneocyte-like mTECs and/or of the Hassall's corpuscles is still poorly understood. The downregulation of MHC class II expression suggests that they are not likely to be efficient at direct antigen presentation to CD4^{+} T cells. However, the fact that they still express a large fraction of AIRE-dependent and AIRE-independent TRA genes suggests that they may still be involved in the induction of immunological tolerance, for example by passing their antigen cargo to DCs for subsequent cross-presentation to thymocytes. Although there is a strong body of evidence that AIRE^{+} mTECs actively transfer a fraction of their self-antigen repertoire to DCs[105–107] (**FIG. 4a**), it is unclear whether the corneocyte-like mTECs also use such an antigen-transfer mechanism (**FIG. 5b**). In support of this hypothesis, it was shown that Hassall's corpuscles in the human thymus express thymic stromal lymphopoietin (TSLP), which can subsequently induce expression of the costimulatory molecules CD80 and CD86 in thymus-resident DCs. Moreover, these TSLP-conditioned DCs could induce FOXP3^{+} T_{reg} cells[108], which suggests that Hassall's corpuscles might have an important role in tolerance induction. Interestingly, however, corneocyte-like mTECs in the mouse thymus do not seem to express TSLP[5], nor do they seem to be associated with FOXP3^{+} T_{reg} cells and/or DCs[109]. Given that Hassall's corpuscles are morphologically defined cellular islands that are scattered throughout the thymic medulla, it is likely that their primary role is to create local cellular microenvironments that provide specific signals for other cell types. Indeed, it has been shown by several independent studies that Hassal's corpuscles and/or corneocyte-like mTECs are primarily surrounded by other cell subsets, including AIRE^{+} mTECs[11], thymic tuft cells[13] or thymic B cells[110]. However, the functional relevance of these physical associations remains elusive (**FIG. 5b**).

[H2] ***Thymic tuft cells.*** The other main subset of terminally differentiated mTECs that was recently identified in both mouse and human thymi comprises thymic tuft cells[5,13–15]. Tuft (or brush) cells are flask-shaped epithelial cells with chemosensory properties that were originally identified in mucosal tissues owing to their characteristic tuft-like microvilli on their apical sides[111,112]. Interestingly, cells with some morphological features similar to those of tuft cells have been described in the thymus as early as three decades ago[113,114], but a population of thymic tuft-like cells was only recently identified and characterized[5,13–15]. This discovery suggests that tuft cells are not exclusive to mucosal and polarized tissues, but might also have an important role in primary lymphatic organs, such as thymus.

The development of thymic tuft cells is, similarly to their mucosal counterparts and unlike other TEC subsets, controlled by the transcription factor POU2F3[5,13,115–117]. Moreover, during ontogeny, both mucosal tuft cells[118–121] and thymic tuft cells[5] are found only during late embryonic development or after birth. Importantly, lineage tracing experiments carried out by two independent groups have suggested that whereas more than half of the thymic tuft cells arises from mature mTEC^{hi} cells[5,13], the remaining 40-50% does not seem to pass through the AIRE-expressing mTEC stage. Specifically, diphtheria toxin treatment of mice in which diphtheria toxin receptor (DTR) expression is driven by the *Aire* promoter resulted in only a partial (yet significant) depletion of thymic tuft cells[13]. Furthermore, AIRE deficiency does not result in marked changes in thymic tuft cell development and/or their gene expression profile[5]. Finally, scRNA-seq and chromatin immunoprecipitation followed by sequencing (ChIP-seq) analyses show that thymic tuft cells are very distant from AIRE^{+} mTECs in terms of both their transcription profile and their epigenetic landscape, with no clear cell subset that could be a putative transitional state between the two[5]. Therefore, additional studies are required to better delineate the mechanisms underlying tuft cell development in the thymus and their relationship to mature mTECs.

In addition to their unique morphological features, tuft cells are characterized by a unique gene expression signature[122,123]. Both mucosal and thymic tuft cells express DCLK1, which is one of the most commonly-used markers for these cells[5,13,119], as well as several key genes that are involved in the biosynthesis of various secreted molecules with diverse biological functions. For example, they express high levels of IL-25 or genes encoding enzymes involved in acetylcholine synthesis

(such as *ChAT*) or prostaglandin and leukotriene synthesis (such as *Ptgs1, Hpgds* and *Alox5*) (**FIG. 6**). The capacity of tuft cells to express these specific genes probably reflects their putative biological roles in regulating immune responses and/or tissue homeostasis[124]. In keeping with their mucosal counterparts, thymic tuft cells express genes such as *Plcb2, Trpm5, Gnb3* and *Gng13*, which are involved in the taste reception signalling pathway. Although it is not clear why tuft cells express such genes, it is probably linked to their natural chemosensory capacity. Despite these striking molecular similarities between thymic tuft cells and mucosal tuft cells, there are also several clear differences. Indeed, a recent RNA-seq analysis compared tuft cells isolated from different tissues and found that the transcriptional signature of thymic tuft cells was the most distinct[125]. Unlike other types of tuft cell, thymic tuft cells are characterized by relatively high levels of expression of several members of the taste receptor 2 (*Tas2r*) locus (which encodes a family of receptors for bitter taste), *Gnat3*, *L1cam*, or genes encoding MHC class II and CD74, which are related to antigen presentation[5,13] (**FIG. 6**). By contrast, thymic tuft cells do not express various genes that are characteristic of intestinal tuft cells (such as *Lgals2, Lgals4, Muc13, Fabp1* and *Apoa1*).

Importantly, based on their relatively high levels of expression of genes encoding the antigen presentation machinery, their medullary localization and expression of various TRA genes, it has been hypothesized that thymic tuft cells may be involved in the induction of immunological tolerance[13]. Indeed, IL-25 immunization of mice transplanted with wild-type or tuft cell-deficient thymi showed that the presence of tuft cells was essential to prevent the generation of IL-25-specific antibodies[13]. However, it is of importance to stress that although thymic tuft cells express MHC class II molecules, their expression levels are more than an order of magnitude lower than those of AIRE$^+$ mTECs, which suggests that tuft cells are not likely to be very potent as antigen-presenting cells. In addition, unlike AIRE$^+$ mTECs, thymic tuft cells do not have any features of PGE[5]. Finally, it is also not clear why a tuft cell-specific gene signature would preferentially be selected for tolerance induction in the thymus over other tissue- and/or cell-specific antigens. Therefore, further experiments in more physiological settings are required to determine whether tolerance induction is indeed the primary role of thymic tuft cells or whether they might have alternative and/or additional roles.

Several independent studies recently showed that mouse intestinal tuft cells are the prime activators of the type 2 immune response **[G]** to parasitic infection and are essential for parasite clearance[115,125–129]. To this end, they secrete pre-synthesized IL-25 in response to parasites, which in turn activates gut-resident group 2 innate lymphoid cells **[G]** (ILC2s), eventually resulting in the initiation and amplification of the type 2 cytokine response with subsequent parasite clearance. As thymic tuft cells also express high basal levels of IL-25, it has been speculated that they may also promote a type 2 response in the thymus. Indeed, tuft cell deficiency is associated with reduced numbers of type 2 natural killer T (NKT2) cells and EOMES^{+}CD8^{+} SP thymocytes in the thymus[13], and by increased frequencies and numbers of thymic ILC2s[5]. However, the actual role of the type 2 immune response in the thymus, and in particular the role of thymic NKT2 cells and/or ILC2s, is still largely unclear (**FIG. 6**). Correspondingly, it remains to be determined whether and how thymic tuft cells activate a type 2 immune response and, most importantly, why such a response is required in a non-mucosal organ that is not exposed to parasites.

[H1] Concluding remarks

Recent studies have provided clear evidence that the division of TECs into cTECs and mTECs is insufficient, as each of these two main TEC populations is, in fact, highly heterogeneous and comprised of multiple sub-populations with distinct developmental, molecular, morphological and functional characteristics.

Although recent years have seen tremendous progress in a comprehensive decomposition and detailed molecular characterization of the TEC compartment, including the identification of thymic tuft cells and corneocyte-like mTECs, an understanding of their exact functional roles and physiological relevance remains largely elusive. Therefore, the challenges in the future will likely revolve around the functional characterization of these TEC subsets and elucidating their interactions with other cell types resident in the thymus. Corneocyte-like mTECs are likely to complement AIRE^{+} mTECs in the induction of central tolerance, whereas thymic tuft cells might, owing to their putative cholinergic and chemosensory capacities, have other functional roles that are unrelated to tolerance induction. An additional challenge will be to map the developmental paths taken by different TEC subpopulations and further delineate and validate their developmental hierarchy. This will include resolving the controversy regarding the identity of the

unipotent and/or bipotent TEPCs and uncovering the differentiation pathways that lead to the generation of mature antigen-presenting mTECs and cTECs, corneocyte-like TECs and thymic tuft cells. Therefore, a key prerequisite to successfully address these questions will likely be the identification of the specific signals and/or transcription factors that are crucial for the development of the individual TEC subsets. An additional point to be addressed is the elucidation of the complex molecular mechanisms that are used by AIRE and FEZF2 to mediate PGE for the induction of central tolerance. Finally, our still limited understanding of cTEC heterogeneity, as well as of the functional relevance of thymic nurse cells, begs for additional cutting-edge studies based on single-cell genomics and/or imaging in the future.

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Figure 1 | Thymic epithelial cell heterogeneity and development. a | Historical versus current views of thymic epithelial cell (TEC) heterogeneity. Historically (left panel), medullary thymic epithelial cells (mTECs) and cortical thymic epithelial cells (cTECs) were differentiated using only a small number of surface markers, such as UEA1 (for mTECs), LY51 (for cTECs), or MHC class II and/or CD80 and CD86 (for the segregation of less mature TECs from more mature TEC

developmental states). Recent technological advancements have been used to show that there is a high level of TEC heterogeneity (right panel), exemplified by the existence of various cTEC and mTEC subpopulations with distinct molecular and functional characteristics. **b |** Current models for TEC development. The development of TECs from a thymic epithelial progenitor cell (TEPC) differs between the embryonic mouse thymus and the adult mouse thymus. In the embryonic thymus (left panel), bipotent β5t^{+} TEPCs can ultimately give rise to both mTEC and cTEC lineages, a capacity that is maintained during neonatal life. The bipotent TEPCs commit to the cTEC lineage by default (thick arrow), whereas entry into the mTEC lineage (thin arrow) requires activation of an mTEC-specific transcriptional programme, including activation of the nuclear factor-κB (NF-κB) signalling pathway. In the adult thymus (right panel), the existence of a bipotent TEPC remains controversial, with a prevailing assumption that even if such bipotent progenitors exist, the contribution of lineage-restricted TEPCs to the maintenance of both cTECs and mTECs increases with age. **c |** Similarities and differences between embryonic TEPCs and differentiated cTECs. Although embryonic TEPCs have a strong molecular 'footprint' of differentiated cTECs, including high levels of expression of β5t and CD205 (similarly expressed genes are shown in green), they differ from differentiated cTECs in terms of increased proliferation and decreased antigen-presentation capacities, as well as differential expression of various key genes (shown in red). AIRE, autoimmune regulator; APOE, apolipoprotein E; CCL21, CC-chemokine ligand 21; CTSL, cathepsin L1; DCLK1, serine/threonine protein kinase; DLL4, delta-like ligand 4; FN1, fibronectin 1; IGFBP5, insulin-like growth factor-binding protein 5; jTEC, junctional TEC; KRT10, keratin type I cytoskeletal 10; PDPN, podoplanin; PKM, pyruvate kinase 2.

Figure 2 | Function and diversity of cortical thymic epithelial cells. a | Cortical thymic epithelial cells (cTECs), characterized by surface expression of LY51 and LY75, have important roles in the initial stages of T cell development and selection, including: 1) homing of blood-borne lymphoid progenitor cells into the thymus, through the expression of CC-chemokine ligand 25 (CCL25) and CXC-chemokine ligand 12 (CXCL12); 2) commitment of the lymphoid progenitors to the T cell lineage, through expression of the Notch ligand delta-like ligand 4 (DLL4); 3) expansion of the developing thymocyte clones, through secretion of IL-7 and stem cell factor (SCF; also known as KIT ligand); 4) positive selection of thymocytes; and 5) death by neglect of thymocytes bearing a

T cell receptor (TCR) that does not recognize peptide presented by an MHC complex. Positive selection of thymocytes is supported by cTEC expression of a specific antigen processing and presentation machinery, including the use of autophagy to obtain self-proteins for processing using the thymoproteasome, which uniquely expresses the β5t subunit, in addition to the proteases cathepsin L1 (CTSL) and thymus-specific serine protease (TSSP), and CD83, which regulates the turnover of surface MHC class II molecules. **b |** cTEC heterogeneity. In the adult thymus, two major cTEC subsets were recently described by single-cell RNA-sequencing analysis, based on the differential expression of DLL4 as well as of additional key genes, including *Ccl25, Cxcl12, Cd83, Ly6a* (which encodes SCA1) and *Ccrl1.* The heterogeneity of cTECs is further increased by the existence of thymic nurse cells, which engulf and subsequently 'nurture' developing thymocytes. A single thymic nurse cell can engulf a large number of thymocytes**.**

Figure 3 | Model for the differentiation of medullary thymic epithelial cells. Several lines of evidence suggest that in the adult mouse thymus, PDPN$^+$ junctional thymic epithelial cells (jTECs), which are localized at the cortico-medullary junction, are the earliest subset of medullary thymic epithelial cells (mTECs) and give rise to other mTEC subsets including CCL21$^+$ mTEC^{lo} cells and AIRE$^+$ mTEC^{hi} cells. The differentiation into AIRE$^+$ cells is mainly driven by RANK signalling, and the differentiation into CCL21$^+$ mTECs was shown to require lymphotoxin-β (LTβ). The CCL21$^+$ mTECs were shown to facilitate recruitment of positively selected single-positive (SP) CCR7$^+$ thymocytes into the medulla through the secretion of CCL21Ser. AIRE$^+$ mTECs were found to differentiate further into KRT10$^+$ corneocyte-like mTECs and possibly also into DCLK1$^+$ thymic tuft cells (tuft mTECs). Although the PDPN$^+$ jTECs and CCL21$^+$ mTECs are not developmentally derived from AIRE$^+$ mTECs, their cellularity is markedly reduced in AIRE-deficient thymi, which suggests that their survival and/or proliferation are regulated by their AIRE$^+$ counterparts. Both PDPN$^+$ jTECs and CCL21$^+$ mTECs share expression of several key genes, including *Ccl21a, Ccl21c, Itgb4, Sox4* and *Ascl1*. AIRE, autoimmune regulator; CCL21, CC-chemokine ligand 21; CCR7, CC-chemokine receptor 7; KRT, keratin; PDPN, podoplanin.

Figure 4 | The heterogeneity of mature medullary thymic epithelial cells stems from differential expression of tissue-restricted antigens. a | Mature medullary thymic epithelial cells (mTECs) facilitate either negative selection or agonist selection of self-reactive thymocytes.

To this end, mTECs are characterized by promiscuous gene expression (PGE) of thousands of tissue-restricted antigens (TRAs), which is mainly orchestrated by AIRE and to some extent by FEZF2. Mature mTECs also upregulate expression of costimulatory molecules, such as CD80 and CD86 and of MHC class II, which is required for presentation of self-antigens. Antigen presentation on MHC class II molecules is mediated by high and constitutive levels of autophagy in mature mTECs, as well as by immunoproteasome activity. Ultimately, self-antigens are presented to single-positive (SP) thymocytes either directly by mature mTECs or indirectly by antigen transfer to dendritic cells (DCs), which express the chemokine receptor XCR1 and are attracted to the thymus by AIRE-dependent expression of XCL1. **b |** At a population level, mTECs express ~90% of the self-proteome, such that the antigen repertoire in the thymus medulla mirrors the highly diverse and tissue-specific repertoire (highlighted by different colours) of the entire body. However, at the single-cell level, each individual mTEC expresses only a fraction of the entire antigen 'inventory', which suggests that a mosaic coverage is required to achieve maximal selection efficiency. It has been shown in several studies that AIRE-driven gene expression of TRAs by mTECs follows rules of 'ordered stochasticity', such that certain genes tend to be co-expressed in an individual mTEC. AIRE, autoimmune regulator; T_{reg} cell, regulatory T cell.

Figure 5 | Corneocyte-like medullary thymic epithelial cells and Hassal's corpuscles. a | The terminal differentiation of $AIRE^+$ medullary thymic epithelial cells (mTECs) (right panel) is highly similar to skin keratinocyte differentiation (left panel). The differentiation of mTECs is accompanied by downregulation of AIRE and MHC class II expression and a concomitant upregulation of expression of LY6D and/or polymeric immunoglobulin receptor (PIgR), as well as of various genes with high specificity for terminally differentiated keratinocytes (corneocytes) such as the genes encoding keratin type II cytoskeletal 1 (KRT1) and keratin type I cytoskeletal 10 (KRT10), involucrin, desmogleins, clusterin, dermokine, serine protease inhibitor Kazal-type 5 (*Spink5*) and retroviral-like aspartic protease 1 (*Asprv1*). Further similarities between corneocyte-like mTECs and corneocytes include the loss of nuclei and cornification as corneocyte-like mTECs differentiate into Hassall's corpuscles. **b |** The functional role(s) of corneocyte-like mTECs and/or of Hassall's corpuscles are still poorly understood. As corneocyte-like mTECs downregulate MHC class II expression but still express many AIRE-dependent and AIRE-independent tissue-restricted antigens (TRAs), they may potentially transfer these antigens to

dendritic cells (DCs) for subsequent cross-presentation. The Hassall's corpuscles and/or corneocyte-like mTECs were found to be surrounded by several other cell subsets in the medulla, including AIRE$^+$ mTECs, thymic tuft cells, thymic B cells and perhaps additional cell types; however, the functional relevance of these physical associations has yet to be determined.

Figure 6 | Thymic tuft cells. Thymic tuft cells are chemosensory cells whose development depends upon the transcription factor POU2F3. They have been shown to express neural cell adhesion molecule L1 (L1CAM), in addition to keratin type II skeletal 8 (KRT8), keratin type I cytoskeletal 18 (KRT18) and the transcription factor SOX9. They also express taste receptors and components of their downstream signalling cascade (such as gustducin, 1-phosphatidylinositol 4,5-bisphosphate phosphodiesterase β2 (PLCB2), IP3 and TRPM5), although the signals to which thymic tuft cells respond are currently unknown. Thymic tuft cells also express key enzymatic machineries for the biosynthesis of various biologically active molecules, including acetylcholine, leukotrienes and prostaglandins, which suggests that they can affect the homeostasis of neighbouring cells. Similarly to their mucosal tuft cell counterparts, thymic tuft cells express high levels of IL-25, which can act on cells expressing IL-25 receptor (IL-25R), including group 2 innate lymphoid cells (ILC2s) and type 2 natural killer T (NKT2) cells. Indeed, the absence of tuft cells in *Pou2f3*-knockout mice resulted in increased numbers of thymic ILC2s and decreased numbers of thymic and splenic NKT2 cells and thymic EOMES$^+$CD8$^+$ T cells. Thymic tuft cells were shown to localize next to corneocyte-like mTECs, although the nature of this proximity has yet to be elucidated.

Box 1 | T cell education

The thymus is the only tissue in the body that can support the development of lymphoid progenitors into mature and immunologically competent naive T cells. Specifically, early thymic progenitors are recruited to the thymus where, upon encountering Notch ligands (in particular delta like ligand 4 (DLL4)) provided by cortical thymic epithelial cells (cTECs), they irreversibly commit to the T cell lineage. Moreover, cTECs have a crucial role in controlling the subsequent steps of the T cell developmental programme (until the CD4$^+$CD8$^+$ double-positive (DP) thymocyte stage), including clonal expansion and positive selection of T cell clones that express functional αβ T cell receptors (TCRs). Specifically, the highly broad and diverse repertoire of

TCRs that is generated by the semi-random process of VDJ recombination is tested for its ability to bind peptide–MHC (pMHC) complexes that are exclusively presented by cTECs. To this end, cTECs express a unique proteasome (known as the thymoproteasome) and lysosomal proteases that enable distinctive cleavage and subsequent presentation of the self-antigen repertoire. Interactions between a presented antigenic peptide and its corresponding TCR result in survival of the T cell clone and its progression to either the $CD4^+$ or $CD8^+$ single-positive (SP) subset, whereas failure of a thymocyte to bind such antigens leads to its death by neglect. The surviving SP thymocytes migrate into the thymic medulla, where potentially dangerous clones expressing self-reactive TCRs are pruned from the repertoire through a process known as negative selection. This step is mainly mediated by dendritic cells (DCs) and medullary thymic epithelial cells (mTECs). Importantly, to effectively screen for as many self-reactive thymocyte clones as possible, mTECs are equipped with a unique capacity to express almost 90% of the coding genome, including tissue-restricted antigens. Peptides derived from these self-antigens are presented on MHC molecules on the surface of mTECs or are transferred to neighboring DCs for cross-presentation, which further potentiates the selection process. High-affinity interaction between a TCR and self-pMHC most commonly results in clonal deletion, whereas interactions of low to intermediate affinity typically divert a given clone into the $FOXP3^+CD4^+$ regulatory T cell lineage, a process known as agonist selection. Functional defects in either cTECs and/or mTECs are associated with diverse immunopathologies, ranging from severe immunodeficiency to autoimmunity.

Glossary

Thymic epithelial cells

(TECs). Specialized stromal cells found in the thymus. They have the ability to present antigens on MHC class I and class II molecules to developing T cells (thymocytes). Their main known functions include the induction of T cell lineage commitment, positive selection of functional T cell clones and negative selection of self-reactive T cell clones.

Positive selection

A crucial checkpoint in αβ T cell development, exclusively facilitated by cortical thymic epithelial cells, that ensures only functionally competent T cell clones capable of recognizing peptide–MHC complexes with adequately high affinity continue in the developmental process. T cell clones that do not recognize peptide–MHC with sufficient affinity die by neglect.

Negative selection

A selection process mediated by thymus-resident antigen-presenting cells (for example, mTECs, dendritic cells and B cells) that ensures T cell clones that recognize self-peptide–MHC complexes with very high affinity are eliminated from the repertoire. This process occurs mainly in the thymic medulla, although there is some evidence that negative selection can also occur in the cortex.

Agonist selection

A selection process that ensures $CD4^+$ T cell clones that recognize self-peptide–MHC class II complexes with medium to high affinity differentiate into $CD4^+CD25^+FOXP3^+$ regulatory T cells. It has been suggested that this process is mediated by medulla-resident antigen-presenting cells (such as mTECs, dendritic cells and B cells).

Autoimmune regulator

(AIRE) A transcriptional regulator, expressed almost exclusively in mature $MHCII^{hi}$ mTECs, which induces expression of most tissue-restricted antigen genes in the thymus, a step that is necessary for purging of self-reactive T cells and induction of central tolerance.

Thymoproteasome

A specialized form of the proteasome that is found exclusively in cortical thymic epithelial cells and that is essential for the generation of a unique peptide repertoire to support positive selection of T cell clones. The thymoproteasome uniquely incorporates the β5t subunit (encoded by *Psmb11*).

Reaggregate thymic organ cultures

(RTOCs). An experimental method that enables the ex vivo generation of three-dimensional thymic organoids from purified fetal thymic epithelial cells and other thymic cell subsets. The resulting organoids can also be used in transplantation studies for a longer period of time.

Thymic nurse cells

(TNCs). Large cortical thymic epithelial structures that internalize developing thymocytes through extensions of the plasma membrane. Thymic nurse cells can internalize up to 200 double-positive thymocytes and have been shown to be crucial for secondary T cell receptor α-chain rearrangement.

Nude mice

A mouse strain having a naturally occurring loss of function mutation in the Forkhead box N1 (*Foxn1*) gene, which encodes a transcription factor that is crucial for the development of hair follicles, mammary glands and thymic epithelial cells. As a result, nude mice develop no hair and have a dysfunctional thymic rudiment, which is unable to support normal T cell development resulting in severe immunodeficiency.

Csn2^{Cre+}*Rosa26*^{tdTomato} reporter mice

Csn2^{Cre+} mice are a transgenic mouse model in which the coding sequence for Cre recombinase is inserted downstream of the *Csn2* gene promoter. *Rosa26*^{tdTomato} mice are a transgenic mouse model in which the tdTomato reporter gene, together with a stop cassette flanked by loxP sites, is inserted into the *Rosa26* locus. As *Csn2* is specifically expressed in most mTEC^{hi} cells, the *Csn2*$^{Cre+}$*Rosa26*^{tdTomato} reporter mice enable lineage tracing of mTEC^{hi}-derived cells.

CCL21Ser-deficient mice

CCL21Ser-deficient mice are a transgenic mouse model in which the tdTomato reporter gene is inserted at the translation initiation site of the *Ccl21a* gene promoter without affecting the expression of *Ccl21b* or *Ccl21c* genes. Mice homozygous for the insertion are therefore specifically deficient for CCL21Ser, but not for CCL21Leu, which is encoded by *Ccl21b* and/or *Ccl21c* genes.

Tissue-restricted antigens

(TRAs). Proteins that are expressed, processed and presented by thymic epithelial cells to developing thymocytes for the purpose of selection that are otherwise specifically expressed only in five or fewer peripheral tissues.

AIRE-dependent genes

Genes that require AIRE for their expression.

AIRE-enhanced genes

Genes that have low levels of expression in the absence of AIRE, but expression of which is significantly increased by AIRE.

Ordered stochasticity

The autoimmune regulator (AIRE) protein is said to operate with ordered stochasticity, such that the genes it activates in individual mTECs are stochastically selected, but the process is not completely random as co-expression groups of genes within cells are found.

Autoimmune polyendocrine syndrome type 1

(APS1). Also known as autoimmune polyendocrinopathy candidiasis ectodermal dystrophy (APECED). A genetic disorder, caused by mutations in the *AIRE* gene, that leads to a devastating multi-organ autoimmune syndrome. It is diagnosed when patients present with at least 2 out of 3 of the classical symptoms, which include chronic mucocutaneous candidiasis, hypoparathyroidism and adrenocortical insufficiency.

Hassall's corpuscles

Islet-like structures found in the medullary region of the thymus that are composed of squamous epithelial cells expressing high levels of various keratins (for example, KRT10) and involucrin.

Type 2 immune response

An immune response characterized by an increased production of various cytokines (e.g. IL-4, IL-5 and IL-13) and concomitant activation of distinct immune cell populations including, type-2

helper T cells (Th2 cells), eosinophils, basophils, mast cells, type-2 innate lymphoid cells (ILC2) and type-2 natural killer T cells (NKT2). The type-2 immune response plays an important role in host defense against parasites, but when dysregulated, may underlie the development of diverse allergic disorders.

Group 2 innate lymphoid cells

(ILC2s). A population of lymphoid-derived cells that are defined by the absence of key lymphoid, myeloid and dendritic cell markers and by expression of the transcription factor GATA3 and various type 2 cytokines, such as IL-4, IL-5, IL-9 and IL-13. They have been identified in many tissues, including the skin, intestinal tract and respiratory tract, and they have been suggested to have a role in immune responses against parasites, as well as in allergy and asthma.

Table of Contents

Recent studies using single-cell genomic technologies and in vivo fate mapping have shown that thymic epithelial cells are far more heterogeneous than previously thought, comprising multiple sub-populations with distinct molecular and functional characteristics.

Fig 1

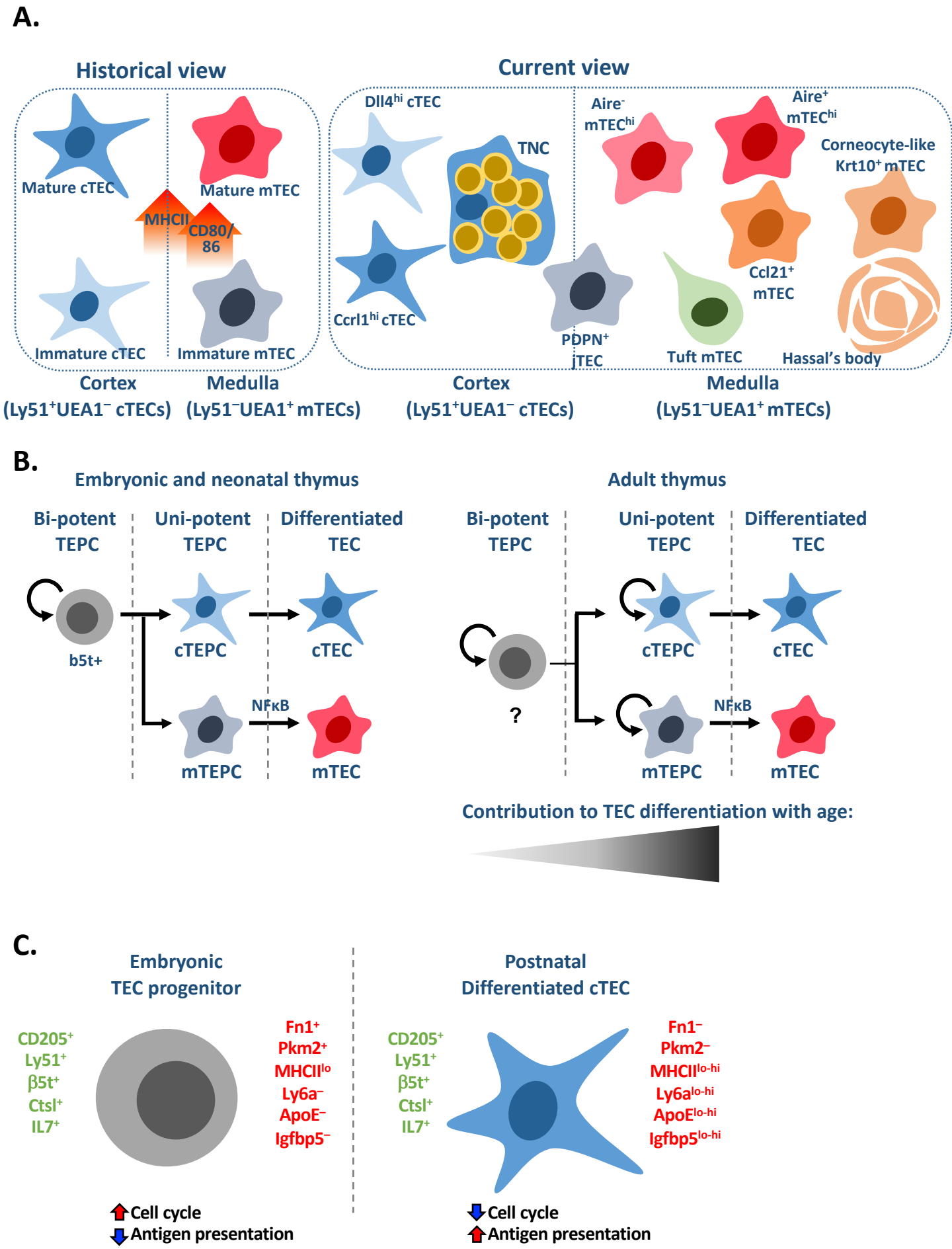

A.
Historical view
Current view
Mature cTEC
Mature mTEC
MHCII
CD80/86
Immature cTEC
Immature mTEC
Cortex (Ly51+UEA1− cTECs)
Medulla (Ly51−UEA1+ mTECs)
Dll4hi cTEC
TNC
Ccrl1hi cTEC
Aire- mTEChi
Aire+ mTEChi
Corneocyte-like Krt10+ mTEC
Ccl21+ mTEC
PDPN+ iTEC
Tuft mTEC
Hassal's body
Cortex (Ly51+UEA1− cTECs)
Medulla (Ly51−UEA1+ mTECs)
B.
Embryonic and neonatal thymus
Bi-potent TEPC
Uni-potent TEPC
Differentiated TEC
b5t+
cTEPC
cTEC
NFκB
mTEPC
mTEC
Adult thymus
Bi-potent TEPC
Uni-potent TEPC
Differentiated TEC
?
cTEPC
cTEC
NFκB
mTEPC
mTEC
Contribution to TEC differentiation with age:
C.
Embryonic TEC progenitor
CD205+
Ly51+
β5t+
Ctsl+
IL7+
Fn1+
Pkm2+
MHCIIlo
Ly6a−
ApoE−
Igfbp5−
Cell cycle
Antigen presentation
Postnatal Differentiated cTEC
CD205+
Ly51+
β5t+
Ctsl+
IL7+
Fn1−
Pkm2−
MHCIIlo-hi
Ly6alo-hi
ApoElo-hi
Igfbp5lo-hi
Cell cycle
Antigen presentation

Fig 2

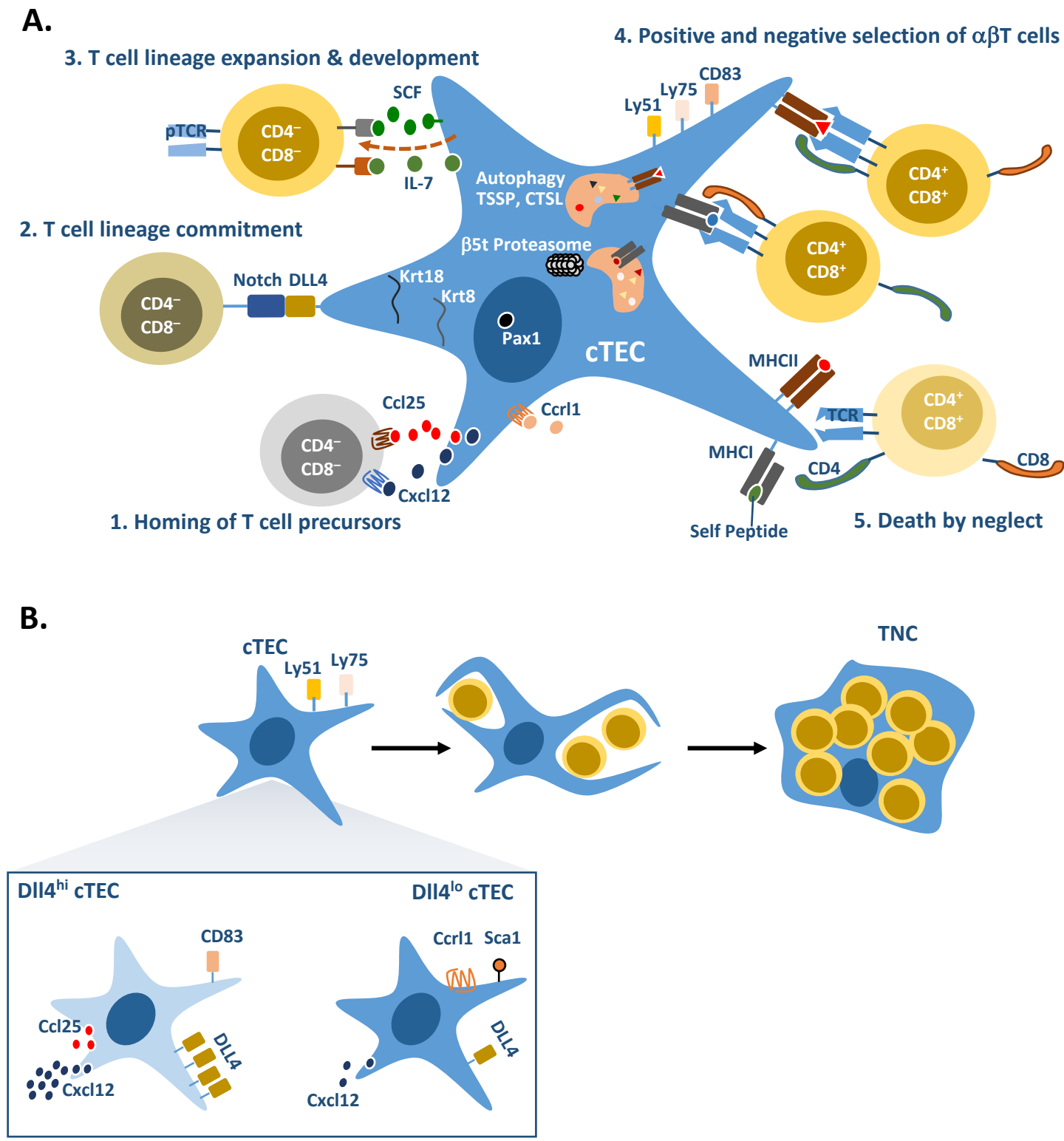

A.
3. T cell lineage expansion & development
4. Positive and negative selection of αβT cells
pTCR
CD4⁻ CD8⁻
SCF
IL-7
CD83
Ly75
Ly51
Autophagy
TSSP, CTSL
β5t Proteasome
2. T cell lineage commitment
Notch DLL4
Krt18
Krt8
Pax1
cTEC
CD4⁺ CD8⁺
MHCII
TCR
MHCI
CD4
CD8
Self Peptide
5. Death by neglect
Ccl25
Ccrl1
Cxcl12
1. Homing of T cell precursors
B.
cTEC
Ly51
Ly75
TNC
Dll4hi cTEC
Dll4lo cTEC
CD83
Ccl25
Cxcl12
DLL4
Ccrl1
Sca1

Fig 3

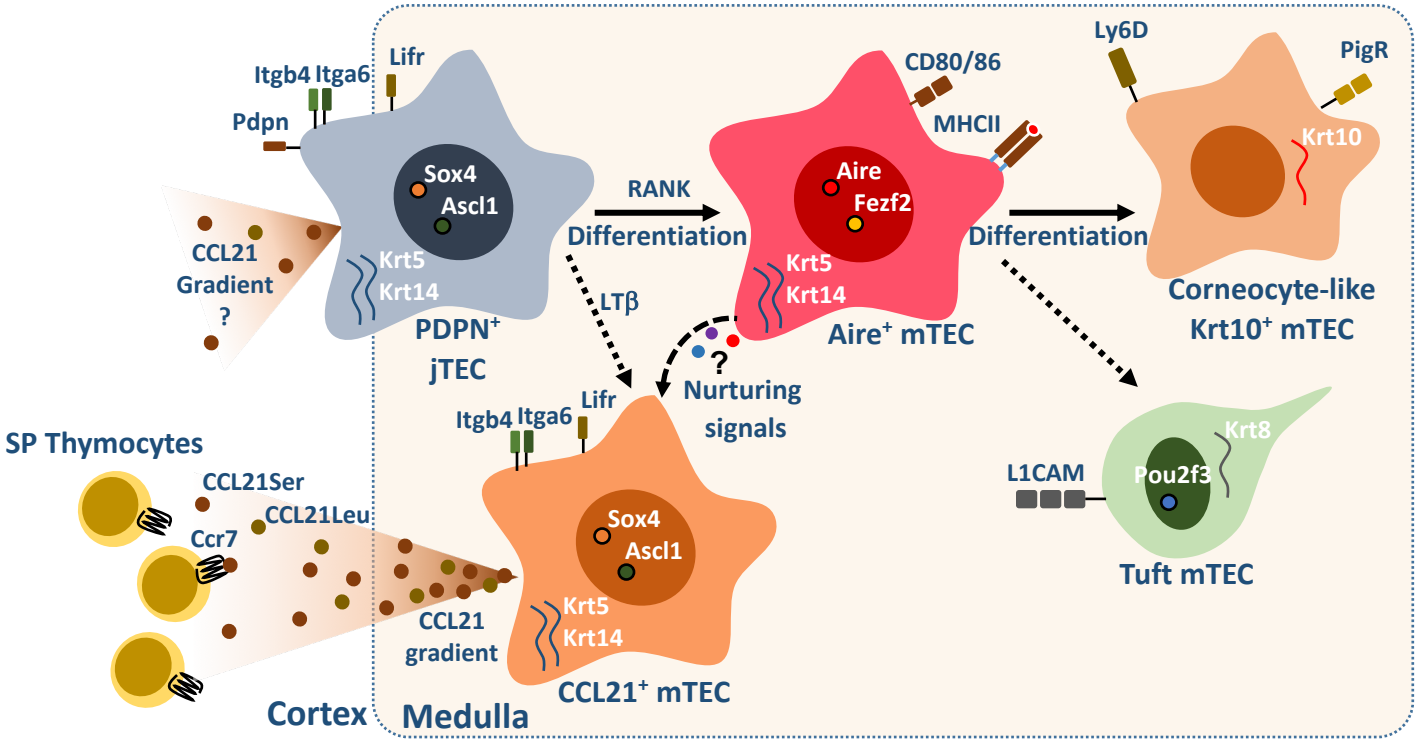
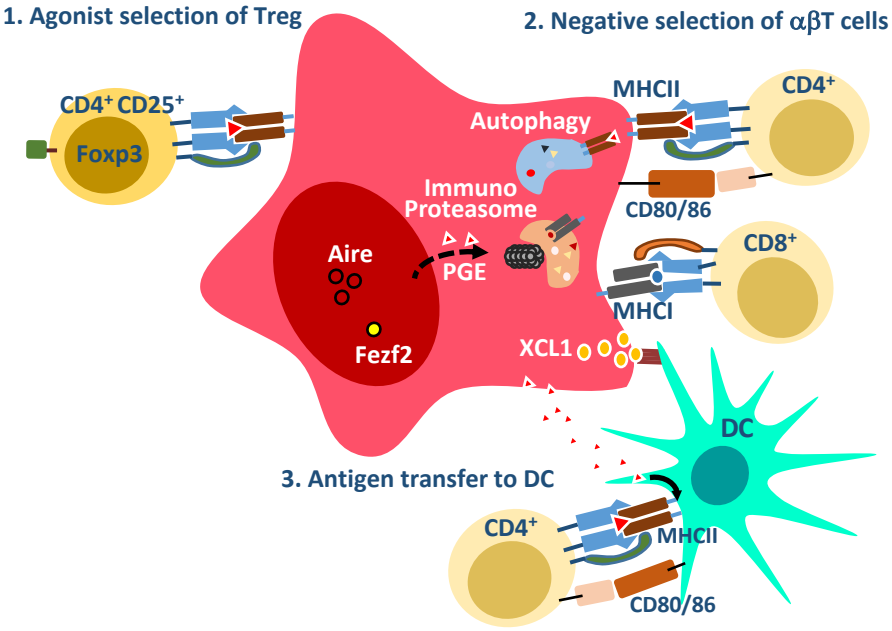

Itgb4 Itga6
Lifr
Pdpn
Sox4
Ascl1
Krt5
Krt14
PDPN+
jTEC
CCL21
Gradient
?
RANK
Differentiation
LTβ
CD80/86
MHCII
Aire
Fezf2
Krt5
Krt14
Aire+ mTEC
Differentiation
Ly6D
PigR
Krt10
Corneocyte-like
Krt10+ mTEC
?
Nurturing
signals
Itgb4 Itga6
Lifr
Sox4
Ascl1
Krt5
Krt14
CCL21+ mTEC
L1CAM
Pou2f3
Krt8
Tuft mTEC
SP Thymocytes
CCL21Ser
CCL21Leu
Ccr7
CCL21
gradient
Cortex
Medulla

Fig 4

A.


1. Agonist selection of Treg
2. Negative selection of αβT cells
CD4+ CD25+
Foxp3
Autophagy
MHCII
CD4+
Immuno
Proteasome
CD80/86
Aire
PGE
CD8+
MHCI
Fezf2
XCL1
DC
3. Antigen transfer to DC
CD4+
MHCII
CD80/86

B.

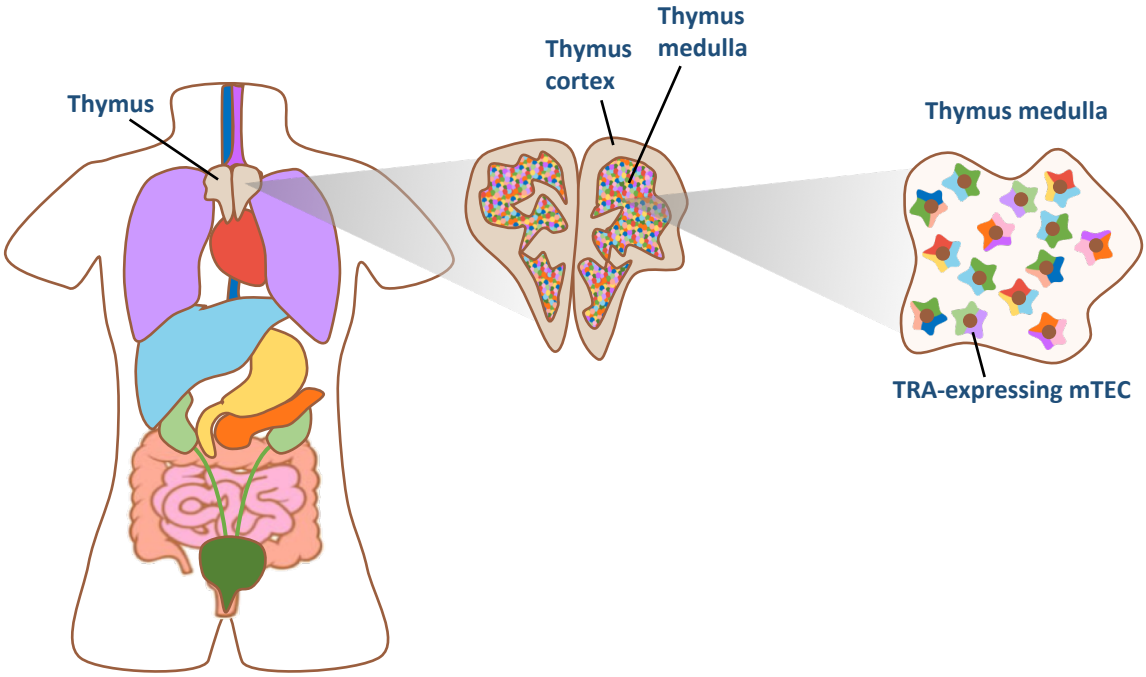
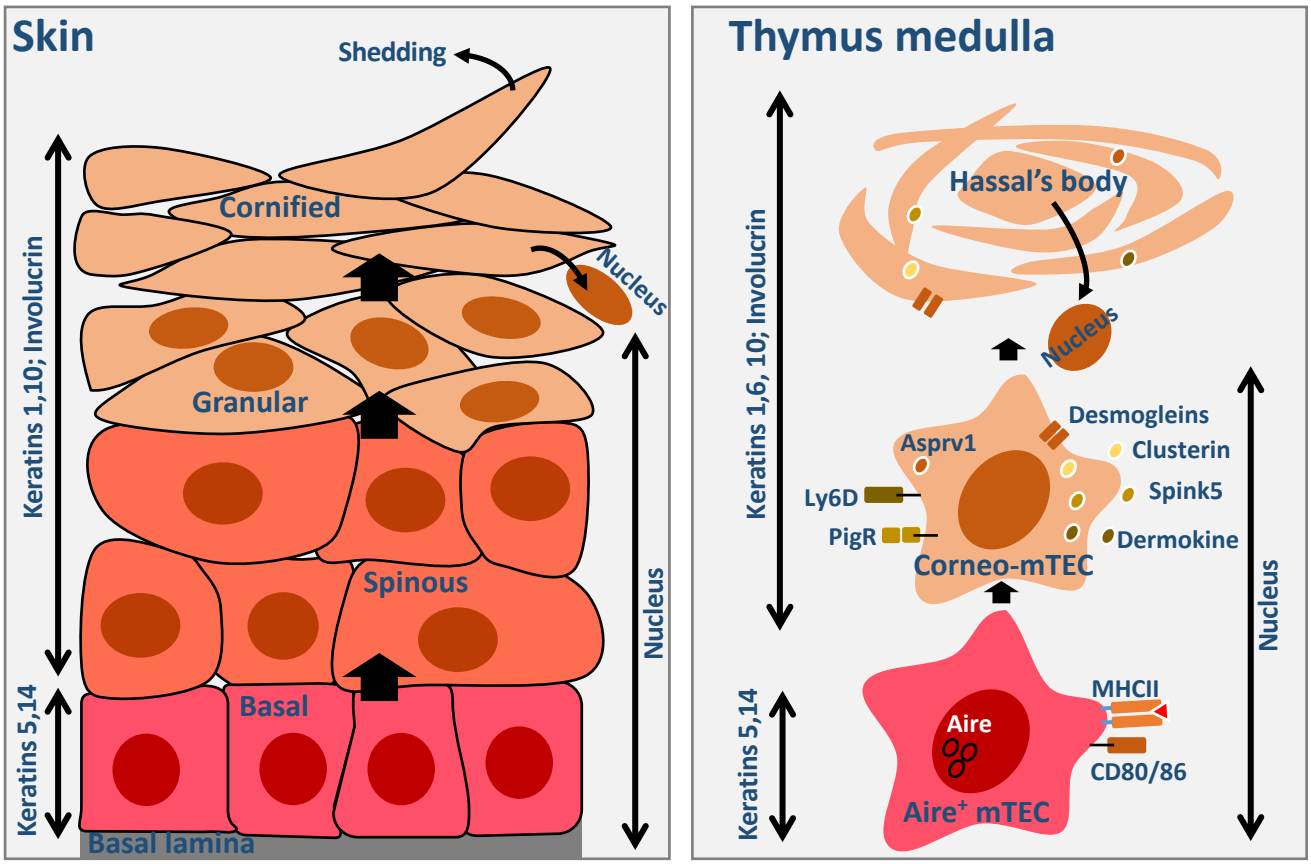

Thymus
Thymus
cortex
Thymus
medulla
Thymus medulla
TRA-expressing mTEC

Fig.5

A.


Skin
Shedding
Cornified
Nucleus
Granular
Spinous
Basal
Basal lamina
Keratins 1,10; Involucrin
Keratins 5,14
Nucleus
Thymus medulla
Hassal's body
Nucleus
Keratins 1,6, 10; Involucrin
Desmogleins
Asprv1
Clusterin
Ly6D
Spink5
PigR
Dermokine
Corneo-mTEC
Nucleus
Keratins 5,14
MHCII
Aire
CD80/86
Aire+ mTEC

B.

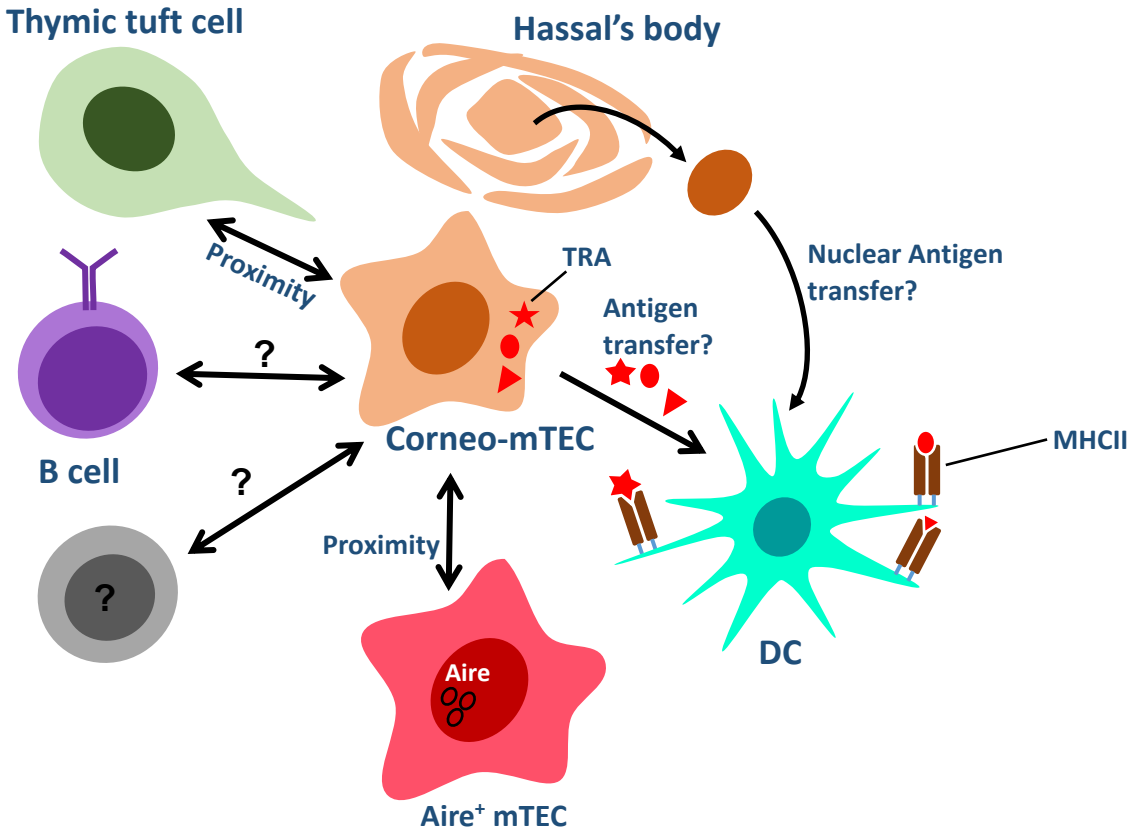
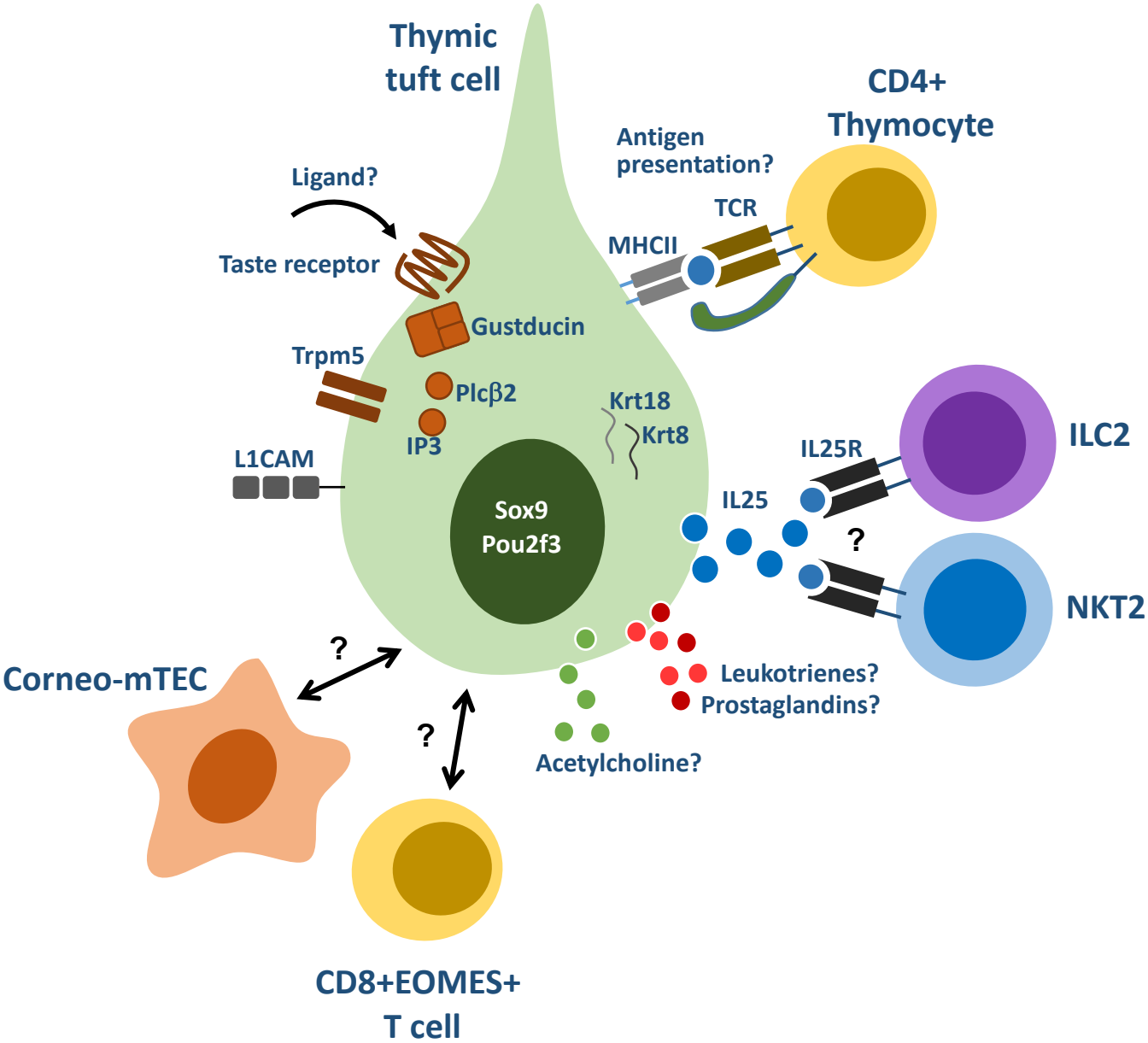

Thymic tuft cell
Hassal's body
Proximity
TRA
Nuclear Antigen transfer?
Antigen transfer?
?
B cell
Corneo-mTEC
MHCII
?
Proximity
?
DC
Aire
Aire+ mTEC

Fig.6


Thymic
tuft cell
CD4+
Thymocyte
Antigen
presentation?
Ligand?
TCR
MHCII
Taste receptor
Gustducin
Trpm5
Plcβ2
Krt18
Krt8
IP3
L1CAM
ILC2
IL25R
IL25
Sox9
Pou2f3
?
NKT2
Corneo-mTEC
?
Leukotrienes?
Prostaglandins?
?
Acetylcholine?
CD8+EOMES+
T cell