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REVIEW

Thymic stromal cell subsets for T cell development

Takeshi Nitta[1] · **Harumi Suzuki**[1]

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Abstract The thymus provides a specialized microenvironment in which a variety of stromal cells of both hematopoietic and non-hematopoietic origin regulate development and repertoire selection of T cells. Recent studies have been unraveling the inter- and intracellular signals and transcriptional networks for spatiotemporal regulation of development of thymic stromal cells, mainly thymic epithelial cells (TECs), and the molecular mechanisms of how different TEC subsets control T cell development and selection. TECs are classified into two functionally different subsets: cortical TECs (cTECs) and medullary TECs (mTECs). cTECs induce positive selection of diverse and functionally distinct T cells by virtue of unique antigen-processing systems, while mTECs are essential for establishing T cell tolerance via ectopic expression of peripheral tissue-restricted antigens and cooperation with dendritic cells. In addition to reviewing the role of the thymic stroma in conventional T cell development, we will discuss recently discovered novel functions of TECs in the development of unconventional T cells, such as natural killer T cells and γδT cells.

Keywords Thymus · T cell · Repertoire selection · Thymic epithelial cell · cTEC · mTEC

✉ Takeshi Nitta
nit-im@m.u-tokyo.ac.jp

✉ Harumi Suzuki
hsuzuki@ri.ncgm.go.jp

[1] Department of Immunology and Pathology, Research Institute, National Center for Global Health and Medicine, Chiba 272-8516, Japan

Introduction

T lymphocytes (T cells) are central players in the adaptive immune system. Specific antigen recognition by T cells is dependent on their T cell antigen receptors (TCRs), αβTCR and γδTCR. T cells that express an αβTCR (αβT cells), as well as a coreceptor CD4 or CD8, are considered 'conventional' T cells in human and mouse. They are termed as such because αβTCR recognition of peptide antigens displayed by major histocompatibility complex (MHC) proteins plays a major role in immune responses against foreign antigens. Therefore, in this article, the terms 'T cell' and 'TCR' refer to αβT cell and αβTCR, respectively, unless otherwise specified. The specificity of antigen recognition by TCR is stringently established, such that T cells are reactive to foreign antigens but are tolerant to self-antigens [1, 2].

T cell development and TCR repertoire formation occur primarily in the thymus [3]. The thymus is an organ that provides a unique microenvironment composed of a variety of stromal cells, including thymic epithelial cells (TECs), endothelial cells, fibroblasts, and hematopoietic stromal cells such as dendritic cells (DCs) [4, 5]. These thymic stromal cells coordinate a three-dimensional meshwork architecture that hosts hematopoietic stem cell-derived T-lineage cells, called thymocytes, and critically supports their development. The thymus is subdivided into two histologically discrete regions, the cortex and medulla. The cortex is the outer region of the thymus, where a stromal meshwork houses densely packed immature thymocytes, while the medulla is the inner region with less densely localized mature thymocytes and enriched stromal cells. The most characteristic stromal components that distinguish cortical and medullary microenvironments are two different subsets of TECs: cortical TECs (cTECs) and

medullary TECs (mTECs). cTECs and mTECs are both derived from endodermal epithelium, yet they display distinct phenotypes and functions in the regulation of T cell development. These TECs, along with other stromal cells, provide multiple signals to guide the differentiation, migration, proliferation, survival, and death of developing thymocytes, thus playing pivotal roles in forming the adaptive immune system [6, 7].

In this review, we first provide an overview of the stepwise process of T cell development in the thymus, and then review historical and recent studies on the development and function of TECs, particularly focusing on the contrasting roles of cortical and medullary microenvironments. We will also highlight the bidirectional interplay between TECs and developing thymocytes that is required for optimal development and repertoire formation of T cells. In addition, recently discovered functions of TEC subsets in controlling unconventional T cell development are also discussed.

Overview of thymic T cell development

T cell development begins with seeding of the thymus by early T cell progenitors (ETPs) derived from hematopoietic stem cells in fetal liver or adult bone marrow [8]. These ETPs belong to CD4/CD8 double negative (DN) thymocytes and undergo developmental programs through DN1 ($CD44^+CD25^-$), DN2 ($CD44^+CD25^+$), DN3 ($CD44^-CD25^+$), and DN4 ($CD44^-CD25^-$) stages. During DN2 and DN3 stages, V(D)J rearrangement at TCRγ, δ and β loci occurs. Production of a successfully rearranged TCR β-chain leads to further differentiation into the DN4 stage. This process, called 'β selection', ensures commitment to the αβT cell lineage, and DN4 thymocytes proliferate and express CD4 and CD8 coreceptors, giving rise to CD4/CD8 double positive (DP) thymocytes. These differentiation processes are associated with relocation of thymocytes [9]: in adult thymus, ETPs first arrive at the cortico-medullary junction, developing DN2 and DN3 thymocytes migrate through the cortex toward the subcapsular region, and the generation of DP thymocytes occurs in the outer cortex.

In the cortex, DP thymocytes undergo TCRα-VJ rearrangement, thereby expressing αβTCR on the cell surface. Interaction of αβTCR with peptide-MHC (pMHC) complexes presented in the cortical microenvironment leads to the fate decision of DP thymocytes. DP thymocytes that receive low avidity TCR interactions with self pMHC survive and differentiate into CD4 single positive (SP) or CD8SP thymocytes, in a process referred to as positive selection. In contrast, DP thymocytes expressing TCR strongly reactive to self pMHC (self-reactive cells) die by apoptosis, a process referred to as negative selection. Positively selected CD4SP or CD8SP thymocytes relocate to the medulla by chemotactic migration. In the medulla, mTECs express a variety of peripheral tissue-restricted antigens (TRAs) that are presented autonomously by mTECs or indirectly by DCs, such that SP thymocytes reactive to TRAs are deleted by negative selection or induced to differentiate into $Foxp3^+$ regulatory T cells (Tregs). These medullary controls of T cell development are crucial for establishment of self-tolerance and preventing autoimmunity, and largely depend on autoimmune regulator (Aire), a nuclear factor expressed in mTECs. Consequently, mature SP thymocytes that have completed cortical and medullary selection processes—and which thereby express diverse yet self-tolerant TCRs—are released to the circulation as naïve T cells (Fig. 1).

In addition to the mainstream conventional αβT cell development, the thymus also supports the development of unconventional (non-classical) T cells. γδT cells form a distinct T cell lineage expressing γδTCR that recognizes native non-peptide and peptide antigens such as stress-induced proteins. αβT and γδT cell development diverge at the DN2 and DN3 stages. Unlike αβT cell development, γδT cell development does not require antigen-specific interactions in the thymus [10]. γδT cell subsets expressing different TCR-Vγ chains are generated at defined periods during ontogeny and distribute to different epithelial and mucosal tissues [11]. Invariant natural killer T (iNKT) cells represent an unconventional αβT cell subset expressing invariant Vα14-Jα18 TCR that recognize glycolipid antigens presented by MHC-like CD1d molecules, and play roles in controlling innate and adaptive immune responses [12]. These iNKT cells are positively selected by CD1d/glycolipid complexes expressed on the surface of DP thymocytes [13].

T cell development as described above is controlled in the thymic microenvironment, mainly by TECs, and in turn, developing T cells critically regulate the development of TECs, such that T cell immunity can be finely tuned for optimal immune responses.

Generation of thymic epithelium

Thymic epithelial cells are derived from endodermal epithelium from the third pharyngeal pouch [14]. Early TEC development is controlled by specific transcription factors including FoxN1 (Whn), Tbx1, and Pax1 [15, 16]. FoxN1 is a major mediator of TEC development and function, as FoxN1 deficiency completely disrupts thymic T cell development in animals and human [17–20]. FoxN1 regulates the transcription of various target genes essential for hematopoietic function of the thymus, including cytokines, chemokines, and Notch ligands. FoxN1

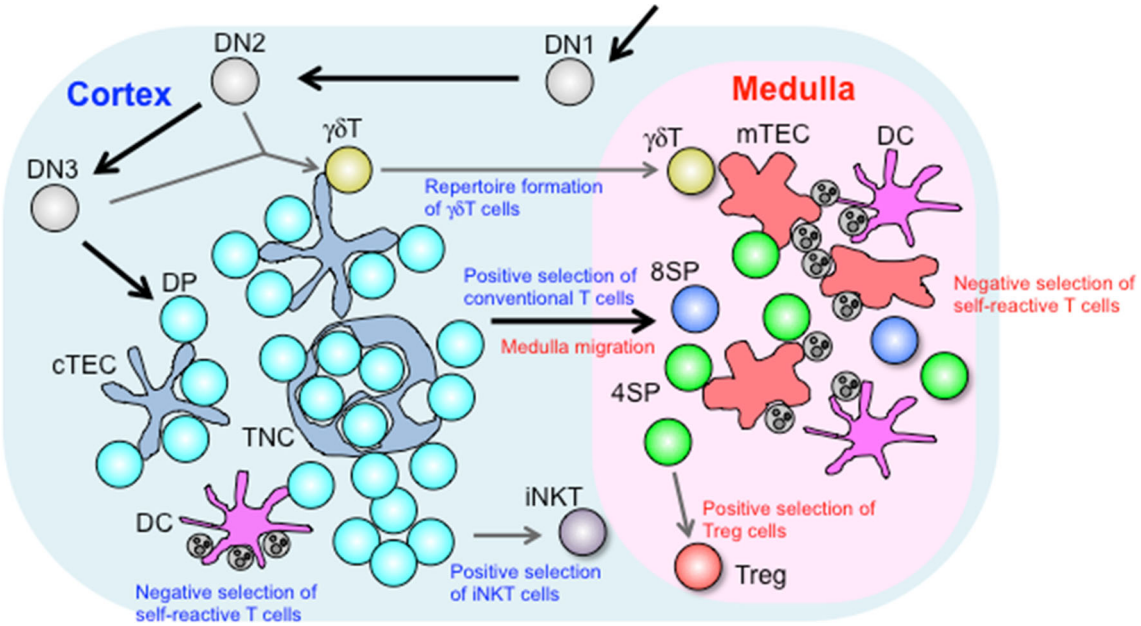

Fig. 1 Roles of thymic stromal cells in T cell development. Developing DN thymocytes migrate from the cortico-medullary junction toward the subcapsular region, and then back toward the medulla upon development to DP thymocytes. In the cortex, DP thymocytes begin to express TCR on their surface and are selected upon interaction with pMHC complexes displayed in the microenvironment. cTECs produce a unique set of MHC-bound peptides that are essential for inducing positive selection, while DCs critically contribute to induction of negative selection. Intimate interaction between DP thymocytes and cTECs results in formation of TNCs, which facilitate prolonged survival of inner DP thymocytes and secondary TCRα recombination. Positively selected cells migrate into the medulla in response to chemokines produced by mTECs. γδT cells diverge from the αβT cell lineage at the DN stage, and their repertoire formation is regulated by cTECs through unknown mechanisms. The cortical microenvironment is also important for positive selection of iNKT cells that depends on cell–cell interaction among DP thymocytes. In the medulla, SP thymocytes are screened for self-reactivity. SP thymocytes reactive to TRAs presented by mTECs or DCs are deleted by negative selection or induced to differentiate into Foxp3$^+$ Treg cells

expression is detected in almost all TECs during embryogenesis [21], in a manner dependent on Wnt signaling [22], while a fraction of TECs from adult mice lose the expression of FoxN1 but maintain the expression of its target genes [23], suggesting a FoxN1-independent mechanism for maintenance and function of postnatal TECs.

Other factors that regulate TEC development include the transcription factor p63 and its interacting partner Polycomb protein Cbx4. Both are strongly expressed in TECs and required for proliferation and maintenance of cTECs and mTECs [24–26].

Thymic cortex

cTEC development

The stromal architecture in the thymic cortex is mainly composed of cTECs. cTECs can be identified by expression of marker proteins such as Keratin-8, Keratin-18, Cerebellar degeneration-related antigen 1 (CDR1), CD205, CD249 (Ly51), Interleukin (IL)-7, the thymoproteasome subunit β5t, and the atypical non-signaling chemokine receptor CCRL1 (CCX-CKR1). In contrast, mTECs are characterized by a different set of markers such as Keratin-5, Keratin-14, CD80, and Aire. Both cTECs and mTECs are derived from common endodermal progenitor cells identified in the third pharyngeal pouch [27, 28]. These common TEC progenitors progress to a transitional progenitor stage, a process dependent on the transcription factor FoxN1 [29, 30]. Such transitional TEC progenitors express cTEC-associated genes such as CD205, β5t, CCRL1, and IL-7, and give rise to both cTECs and mTECs, including the Aire$^+$ subset [30–33]. It is still unclear how cTEC and mTEC lineage determination progresses: whether asymmetrically, in that the transitional progenitors undergo maturation into cTECs by default or lose cTEC traits upon differentiation to mTECs, or symmetrically, in that the transitional progenitors coexpress cTEC- and mTEC-associating genes and acquire enhanced expression of lineage-associating genes or lose ones for another lineage during lineage determination [34].

Generation of transitional TEC progenitors does not require lymphocyte-derived signals, since Keratin-8$^+$ cells are detected in Rag2/γc double-deficient mice, in which the development of T, B, and NK cells is completely impaired

[35], and β5t expression in TECs is readily detectable in CD3εTg26 mice, in which thymocyte development arrests at DN1 due to the unidentified mechanism caused by genomic insertion of multiple copies of a human CD3ε transgene [36]. The expression of cTEC-specific markers or functional proteins, such as CD249, CCRL1, β5t and MHC class II, gradually increases along cTEC ontogeny [29, 33, 36, 37]. Mature MHC class II^{hi} cTECs are detectable in Rag1-deficient mice (arrested at DN3) but not in CD3εTg26 mice (arrested at DN1) [29], indicating that the functional maturation of cTECs requires thymocyte development beyond the DN1 stage. This is consistent with an early report that the meshwork architecture of thymic cortical epithelium is disturbed in CD3εTg26 mice [38]. Maturation of cTECs was restored in CD3εTg26 mice by transfer of wild-type T-progenitor cells [39]. Together, these results indicate that maturation of cTECs requires signals delivered by developing thymocytes, likely through as of yet unidentified intercellular signals (Fig. 2).

Early T cell development and migration in the cortex

Cortical TECs are the predominant source of Notch ligands, cytokines, and chemokines required for early T cell development. Delta-like 4 (Dll4), a Notch ligand expressed by cTECs, is essential and sufficient for T-lineage determination of early lymphoid progenitors in the thymus [40–42]. IL-7 is also predominantly produced by cTECs [43] and promotes survival, proliferation, and differentiation of thymocytes [44–46]. Outward migration of DN thymocytes from the corticomedullary junction to the subcapsular region is mediated by chemokines CCL25 and CXCL12, produced by cTECs, and their receptors CCR9 and CXCR4, respectively, expressed on DN thymocytes [47–50]. CXCL12-CXCR4 signaling also promotes β selection [51]. CCRL1, an atypical non-signaling chemokine receptor highly expressed in cTECs [52], promotes outward migration of DN thymocytes via still-unknown mechanisms [53, 54]. Vascular-cell adhesion molecule-1

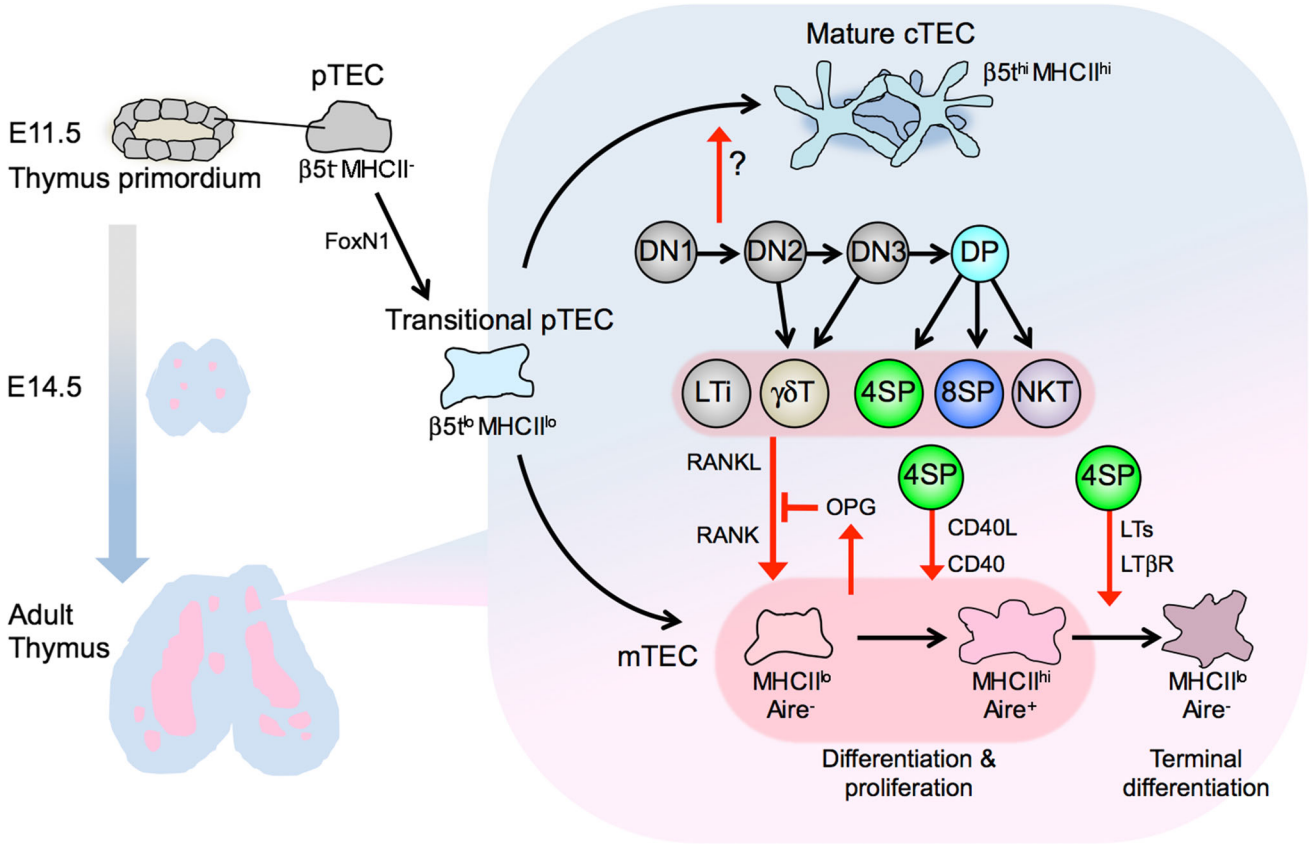

Fig. 2 Development of thymic epithelial cells is induced by various subsets of thymocytes. cTECs and mTECs arise from common progenitor TECs (pTECs) in endodermal epithelium from the third pharyngeal pouch. pTECs differentiate into 'transitional' pTECs that express cTEC-associated genes such as β5t and IL-7. This process is critically regulated by the transcription factor FoxN1 but independent of lymphocytes. Thymocyte development beyond DN1 induces maturation of cTECs expressing high levels of β5t. The development of mTECs is triggered in embryonic thymus by LTi cells and γδT cells that express RANKL. In postnatal thymus, SP thymocytes and NKT cells express RANKL to promote the differentiation and proliferation of Aire-expressing mTECs. RANKL-stimulated mTECs produce OPG to self-tune their development. CD40L expressed in CD4SP thymocytes cooperates with RANKL to promote mTEC development. CD4SP thymocytes also express LTs, which induce terminal differentiation of mTECs

(VCAM-1), expressed by cTECs, and its receptor integrins α4β1 and α4β7, expressed by DN thymocytes, are also important for intimate stromal interaction and outward migration of DN thymocytes [55]. DN thymocytes turn back inward and differentiate into DP thymocytes at the subcapsular region, where transforming growth factor (TGF) β is expressed and exerts negative feedback on DN to DP differentiation [56].

The majority of DP thymocytes move randomly in the cortex, likely scanning pMHC ligands for with newly generated TCR in the cortical microenvironment [57]. Successful TCR interaction with pMHC ligands leads to a stop of the 'random walk' migration and a prolonged duration of thymocyte–stromal interaction [58], which is required for efficient positive selection. Indeed, DP thymocytes with enhanced migratory activity to cortical chemokines CCL25 and CXCL12 in vitro and dysregulated migration in in vivo thymic cortex undergo less positive selection but unaltered negative selection [59, 60], indicating that properly regulated migration and stromal interaction of DP thymocytes is distinctly required for positive selection.

Positive selection in the cortex

The most recognized function of cTECs is the induction of T cell-positive selection. As described above, low affinity TCR engagement by pMHC complexes induces positive selection of functional T cells, whereas high affinity TCR-pMHC interaction leads to negative selection of self-reactive (potentially harmful) T cells. Recent studies support the idea that cTECs have unique proteolytic and antigen-processing capabilities to produce MHC-associating peptides that are essential for positive selection.

For the MHC class I system, cTECs are equipped with a unique type of proteasome. Proteasomes are multi-subunit protease complexes responsible for producing MHC class I-associating peptides as well as for turnover of intracellular proteins [61]. Peptides with C-terminal hydrophobic anchor residues are produced by chymotrypsin-like activity of the proteasomes, which is mediated by β5 catalytic subunits. Unlike most somatic cells that express 'standard proteasomes' containing β5 subunits or immune cells and interferon (IFN) γ-stimulated cells that express β5i subunit-containing 'immunoproteasomes' [62, 63], cTECs express a specialized type of proteasome, called a 'thymoproteasome', that contains the β5t subunit [64, 65]. β5t is exclusively expressed by cTECs throughout the lifespan of mice [36], thus representing a specific marker of cTECs. In mice deficient for β5t, cTECs express β5- and β5i-containing proteasomes and display a spectrum of MHC class I-associating peptides that are different from those in β5t-sufficient cTECs [66, 67]. In these mice, positive selection of MHC class I-restricted thymocytes is substantially reduced, leading to a marked reduction (20 % of wild-type) and altered repertoire of CD8 T cells, indicating that optimal positive selection of CD8 T cells requires the β5t-dependent peptide repertoire in cTECs. The β5t-dependent peptides are also essential for functionally conditioning antigen responsiveness of positively selected CD8 T cells [68]. A recent study identified unique cleavage motifs in β5t-dependent MHC class I-associating peptides that confer low affinity TCR interaction and capabilities to efficiently induce positive selection [69]. This uniqueness of the peptide motifs might be attributed to the peptide cleavage preference between β5t and the other subunits β5 and β5i [64, 69]. Collectively, these aspects indicate that cTECs regulate positive selection of CD8 T cells by producing a unique set of MHC class I-associating peptides that exhibit low affinity for TCR [70].

In the MHC class II system, various lysosomal proteases produce peptide antigens [71]. cTECs highly express lysosomal proteases cathepsin L and thymus-specific serine protease (TSSP) [71, 72]. Mice deficient for cathepsin L show a reduced positive selection of polyclonal CD4 T cells [73, 74]. TSSP-deficient mice show a defective positive selection of CD4 T cells with certain TCR specificities [75, 76], including diabetogenic self-reactive CD4 T cells [77]. It was also shown that cTECs exhibit high levels of constitutive macroautophagy [78], a cellular process that facilitates loading of endogenously generated peptides onto MHC class II molecules. Mice with defective macroautophagy induction, specifically in TECs, show altered repertoire selection of certain CD4 T cells [79]. These data strongly support the idea that cTECs have unique protein degradation and antigen-processing machineries for inducing positive selection of CD4 T cells, although the nature of MHC class II-associating peptides produced by cTECs remains to be elucidated.

Negative selection in the cortex

Thymic cortex is also the place where self-reactive thymocytes are deleted by negative selection. A recent study estimated that nearly 6 times as many thymocytes undergo negative selection compared with positive selection, and 75 % of negative selection occurs in the cortex [79], most frequently in the inner cortical region [80]. However, negative selection, by any experimental model tested, was observed to be normal in β5t-deficient mice [66] and TSSP-deficient mice [75], indicating that cTEC-specific peptides are not required for cortical negative selection. Involvement of cTECs in negative selection in the cortex was also challenged by our recent finding that negative selection was not affected in the *TN* mutant mice that intrinsically lack mature cTECs (see below) [37]. Rather, it is the cortex-resident DCs that appear to be responsible for negative selection in the cortex [80].

Thymic nurse cell

Recently, a long-argued topic in cTEC function was revisited. In 1980, a group reported the discovery of unique multicellular complexes in cell suspensions prepared by enzymatically dissociating thymus tissues [81, 82]. These complexes were termed 'thymic nurse cells' (TNC) for the large thymic epithelial cells that had engulfed multiple (up to 50) living lymphocytes within their intracellular vesicles. These studies, as well as many later studies (reviewed in [83]), hypothesized that TNCs provide a unique microenvironment for T cell selection, although the precise cell lineage and function of TNC-forming thymic epithelium had long remained elusive. A recent report found that approximately 10–15 % of β5t-expressing cTECs, but not mTECs, form thymocyte-wrapping complexes in adult mouse thymus that are identical to previously described TNCs [84]. The formation of TNC requires normal development of cTECs, as cTEC-deficient mice have no TNCs in the thymus ([37] and our unpublished data). TNCs are poorly formed in embryonic thymus from normal mice or in adult thymus from 'positive-selector' TCR transgenic mice, but readily detectable in the 'null-selector' mouse thymus. The majority of TNC-enveloped lymphocytes are long-lived, unselected DP thymocytes undergoing secondary TCRα-VJ rearrangements. Thus, TNCs are formed upon persistent cTEC-DP thymocyte interactions and facilitate secondary TCRα rearrangements. Given that the efficiency of secondary TCRα rearrangements is controlled by DP thymocyte survival [85], the microenvironments within intra-TNC vesicles may ensure survival of enclosed DP thymocytes. Secondary TCRα rearrangement is required for multiplying the opportunities for positive selection and thereby maximizing the developmental efficiency of functional T cells [86]. The mechanisms by which unselected thymocytes are enclosed into and positively selected thymocytes are released from the TNC complexes, and how intra-TNC microenvironments promote survival and/or continued TCR rearrangement in DP thymocytes, remain to be studied.

Taken together, SP thymocytes that passed positive and negative selection in the cortex set out on a new journey toward the medulla, to be further screened for TCR reactivity to self.

New aspects of cTEC function: unconventional T cell development

To date, a few studies have reported mutant mice lacking normal cTEC development. Preferential loss of cTECs and disorganized thymic cortical architecture were observed in Keratin-5-driven Stat3-deficient mice [87], Eph4-deficient mice [88], and transgenic insertional mutant mice called Tg66 [89], although the molecular basis of the cTEC deficiency in these mice remains unclear. A study using mice transgenic for the human diphtheria toxin receptor under the control of the CCRL1 promoter demonstrated that diphtheria toxin-inducible depletion of cTECs resulted in nearly complete loss of DN and DP thymocytes, confirming that cTECs are essential for thymic cortical architecture and thereby maintenance of cortical thymocytes [52].

Recently, we established a spontaneous mutant mouse line, called *TN* that exhibits an almost complete loss of mature cTECs yet only a modest effect on mTECs [37]. A missense mutation in the gene encoding β5t was responsible for this phenotype. The mutant β5t inhibits normal proteasome assembly and cell survival, resulting in substantial loss of $\beta5t^{high}$ mature cTECs and accumulation of $\beta5t^{low}$ transitional TEC progenitors. Therefore, the *TN* mouse is a novel animal model that intrinsically and specifically lacks mature cTECs. The thymus from *TN* mice shows a disorganized cortical architecture, massive loss of thymic cellularity, impaired positive selection, and altered αβTCR repertoire: all in agreement with the above-described functions of cTECs in forming the cortical microenvironment and inducing positive selection. cTEC deficiency also caused a reduction in iNKT cell development, possibly due to inefficient cell–cell interaction among DP thymocytes in the disorganized cortical microenvironment.

The most unexpected and significant finding from the study of *TN* mice is the influence of mature cTEC deficiency on development and repertoire formation of γδT cells. It has been known that thymic development of γδT cell subsets is ontogenically regulated and that γδT subsets show different tissue distribution and effector functions [11]. Recent studies have highlighted an IL-17-producing subset of γδT (γδT17) cells, which includes $V\gamma4^+$ and $V\gamma6^+$ cells in mice, as being essential for various infections, inflammations, and malignancies [90], although regulation of thymic development of γδT17 cells remains unclear. In the thymus from cTEC-deficient *TN* mice, while the frequency of total γδT cells is unaltered, the proportion of γδT17 cells is greatly increased [37]. Among these γδT17 cells, the $V\gamma6^+$ subset robustly increased, whereas the $V\gamma4^+$ subset decreased, resulting in the marked skewing from Vγ4 to Vγ6 in the TCR repertoire of γδT17 cells and the perturbation of γδT17-dependent inflammatory responses in peripheral tissues. The γδT17 repertoire is unaffected by β5t deficiency and mTEC development. Thus, normal cTEC development contributes to optimal repertoire formation not only of conventional αβT cells but also of unconventional 'innate type' γδT cells. The thymus from *TN* mice may provide a 'fetal type' microenvironment that specifically supports the predominant development of

$V\gamma6^+$ γδT17 cells [91, 92]. It is also possible that the thymus lacking mature cTECs has altered expression of as of yet unidentified selecting ligand molecule(s) or cell-surface proteins that mediate differentiation or deletion of γδT17 cell subsets: for example, as mTECs regulate development of $V\gamma5^+V\delta1^+$ γδT cells via expression of a B7-family protein called 'selection and upkeep of intraepithelial T cells (Skint1)' [93].

Thymic medulla

mTEC development

Medullary TECs emerge from TEC progenitors expressing cTEC-associated genes, and are distinguished by the expression of proteins such as Keratin-5, Keratin-14, CD80, Aire, Claudin-3, and Claudin-4 and their reactivity with the fucose-binding lectin Ulex europaeus agglutinin 1 (UEA1) [94, 95]. mTECs are further classified into two subsets, $mTEC^{hi}$ ($MHC\ class\ II^{hi}\ CD80^{hi}$) cells and $mTEC^{lo}$ ($MHC\ class\ II^{lo}\ CD80^{lo}$) cells [94], and the $mTEC^{hi}$ cells represent functionally mature mTECs expressing Aire (Fig. 2). Several reports show that $mTEC^{lo}$ cells can give rise to $mTEC^{hi}$ cells [96–98], but recent lineage tracing studies show that $Aire^+$ $mTEC^{hi}$ cells progress to an $Aire^-$ $mTEC^{lo}$ stage [99, 100], indicating that $mTEC^{lo}$ is a heterogeneous cell population including developing immature mTECs and developed mature 'post-Aire' mTECs. The 'post-Aire' $mTEC^{lo}$ cells represent a distinct mTEC subpopulation expressing chemokines such as CCL21 [101]. This cell subset also includes terminally differentiated mTECs, characterized by the expression of Involucrin and the stratified squamous epithelia resembling Hassall's corpuscles, as observed in the human thymus [102, 103].

Early studies, mostly conducted in the 1990s, indicated that thymic medulla formation is defective in mice with T cell development arrested at early stages [104]. Particularly, mice deficient for positive selection showed a marked reduction of thymic medullary regions and mTEC cellularity without affecting overall thymus size and cortical architecture [94, 105–107], indicating that the positively selected SP thymocytes induce the development of mTECs, which, in turn, provide a microenvironment for selection and maturation of SP thymocytes. This mTEC-thymocyte interdependency is referred to as 'thymic crosstalk'.

Over a span of two decades, a series of studies has revealed that the signaling pathways for the activation of nuclear factor-κB (NF-κB) are required for mTEC development. Mice deficient for TNF receptor-associated factor 6 (TRAF6), NF-κB-inducing kinase (NIK), IκB-kinase α (IKK α), Bcl-3, NF-κB2 (p52), or RelB, exhibit defective development of $Aire^+$ mTECs and thymic medulla formation in an mTEC-autonomous manner [108–116]. These NF-κB pathways for thymic medulla formation are activated by TNFR superfamily receptors, receptor for activating NF-κB (RANK), CD40, and lymphotoxin β receptor (LTβR), expressed on mTECs, and their TNF superfamily ligands RANKL, CD40L, and lymphotoxins (LTs), respectively, are expressed by lymphoid cells, mostly SP thymocytes [117–120]. This configuration of receptor-ligand expression provides an explanation for early observations of 'thymic crosstalk' and mechanism for later findings of NF-κB involvement. Indeed, TNF superfamily ligand-mediated mTEC development is ensured by TCR–ligand interactions between self-reactive SP thymocytes and mTECs [121–125].

RANKL, a major mediator of mTEC development, is produced by lymphoid tissue inducer (LTi) cells and γδT cells in the embryonic thymus and by SP thymocytes and iNKT cells in postnatal thymus [118, 120, 126, 127] (Fig. 2). CD40L and LTs are expressed predominantly by SP thymocytes [117, 120, 126]. These TNFSF ligands have cooperative as well as distinct non-redundant functions in mTEC development. RANKL and CD40L synergistically promote development and proliferation of $Aire^+$ mTECs [119, 120], while LTs regulate the development of a distinct subset of mTECs expressing CCL21 [101, 128, 129]. LT signals also regulate the expression of RANK in mTECs [130] and the terminal differentiation of mTECs [103].

RANKL signaling in mTECs up-regulates the transcription factor Spi-B, which in turn induces the expression of some TRAs, co-stimulatory molecules, and osteoprotegerin (OPG) [131]. OPG is an inhibitory decoy receptor for RANKL and represses RANKL-mediated mTEC development and expansion [98, 120, 131]. The fact that mTEC development is primarily dependent on interaction with SP thymocytes and controlled by the RANKL-OPG negative feedback system indicates that the cellularity and function of mTECs must be properly adjusted, such that self-reactive SP thymocytes can be moderately, not excessively, deleted in the thymic medulla.

mTEC development is promoted by coordination between RANKL and type I interferon signals [132] and negatively regulated by TGFβ signaling [133]. It was also reported that the microRNA production by the endoribonuclease Dicer, and specifically microRNA miR-29a, was essential for postnatal maintenance of mTECs [134, 135].

Medulla migration and emigration of thymocytes

Double positive thymocytes that received positive selection signals differentiate into CD4SP or CD8SP thymocytes and express the chemokine receptor CCR7 on the cell surface

[136]. CCR7 ligand chemokines, CCL19 and CCL21, are produced by mTECs [136] and medullary fibroblasts [137], and attract CCR7-expressing SP thymocytes from the cortex to the medulla [136, 138, 139]. During medullary residency, which is estimated to be 4–5 days [140], SP thymocytes are exposed to antigens presented by mTECs and DCs. CCR7-mediated medullary migration is required to ensure negative selection of self-reactive SP thymocytes [128, 141]. Indeed, mice deficient for CCR7 or CCR7 ligand chemokines exhibit organ-specific autoimmunity [139, 142, 143]. CCR7 signals also direct the migration of γδT cells to the medulla [144]. mTECs produce another chemokine XCL1 that mediates medullary accumulation of thymus-resident DCs [145].

SP thymocytes that have completed developmental programs and repertoire selection are exported from the thymus into circulation. Export of thymocytes is controlled by chemotactic signaling via sphingosine-1 phosphate (S1P) and its receptor S1PR1. Mature SP thymocytes express high levels of S1PR1 and then migrate toward a gradient of S1P [139, 146, 147], which is provided by neural crest-derived perivascular cells (pericytes) in the cortico-medullary junctions [148] and circulating blood [149]. However, the mechanism that determines the timing of thymocyte emigration such that only mature yet self-tolerant SP thymocytes are permitted to exit the thymus remains largely unclear. It is speculated that, in mature SP thymocytes that have completed self-reactivity screening, cessation of TCR signaling leads to down-regulation of CD69—an inhibitor of S1PR1 surface expression—resulting in up-regulation of S1PR1 expression, thereby rendering SP thymocytes responsive to S1P and primed for thymic exit [150].

TRA expression by mTECs

In the medulla, a diverse array of TRAs—whose expression is primarily restricted to peripheral tissues—are transcribed in mTECs, particularly $mTEC^{hi}$ cells [151–154], in a phenomenon termed 'promiscuous gene expression'. SP thymocytes reactive to these TRAs are ejected from the conventional T cell pool through deletion by negative selection or differentiation to $Foxp3^+$ Tregs (see below). As shown by many studies, T cells produced in mice lacking normal mTEC development caused autoimmune disorders, indicating that mTECs are essential for establishing central tolerance [99, 109, 111–119, 155]. TRA expression represents a mosaic pattern, as each TRA protein is expressed in only 1–3 % of mTECs, such that a maximal number and sufficient epitope density of TRAs can be displayed to SP thymocytes [7]. A single mTEC expresses a set of TRA genes, which are clustered in chromosomes and colocalized to nuclear subdomains [156].

A substantial fraction of TRAs is controlled by Aire [157], a nuclear protein predominantly expressed in mTECs [158–160]. Aire-driven TRA expression is crucial for negative selection of TRA-reactive SP thymocytes [161–163] and generation of $Foxp3^+$ Tregs [164–166] in the medulla. Genetic deficiency of Aire results in autoimmune polyendocrinopathy syndrome type 1 (APS1) or autoimmune polyendocrinopathy–candidiasis–ectodermal dystrophy (APECED) in human [167, 168], and similar organ-specific autoimmune disorders in mice [157, 169, 170], indicating that Aire is essential for establishment of self-tolerance. Accruing evidence indicates that Aire has no obvious DNA binding domain [171, 172] but instead epigenetically regulates transcriptional elongation and pre-mRNA processing of target TRA genes [154, 173–175]. However, some groups propose another mechanistic view whereby Aire controls the differentiation program of mTECs to enable TRA expression. This is because Aire deficiency in mice causes abnormal medulla organization and mTEC development [97, 100, 102, 176–178] and defective T cell tolerance against transcriptionally unrepressed TRAs [163, 170]. Indeed, Aire also regulates expression of a large number of non-TRA proteins such as cytokines, chemokines, MHC class II peptide-loading factors, posttranslational modifiers, and proteases [145, 163, 179–181]. Several studies showed that Aire regulated the expression of some microRNAs, including the miR-376 family members, which were located in the genome within an Aire-dependent TRA gene [182], and miR-29a, which affected Aire-dependent TRA expression and maintenance of TEC cellularity in aged mice [134, 183].

Also, it should be noted that more than half (60 %, estimated by [181]; 64 %, estimated by [184]) of total TRAs expressed in mTECs are Aire-independent [154, 157], indicating that additional transcriptional or epigenetic mechanisms must be responsible for the induction of Aire-independent gene expression in mTECs. Some Aire-independent TRAs are regulated by LTβR signaling [129, 185], although the downstream regulator(s) remain to be identified.

Although mTEC-dependent tolerance induction is essential for protection against autoimmunity, this system has a possible demerit in anti-tumor immunity. Because self-antigens expressed in mTECs include tumor-associated antigens, tumor-specific T cells can be deleted in the thymic medulla and fail to reach peripheral targets [165, 186–188]. Given the double-edged potential of mTECs and the medullary microenvironment, it seems reasonable that the capacity and function of the thymic medulla is finely modulated by the RANKL-OPG feedback mechanism [98, 120, 131]. It has been experimentally shown that suppression of RANKL-mediated mTEC development and maintenance can rescue tumor-specific T cells from

medullary deletion and attenuate tumor progression in mice [98, 131]. Further study may lead to the development of new therapeutic approaches to control T cell tolerance and anti-tumor immunity.

mTEC-DC interactions for central tolerance

In addition to mTECs as the lead player, thymic DCs also play a pivotal role in inducing T cell tolerance in the thymic medulla. Thymic DCs are predominantly localized in the medulla, with a small fraction sparsely localized in the cortex [138, 144]. As well as peripheral DCs, thymic DCs are derived from hematopoietic precursor cells [189], some through intrathymic differentiation and others from peripheral circulation [190].

It has been shown that thymic DCs contribute to T cell tolerance, through direct presentation of endogenously expressed antigens and indirect presentation of antigens expressed by other cells. Mtv-encoded superantigens as well as TRAs expressed by mTECs and blood-borne antigens can be presented by thymic DCs to developing thymocytes, to induce negative selection [191–194]. Furthermore, a subpopulation of peripheral DCs can be recruited to the thymic medulla and present peripheral antigens to induce negative selection [190, 195, 196].

Antigen presentation by mTECs and thymic DCs also induces development of Foxp3$^+$ Treg cells, which are essential for protection from autoimmunity [197, 198]. Foxp3$^+$ Treg cells differentiate from Foxp3$^-$ CD25$^+$ CD4SP or Foxp3$^+$ CD25$^-$ CD4SP precursor cells, a process that requires cytokine signals and TCR-CD28 co-stimulatory signals [199–201]. In the thymus, the majority of Foxp3$^+$ Treg cells are detected in the medulla [164, 202], where mTECs and DCs present antigens with co-stimulatory molecules. In mice deficient for mTEC development or expression of MHC class II on mTECs, thymic development of Foxp3$^+$ Treg cells is impaired [111, 112, 116, 203]. Studies using neo-self antigen transgenic mice showed the generation of Foxp3$^+$ Treg cells specific for self-antigen expressed by Aire$^+$ mTECs [164, 204]. It was also reported that Foxp3$^+$ Treg cells reactive to endogenous self-antigens are generated in an Aire-dependent manner [165, 166]. These data provide a link between Aire-dependent TRA expression and development of TRA-specific Treg cells. A recent report estimated that a substantial portion (about half) of Aire-dependent negative selection and Treg development are mediated by indirect presentation of TRAs by thymic DCs [205]. This mTEC-DC cooperation might be dependent on unidirectional, intercellular transfer of mTEC-derived proteins to DCs [206, 207]. For optimal Treg cell induction, these tripartite interactions among mTECs, DCs, and CD4SP thymocytes, require medullary accumulation of thymic DCs, which depends on the chemokine XCL1 [145], as well as CCR7-mediated medullary migration of CD4SP thymocytes [208]. Thymic DCs can also induce development of Foxp3$^+$ Tregs reactive to blood-borne antigens [194, 195].

Unique swirled epithelial structures composed of terminally differentiated mTECs, called 'Hassall's corpuscles', may provide the microenvironment for the generation of Treg cells. Hassall's corpuscles produce thymic stromal lymphopoietin (TSLP) [209], which was shown to activate immature thymic DCs to promote the expression of co-stimulatory molecules [210, 211]. TSLP-activated thymic DCs induce differentiation of CD4SP thymocytes into Foxp3$^+$ Tregs [209].

The number of thymic Foxp3$^+$ Tregs is likely controlled by the mTEC cellularity and size of the medulla, as the thymus from OPG-deficient mice contains the increased number of Foxp3$^+$ Tregs [131]. A recent report showed that the increased Foxp3$^+$ Tregs in OPG-deficient thymus included a substantial number of recirculating Tregs that re-entered the thymus from the periphery [212], suggesting that mTECs provide intrathymic niches for peripheral Tregs.

Unconventional T cell development in the medulla

Medullary TECs play a role in γδT cell development, in a manner different from that of cTECs. mTECs from fetal thymus express Skint1, a B7-family protein required for intrathymic maturation of Vγ5Vδ1$^+$ epidermal γδT cells [93, 213, 214]. Skint1 is considered to induce strong, agonist-like signals to Vγ5Vδ1 TCR and a differentiation program toward an IFNγ-producing lineage [215]. Given that cTECs and mTECs regulate distinct subpopulations of γδT cells, it is possible that cTECs and mTECs provide a distinct set of putative ligands or selecting molecules for modulating γδT cell immunity. In fetal mouse thymus, Vγ5Vδ1$^+$ γδT cells closely associate with mTECs, and foster Aire$^+$ mTEC development by expression of RANKL [93], implying a bidirectional crosstalk as well between RANKL-expressing γδT cells and Skint1-expressing mTECs.

It was also shown that mTECs were required for optimal maturation of iNKT cells and that developing iNKT cells express RANKL and CD40L to promote development of Aire$^+$ mTECs [127], suggesting that thymic crosstalk interactions also occur between iNKT cells and mTECs, although the intrathymic distribution of developing iNKT cells remains to be determined. Mature iNKT cells express the chemokine receptor CXCR3, which is required for thymic retention in response to its ligand CXCL10 produced in the medulla [216].

A series of studies demonstrated that the thymic medulla supports the development of natural IL-17-producing T

helper (nTh17) cells, a recently described unconventional $CD4^+$ αβT cell subset that potentially contributes to protective and pathological inflammatory responses. Intrathymic development of nTh17 cells requires MHC class II expression on mTECs but not on cTECs [217], and is induced by self-antigen recognition and the cytokines IL-6 and TGFβ [218]. It was also reported that RelB-dependent $Aire^+$ mTECs are required for nTh17 cell development [219], suggesting a novel mTEC-mediated regulatory mechanism of inflammatory and autoimmune responses.

Concluding remarks

Here, we focused on the developmental mechanisms and functions of thymic stromal cells—namely, TECs. cTECs and mTECs are derived from common progenitors and upon differentiation and maturation acquire distinct functional characteristics essential for supporting T cell development. cTECs shape the functional T cell repertoire through positive selection, whereas mTECs trim the self-reactive repertoire through negative selection and cell fate conversion into Tregs. The development of TEC subsets is largely dependent on the signals from developing T cells, and these crosstalk interactions are indispensable for organization and fine-tuning of the thymic microenvironment. It should also be noted that cTECs and mTECs are important for the development not only of conventional T cells but also of unconventional T cells that bridge innate and adaptive immunity. Given this cellular and molecular basis for orchestrating the development and function of thymic stromal cells, current and forthcoming studies will provide invaluable information toward in vivo regeneration and reconstitution of thymic tissue for future therapeutic application.

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