



In Pursuit of Adult Progenitors of Thymic Epithelial Cells

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Peripheral T cells capable of discriminating between self and non-self antigens are major components of a robust adaptive immune system. The development of self-tolerant T cells is orchestrated by thymic epithelial cells (TECs), which are localized in the thymic cortex (cortical TECs, cTECs) and medulla (medullary TECs, mTECs). cTECs and mTECs are essential for differentiation, proliferation, and positive and negative selection of thymocytes. Recent advances in single-cell RNA-sequencing technology have revealed a previously unknown degree of TEC heterogeneity, but we still lack a clear picture of the identity of TEC progenitors in the adult thymus. In this review, we describe both earlier and recent findings that shed light on features of these elusive adult progenitors in the context of tissue homeostasis, as well as recovery from stress-induced thymic atrophy.

Keywords: thymus, thymic epithelial cells, immunodeficiency, immune dysregulation, single cell RNA-sequencing

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INTRODUCTION

Once regarded as a vestigial organ that had lost its function during evolution, the thymus is now recognized as a primary lymphoid organ that performs irreplaceable functions in differentiation and selection of self-tolerant T cells (1). The thymus “educates” T cells by allowing those that possess a T-cell-receptor capable of interacting with major histocompatibility complex class I or II (MHCI and MHCII) molecules to survive, while eliminating those that recognize self-antigens presented by MHCI or MHCII molecules. This event is orchestrated by two types of thymic epithelial cells (TECs) that reside in the thymic cortex (cortical thymic epithelial cells, cTECs) and medulla (medullary thymic epithelial cells, mTECs). Functionally, cTECs are mainly required for lineage commitment, expansion, and positive selection of thymocytes, while mTECs promote negative selection of self-reactive T cells or promote their diversification into the regulatory T cell lineage by ectopically expressing self-derived tissue-specific antigens (2–4). The expression of tissue-specific antigens is partially regulated by the autoimmune regulator (AIRE), the mutation and dysfunction of which contribute to severe autoimmune diseases (5).

In the past several years, intense effort has focused on understanding the development of TECs, and bipotent and unipotent progenitors of cTECs and mTECs have been rigorously studied. The presence of embryonic bipotent progenitors of cTECs and mTECs was evidenced by transplanting a single early embryonic TEC (day 12.5) into the fetal thymus to generate both cTECs and mTECs (6), and by neonatal reactivation of developmentally arrested fetal bipotent progenitors (7). These embryonic bipotent progenitors can be identified by expression of placenta-expressed transcript 1 (PLET1), and resemble the phenotype of cTECs expressing CD205, β 5t, and IL-7 (8–12). Although evidence regarding embryonic unipotent progenitors of cTECs is limited (13), equivalent progenitors of mTECs have been well-studied (14–16). Overall, it is most likely that mTEC progenitors and cTECs are derived from bipotent progenitors in the embryonic thymus, although mechanisms underlying fate decisions of bipotent TEC progenitors remain elusive.

In contrast to embryonic TEC progenitors, little is known about the corresponding progenitors that maintain thymic tissue function in adults. In this review, we first highlight some reported properties of adult progenitors under homeostatic conditions, and then review how the putative adult progenitors contribute to thymic regeneration, from both the cellular and molecular perspectives, following stress-induced damage to the thymus.

ADULT TEC PROGENITORS UNDER HOMEOSTATIC CONDITIONS

In vivo cell labeling and ablation studies suggested that TECs are able to undergo turnover in the adult thymus (14, 17, 18). Adult progenitors or stem cells of TECs should exist to maintain the steady-state functions of the thymus so as to repopulate the periphery with immunologically competent T cells. In this section, we describe some recent findings on the identity of these elusive adult TEC progenitors under steady-state conditions.

Differences Between Embryonic and Adult TEC Progenitors

Earlier studies have suggested that some molecular features of TEC progenitors may differ between embryonic and adult thymus. Although mTEC-restricted progenitors are enriched in cells expressing the embryonic stem cell marker, SSEA-1, within the CLAUDIN-3/4-positive (CLD3/4^{hi}) population of the fetal thymus (16), these fractions appear to lose their self-replicating capacity in the adult thymus (16). Most SSEA-1⁺ CLD3/4^{hi} TECs in the adult thymus are MHCII^{lo/-} cells and express keratin 10, a marker of terminally differentiated mTECs known as Post-AIRE mTECs (16, 19–22). Moreover, unlike $\beta 5t^+$ embryonic bipotent progenitors, adult $\beta 5t^+$ cells contribute minimally to maintenance of TECs (23, 24). In light of these findings, Ohigashi et al. (23) argued that although adult progenitors are derived from embryonic $\beta 5t^+$ bipotent progenitors, they develop into mTEC-restricted SSEA-1⁺ CLD3/4^{hi} progenitors after losing $\beta 5t$ expression.

Adult Bipotent Progenitors

The existence of an adult bipotent progenitor capable of supplying cTECs and mTECs was tested using the thymic reaggregation/transplantation approach (25, 26). Ulyanchenko et al. demonstrated that TECs expressing LY51 (a marker of cTECs) and PLET1 had progenitor activities. Notably, the activity of bipotent progenitors generating both cTECs and mTECs were present in the MHCII⁺ fraction of Ly51⁺PLET1⁺ TECs (hereafter referred to as PLET1⁺ TECs) (25). On the other hand, Wong et al. (26) proposed that bipotent progenitors are present in a subset of TECs expressing low levels of MHCII and LY51 and lacking the mTEC marker UEA-1 ligand (referred to as TEC^{lo}). They both found that bipotent progenitors were present in UEA-1-negative TEC fractions, and that they express surface LY51 and *Pax1* mRNA, suggesting their similarity to cTECs. Unfortunately, there are some discrepancies between these studies. For example, PLET1⁺ TECs are enriched in the MHCII^{hi} fraction, but TEC^{lo} belongs to MHCII^{lo} fraction (25, 26). PLET1⁺ TECs comprise

<1% of all TECs, and limiting dilution analysis suggested their bipotency at nearly clonal resolution (25). In contrast, TEC^{lo} comprises about 20% of all TECs (26). Therefore, it may be possible that both unipotent cTEC and mTEC progenitors could be present in TEC^{lo} (25, 26). Importantly, as both studies verified their differentiation potential using reaggregation with fetal thymic cells, such conditions may not be suitable to address adult progenitors. Moreover, details of experimental conditions for thymic reaggregation differed slightly between two studies, which may explain the discrepancy. *In vivo* fate mapping needs to be performed in the adult thymus to evaluate their physiological fate.

A Subset of mTEC^{lo} Cells Represents mTEC Lineage-Restricted Adult Progenitors

Previously, mTECs were categorized as mTEC^{lo} or mTEC^{hi}, depending on expression levels of AIRE, CD80, and MHCII. mTEC^{lo} cells expressing lower levels of AIRE, CD80, and MHCII have been considered as an immature stage of mTEC^{hi} (17, 27–29). However, recent findings demonstrated that the mTEC^{lo} fraction contains multiple subsets. Several studies showed that AIRE⁺ mTEC^{hi} can further differentiate into mTECs with lower expression of AIRE, CD80, and MHCII (Post-AIRE mTECs), which are included in the mTEC^{lo} fraction (19–22). Moreover, Lucas et al. (30) demonstrated that the mTEC^{lo} fraction can be segregated by expression of the chemokine, CCL21, into CD104⁺ CCL21⁺ and CD104⁻ CCL21⁻ subsets. Since CCL21 recruits positively selected thymocytes to the thymic medulla, the CD104⁺ CCL21⁺ mTEC^{lo} subset may be considered functionally mature cells (31). Onder et al. (32) reported that within CD80⁻ TECs, there is a population of podoplanin (Pdpn)-expressing mTEC-restricted progenitors localized in the cortical-medullary junction (junctional TECs). In summary, it is likely that a limited population of mTEC^{lo} cells should be unipotent progenitors of mTEC^{hi}.

New Insights Gained From Single-Cell RNA-Sequencing Studies

Recent progress in single-cell RNA-sequencing (scRNA-seq) technology has uncovered a previously unknown degree of heterogeneity among TECs and has provided new insights into both the developmental pathway and mechanism of TECs, especially mTECs under homeostatic conditions (33–36). Based on the cell type clusters obtained from scRNA-seq results, Bornstein et al. categorized mTECs into four subsets: mTEC I, mTEC II, mTEC III, and a newly identified mTEC IV or tuft cells with chemosensory properties (33, 35). With respect to previous mTEC classifications, the mTEC I, mTEC II, and mTEC III subsets are equivalent to CCL21⁺ mTEC^{lo}, AIRE⁺ mTEC^{hi}, and Post-AIRE mTEC subsets, respectively. Notably, Lucas et al. (30) showed that DCLK1⁺ mTEC IV/tuft cells are enriched in the CD104⁻ CCL21⁻ mTEC^{lo} subset. Additionally, Dhalla et al. (34) used scRNA-seq to more deeply interrogate mTEC heterogeneity. These authors identified a “Proliferating mTEC” cluster that seemed to bridge the clusters representing

mature AIRE⁺ mTEC^{hi} and CCL21⁺ mTEC^{lo} (34). Cells in the “Proliferating mTEC” cluster exhibited upregulation of genes involved in proliferation, such as *Mki67*, and expressed *Aire*, suggesting that it could represent proliferating mTECs previously reported within mTEC^{hi} (14, 26, 34). The trajectory of diffusion pseudotime analysis suggested that cells in the “Proliferating mTEC” cluster could act as bipotent mTEC progenitors that differentiate into both AIRE⁺ mTEC^{hi} and CCL21⁺ mTEC^{lo} lineages (34). However, analysis of the same data using RNA velocity, a different trajectory method that relies on pre- and post-spliced RNA reads (37), produced conflicting results. The latter analysis indicated that rather than differentiating into the CCL21⁺ mTEC^{lo} cluster, the “Proliferating mTEC” cluster seemed to be derived from CCL21⁺ mTEC^{lo} and junctional TEC clusters (34). In a different study, Baran-Gale et al. (38) conducted scRNA-seq of mouse TECs throughout the 1st year of life and studied their various trajectories using genetic fate mapping under control of the $\beta 5t$ promoter. They also identified a cluster equivalent to the “Proliferating mTECs” (38). However, their diffusion pseudotime analysis failed to suggest that the “Proliferating mTEC” cluster was positioned at the branch point between mTEC differentiation into AIRE⁺ mTEC^{hi} and CCL21⁺ mTEC^{lo} lineages (38). Instead, they showed that the “Intertypical TEC” cluster, which encompassed cTEC, CCL21⁺ mTEC^{lo}, and junctional TEC, could bifurcate into two mTEC trajectories that both progressed toward AIRE⁺ mTEC^{hi} via the “Proliferating mTEC” cluster (38). In a more recent study, Wells et al. (39) showed that the “TAC-TEC” cluster, a cluster equivalent to the “Proliferating mTECs,” could give rise to both AIRE⁺ mTEC^{hi} and CCL21⁺ mTEC^{lo} lineages, using RNA velocity. In summary, *in silico* predictions of mTEC differentiation dynamics deduced from scRNA-seq data were split. These discrepancies could be due to differences in cell coverages and sequencing depths detected using different scRNA-seq methods (Table 1). For instance, low numbers of detected mRNA species could affect RNA velocity analysis, which relies on detection of unspliced mRNAs occupying 15–25% of total sequencing reads in scRNA data (37).

Notably, the “Intertypical TEC” cluster contained PLET1⁺ TECs and expressed markers associated with the bipotent TEC^{lo} progenitors, which were identified by cytometry-based analysis as described above (25, 26, 38). Diffusion pseudotime analysis showed that the “Intertypical TEC” cluster could not only contribute to the mTEC lineage, but also to the cTEC lineage, suggesting its bipotency (38). Additionally, these authors suggested that the aging “Intertypical TEC” cluster displayed

features of progressive quiescence, and that it could arise from either $\beta 5t^+$ or $\beta 5t^-$ progenitors independently (38). This proposal contradicts the argument put forth by Ohigashi et al. (23) that $\beta 5t^+$ and $\beta 5t^-$ progenitors possess a precursor-product relationship.

Importantly, all of the clusters described above, which are identified in mouse analyses, can also be identified in scRNA-seq data obtained from the human thymus, indicating that the cluster-based classification is not restricted to mice (38, 40). Nevertheless, we await experimental verification of the existence and function of putative adult progenitors inferred from computationally defined clusters.

ADULT TEC PROGENITORS DURING RECOVERY FROM STRESS-INDUCED DAMAGE

Adult progenitors would be integral not only to maintaining tissue homeostasis, but also to recovery of the thymus from stress-induced damage. In the following sections, we describe how putative adult TEC progenitors could contribute to thymic recovery at both the cellular and molecular levels, based on studies using mouse models that mimic insults.

Repair Potential of the Damage-Sensitive Thymus

The thymus is extremely sensitive to damage and exposure to acute or chronic insults results in a pronounced decline in cellularity, a phenomenon known as thymic atrophy (41, 42). For example, we recently demonstrated thymic atrophy displayed by mice under microgravity (0g) conditions during spaceflight, which was partially mitigated by exposure to 1g during spaceflight (43). After resolution of acute insults such as infections, cytoreductive therapies, and emotional and physical discomfort, the thymus is able to regenerate, although its capacity declines with age (41, 42). To study this endogenous thymic regeneration, researchers have employed viral and bacterial infections, sub-lethal irradiation, and synthetic corticosteroid injections to model acute insults (44–50). Chronic insults, such as aging, hamper the ability of the thymus to regenerate, but age-induced defects in recovery can be transiently reversed by ablation of sex steroids (51–53). This can be explained by the ability of sex hormones to induce apoptosis and to inhibit proliferation of developing T cells (54, 55). In fact, the

TABLE 1 | Comparison of scRNA-seq methods (FACS sorted cells in wells or droplet-based), cell numbers used after quality control, sequencing depths, and methods of trajectory analysis used by different scRNA-seq studies from postnatal thymus glands of wild-type mice under homeostasis.

	scRNA-seq method	Number of cells used in analysis	Sequencing depth per cell	Trajectory analysis
Bornstein et al. (33)	MARS-seq (FACS)	1,825 CD45 ⁺ cells and 1,716 TECs	Median of 1,711 UMIs	None
Dhalla et al. (34)	10X Genomics (Droplet)	6,894 mTECs	Median of 1,830 genes	Diffusion pseudotime RNA velocity
Baran-Gale et al. (38)	SMART-Seq2 (FACS)	2,327 TECs	Not mentioned	Diffusion pseudotime
Wells et al. (39)	10X Genomics (Droplet)	2,434 TECs	200–7,500 genes	RNA velocity

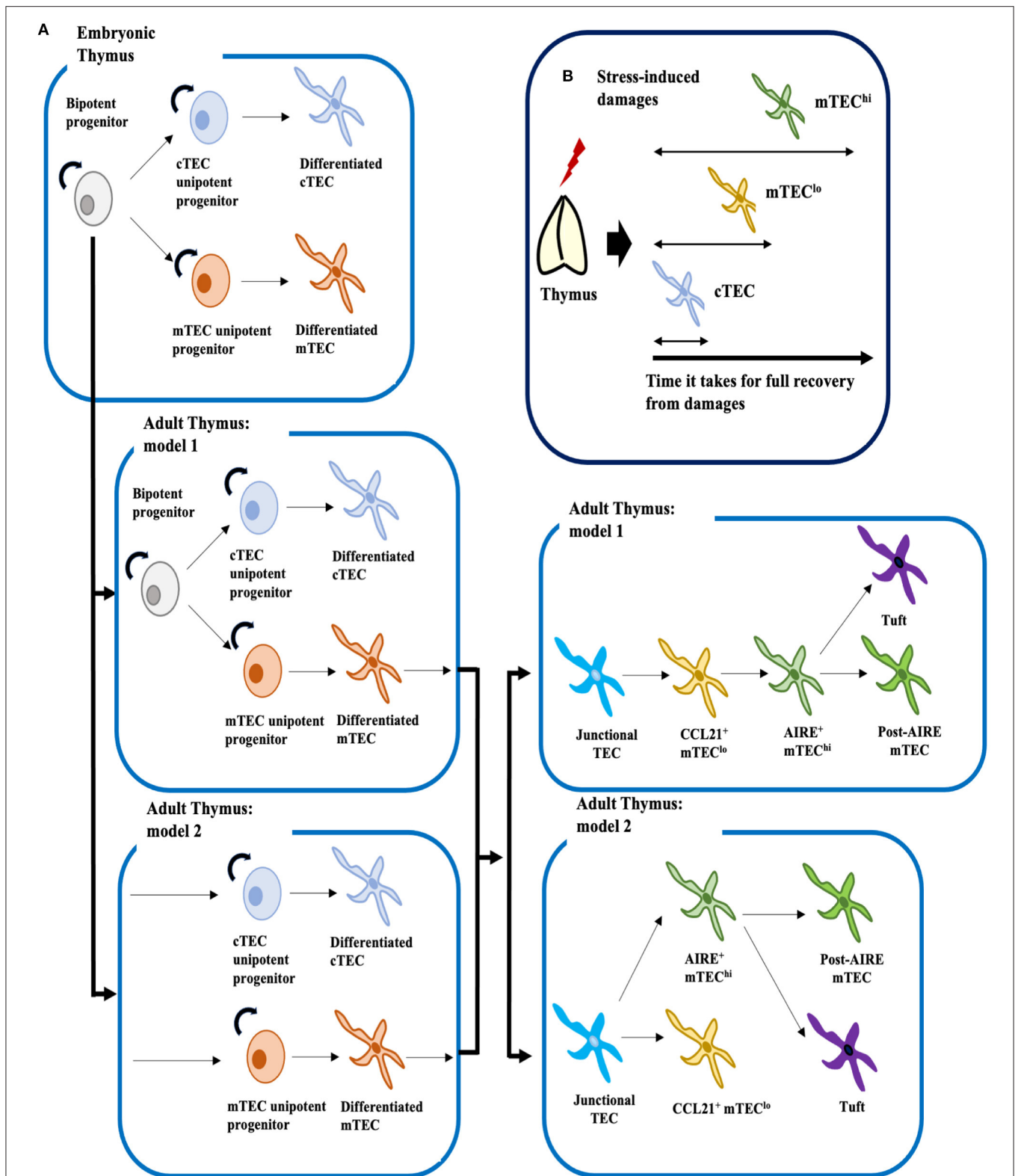


FIGURE 1 | Proposed mechanistic models for development of TECs under homeostatic conditions, and cellular mechanisms of TEC recovery after insults. **(A)** Embryonic bipotent progenitors in the thymus can generate both mature cTECs and mTECs. In the adult thymus, two models for development of cTECs and mTECs can be proposed. The first model, by analogy to the fetal thymus, posits that LY51⁺ MHCII^{lo} or LY51⁺ PLET1⁺ MHCII^{hi} adult bipotent progenitors, though their
(Continued)

FIGURE 1 | identities remain controversial, supply both mature cTECs and mTECs. The second model suggests that adult bipotent progenitors are lost with age, and that unipotent progenitors committed to either the cTEC or mTEC lineages supply mature cTECs and mTECs. However, these two models are not necessarily mutually exclusive. Similarly, two models can be proposed for development of mTEC^{lo} and mTEC^{hi}: one supports linear differentiation from junctional TECs to mature mTEC^{hi} via CCL21⁺ mTEC^{lo}, and the other suggests bifurcation of junctional TECs into mature CCL21⁺ mTEC^{lo} and AIRE⁺ mTEC^{hi}. Mature AIRE⁺ mTEC^{hi} would then differentiate into Post-AIRE mTECs or tuft cells (4, 35) **(B)** Mouse models of thymic stress have shown that cTECs are the first cell type to recover from insults, followed by mTEC^{lo} and then mTEC^{hi}. Therefore, it is possible that the adult progenitor population may be confined to cTECs.

regenerative capacity of the thymus was known well before its function as a lymphoid organ was discovered (56, 57).

Cellular Mechanisms of TEC Regeneration

Several studies have shed light on cellular mechanisms of TEC regeneration, which appear to initiate from the putative adult progenitors. Using an irradiation-induced stress model, we recently performed a quantitative analysis of TEC regeneration and its mathematical modeling (58). We showed that full recovery was reached earlier by cTECs than mTECs, and that mTEC recovery might be negatively regulated by CD4⁺ CD8⁺ double-positive T cells (58). Similar results were obtained by Dudakov et al. (59), suggesting that cTECs and mTEC^{lo} subsets were the major contributors to TEC recovery. Dumont-Lagacé et al. (60) used the tetracycline-inducible H2B-GFP mouse model to tag slow-cycling label-retaining cells (LRCs), which hypothetically label quiescent stem cells in the adult thymus. Following induction of acute injury by exposure of mice to irradiation, UEA⁻ LRCs displayed a significant increase in proliferation (60). Interestingly, these LRCs were localized in the cortical-medullary junction and were proposed as adult progenitors (60). Recently, Lepletier et al. (61) used the aging/sex steroid-ablation model to demonstrate that during recovery, there was a decrease in the ratio of MHCII^{lo} cTEC to MHCII^{hi} cTEC, followed by a decrease in the ratio of mTEC^{lo} to mTEC^{hi}. Taken together, these studies consistently showed that cTECs sharing similar phenotypes to the putative adult bipotent progenitors of TECs can initiate thymic recovery from stress, and potentially could contain a subpopulation of adult progenitors (25, 26).

Molecular Mechanisms of TEC Regeneration

Many soluble factors are involved in endogenous regeneration of TECs after damage. Following sublethal irradiation, depletion of radiosensitive CD4⁺ CD8⁺ double-positive T cells triggers radioresistant innate lymphoid cells to produce IL-22 (59, 62, 63). IL-22 binding to the IL-22 receptor on TECs then promotes TEC recovery through phosphorylation of STAT3 and STAT5 (59, 63). Of note, cTECs and mTEC^{lo}, but not mTEC^{hi} subsets showed significant early responses to administration of IL-22 during recovery (59).

An alternative mechanism of recovery involves keratinocyte growth factors (KGF). KGF is mainly expressed by fibroblasts and its cognate receptor, Fgfr2IIIb, is exclusively expressed by TECs in the thymus (64–67). Importantly, administration of KGF to mice exposed to irradiation accelerated the recovery of TECs by enhancing their proliferation (67).

Bone morphogenetic protein 4 (BMP4) also participates in mediating thymic regeneration. BMP4 is predominantly expressed by fibroblasts and radioresistant endothelial cells, and its expression increases soon after radiation exposure (68). Remarkably, administration of thymus-derived *ex vivo*-propagated endothelial cells, but not of endothelial cells derived from other organs, rescued the damage to TECs in mice exposed to radiation (68). Such rescue was driven by increased proliferation of cTECs, reflecting their higher expression of the non-redundant receptor, BMPR2, compared with mTECs (68).

CONCLUSIONS AND FUTURE PERSPECTIVES

Despite striking progress owing to advances in scRNA-seq technology, the exact mechanisms by which TECs develop remain far from clear, especially in postnatal and adult thymus. Based on our current understanding, we can propose two potential models for the development of cTECs and mTECs (**Figure 1A**). Given that early progenitors in the adult could arise from $\beta 5t^+$ embryonic bipotent progenitors, mature cTECs and mTECs could be replenished by bipotent progenitors or/and by two types of unipotent progenitors separately committed to the cTEC or mTEC lineages (8, 9, 11, 23). Studies of TEC development under homeostatic and recovery conditions suggest that these early progenitors bear cTEC phenotypes (**Figure 1B**) (25, 26, 58–61).

Recent findings also suggest that similar to mTEC^{hi}, mTEC^{lo} could also contain functionally mature cells (30, 31, 34). Thus, two models can be proposed for development of the mTEC lineage: one supports bifurcation from junctional TECs into terminally differentiated mTEC^{hi} and CCL21⁺ mTEC^{lo}, and the other, which is grounded in the traditional view that mTEC^{lo} are immature, supports the linear differentiation pathway from junctional TECs to mature mTEC^{hi} via CCL21⁺ mTEC^{lo} (**Figure 1A**). Further experimentation will be necessary to verify developmental trajectories proposed from *in silico* analyses. Notably, cell type clusters identified in scRNA-seq analyses by Baran-Gale et al. (38) contained mixtures of cells from TEC subsets defined by flow cytometry. Hence, new cell markers will be needed to equate cytometrically defined cell types with the computationally derived cell clusters.

Another key point is the possibility that progenitors identified under homeostatic conditions could differ from those present during TEC recovery. Interestingly, whereas IL-22 is critical for TEC recovery from radiation-inducing damage, deletion of IL-22 did not significantly affect TEC cellularity in steady state conditions. This implies that the TEC progenitor potential

may depend on thymic microenvironments. Moreover, the discrepancy in the two studies reporting bipotent adult TEC progenitors may be explained by possible variations and plasticity of TEC progenitors. Indeed, in other epithelial tissues such as the small intestine, skin, mammary gland, and lung, the potential of stem cells appears to change depending on the microenvironment (e.g., niches) and conditions in tissues (69). Cells that lack stemness during steady-state conditions have the capacity to acquire features of stem cells under different settings (70). To resolve similar complexity of TEC progenitors, an *in vivo* fate-mapping study using a specific progenitor marker driving CRE should be useful.

Finally, identification of TEC progenitors has crucial clinical implications. For example, therapeutic targeting of these cells would provide new opportunities for reversal of thymic aging, thereby boosting the responsiveness of an individual's immune

system. Ultimately, such a discovery could also contribute to the field of regenerative medicine by allowing creation of thymic organoids, which could open up a new avenue of treatment for immunological disorders.

AUTHOR CONTRIBUTIONS

TI wrote the first draft of the manuscript and NA and TA critically reviewed the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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