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Development and developmental potential of cortical thymic epithelial cells

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For Review Only

Summary

The thymic cortex provides a microenvironment for the development and positive selection of immature T cells. Cortical thymic epithelial cells (cTECs), which structurally and functionally support the thymic cortical microenvironment, originate from endodermal epithelial progenitors that arise in the third pharyngeal pouch. Recent studies have revealed that thymic epithelial progenitors pass through a stage where the cells express cTEC-associated molecules prior to lineage separation into cTECs and medullary TECs (mTECs). Here we review the molecular signatures of cTECs and highlight the development and developmental potential of cTECs.

Keywords

Thymus, cortex, cortical thymic epithelial cell, positive selection, thymic epithelial progenitor

Running title

Cortical thymic epithelial cells

Introduction

The thymus is a primary lymphoid organ that supports the development and repertoire selection of T cells. The thymic architecture is mostly divided into two distinct microenvironments, the cortex and the medulla, which are characterized by the presence of cortical thymic epithelial cells (cTECs) and medullary thymic epithelial cells (mTECs), respectively. The cortical and medullary thymic microenvironments differently contribute to T cell development; i.e., the cortex supports early T cell development and positive selection of immature thymocytes, whereas the medulla supports the establishment of self-tolerance in T cells.

T-lymphoid progenitors that migrate into the thymus parenchyma are induced to differentiate into T cells through the signals through Notch ligand DLL4 and γ c-cytokine IL-7, which are highly expressed in cTECs (1-4). Immature thymocytes are primarily detectable in the thymic cortex (5, 6), where the thymocytes are induced by DLL4 and IL-7 to express T-cell antigen receptor TCR $\alpha\beta$ as well as co-receptors CD4 and CD8. The V(D)J rearrangement in the TCR α and TCR β genomic loci in cortical thymocytes is responsible for the diversity in TCR recognition specificities carried by the pool of T cells. cTECs are also reported to contribute to the development of $\gamma\delta$ T cells (7).

Newly generated thymocytes that express TCRs carrying individual recognition specificities are selected for life or death according to their TCR recognition specificities, initially in the cortical microenvironment through the interaction with cTECs that express self-peptide-associated class I and class II MHC molecules (8). Only thymocytes that are signaled with low-affinity TCR engagement are selectively induced for cell survival. This process is termed positive selection. Positively selected

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4 thymocytes begin expressing chemokine receptor CCR7 and migrate to the medullary
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6 parenchyma where mTECs abundantly produce CCR7 ligand chemokines (9, 10). The
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8 migration to the medulla is important for T cells to establish tolerance to
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10 self-components, including tissue-restricted self-antigens (11-15). Only thymocytes that
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12 survive multiple layers of positive and negative selection in the cortical and medullary
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14 microenvironments are entitled to export to the circulation.
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18 Thus, cTECs are chiefly responsible for the early induction of T cell
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20 generation and the positive selection of newly generated T cells. Here we will initially
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22 provide a brief summary of molecular signatures expressed by cTECs, focusing on the
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24 functions and heterogeneity of cTECs. We will then discuss the development of cTECs
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26 as well as their developmental potential to give rise to mTECs.
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30 31 **cTECs provide microenvironment for early T cell development**

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33 cTECs express various molecules that support T cell lineage specification and
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35 regulate early T cell development in the thymic cortical microenvironment. As these
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37 aspects of cTEC functions have been reviewed previously (10, 15-17), here we only
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39 briefly list several molecules in this regard.
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43 44 *DLL4*

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46 The pioneering study by Schmitt and Zuniga-Pflücker unveiled the ability of
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48 Delta-mediated Notch signals to induce the lineage specification of early lymphoid
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50 progenitors to become T cells (18). It was later identified that among five mammalian
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52 Delta-like ligands, DLL4 is abundant in cTECs and responsible for T cell lineage
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54 commitment of early lymphoid progenitors and subsequent development to the
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4 CD4⁺CD8⁺ stage (1, 2). The expression of DLL4 in cTECs is negatively correlated
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6 with ontogeny, so that DLL4 expression in the thymic cortex decreases with age (19).
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9 Low levels of DLL4 may be sufficient for the maintenance of T-lymphopoiesis in the
10
11 adult thymus.

12 13 14 15 *Cytokines*

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17 Interleukin-7 (IL-7) is a γ c cytokine essential for the survival and
18
19 differentiation of immature lymphoid cells, including immature thymocytes. IL-7 is
20
21 produced by cTECs and mTECs, but is more abundant in cTECs than mTECs (20).
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23 Another cytokine kit-ligand (KL), also known as stem cell factor (SCF), which
24
25 promotes the survival and proliferation of immature thymocytes, is also more highly
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27 expressed in cTECs than mTECs (21).
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31 Transforming growth factor (TGF) β proteins, which are abundant in cTECs,
32
33 contribute to regulating the rate of the generation of CD4⁺CD8⁺ thymocytes from
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35 intermediate CD8^{low} precursor cells (22).
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40 41 *Chemokines*

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43 cTECs highly express chemokines CCL25 and CXCL12 as well as
44
45 chemokine-binding protein CCRL1 (15, 23, 24).
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47 During embryonic development, CCR9 ligand CCL25 produced by TECs in
48
49 the thymus primordium critically regulates the colonization of lymphoid progenitors, in
50
51 coordination with CCR7 ligand CCL21 produced by the neighboring parathyroid
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53 primordium, particularly before the vascularization of the thymus (25). The role of
54
55 chemokine signals through CCR9 and CCR7 ligands in the thymus seeding of T cell
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4 progenitors can also be detected in postnatal mice when lymphoid progenitor cells are
5 competitively transferred in radiation bone marrow chimera experiments (26, 27).
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8 CXCR4 ligand CXCL12 is detectable in cTECs throughout the cortex, and is
9 most abundantly expressed in the outer cortex (23). CXCL12 critically regulates early
10 thymocyte development by promoting the survival of immature thymocytes (28).
11 CXCL12 also plays a role in the appropriate positioning of immature thymocytes in the
12 thymic cortex (23).
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19 CCRL1, also known as Ccx-ckr1, is a non-signaling receptor for chemokines
20 CCL19, CCL21, and CCL25, and is more abundant in cTECs than mTECs (24). It was
21 reported that CCRL1 regulates thymus colonization before vascularization in fetal mice
22 (29) as well as optimal thymus homeostasis and normal thymocytes development in
23 adult mice (30).
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33 **cTECs organize microenvironment for T cell positive selection**

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35 cTECs provide the microenvironment for not only generating TCR-expressing
36 CD4⁺CD8⁺ thymocytes but also inducing positive selection of newly generated
37 CD4⁺CD8⁺ thymocytes. In addition to expressing self-peptide-associated class I and
38 class II MHC molecules for TCR recognition by CD4⁺CD8⁺ thymocytes, cTECs carry
39 unique protein degradation machineries that provide MHC-associated self-peptides that
40 optimize positive selection of thymocytes. These functions of cTECs have been
41 extensively reviewed elsewhere (14, 15, 31-34). Here we briefly provide an update of
42 the molecules involved in self-antigen presentation in cTECs.
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53 *Thymoproteasome*

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4 The thymoproteasome is a cTEC-specific form of the proteasome, which
5 cytoplasmically produces class I MHC-associated peptides. The thymoproteasome is
6 specifically expressed in cTECs, as its $\beta 5$ subunit, $\beta 5t$ encoded by *Psmb11*, is
7 exclusively abundant in cTECs and not in any other cells (35). Cells that express
8 thymoproteasomes display a unique repertoire of class I MHC-associated peptides (36).
9 An analysis of $\beta 5t$ -deficient mice suggested that thymoproteasome-dependent peptides
10 associated with class I MHC displayed by cTECs are enriched with low-affinity TCR
11 ligands, so that thymoproteasome-expressing cTECs are capable of inducing optimal
12 positive selection of functionally competent $CD8^+$ T cells (36, 37). A recent study using
13 monoclonal TCR-transgenic mice further revealed a novel aspect of
14 thymoproteasome-dependent positive selection, in which thymoproteasome-expressing
15 cTECs are crucial for not only shaping an immunocompetent TCR repertoire but also
16 fine-tuning TCR responsiveness in positively selected $CD8^+$ T cells (38).
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36 *Cathepsin L and thymus-specific serine protease*

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38 Cathepsin L is a ubiquitously expressed lysosomal endopeptidase. In the
39 thymus, cTECs abundantly express cathepsin L, whereas other antigen-presenting cells,
40 including mTECs, predominantly express cathepsin S rather than cathepsin L. In
41 addition to its role in the degradation of invariant chain Ii, which is assembled with
42 class II MHC molecules (39, 40), cathepsin L in cTECs is involved in the generation of
43 class II MHC-associated self-peptides for positive selection of $CD4^+$ T cells (31, 41).
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51 Thymus-specific serine protease (Tssp), which is encoded by *Prss16*, was
52 initially reported in the human genome for its association with the susceptibility to type
53 I diabetes (42). Tssp is highly expressed in cTECs (43). Tssp-deficient mice exhibit a
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4 decrease in class II MHC expression in cTECs and partial impairment in positive
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6 selection of CD4⁺ T cells (44-46). The role of Tssp in tumor regulation is also noted
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8 (47).
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10 11 12 13 *Autophagy*

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15 Autophagy, or macroautophagy, is an intracellular protein degradation system
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17 that is activated by nutrient starvation (48, 49). However, autophagy is constitutively
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19 active in the thymus, especially among TECs including many cTECs, even without the
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21 starvation (49, 50). As autophagosomes fuse with lysosomes for proteolysis, autophagy
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23 contributes to providing cytosolic protein antigens to the class II MHC presentation
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25 pathway. Indeed, autophagy in cTECs has been shown to contribute to the optimal
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27 positive selection of CD4⁺ T cells (50).
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33 **Heterogeneity in cTECs and thymic nurse cells**

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35 Considering that cTECs play multiple roles in T cell development by
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37 promoting the early induction of T cell generation and by supporting positive selection,
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39 it is tempting to speculate that cTECs consist of functionally distinct subpopulations
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41 that individually play different roles in T cell development. It was reported that mTECs
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43 consist of at least two clearly distinct and functionally potent subpopulations, namely,
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45 Aire-expressing self-antigen-producing mTECs and CCR7-ligand-expressing
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47 thymocyte-attracting mTECs (51). However, it has been shown that the majority of
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49 cTECs express DLL4, IL-7, class II MHC, β 5t, and CD205 (1, 4, 52, 53), suggesting
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51 that T cell development-inducing cTECs and positive selection-inducing cTECs are
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53 overlapped with each other.
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4 cTECs are defined as epithelial cells localized in the cortex of the thymus.
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6 Thanks to recent progress in the molecular biology of thymic non-hematopoietic cells,
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8 as outlined above, cTECs can now be identified and isolated on the basis of the
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10 expression of CD205, Ly51 (CD249), and EpCAM (CD326). Other classical markers,
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12 such as class II MHC, keratin 8, and ER-TR4, as well as more recently identified
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14 functional molecules, such as DLL4, IL-7, CCRL1, and $\beta 5t$, are additionally useful for
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16 the identification and characterization of cTECs. The undetectable expression of mTEC
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18 markers, such as keratin 5, keratin 14, MTS-10, ER-TR5, and Aire, as well as the
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20 reactivity to the lectin *Ulex europaeus* agglutinin 1 (UEA-1), can also offer clues for the
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22 identification of cTECs. Measuring the expression of these molecules has inspired
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24 studies of the heterogeneity in cTECs on a single cell basis. By detecting the expression
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26 levels of these molecules, it has been shown that cTECs are heterogeneous most
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28 obviously in class II MHC expression, consisting of class II MHC^{low} and class II
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30 MHC^{high} populations (54). Heterogeneity in the expression levels of DLL4, IL-7,
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32 CD205, and other molecules has also been noted (4, 11, 52, 55).
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38 It is interesting to note that thymic nurse cells (TNCs) represent a functionally
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40 distinct subpopulation of cTECs. TNCs are large TECs that envelop many thymocytes
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42 (56). Since their discovery more than 30 years ago (57, 58), many researches have tried
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44 to uncover their functions. There have been suggestions that TNCs provide the
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46 microenvironments that support the proliferation and differentiation of cortical
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48 thymocytes (59-63) as well as positive and negative selection of thymocytes (64-66).
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50 Recently, Nakagawa, et al. (67) reported that approximately 10% of $\beta 5t$ -expressing
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52 cTECs in adult mouse thymus can be defined as TNCs that completely envelop many
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54 CD4⁺CD8⁺ thymocytes. It was shown that TNCs are not necessary for thymocyte
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4 positive and negative selection, as TNCs were hardly detected in the thymus of
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6 TCR-transgenic mice in that positive or negative selection was readily detectable (67).
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8 Rather, thymocytes confined within TNCs were enriched with long-lived CD4⁺CD8⁺
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10 thymocytes that underwent secondary TCR α rearrangement (67). Therefore, it was
11
12 suggested that TNCs, which represent a subpopulation of cTECs, provide a
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14 microenvironment for the optimal TCR repertoire selection of CD4⁺CD8⁺ thymocytes
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16 through the secondary TCR α rearrangement. Heterogeneity in gene expression profiles
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18 between TNCs and non-TNC cTECs has also been noted (67).
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24 **Ontogeny of cTECs**

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26 Like mTECs, cTECs originate from the endodermal epithelium of the third
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28 pharyngeal pouch (68, 69). Bipotent thymic epithelial progenitors (pTECs) that give rise
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30 to cTECs and mTECs have been detected in embryonic and postnatal thymus (70-72).
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32 In mouse, TECs are detectable as early as embryonic day 11 (E11) by the landmark
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34 expression of Foxn1, a member of the forkhead family of transcription factors that
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36 specify TECs and hair follicle cells (68, 73-75). Spontaneous mutations in Foxn1 lead to
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38 congenital thymic hypoplasia accompanied by severe T cell deficiency in mouse and
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40 human (74-77). Even without Foxn1, keratin-expressing epithelial cells are detectable in
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42 the third pharyngeal pouch, although thymic architecture supported by cTECs and
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44 mTECs is not subsequently formed (75), indicating that Foxn1 is indispensable for the
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46 development of TECs rather than the formation of the third pharyngeal pouch or its
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48 epithelial layers. The differentiation of cTECs from bipotent progenitors is initiated as
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50 early as embryonic day 12 (E12) in mouse (52, 53). Two recent papers have described
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52 the ontogenetic development of cTECs.
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4 Shakib, et al. (52) reported the successive developmental stages of cTECs
5 defined by the expression of CD205 and co-stimulatory molecule CD40 during mouse
6 ontogeny. In Foxn1-deficient mice, the thymus primordium expressed neither CD205
7 nor CD40. CD205⁺ TECs emerged at E12 without detectable CD40 and gradually
8 acquired CD40 expression along with the increase in CD205 expression level during
9 embryogenesis. Isolated CD205⁺ CD40⁻ embryonic TECs expressed β 5t and cathepsin
10 L genes, but were heterogeneous in the expression of class II MHC. Neither CD40 nor
11 class II MHC was expressed in CD205⁺ cTECs in hCD3 ϵ tg26 mice, in which early
12 thymocytes development was defective (52, 78). These results suggest that cTEC
13 development occurs initially through the CD205⁺ CD40⁻ stage and the subsequent
14 elevation of CD40 and class II MHC to give rise to CD205⁺ CD40⁺ class II MHC^{high}
15 cTECs, and that thymocyte development influences the late phase of cTEC
16 development (*Fig. 1*).

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34 Mat Ripen, et al. (53) reported the ontogeny of β 5t-expressing cTECs in
35 mouse. β 5t-expressing cells were detectable as early as E12.5, specifically in the
36 thymus and in CD205-expressing cTECs. The expression levels of CD205 and CD249
37 as well as class II MHC were gradually elevated in β 5t-expressing TECs during
38 ontogeny, suggesting that β 5t is expressed by cTECs at both immature and mature
39 stages. In support of this finding, β 5t expression in cTECs was detectable even in
40 hCD3 ϵ tg26 mice. β 5t was undetectable in the thymus primordium of Foxn1-deficient
41 mice, whereas β 5t was present in abundance in relB-deficient mice that lacked mTECs.
42 Thus, like CD205, β 5t is expressed at the initial appearance stage of cTECs in
43 Foxn1-dependent manner, but independent of thymocytes or mTECs (*Fig. 1*).

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57 The molecular mechanisms regulating cTEC development are vague, in
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4 contrast to the roles of TNFSF cytokine receptors, including RANK, CD40, and
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6 lymphotoxin β receptor, and the downstream signaling pathways for the activation of
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8 NF- κ B transcription factors, which have been extensively documented in mTECs
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10 (79-85). Experiments conducted in hCD3 ϵ tg26 mice, in which thymocyte development
11
12 is arrested at the very early DN1 stage, have shown that the thymic cortex is
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14 disorganized and cTECs are arrested at the CD40⁻ MHC II^{low} stage (52, 86, 87). In
15
16 contrast, cTECs are fully capable of giving rise to the CD40⁺ class II MHC^{high} stage
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18 even in Rag1-deficient mice, in which T cell development is arrested at the DN3 stage
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20 (52). Thus, cTECs require signals from developing thymocytes beyond the DN1 stage
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22 for optimal development (*Fig. 1*).
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27 In addition to developing thymocytes, mesenchymal cells in the thymus
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29 contribute to the development of cTECs. Fibroblast growth factor (FGF)-7 (also known
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31 as KGF), FGF-10, and insulin-like growth factor 1 (IGF-1) produced by mesenchymal
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33 cells promote the proliferation of cTECs and mTECs (88-92). In contrast, mesenchymal
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35 cell-derived retinoic acid (RA) negatively affects the cellularity of cTECs and mTECs,
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37 whereas the blockade of RA signaling increases the cellularity of cTECs and elevates
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39 the expression of DLL4, β 5t, and Tssp in fetal thymus organ culture (93) (*Fig. 1*). It
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41 remains unclear how RA affects cTECs.
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47 **Cells that express cTEC-associated molecules give rise to cTECs and mTECs**

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49 Through further analysis of cTEC development, recent studies have
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51 independently and unexpectedly reported that mTECs are derived from bipotent
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53 progenitors that express cTEC-associated molecules.
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56 Baik, et al. (94) examined the developmental potential of embryonic TECs
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4 expressing cTEC-associated molecule CD205. They cultured fetal thymuses isolated
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6 from E11 and E12 mouse embryos in the presence or absence of agonistic anti-RANK
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8 antibodies that were capable of promoting the development of mTECs, and analyzed the
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10 expression of TEC maturation markers CD40 and MHC II in CD205^{negative},
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12 CD205^{low}, and CD205^{high} TEC populations. All of these populations responded to
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14 RANK stimulation by expressing CD40 and MHC II in 1-day culture of E12 thymuses,
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16 and the frequency of CD40⁺ MHC II⁺ cells progressively increased with the elevation
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18 of CD205 expression levels. On the other hand, E11 thymus cells did not respond to
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20 RANK stimulation; the cells failed to express CD40 and MHC II in the 1-day culture
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22 experiments. These results suggest that CD205⁺ embryonic TECs serially acquire the
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24 potential to give rise to CD40⁺ MHC II⁺ mTECs. They further showed that highly
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26 purified CD205⁺ CD40⁻ embryonic TECs, which expressed a set of cTEC-associated
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28 molecules (52), could differentiate into both Aire-expressing mTECs and
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30 β 5t-expressing cTECs in *in vitro* reaggregate thymus organ culture followed by
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32 transplantation of the aggregates under mouse kidney capsules (94). Thus, CD205⁺
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34 embryonic TECs, which resemble cTECs, carry bipotent progenitor capability that gives
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36 rise to both cTECs and mTECs.
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44 Ribeiro, et al. (20) studied the developmental potential of TECs expressing
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46 IL-7 in IL-7-reporter transgenic mice, in which the IL-7 promoter drove the gene
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48 encoding yellow fluorescence protein (YFP). They found that the majority of YFP⁺
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50 cells were enriched in CD205⁺ Ly51⁺ cTECs through the ontogeny, whereas CD80⁺
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52 mTECs were predominantly detectable in YFP⁻ cells. In addition to those cell-surface
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54 molecules, YFP⁺ cells expressed other cTEC-associated genes, including DLL4, β 5t,
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4 and Tssp, whereas YFP⁻ cells contained other mTEC-associated genes, including Aire
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6 and RANK. Thus, YFP⁺ and YFP⁻ thymic cells predominantly contained cTECs and
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8 mTECs, respectively. They further looked into the developmental potential of YFP⁺
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10 and YFP⁻ TECs isolated from embryonic thymus in *in vitro* reaggregate thymus organ
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12 cultures, and found that both YFP⁺ and YFP⁻ TECs gave rise to Ly51⁺ cTECs and
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14 CD80⁺ mTECs. These results indicate that embryonic TECs that express high levels of
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16 IL-7 and so resemble cTECs retain the differentiation potential into mTECs.
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18 Subsequently, they also examined the developmental potential of embryonic cTECs by
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20 detecting another cTEC-associated molecule, CCRL1, using CCRL1-EGFP-knockin
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22 mice, in which EGFP is expressed under the control of CCRL1 gene expression (95).
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24 The expression of CCRL1-dependent EGFP in the thymus was detectable as early as
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26 E13.5, and gradually increased during ontogeny. *In vitro* reaggregate thymus organ
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28 culture experiments demonstrated that CCRL1-EGFP⁺ TECs gave rise to
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30 UEA1⁺CD80⁺ mTECs in the presence of RANK and CD40 stimulation (95). Therefore,
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32 CCRL1⁺ embryonic TECs, which resemble cTECs, retain developmental potential to
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34 give rise to mTECs.
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42 Those studies by Baik, et al. (94) and Ribeiro, et al. (20, 95) examined the
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44 developmental potential of embryonic cTECs essentially by *in vitro* cell culture
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46 experiments with or without subsequent *in vivo* transplantation in mice. In contrast, in
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48 our recent study, we examined the developmental potential of cTECs by *in vivo* fate
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50 mapping experiments, which enabled the characterization of normally developed
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52 mTECs without employing *in vitro* cell cultures or invasive transplantation surgeries.
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54 To do so, we engineered mice in that the coding sequence of the cTEC-specific gene,
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4 $\beta 5t$, was replaced with Cre recombinase, and crossed those mice with
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6 CAG-loxP-stop-loxP-EGFP-transgenic reporter mice, in which EGFP would be driven
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8 under the control of the CAG promoter only when the loxP-flanked stop sequences were
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10 excised by Cre expression (96). In those mice, EGFP expression reflected present
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12 and/or past expression of $\beta 5t$ in cells. We found that $\beta 5t$ -Cre-mediated EGFP
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14 expression could be detected in TECs but not in other cells in the thymus or other
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16 organs. Among TECs, $\beta 5t$ -Cre-mediated EGFP was expressed in almost all mTECs as
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18 well as in almost all cTECs throughout the ontogeny. As mTECs do not presently
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20 express $\beta 5t$, these results indicate that the majority of mTECs originate from cells that
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22 express $\beta 5t$ (96).
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27 The expression of $\beta 5t$ -Cre-mediated EGFP is detectable in TECs as early as
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29 E12.75, approximately half a day after the first detection of $\beta 5t$ protein in TECs (53, 96).
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31 However, $\beta 5t$ protein is no longer detectable in EGFP⁺ mTECs localized in the central
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33 region of E12.75 thymus. These results suggest that mTECs derived from $\beta 5t$ ⁺ TEC
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35 progenitors lose the ability to express $\beta 5t$ soon after the commitment to become mTECs
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37 (96). Perinatal $\beta 5t$ ⁺ TECs, which resemble cTECs, are indeed bipotent, giving rise to
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39 cTECs and mTECs as shown in the reaggregate organ culture and kidney
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41 transplantation experiments (97).
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46 These studies from at least three independent laboratories have collectively
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48 proposed a novel concept for the TEC differentiation pathways, particularly the
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50 mechanisms of how cTECs and mTECs are diversified from their common progenitors;
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52 i.e., bipotent TEC progenitors progress through the stage that exhibits the molecular
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54 signatures of cTECs, including the expression of CD205, IL-7, and $\beta 5t$, prior to
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4 commitment to the cTEC and mTEC lineages (98) (*Fig. 2*). As these bipotent TEC
5 progenitors express cTEC-associated molecules, these progenitors can be viewed by
6 definition as a fraction of cTECs. Previously reported embryonic cTECs may contain,
7 or even represent, the bipotent TEC progenitors expressing cTEC-associated molecules.
8 In addition, it can be interpreted that a fraction of cTECs carry developmental potential
9 to give rise to mTECs. The concept of “serial progression” of cTECs and mTECs,
10 agrees with the earlier development of cTECs than mTECs in ontogeny and with the
11 necessity for mTECs to establish medullary self-tolerance only when cTECs are
12 functionally competent to induce and positively select T cells.
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26 **Perinatal and postnatal mTEC progenitors that express cTEC molecules**

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28 Several studies have reported the existence of bipotent TEC progenitors in the
29 adult thymus (71, 72, 99). Accordingly, Ohigashi, et al. (100) and Mayer, et al. (97)
30 examined the developmental potential of $\beta 5t^+$ bipotent TEC progenitors in a given
31 period by employing $\beta 5t$ -rtTA knock-in mice, in which reverse tetracycline
32 transactivator (rtTA)-encoding sequence was inserted in the $\beta 5t$ locus. $\beta 5t$ -rtTA
33 knock-in mice were crossed with Tet operator-driven Cre transgenic mice along with
34 loxP-dependent EGFP or ZsGreen reporter mice, which allowed *in vivo* tracing of cells
35 that transcribed $\beta 5t$ during a given period by tracing fluorescent cells labeled by
36 doxycycline (Dox) administered during that period (97, 100). The tracing of
37 fluorescence-labeled mTECs in adult mice revealed that approximately 60% of cells
38 were embryonically labeled, and approximately 30% of mTECs were
39 fluorescence-labeled during the first week of life (100). In sum, at least 90% of mTECs
40 in the adult thymus are derived from progenitors that transcribe $\beta 5t$ during
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4 embryogenesis and the neonatal period up to 1 week of age. These frequencies of
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6 embryonically and neonatally labeled cells within mTECs remained unchanged at least
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8 up to 45 weeks of age (100). The fluorescence-labeled mTECs included class II
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10 MHC^{high} mTECs, which contained Aire⁺ cells, and class II MHC^{low} mTECs, which
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12 contained CCR7-ligand-expressing cells (51, 100). Embryonically and neonatally
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14 labeled mTECs similarly expressed genes that were functionally relevant in mTECs, but
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16 were not identical with respect to the spectrum of promiscuously expressed
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18 self-antigens, including the fetal antigen, α -fetoprotein (100). These results indicate that
19
20 embryonic and neonatal $\beta 5t^+$ progenitors are capable of forming functional mTEC
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22 subpopulations.
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27 In contrast to these perinatal $\beta 5t^+$ progenitors, the contribution of adult $\beta 5t^+$
28
29 progenitors to the *de novo* generation of mTECs in adult thymus was minor. The
30
31 frequency of fluorescence-labeled mTECs dropped to approximately 3-5% in mice that
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33 were Dox-treated after 1 week of age (100). These fluorescence-labeled mTECs might
34
35 in part reflect the promiscuous expression of $\beta 5t$ gene in a small fraction of mTECs.
36
37 However, the fluorescence in mTECs remained detectable even several months after
38
39 Dox treatment and so possibly reflected the contribution of adult $\beta 5t^+$ progenitors in the
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41 long-term maintenance of mTECs in the adult period albeit at a low frequency (97).
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43 Nevertheless, these results indicate that unlike perinatal $\beta 5t^+$ progenitors, adult $\beta 5t^+$
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45 progenitors play only a minor role in the maintenance of mTECs in the adult thymus
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50 (*Fig. 3*).
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53 Considering the active proliferation and rapid turnover of mTECs in the adult
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55 thymus (101-103), it is conceivable that mTEC-lineage committed cells that exceed the
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4 $\beta 5t^+$ bipotent stage during early ontogeny, rather than postnatal bipotent TEC
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6 progenitors, mainly maintain adult mTECs via active proliferation throughout life (*Fig.*
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8 3). It is even possible that mTECs are actually maintained by the continuous
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10 self-duplication of mTECs (*Fig. 3*), as observed in other epithelial tissues, such as
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12 pancreas and liver (104, 105).
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16 In this regard, Sekai, et al. (106) recently reported that
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18 mTEC-lineage-restricted progenitor/stem cells, which are capable of maintaining
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20 functional mTECs, can be defined as claudin-3/4⁺ SSEA1⁺ cells. In collaborative
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22 experiments with their laboratory, we have shown that the majority of those
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24 claudin-3/4⁺ SSEA1⁺ mTEC-lineage-restricted progenitor/stem cells detectable in the
25
26 adult thymus are derived from perinatal $\beta 5t^+$ progenitors (100). Thus, it is possible that
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28 mTEC-lineage-restricted progenitor/stem cells contribute to the maintenance of mTECs
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30 in the adult thymus (*Fig. 3*).
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35 Where do bipotent TEC progenitors localize in the thymus? Classically, it was
36
37 shown that K5⁺ K8⁺ TECs, which were presumed to contain bipotent TEC progenitors,
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39 were enriched at the cortico-medullary junction in the adult thymus (87, 107). More
40
41 recently, perinatally labeled $\beta 5t^+$ TEC progenitors were detected at the
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43 cortico-medullary junction in the adult thymus (97). It is therefore possible that bipotent
44
45 TEC progenitors localize at the cortico-medullary junction in the adult thymus, to
46
47 supply cTECs to the cortex and mTECs to the medulla (*Fig. 4*). Alternatively, the
48
49 localization of bipotent TEC progenitors in the adult thymus is not limited to the
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51 cortico-medullary junction in the thymus. Rather, they produce cTECs and mTECs to
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53 newly generate the microenvironments of the cortex and the medulla, respectively, in
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55 the areas neighboring the place where the progenitors localize, so that the
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4 cortico-medullary junction is consequently formed wherever bipotent TEC progenitors
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6 are present in the thymus parenchyma (*Fig. 4*).
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10 **Postnatal maintenance of cTECs**

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12 The use of $\beta 5t$ -rtTA knock-in-dependent TetO-Cre-mediated fluorescence
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14 reporter mice enabled the analysis of the postnatal maintenance of cTECs with respect
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16 to the contribution and decay of cTECs that express $\beta 5t$ in a given time period. The
17
18 fluorescence labeling of cTECs in these mice could reflect either the current expression
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20 of $\beta 5t$ in cTECs or the past expression of $\beta 5t$ during the differentiation from $\beta 5t^+$
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22 bipotent TEC progenitors. We found that the majority of cTECs in the adult thymus are
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24 fluorescence-labeled by Dox administered during either embryogenesis or the neonatal
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26 period (97, 100). The frequency of the embryonically and neonatally labeled cTECs
27
28 gradually decreased to approximately 70% by 45 weeks old, suggesting that
29
30 approximately two-thirds of cTECs in the adult thymus are maintained by cells that are
31
32 derived from embryonic or neonatal $\beta 5t$ -expressing cells, whereas approximately
33
34 one-third of adult cTECs are *de novo* generated in adult mice (100). Thus, the postnatal
35
36 dynamics for the generation and maintenance of cTECs appears to differ from that of
37
38 mTECs. Unlike mTECs, a considerable portion of cTECs in the adult thymus may be *de*
39
40 *novo* generated in adult life.
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49 **Age-dependent damage in cTECs**

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51 The thymus is one of the most susceptible organs to age-dependent atrophy, or
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53 involution. Thymic involution leads to a decline in *de novo* T cell production and in the
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55 diversity of T cell repertoires, thereby resulting in the deterioration of the immune
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4 system (108). The cortical compartment of the thymus, which is predominantly
5
6 composed of cTECs and CD4⁺CD8⁺ thymocytes, is highly susceptible to the involution
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8 (108, 109). A recent study by Griffith, et al. (110) revealed that reactive oxygen species
9
10 contribute to the early senescence of cTECs during age-dependent thymic involution.
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12 Through global transcriptome analysis and transgenic overexpression experiments, they
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14 showed that the expression of the antioxidant enzyme, catalase, is reduced in TECs,
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16 particularly in cTECs, and that either the transgenic overexpression of catalase or the
17
18 administration of antioxidants diminishes thymic atrophy. These results suggest that
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20 metabolic damage in cTECs by catalase deficiency and thereby by the accumulation of
21
22 reactive oxygen species plays an important role in the age-dependent loss of cTECs.
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27 Age-dependent thymic involution is correlated with the decrease in Foxn1
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29 expression in TECs (111). Genetic manipulation to reduce Foxn1 expression in the
30
31 postnatal thymus leads to a decrease in TEC cellularity, whereas overexpression of
32
33 Foxn1 in aged mice restores the number of TECs (112-115). Inactivation of
34
35 retinoblastoma (RB) protein enhances Foxn1 expression by activating E2F transcription
36
37 factors, so that the RB-E2F transcriptional pathway regulates Foxn1 expression.
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39 Inactivated RB protein in TECs decreases with age (116), whereas the decrease in E2F3
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41 transcription activity in cTECs and MHC II^{low} mTECs is correlated with thymic
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43 involution (21). Age-associated decrease in Wnt4, which promotes Foxn1 expression, is
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45 also correlated with the decrease in Foxn1 expression (21, 117, 118).
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50 In the human thymus, the secretion of proinflammatory cytokines, including
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52 IL-6, is elevated in an age-dependent manner (119). In mouse, many proinflammatory
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54 cytokine genes, including *Il1a*, *Il1b*, *Cxcl2*, *Il6*, *Il12b*, *Il18*, and *Tnf*, are elevated in
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56 thymic dendritic cells in aged thymus, whereas cTECs and thymic fibroblasts express
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4 IL-1 activating receptor gene *Il1r1* rather than IL-1 antagonists *Il1rn* and *Il1r2*, which
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6 are detectable in mTECs (21). In mice deficient in inflammasome *Nlrp3*, active IL-1 β is
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8 reduced and cTECs are maintained in aged thymus (120). Thus, the elevated expression
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10 of proinflammatory cytokines may contribute to the age-dependent damage in cTECs.
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14 15 **Injury and regeneration of cTECs**

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17 Thymic involution is induced not only by the ageing but also by other stresses,
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19 including irradiation, infection, and chemotherapeutic drugs. In contrast to
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21 age-dependent thymic involution, stress-induced thymic injuries are transient and
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23 regenerable. Rode and Boehm (24) engineered *Ccx-ckr1*-diphtheria toxin receptor
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25 (DTR)-transgenic mice, in which the *Ccx-ckr1* promoter drives DTR expression. In
26
27 these mice, transient treatment with diphtheria toxin (DT) efficiently ablated cTECs
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29 accompanied by the decrease in thymocytes, and cessation of the DT treatment led to
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31 the recovery of both cTECs and thymocytes. These results indicate that cTECs carry
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33 regenerative potential to counter injury-triggered thymic involution. Upon irradiation,
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35 radio-resistant lymphoid tissue inducer cells promote the production of IL-22 in an
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37 IL-23-dependent manner (121). The IL-22 receptor is expressed in cTECs and mTECs,
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39 and IL-22 promotes the increase in the number of cTECs and MHC II^{low} mTECs in
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41 irradiated thymus, contributing to the repair of the thymic cortex and medulla (121).
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47 During the post-injury thymic repair, cTECs and mTECs are regenerated from
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49 injury-resistant cells that could be either bipotent progenitors or lineage-restricted cells.
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51 We recently examined the contribution of $\beta 5t^+$ bipotent progenitors during
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53 injury-triggered thymic regeneration, by employing $\beta 5t$ -rtTA x tetO-Cre x GFP-reporter
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55 mice. We found that embryonic and neonatal, rather than adult, $\beta 5t^+$ progenitors
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4 contributed to the majority of mTECs in the thymus regenerated from either total body
5 irradiation or polyinosinic-polycytidylic acid treatment, which mimicked viral
6 double-stranded RNAs and induced interferon- α -mediated injury in TECs. Similar to
7
8 mTECs, the *de novo* generation of cTECs was not enhanced during the injury-triggered
9
10 thymus regeneration (100). Therefore, the injury-triggered regeneration of mTECs is
11
12 mainly mediated by cells that are derived from perinatal $\beta 5t^+$ TEC progenitors, rather
13
14 than by cells derived from adult $\beta 5t^+$ TEC progenitors (*Fig. 5*). Self-duplication or
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16 lineage-committed progenitors likely contribute to the regeneration of most cTECs and
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18 mTECs.
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27 **Sex hormones affect cTECs**

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29 TECs are highly dynamic and exhibit continuous turnover. It was estimated
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31 that approximately 10% of TECs in the adult thymus are newly supplied daily, and
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33 TECs turnover occurs every 10 to 14 days (101). cTECs and mTECs proliferate at a
34
35 similar rate in young mice, but the proliferation rate of cTECs become lower than that
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37 of mTECs in aged mice (122). The ablation of male sex hormones by castration
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39 promotes the regeneration of the thymus in aged mice by enhancing the proliferation of
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41 TECs and thymocytes (101).
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45 Dumont-Lagacé, et al. (123) reported that the expression in cTECs of
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47 molecules associated with cTEC functions, including Foxn1, DLL4, CCL25, $\beta 5t$, and
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49 cathepsin L, was lower in males than females or castrated males. The proliferation of
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51 cTECs was less active in males than females or castrated males, whereas mTEC
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53 proliferation was little affected by gender. They also reported that the cellularity of
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55 cTECs was higher in males than females or castrated males, which could be associated
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4 with the higher expression of cell death inhibitors and the lower expression of cell death
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6 activators (123). However, we detected lower cellularity of cTECs in male mice than
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8 female mice (100). The regeneration of cTECs in DT-treated Ccx-ckr1-DTR-transgenic
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10 mice was reduced in males compared with females or castrated males (24). Nonetheless,
11
12 the contribution of embryonic, neonatal, and adult $\beta 5t^+$ progenitors in the generation,
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14 maintenance, and injury-triggered regeneration was essentially comparable between
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16 female and male mice (100). Thus, gender and sex hormones strongly affect cTECs.
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22 **Epithelial-mesenchymal transition in the thymus**

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24 Epithelial-mesenchymal transition (EMT) is a process that allows an epithelial
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26 cell to change into a mesenchymal cell, and contributes to various phases of
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28 development, tumor metastasis, and tissue repair fibrosis (124). In the thymus, EMT has
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30 been suggested to contribute to tissue adipogenesis associated with age-dependent
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32 involution (125-127).
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36 The $\beta 5t$ -Cre x loxP-EGFP mice enabled efficient labeling of virtually all
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38 TECs (96) and were so useful for the quantitative analysis of EMT in young and aged
39
40 mice. Immunofluorescence analysis of thymic sections showed that a fraction of
41
42 MTS15⁺ mesenchymal fibroblasts were EGFP⁺ in 2-week-old and 11-month-old mice
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44 (*Fig. 6A*). Flow cytometric analysis indicated that the frequency of EGFP⁺ cells in
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46 CD45-PDGFR α^+ mesenchymal cells was approximately 10% and comparable between
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48 2-week-old mice and 11-month-old mice (*Fig. 6B*). These results suggest that EMT is
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50 detectable in young mice and is not greatly elevated by age, at least up to 11 months
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52 old.
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Concluding remarks

In this review, we summarized current knowledge of cTEC biology, focusing on the development and developmental potential of cTECs. Despite the importance of cTECs in the development and selection of T cells, little is known about the molecular mechanisms underlying the development of cTECs. Important issues to be addressed in this regard include the molecular mechanisms that specify cTEC lineage from bipotent progenitors and that induce thymocyte-dependent maturation of cTECs. The recent finding that bipotent TEC progenitors transiently express cTEC-associated molecules, such as $\beta 5t$ and CD205, may provide a useful clue useful to unravel the molecular mechanisms that regulate the bifurcation of cTECs and mTECs as well as the subsequent development of cTECs and mTECs.

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Figure legends

Fig. 1. Phenotypic progression of cTECs during ontogeny. cTEC expression of cell-surface molecules including MHC II, CD40, CD205, Ly51, and CCRL1 increases along the ontogeny, whereas EpCAM cell-surface expression declines. The expression of $\beta 5t$ and high levels of IL-7 is also detectable in cTECs throughout the ontogeny. The development of cTECs is regulated by signals provided by developing thymocytes. FGF-7, FGF-10, and IGF-1 produced by mesenchymal cells promote the proliferation of cTECs, whereas mesenchymal cell-derived RA negatively affects the cellularity of cTECs.

Fig. 2. mTECs are derived from cells that express cTEC-associated molecules. Bipotent TEC progenitors progress through the stage in that cells express cTEC-associated molecules, including $\beta 5t$, CD205, CCRL1, and high levels of IL-7, prior to the lineage specification into cTECs and mTECs. cTECs retain the expression of these molecules, which is down-regulated in mTECs.

Fig. 3. Adult mTECs are maintained by mTEC-lineage-restricted cells that pass beyond the bipotent stage during early ontogeny. Adult mTECs are maintained by cells that pass through the $\beta 5t^+$ bipotent stage rather than by bipotent progenitors. It is possible that adult mTECs are maintained by the continuous self-duplication of mTECs. Alternatively, mTEC progenitor/stem cells may contribute to the maintenance of mTECs in the adult thymus.

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4 **Fig. 4. Two hypotheses regarding the localization of bipotent TEC progenitors. (A)**

5 Bipotent TEC progenitors in the adult thymus localize at the cortico-medullary junction
6 to supply cTECs into the cortex and mTECs into the medulla. (B) The localization of
7 bipotent TEC progenitors in the adult thymus is not limited to the cortico-medullary
8 junction but can be anywhere in the parenchyma. Bipotent TEC progenitors newly
9 produce cTECs and mTECs, which generate the microenvironments of the cortex and
10 the medulla, respectively. Consequently, the cortico-medullary junction will be formed
11 in the area where bipotent TEC progenitors originally reside.
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24 **Fig.5. Contribution of perinatal versus adult $\beta 5t^+$ TEC progenitors in the**

25 **development, maintenance, and regeneration of adult mTECs.** The majority of adult
26 mTECs are maintained and regenerated by cells that pass beyond the $\beta 5t^+$ bipotent
27 stage during embryogenesis (red line) and neonatal period (blue line). The contribution
28 of adult $\beta 5t^+$ TEC progenitors is minor, even during injury-triggered mTEC
29 regeneration (green line).
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41 **Fig. 6. Epithelial-mesenchymal transition in the thymus. (A)** Immunofluorescence

42 analysis of the thymus obtained from $\beta 5t$ -Cre-knockin mice crossed with
43 CAG-loxP-stop-loxP-EGFP-transgenic reporter mice (abbreviated as $\beta 5t$ -Cre x
44 loxP-EGFP mice). Thymus sections were examined in 2-week-old (wo) and
45 11-month-old (mo) mice for the expression of EGFP (green), K5 (blue), and MTS15
46 (red). Data are representative of at least three separate experiments. Scale bar = 25 μ m.
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54 **(B)** Representative flow cytometry profiles of collagenase-digested thymus cells from

55 $\beta 5t$ -Cre x loxP-EGFP mice. Cells were multi-color-stained for CD45, PDGFR α , and
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4 propidium iodide (PI). Histograms show EGFP expression profiles in
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6 PI-CD45-PDGFR α^+ viable thymic mesenchymal cells (left panels) in β 5t-Cre x
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8 loxP-EGFP mice (solid lines) and littermate control β 5t-Cre-knockin mice (shaded
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10 lines). Numbers in histograms indicate frequencies within the indicated area. Bar graph
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12 shows the frequency (means and SEs, n = 3) of EGFP $^+$ cells in PI-CD45-PDGFR α^+
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14 cells (right panel). n.s., not significant.
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For Review Only

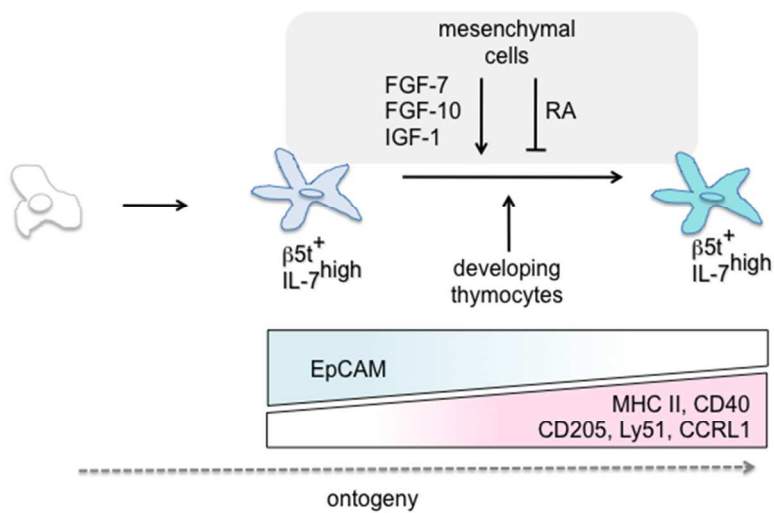


Figure 1
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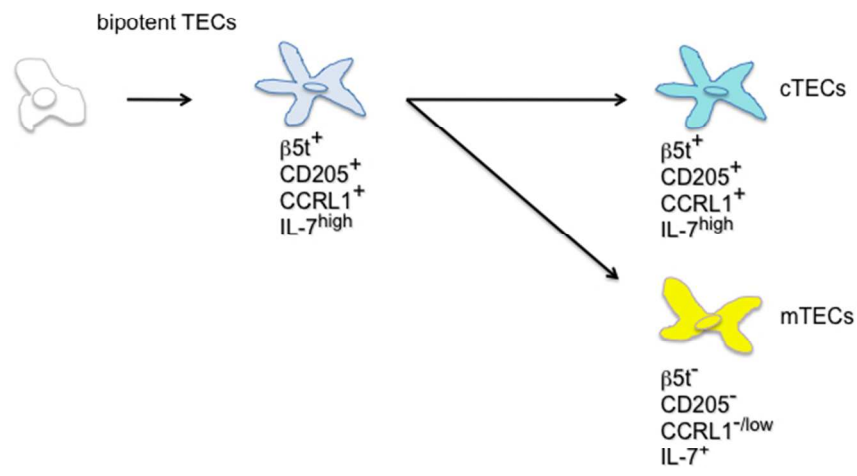


Figure 2
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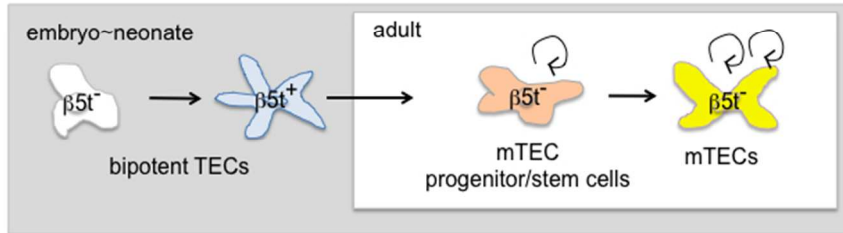


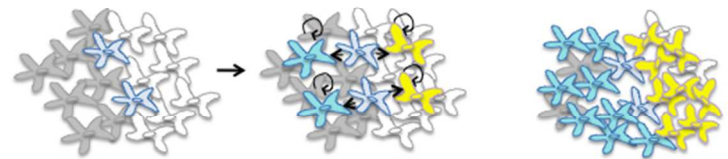
Figure 3
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A Hypothesis 1: bipotent TEC progenitors localize at cortico-medullary junction to supply cTECs and mTECs



B Hypothesis 2: The cortico-medullary junction is formed where bipotent TEC progenitors localize

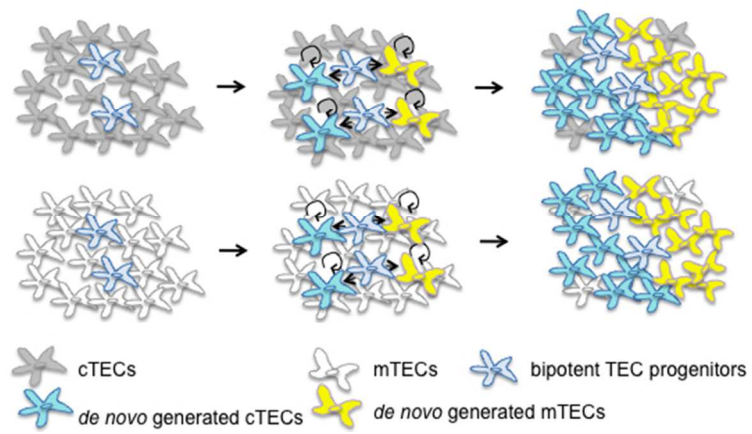


Figure 4
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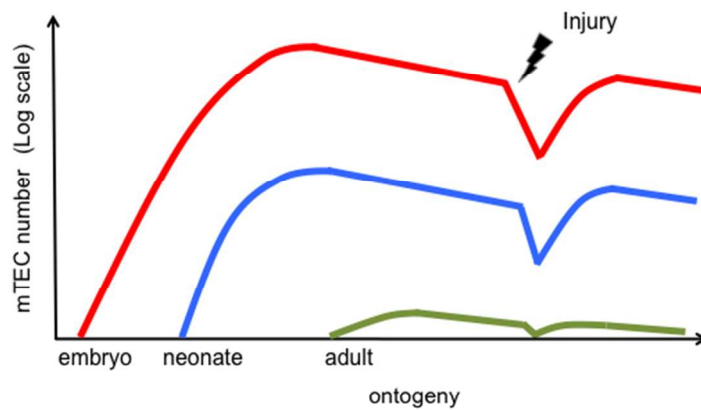


Figure 5
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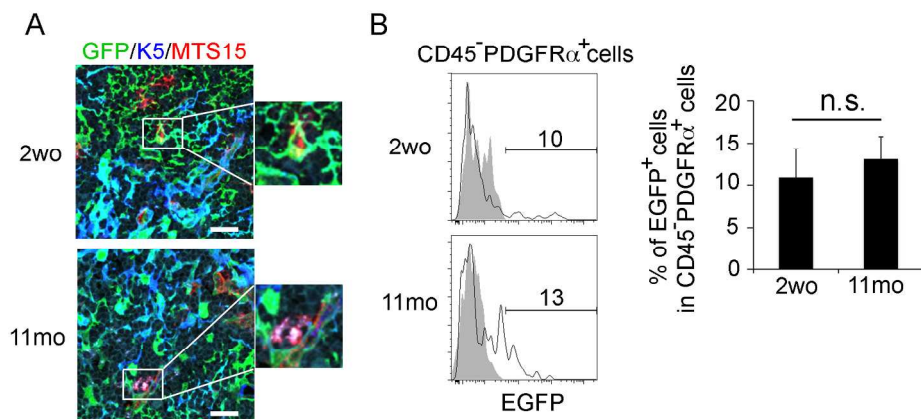


Figure 6
209x104mm (300 x 300 DPI)

Review Only