THYMIC EPITHELIAL CELLS: NEW INSIGHTS INTO THE ESSENTIAL DRIVING FORCE OF T-CELL DIFFERENTIATION

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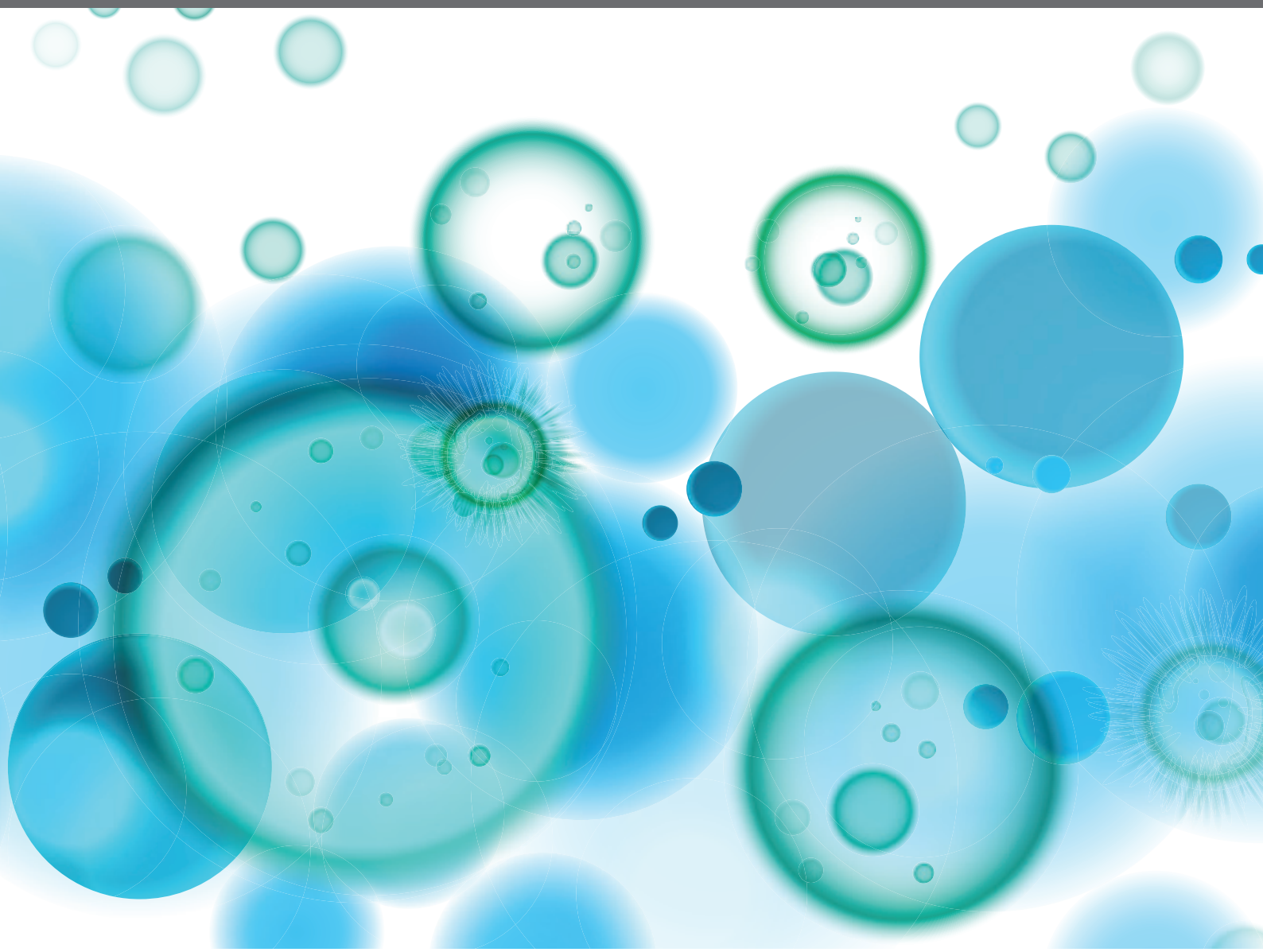

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THYMIC EPITHELIAL CELLS: NEW INSIGHTS INTO THE ESSENTIAL DRIVING FORCE OF T-CELL DIFFERENTIATION

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EDITORIAL
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Editorial: Thymic Epithelial Cells: New Insights Into the Essential Driving Force of T-Cell Differentiation

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Editorial on the Research Topic

Thymic Epithelial Cells: New Insights into the Essential Driving Force of T-Cell Differentiation

The thymus is the primary lymphoid organ specialized in the development of a diverse repertoire of non-self-reactive T lymphocytes. Thymic epithelial cells (TEC)s represent the main stromal cell type of the thymus regulating virtually all stages of T-cell development, from T-cell lineage commitment to positive, negative and T regulatory (Treg) cell selection (1, 2). These processes depend on the interaction between T-cell precursors with highly specialized subsets of TEC located within either the cortex; cortical (c)TECs, or medulla; medullary (m)TECs. While hematopoietic-intrinsic defects have been well characterized throughout the years, the study of TECs has been more complex, particularly in humans, due to their low cell density and difficulty in their isolation. Herein, we host a special issue focused on the latest discoveries in TEC biology.

Notably, proper TEC differentiation depends on reciprocal signals provided by developing thymocytes, a process commonly known as thymic cross-talk. Among the signals involved in thymic cross-talk, the interaction between the TNF receptor (TNFR) family member receptor activator of nuclear factor kappa-B (RANK), expressed by TECs, its ligand RANKL, expressed by positively selected CD4 thymocytes and a subset of group 3 innate lymphoid cells, controls mTEC proliferation/differentiation and TEC regeneration. In her review, Irla discusses recent advances in the complexity of the mTEC compartment, the role of the axis RANK/RANKL in TEC differentiation and regeneration and how the specific targeting of this axis may open the way to novel therapeutic approaches aimed at thymic regeneration and T-cell recovery.

Montero-Herradón et al. explored the minimal requirements of lympho-epithelial interactions to guarantee regular thymopoiesis. Using reaggregate thymic organ cultures comprising differing numbers of fetal TECs, grafted under the kidney capsule of FoxN1$^{-/-}$ hosts, authors investigated how many TECs are necessary to support thymocyte maturation. The results demonstrated reduced T-cell-TEC cross-talk due to low TEC numbers is sufficient to support proper T-cell differentiation.

The establishment of central tolerance is a major function of the thymus and helps prevent the insurgence of autoimmune diseases. To establish and maintain central tolerance, elimination of self-reactive T lymphocytes through negative selection, and the generation of regulatory T-cells are required. mTECs and other antigen presenting cells, such as dendritic cells, are critical for both mechanisms. Throughout life the thymus undergoes conspicuous changes that affect the

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composition and function of TECs and antigen presenting cells, ultimately leading to alterations in central tolerance induction. Srinivasan et al. discuss how age-related changes to TECs and the thymic microenvironment affect T-conventional and T-regulatory cell selection and responses in humans and mice.

Autoimmune regulator (Aire)-expressing mTECs have an important role in tolerance induction. Indeed, the transcriptional regulator Aire is critical in inducing the expression of tissue-restricted antigens in mTECs, which direct self-reactive thymocytes toward apoptosis or Treg cell differentiation. In the last decade, several studies have indicated that mTECs continue to differentiate beyond Aire expression. However, the function and lineage relationships of these terminally differentiated post-Aire mTEC populations remain unclear. The perspective article by Laan et al. summarizes recent studies in mice and humans about these terminally differentiated mTEC subsets, including Hassall's corpuscles structures, thought to be the final differentiation stage of the post-Aire lineage. Authors discuss their roles in tolerance induction, with a particular focus on their involvement in creating a pro-inflammatory microenvironment within the thymus.

One of the most important roles of TECs is to present MHC-associated antigens to developing thymocytes, in order to generate a wide repertoire of immunocompetent yet self-tolerant T-lymphocytes. Proteasomes degrade ubiquitinated proteins into peptide fragments that are eventually loaded onto MHC class I molecules. cTECs specifically express thymoproteasomes, which are essential for the positive selection of CD8+ T-cells, while other thymic cells, including mTECs, express immunoproteasomes that are critical for the elimination of self-reactive thymocytes. In their review article, Frantzeskakis et al. offer an overview of the functions and types of proteasomes expressed in the thymus, with a special focus on the most recent findings on thymoproteasomes and immunoproteasomes.

Although many aspects of TEC development and function have become increasingly clear in recent years, there are still several features of their basic biology that need further clarification. Biochemical studies on metabolic regulation of TECs have been especially challenging due to technical obstacles in TEC isolation. Semwal et al. discuss three distinct, but interconnected areas of TEC metabolic regulation: mTOR signaling, redox status of the cell, and autophagy. Authors discuss these three aspects in the establishment and maintenance of the thymic stromal compartment, along with age-associated dysfunctions.

Recent advances, particularly in scRNAseq, have added significant knowledge on the phenotypical and functional heterogeneity of TECs. However, there remains a lot of uncertainty about the identity of TEC progenitors (TECp) in the adult thymus. The prospective identification and isolation of adult TECp could present important clinical implications, as potentially they could be exploited to reverse age-associated alterations to the thymic environment or for the generation of thymic organoids in the field of regenerative medicine. In their review article, Ishikawa et al. discuss earlier and more recent studies that could help clarify the distinctive features of adult TECp, both in the homeostatic condition and recovery from thymic involution.

Another report on TECp and their age-related changes is provided by Pinheiro and Alves. The authors focus on the first weeks of murine post-natal life and highlight the timely coordination between the expansion and maturation of TECs and a presumable drop in the bioavailability of TECp during this specific period. Enhanced knowledge in this area offers strategies for reversal of thymic involution.

In addition to TECs, the thymic stromal cell compartment contains mesenchymal cells, including fibroblasts, pericytes, and endothelial cells. Two review articles in this issue discuss current and past knowledge on the function of these less studied, but important, non-epithelial thymic stromal populations. Nitta and Takayanagi consider historical studies and more recent advances in our understanding of the contribution of non-TEC stromal cells in thymic organogenesis and T-cell development. In particular, they highlight the role of fibroblasts on T-cell repertoire selection. James et al. discuss how in recent years, through improvements in the phenotypical identification and functional classification of fibroblasts and endothelial cells, their contribution to thymus development and function is beginning to be better understood.

This special issue on TECs includes original research articles presenting new insights into different phenotypical and functional aspects of TECs, ranging from *in-vitro* approaches, murine models and human samples. Gao et al. evaluate the role of the suppressor of cytokine signaling 3 (SOCS3), which is a negative regulator of cytokine signaling in thymic T-cell differentiation. Cytokines produced by both T-lymphocytes and TECs are crucial mediators for thymic cross-talk and are important for the development and maintenance of both stromal and lymphoid cells. Therefore, cytokine release and response need to be tightly controlled. Authors show that lack of SOCS3 leads to dramatic loss of thymic cellularity and altered cortico-medullary compartmentalization, resulting ultimately in a reduced output of recent thymic emigrants in SOCS3-deficient mice. Interestingly, they show that SOCS3 expression is mainly confined to TECs rather than thymocytes and identify a novel role for SOCS3 in supporting the maturation and anatomical distribution of TECs.

Tao et al. explore the role of TECs in the development of non-conventional, innate like T-cells, specifically iNKT and γδT-cells. This area of research remains much less well understood compared to the involvement of TECs in conventional αβT-cell differentiation. From the analysis of publicly available databases, authors found that transcripts of many cytokines and cytokine receptors are expressed by murine and human TECs. Particularly, they demonstrate that expression of IL-15 and IL-15Rα in TECs is crucial for thymic development of type 1 innate-like T-cells, such as iNKT1 and γδT1 cell, by showing a role for TECs not only as a source of IL-15 but also in the trans-presentation of IL-15 to ensure type 1 innate like T-cell development.

Han and Zúñiga-Pflücker explain the mechanism by which high oxygen in fetal thymus organ cultures (FTOCs) is critical to support efficient thymocyte development. It has been reported that fetal thymus lobes placed in low oxygen submersion (LOS) fail to support thymocyte development, whilst submersion

cultures performed in the presence of high concentrations of ambient oxygen (ranging from 60 to 80%) (HOS) can facilitate normal thymocyte development. However, the mechanisms mediating the increased production of mature T-cells in HOS-FTOCs remained unknown. Here, authors demonstrate that HOS rescues the expression of FOXN1 and its target genes, DLL4 and CCL25, in addition to maintaining high levels of MHCII expression. Additionally, authors showed that increased oxygen availability can promote self-renewal of DN3 cells, resulting in an increased cellularity of submersion FTOCs.

Lastly, two articles from the group led by Villa and Bosticardo presented novel evidence of altered thymic development in Down Syndrome (DS) patients and in mice and patients with MHCII-deficiency. Based on previous studies suggesting immune senescence in DS, Marcovecchio et al., tested the hypothesis that induction of cellular senescence may contribute to early thymic involution and immune dysregulation in this syndrome. Authors showed that immunohistochemical analysis of thymic tissue in DS patients present with signs of accelerated thymic aging. Transcriptomic analysis of TECs revealed enriched expression of genes involved in cellular response to stress, epigenetic histone DNA modifications and senescence in DS samples. This signature was also confirmed in thymocytes and peripheral T-cells of DS patients. These results support a key role of cellular senescence and increased oxidative stress in the onset of immune dysregulation in DS patients. Ferrua et al. set out to explore the contribution of TEC alterations to the pathogenesis of MHCII deficiency, a rare combined immunodeficiency due to mutations in genes regulating the expression of MHCII molecules. The authors observed an overall perturbation of thymic structure and function in both MHCII$^{-/-}$ mice and patients. Transcriptomic and proteomic profiling of TECs from MHCII$^{-/-}$ mice revealed defective mTEC maturation and decreased promiscuous gene expression, causing defects in the establishment of central tolerance. Additionally, authors show peripheral tolerance impairment in MHCII deficiency, likely due to defective Treg cell generation and/or function and B-cell tolerance breakdown.

In summary, this special issue gives an overview of the up do date knowledge on TEC biology and their role in thymocyte development, in addition to providing novel research data that deepen our understanding about this fascinating and unique cell type.

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All authors participated in the editorial reviews of the manuscripts. All authors participated in the writing and editing of the editorial article. All authors contributed to the article and approved the submitted version.

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Non-Epithelial Thymic Stromal Cells: Unsung Heroes in Thymus Organogenesis and T Cell Development

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The stromal microenvironment in the thymus is essential for generating a functional T cell repertoire. Thymic epithelial cells (TECs) are numerically and phenotypically one of the most prominent stromal cell types in the thymus, and have been recognized as one of most unusual cell types in the body by virtue of their unique functions in the course of the positive and negative selection of developing T cells. In addition to TECs, there are other stromal cell types of mesenchymal origin, such as fibroblasts and endothelial cells. These mesenchymal stromal cells are not only components of the parenchymal and vascular architecture, but also have a pivotal role in controlling TEC development, although their functions have been less extensively explored than TECs. Here, we review both the historical studies on and recent advances in our understanding of the contribution of such non-TEC stromal cells to thymic organogenesis and T cell development. In particular, we highlight the recently discovered functional effect of thymic fibroblasts on T cell repertoire selection.

Keywords: thymus, T cell, stromal cell, thymic epithelial cell, fibroblast, mesenchymal cell, repertoire selection

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INTRODUCTION

T cells, which are the central player in the acquired immune system, develop in the thymus (1). Supported by a three-dimensional framework composed of thymic stromal cells, immature T cells (called thymocytes) undergo proliferation, differentiation, and cell fate determination, and consequently give rise to mature T cells expressing a diverse T cell receptor (TCR) repertoire.

Thymus-seeding progenitor cells from the fetal liver or adult bone marrow enter into the thymus and give rise to Early T-cell progenitors (ETPs), followed by their differentiation from the $CD4^{-}CD8^{-}$ (double negative, DN) to the $CD4^{+}CD8^{+}$ (double positive, DP) stage and the expression of rearranged TCR on their cell surface (2). Interaction between the TCR and self-peptide/MHC complexes in the thymus dictates the fate of the developing thymocytes, namely, positive selection of $CD4^{+}CD8^{-}$ (CD4 single positive, CD4SP) or $CD4^{-}CD8^{+}$ (CD8 single positive, CD8SP) thymocytes, along with the negative selection of self-reactive cells or their diversion into regulatory T (Treg) cells (3). Such differentiation and selection events are controlled by the coordinated action of a set of thymic stromal cells that are localized in different areas of the thymus. TECs are one of the most prominent thymic stromal components (4, 5). The thymus is subdivided into two discrete regions, the cortex and medulla.

The cortex is the outer region, where the cortical TECs (cTECs) provide a reticular meshwork that houses densely packed DN and DP thymocytes, while the medulla is the inner region with less densely localized SP thymocytes supported by medullary TECs (mTECs).

cTECs play key roles in the early events of T cell development, such as T cell lineage commitment, proliferation, migration, and the survival of immature thymocytes, by virtue of the production of the Notch ligand (DLL4), cytokines (SCF, IL-7), and chemokines (Cxcl12 and Ccl25) (6). Of particular importance in terms of T cell repertoire formation is that cTECs express a unique proteasome and lysosomal proteases, which enable the production and presentation of a unique set of self-peptides for the positive selection of a diverse TCR repertoire (7–11). In the subsequent steps, that are negative selection and Treg differentiation, mTECs play the dominant role. Functionally mature mTECs express a diverse set of genes that represent almost all of the coding transcripts, including those of the peripheral tissue-restricted antigens (TRAs) (12–14). This unique trait of mTECs ensures the negative selection and/or Treg conversion of self-reactive SP thymocytes that recognize such TRAs (15–20). mTECs also produce the chemokine CCL21 that induces the relocation of SP thymocytes from the cortex to the medulla, which promotes negative selection (21–24). Collectively, the thymic cortex promotes the generation of a diverse TCR repertoire, while the thymic medulla establishes the self-tolerance of T cells.

Several review articles in the current series "Thymic Epithelial Cells: New Insights in the Essential Driving Force of T Cell Differentiation", as well as previous reviews, have provided detailed information on the function and heterogeneity of TECs (5). Although TECs are undoubtedly the key stromal component for controlling T cell development, numerous studies have also been accumulating that demonstrate the importance of thymic stromal cells other than TECs. In this review, we focus on such non-TEC thymic stromal cells in thymus organogenesis and T cell development.

OVERVIEW OF THYMIC STROMAL CELLS

The thymus in mammals is made up of two lobes and is located in the upper anterior part of the chest between the lungs and on the heart. The outmost layer of the mouse thymus is covered with a capsule, which is composed of a monolayer of fibroblasts (capsular fibroblasts, capFbs). The cortical region directly under the capsule is called the subcapsular zone (SCZ), where proliferating DN thymocytes are localized (25). The SCZ contains a unique type of TECs (26, 27), but their function has been poorly elucidated. The cortex of the adult mouse thymus contains a network of cTECs (estimated as comprising ~10^6 cells per mouse) that houses densely packed DP thymocytes (more than 10^8 cells per mouse) (28). These cell number estimates are consistent with the observations that cTECs are very large cells with a three-dimensional reticular form so that a single cTEC adhere to hundreds of DP thymocytes. In particular, a fraction of cTECs form large multicellular complexes, termed 'thymic nurse cells', in that multiple DP thymocytes are enwrapped alive within intracellular vesicles of cTECs (29, 30). These unique cell-in-cell structures facilitate prolonged survival and continued TCRα rearrangement of enclosed DP thymocytes, likely contributing to the production of diverse TCR repertoire. Because of the difficulty in cell sorter-based isolation and single-cell-based analyses of such large cTECs, the functional heterogeneity of cTECs is still poorly understood and most likely underestimated (5). The cortex also contains dendritic cells that are sparsely distributed throughout the region and contribute to cortical negative selection (31).

Traveling inward from the cortex, there is a blood vessel-rich region called the cortico-medullary junction (CMJ), which is the site of the immigration of T-precursor cells and the emigration of mature SP thymocytes. The CMJ is also enriched with the lineage-committed progenitors of mTECs (termed junctional TECs) (32). In the medulla, mTECs and medullary fibroblasts (mFbs) form a reticular network that enmeshes SP thymocytes. The ratio of stromal cells to thymocytes is higher than that in the cortex. The number of mTECs is estimated to be ~2.5×10^6 cells per mouse, which is still less than that of medullary SP thymocytes (estimated as ~1×10^7 cells) but outnumbers the SP thymocytes newly generated in the adult mouse thymus per day (estimated to be ~1×10^6), likely contributing to the efficient screening of SP thymocytes for self-reactivity (28). The medulla is also the place where dendritic cells and B cells are enriched, both of which contribute to the induction of self-tolerance (33–35).

The blood vasculature is also an important parenchymal component of the thymus that supplies oxygen and nutrients and ensures the import and export of cells. The vasculature in the thymus consists of morphologically and functionally distinct types of blood vessels. The cortex contains a network of capillaries, while the CMJ and medulla are enriched with arterioles and postcapillary venules (25, 36). A fraction of thymic endothelial cells are surrounded by pericytes, specialized contractile fibroblast-like cells expressing smooth muscle actin (α-SMA) (37).

In addition to the above types of stromal cells, histological studies have reported a variety of atypical cells that structurally resemble the epidermal epithelium, ciliated epithelium, neuroendocrine cells, muscle cells, or nerve cells in the thymus (38–43). Recent studies demonstrated that these cells can be categorized as subsets of differentiated mTECs that include keratinocyte-like mTECs forming Hassall's corpuscles, thymic tuft cells, and neuroendocrine cell-like mTECs (44–47), suggesting the highly heterogeneous traits of mTECs and their possible contribution to the production of a diverse array of self-antigens for the establishment of self-tolerance. Also, certain neuron-associated genes such as *Nes*, *Pde1a*, and *Pde1b* are predominantly expressed in pericytes in the thymus [unpublished results based on transcriptome data (GEO: GSE147357)], although whether these cells and other nerve-like cells exert neural functions in the thymus is still unknown.

As animals age, the thymus undergoes a progressive atrophy called involution, mainly due to qualitative and quantitative degeneration of thymic stromal cells (4, 48). TEC is the thymic stromal cell type most affected by aging. In particular, mTECs are

markedly compromised in cellularity and gene expression capability during aging (49). The age-dependent decline of TECs parallels with increased adipose tissue in the thymus. It was shown that TECs in aged mice can undergo epithelial-to-mesenchymal transition (EMT) to differentiate into fibroblasts and adipocytes (50, 51). How aging impacts mesenchymal thymic stromal cells and their role in thymic involution and adiposis awaits further studies.

To understand the developmental origin and function of these thymic stromal cells, it is necessary to trace them back to the organogenesis of the thymus.

THYMIC ORGANOGENESIS

The thymus develops from the third pharyngeal pouch (PP), which is formed by evagination of the endoderm-derived epithelial layer from the gut tube around embryonic day (E) 9.5–10.5 in C57BL/6 mice (52). In the third PP, the evaginated epithelial cells are surrounded by neural crest-derived mesenchymal cells (**Figure 1A**). These mesenchymal cells direct the patterning of the third PP through the production of soluble factors such as bone morphogenic protein-4 (BMP4) (53). The third PP is responsible for the origin of the parathyroid gland and the thymus (**Figure 1B**). The transcription factor FoxN1 starts to be expressed at E11.5 in the caudal ventral portion of the epithelial primordium and plays an essential role in thymus organogenesis, while Gcm2, another transcription factor expressed in the cranial dorsal portion, is required for the development of the parathyroid gland. Several other transcription factors expressed in mesenchymal cells (HoxA1, Eya1, Six1, Pax9, Tbx1, and Ripply3) in this region are also required for the patterning of the third PP and the subsequent development of the thymus. Genetic defects in these transcription factors results in thymus hypoplasia and severe immunodeficiency in humans (54).

On E12.5 in mice, the thymus primordium that is composed of epithelial and mesenchymal cells is detached from the pharynx (**Figure 1C**) and begins to migrate down the neck and into the mediastinum, where the two thymic lobes from the two sides fuse together in front of the trachea on E14.5. During these processes, the endodermal layer in the third PP differentiates into TECs. The mesenchymal cells surrounding the thymus primordium form the capsule that covers the surface of the adult thymus, while a fraction of these cells migrate into the epithelial rudiment in the period E12–14 to form the vascular and reticular architecture in the medulla of the adult thymus (**Figures 1D, E**). The thymic epithelial and mesenchymal components spatially and functionally interact in a coordinated manner to control thymus organogenesis and T cell development.

THE FETAL THYMIC MESENCHYME CONTROLS THYMIC EPITHELIAL CELL DIFFERENTIATION

An early study using a reaggregated organ culture technique demonstrated that fetal mesenchymal cells were required for the reaggregation of the thymic stroma and thymocytes and the development of DP thymocytes (55). In this experimental setting, the fetal mesenchymal cells can be replaced by fibroblasts treated so as to be metabolically inactive, suggesting that the fibroblast-associated extracellular matrix is the key element for supporting thymus organogenesis (56). The same group reported that when the mesenchymal layer was removed, the fetal thymic epithelium failed to undergo cellular expansion in both an *in vitro* organ culture (57) and *in vivo* organ transplantation (58). These findings suggest an essential role for thymic mesenchymal cells in controlling the capacity of T cell production.

The differentiation and expansion of fetal TECs are likely regulated by a combination of mesenchymal cell-derived signaling factors. BMP4, a soluble protein secreted by thymic mesenchyme and epithelium, is crucial for the development of both the thymus and parathyroid gland as well as TEC differentiation (53, 59). The fetal thymic mesenchyme also produces fibroblast growth factor-7 (FGF7), FGF10, and Wnt ligands that promote TEC differentiation and proliferation (58, 60–62). The expression of BMP4 and Wnt ligands in the fetal thymic mesenchyme is regulated by the transcription factor MafB (63). Thymic mesenchymal cells also serve as the major source of retinoic acid, which promotes the proliferation of cTECs (64).

Thymic mesenchymal cells diverge at an early stage (E13) to give rise to perithymic and intrathymic cell populations (57, 65) (**Figure 1D**). The perithymic cell population remains outside the organ and forms the thymic capsule, while the other population migrates into the thymus across the epithelial layers and differentiates into mFbs and pericytes (**Figure 1E**). Along with this migration, mesodermal progenitor cells enter into the thymic rudiment and differentiate into blood vessel endothelial cells in order to form the vascular network (66), and as a consequence, the vessels are connected to the peripheral vasculature at E15.5 (67), which switches the route of thymus ingress of T-cell progenitors from intraluminal crawling-dependent to bloodstream-dependent. The molecular mechanisms for generating the mesenchymal cell heterogeneity and the patterning of the vasculature remain unknown. It is also unclear which mesenchymal cell subset(s) are responsible for producing the key factors and controlling the fetal TECs. In the future, single-cell transcriptome analyses will be a powerful tool to decipher the heterogeneity of the fetal thymic mesenchyme in controlling thymus morphogenesis and TEC differentiation (68).

MESENCHYMAL STROMAL CELLS IN THE ADULT THYMUS

In the adult thymus, neural crest-derived mesenchymal cells are found as fibroblastic cells predominantly localized in the capsule and medulla (69, 70) (**Figure 1E**). Some fibroblasts are sparsely distributed within the cortex. capFbs form a monolayer sheet that covers the surface of the thymic parenchyma that is filled with thymocytes and epithelial networks. In the medulla, neural

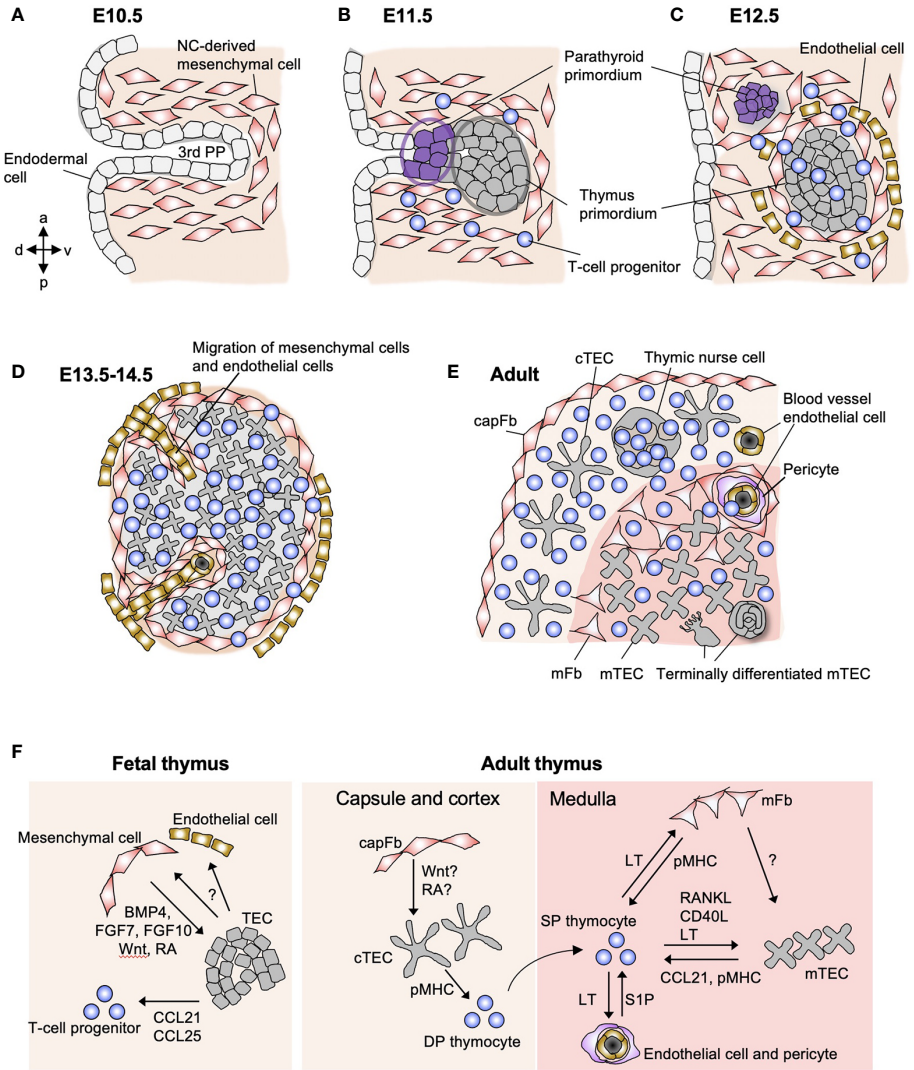

FIGURE 1 | Schematic representation of stromal cells in the fetal and adult thymus. **(A)** The third pharyngeal pouch (PP) is a structure temporary formed in the E9.5–10.5 embryo, whose patterning is regulated by the surrounding NC-derived mesenchymal cells. **(B)** On E11.5, endoderm-derived epithelial cells form primordia of parathyroid gland and thymus. **(C)** Both primordia separate from the foregut on E12.5, being surrounded by NC-derived mesenchymal cells and mesoderm-derived endothelial cells. From E11.5 to E12.5, T-cell progenitors migrate into the thymic primordium by the coordinating action of chemokines, Ccl21 produced by parathyroid epithelium and Ccl25 produced by thymic epithelium. **(D)** On E13.5, endothelial cells and mesenchymal cells begin to migrate into the thymic primordium to form vascular network. **(E)** The intrathymic mesenchymal cells differentiate to reticular mFbs and blood vessel pericytes, while the perithymic mesenchymal cells remain outside of the epithelium and form the thymic capsule. **(F)** Interactions among stromal cells and developing T cells in fetal and adult thymus. In fetal thymus, NC-derived mesenchymal cells produce BMP4, FGF7, FGF10, Wnt ligands, and retinoic acid (RA) to promote the differentiation and proliferation of TECs. Fetal TECs induce inward migration of mesenchymal cells and endothelial cells via unknown mechanism. In adult thymus, mFbs control mTEC development via yet unidentified factors, suggesting that mFbs lie upstream in the hierarchy of stromal interaction in the medulla. The development of mature mFbs with unique gene expression is controlled by lymphotoxin (LT) signals provided by SP thymocytes. SP thymocytes also produce RANKL and CD40L as well as LT to induce differentiation and proliferation of mTECs. Both mFbs and mTECs contribute to the production of self-antigens for the induction of T cell tolerance. mTECs produce CCL21 to attract positively selected SP thymocytes from the cortex to the medulla. The emigration of mature T cells from the thymus is promoted by S1P produced by blood vessel pericytes.

crest-derived cells are subdivided into mFbs that form the reticular structure (71) and blood vessel adventitial layer (72) and pericytes that surround the endothelial cells.

Because of their abundance and unique structure in the thymus, thymic fibroblasts, similar to TECs, have been recognized as an important stromal component. Transcriptome analyses revealed that thymic fibroblasts express a unique set of genes for interactions with epithelial cells and are distinct from skin and bone fibroblasts, suggesting a pivotal role of these cells in the thymic microenvironment (73). Thymic fibroblasts also produce vasculogenic and angiogenic factors, consistent with their close localization and potential interaction with vascular

endothelial cells (72). A series of studies have reported that thymic fibroblasts express a set of structural proteins and functional molecules, such as collagens, podoplanin/gp38, CD34, fibroblast-specific protein-1 (FSP1), platelet-derived growth factor receptor α (PDGFRα), PDGFRβ (58, 65, 71–76), and epitopes for the monoclonal antibodies MTS-15 and ER-TR7 (77, 78).

FSP1 (also known as S100a4) is reportedly not only a marker protein but also is responsible for the function of thymic fibroblast (74). Deletion of FSP1-expressing cells resulted in a prominent reduction in mTECs in both the steady state and regenerative phase after irradiation, suggesting that FSP1-expressing thymic fibroblasts are crucial for the maintenance and regeneration of mTECs. FSP1-deficient mice exhibited a smaller sized thymus and reduced number of mTECs compared with control mice. Furthermore, the addition of FSP1 protein increased the proliferation and maturation of mTECs in fetal thymus organ culture, indicating the role of FSP1 as a direct regulator of mTECs. The capacity of FSP1-expressing fibroblasts to control TECs might also be mediated by their ability to produce large amounts of FGF7. Collectively, this study demonstrates the pivotal role of FSP1-expressing fibroblasts in controlling mTECs.

However, the above mentioned thymic fibroblast-specific proteins or markers, including FSP1, cannot be used to distinguish two remotely localized fibroblast subsets, capFb and mFb. Whether thymic fibroblast subsets are functionally heterogeneous and how they make different contributions to TEC and T cell development remained open questions.

CAPSULAR FIBROBLASTS

Recently, we developed an enzymatic digestion method that allows the fractionation of thymic cells based on their intrathymic localization (79). Thymic fibroblasts were enriched in the fractions that correspond to the capsule and medulla, enabling the isolation of capFbs and mFbs. Among the differentially expressed genes, we identified *Dpp4*, a gene encoding the cell-surface protease dipeptidyl peptidase-4 (also called CD26), which is specifically expressed in capFbs and, consequently, established a means to separate capFbs ($DPP4^+$ $gp38^+$) and mFbs ($DPP4^-$ $gp38^+$) by flow cytometry and by histological analyses (79).

Both capFbs and mFbs highly express certain fibroblast-associated genes such as extracellular matrix proteins (*Col1a1*, *Col3a1*, *Col6a1*, *Dcn*, *Lum*, *Mgp*, and *Sparc*), extracellular proteases (*Htra1*, *Htra3*, *Mmp2*, *Mmp3*, *Mmp14*, and *Mmp23*), and protease inhibitors (*Serping1* and *Serpinh1*). These gene expression signatures are common to fibroblastic cell types in secondary lymphoid organs and are consistent with a previous report (73). capFbs but not mFbs highly express *Dpp4*, *Pi16*, *Sema3c*, *Sema3d*, and *Aldh1a2*. When compared with the transcriptome data of human thymic fibroblasts (46), it is suggested that the $DPP4^+$ capFbs in the mouse thymus still contain (at least) two subpopulations: perilobular and interlobular cells. capFbs also express many Wnt family ligands and regulators (*Wnt2*, *Wnt5a*, *Wnt5b*, *Wnt9a*, *Wnt10b*, *Wnt11*, and *Sfrp2* and *Sfrp4*) at higher levels than those in mFbs and other thymic stromal cells, suggesting a role for capFbs in cTEC development via the regulation of Wnt signaling. The interplay between capFbs and subcapsular cTECs may be important for maintaining thymic architecture and thymopoietic niches, but uncovering its physiological significance and molecular basis still remains a challenge.

DPP4 is a useful marker for the detection and isolation of capFbs in the mouse thymus, although it has not yet been evaluated in the human thymus. It has been reported that DPP4 is expressed by activated fibroblasts in fibrotic tissues such as the skin of systemic sclerosis patients (80, 81). Genetic ablation and pharmacological inhibition of DPP4 ameliorated fibrosis in mice, indicating that DPP4 activity plays a role in fibroblast activation and tissue fibrosis. Whether the DPP4 is involved in the function of the thymic capsule remains to be elucidated.

MEDULLARY FIBROBLASTS

mFbs resemble fibroblastic reticular cells (FRCs) that form conduit-like network in secondary lymphoid organs, and they have also been known as thymic FRCs (71, 82). Transcriptome analyses on mFbs and LN FRCs revealed possible functional differences between these two morphologically similar cell types (79, 83). LN FRCs are subdivided into several types based on their expression of functional genes, including chemokines and cytokines: T-cell zone reticular cell (TRC) expressing *Ccl19*, *Ccl21a*, and *Il7*; follicular dendritic cell (FDC) expressing *Cxcl13*; marginal reticular cell (MRC) expressing *Tnfsf11* (RANKL); Cxcl12-expressing reticular cell (CRC); and medullary reticular cell (medRC) expressing *Cxcl12*, *Il6*, *Tnfsf13*, and *Tnfsf13b*. Most of these FRC-associated genes are not or just barely expressed in mFbs. In the thymus, RANKL is predominantly expressed in SP thymocytes to induce mTEC development (84), and Ccl19, Ccl21, Cxcl12, and IL-7 are produced by TECs so as to control the relocation and survival of thymocytes (85, 86). It is likely that the role played by FRCs in the LN is replaced by TECs in the thymus. mFbs highly express other sets of chemokines as well, such as Cx3cl1 and Cxcl14, that are barely expressed in LN FRCs or capFbs, possibly contributing to the regulation of cell migration in the thymic medulla. Thus, mFbs comprise a thymus-specific subset of fibroblasts that is functionally distinct from LN FRCs. It is possible that mFbs also consist of multiple subsets with different functions. A population of $CD34^+$podoplanin$^+$ mFbs are detected in adventitial layers that surround pericytes and endothelial cells, thus referred to as adventitial cells (72).

A set of genes that include certain collagens (*Col6a5*, *Col6a6*), matrix metalloprotease-9 (*Mmp9*), metabolic enzymes (*Hmgcs2*, *Ltc4s*, and *Qprt*), and TGFβ-binding proteins (*Ltbp1* and *Ltbp2*) are predominantly expressed in mFbs among the thymic stromal cell types. Notably, most of these mFb-associated genes are expressed under the control of the lymphotoxin signal.

Lymphotoxin ($LT\alpha_1\beta_2$), a TNF superfamily ligand, is primarily expressed by SP thymocytes in the thymus and binds to the lymphotoxin β receptor (LTβR) expressed in thymic stromal cells to induce intracellular signal transduction. Early studies reported that the LTβR expressed in thymic stroma is important for the induction of T cell tolerance, followed by later studies demonstrating the requirement of the LTβR in optimal mTEC differentiation (87–89). However, mice lacking LTβR specifically in TECs did not display any impact on T cell tolerance, indicating that the key target of lymphotoxin signaling in the context of tolerance induction should be non-TEC stromal cells (90, 91). We demonstrated that the LTβR expressed in mFbs is pivotal for the induction of T cell tolerance. LTβR-dependent, mFb-associated genes include certain TRAs, and mice specifically lacking LTβR in thymic fibroblasts exhibited a marked production of autoantibodies against those TRAs (79). The fibroblast-specific LTβR-deficient mice displayed signs of autoimmunity against peripheral tissues in a manner that was similar to systemic LTβR-deficient mice. These findings indicate that mFbs act as the primary source of certain self-antigens for the induction of T cell tolerance.

The number of self-antigens expressed in mFbs is less than that in mTECs, but mFbs express a set of cell type-specific antigens that mTECs cannot cover, contributing to the diversity of self-antigen expression so as to ensure the establishment of self-tolerance. Unlike mTECs, mFbs express MHC class I but not MHC class II or co-stimulatory molecules, suggesting that mFbs do not function as professional antigen-presenting cells. It is likely that mFb-derived self-antigens are transferred to and presented by thymic dendritic cells to induce T cell tolerance, just as a substantial portion of mTEC-derived antigens are indirectly presented by thymic dendritic cells (92, 93).

It is also possible that mFbs indirectly control T cell tolerance through the organization of mTEC development, since the fibroblast-specific deficiency of LTβR results in a reduction in the number of mTECs (79). Consistent with this, a previous report showed that the LTβR signal influences the localization of mFbs and their interaction with mTECs (75). In contrast, the loss of mTECs has no influence on mFb cellularity or gene expression (79). These findings indicate that mFbs are upstream in the hierarchy of stromal interactions in the medullary microenvironment. LTβR-dependent, mFb-associated genes such as extracellular matrixes or proteases might play various roles in organizing the cellularity and function of mTECs. LTβR signal also controls the expression of cell adhesion molecules ICAM-1 and VCAM-1 in mFbs, suggesting the role of mFbs in regulating immune cell trafficking in the thymus (72, 94).

CELL–CELL INTERACTIONS IN THYMIC MEDULLA FORMATION

It is known that the formation of the thymic medulla is induced upon the development of SP thymocytes (33, 95). This lympho-stromal interplay is referred to as 'thymic crosstalk'. The major mediator of the thymic crosstalk signal is RANKL, a TNF superfamily ligand expressed by positively selected SP thymocytes. Its receptor, RANK, is expressed predominantly on mTECs in the thymus. Mice lacking either RANKL or RANK exhibit a marked reduction of $Aire^+$ mTECs and signs of autoimmunity, indicating the essential role of the RANKL-RANK axis in the induction of mTEC development and thereby T cell tolerance (84, 96, 97). RANKL is produced as a membrane-bound ligand and cleaved into the soluble form by proteases. As mice specifically lacking soluble RANKL have no phenotype in the thymic medulla, it is strongly suggested that membrane-bound RANKL is pivotal for mTEC differentiation (98). This is consistent with a previous finding that optimal differentiation of mTECs requires self-antigen-specific, cell-to-cell interactions with SP thymocytes (99).

The RANK signal activates the transcription factor NF-κB through signaling pathways mediated by TRAF6, NIK, and IκB-kinase (IKK) (100–104) and thereby induces mTEC differentiation and Aire expression in mTECs (105). RANKL-stimulated mTECs produce osteoprotegerin (OPG), an inhibitory decoy receptor for RANKL, to self-tune their differentiation and Aire expression (84, 106–108). SP thymocytes produce other TNFSF ligands, CD40L and lymphotoxin. CD40L acts cooperatively with RANKL to promote the generation of $Aire^+$ mTECs (97), while lymphotoxin induces the differentiation of $Aire^-$ mTEC subsets, including $CCL21^+$ mTECs, Hassall's corpuscles, and thymic tuft cells (109–113).

Furthermore, lymphotoxin is required for the development of the mature mFbs that express a set of mFb-associated genes. The lymphotoxin signal in mFbs also controls the cellularity of $Aire^+$ mTECs (79). Along with the fact that SP thymocytes are the most abundant source of lymphotoxin as well as RANKL and CD40L in the thymus (84, 87), these series of findings provide a detailed picture of the thymic crosstalk in which developing SP thymocytes act on both mFbs and mTECs to establish the medullary microenvironment for selecting themselves (**Figure 1F**).

THE THYMIC VASCULATURE AND BLOOD–THYMUS BARRIER

After the vascularization takes place in the embryonic thymus, the endothelial cells that form the blood vessel network provide entry sites into the thymus for circulating ETPs. Thymic endothelial cells express a set of adhesion molecules, VCAM-1, ICAM-1, and P-selectin, that enable both the attachment of ETPs and their migration across the endothelium into the thymic microenvironment (114, 115).

A portion of the blood vessels in the CMJ and medullary regions are surrounded by extracellular matrix and pericytes to form perivascular spaces, through which mature T cells exit into the periphery. The T cell egress from the thymus is promoted by sphingosine-1-phosphate (S1P) and its receptor, S1P receptor 1 (S1P1). S1P is a lipid mediator that attracts mature SP thymocytes highly expressing S1P1 from the medullary region into the perivascular space. It has been shown that pericytes are responsible for the production of the S1P that promotes thymocyte egress (116). The trans-endothelial entry of ETPs and exit of mature

T cells are regulated by the LTβR expressed in endothelial cells and pericytes (94, 117). Although the cellular source of LTβR ligands in the context of controlling cell traffic and the significance of this regulatory mechanism are not yet known, the LTβR may be a potential target to efficiently restore T cell production capacity in certain therapeutic situations such as bone marrow transplantation (117). Thymic endothelial cells also produce SCF/KitL to promote the survival of c-Kit-expressing ETPs, and this interaction is bi-directional in that membrane-bound SCF/KitL induces proliferation of thymic endothelial cells (118).

It has been established that the vascular permeability in the thymus is lower than that in other organs, suggesting the existence of a putative blood-thymus barrier (BTB) that limits the penetration of large circulating molecules such as proteins into the thymus (119, 120). Studies with intravenous injection of tracers demonstrated that the BTB is prominent in the cortex but weak in the medulla and there blood-borne molecules, including antigens, can enter the thymus (121). This mechanism may provide peripheral antigens entry into the medullary microenvironment so as to induce tolerance to these antigens, while in the cortex the blockade of peripheral antigens may be essential for the induction of positive selection by the cTEC-derived self-antigens. A recent report demonstrated that the role of the tight junction-forming protein claudin-5 (Cld5) in the BTB (122). Cld5 is expressed in capillaries at the cortex and arterioles at the CMJ, but not in postcapillary venules at the CMJ and medulla. $Cld5^-$ postcapillary venule endothelial cells allow blood-borne molecules penetrate into the medullary parenchyma, while $Cld5^+$ endothelial cells block their thymic influx. The $Cld5^-$ endothelial cells are barely detectable in embryonic thymus but increase along postnatal development and aging, and serve as the site for the egress of mature T cells into the blood circulation in postnatal mice, suggesting that the expression profiles of $Cld5^+$ in thymic endothelial cell subsets control the BTB and T cell gateway. Whether and how the BTB is regulated during infection to avoid the entry of infectious pathogens into the thymus is an important, but still open question (123). Blood-borne antigens and peripheral antigens are also transported to the thymus medulla by dendritic cells, contributing to the deletion of self-reactive T cell clones and the differentiation of Tregs (124–127).

CONCLUSIONS

The studies on TEC development and TEC-specific characteristics conducted over the last two decades have remarkably advanced our understanding of T cell differentiation and repertoire selection in the thymus. On the other hand, non-TEC stromal cells, such as fibroblasts, endothelial cells, and pericytes, have been comparatively less investigated in view of T cell development and may be considered the 'unsung heroes' acting in the shadow of TECs. Based on the success of long-sought observations in histology and embryology, recent progress in and applications of single-cell transcriptome technologies have unveiled the functional diversity of such non-TEC stromal cells and highlighted their immunological functions. Along with pioneering studies on the regeneration of TECs (128–131), a better understanding of the cellular and molecular basis of the entire set of thymic stromal cells will provide valuable insights toward the *in vivo* reconstitution of the thymus for future therapeutic applications.

AUTHOR CONTRIBUTIONS

TN and HT wrote, drafted, and edited the manuscript. All authors contributed to the article and approved the submitted version.

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RANK Signaling in the Differentiation and Regeneration of Thymic Epithelial Cells

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Thymic epithelial cells (TECs) provide essential clues for the proliferation, survival, migration, and differentiation of thymocytes. Recent advances in mouse and human have revealed that TECs constitute a highly heterogeneous cell population with distinct functional properties. Importantly, TECs are sensitive to thymic damages engendered by myeloablative conditioning regimen used for bone marrow transplantation. These detrimental effects on TECs delay *de novo* T-cell production, which can increase the risk of morbidity and mortality in many patients. Alike that TECs guide the development of thymocytes, reciprocally thymocytes control the differentiation and organization of TECs. These bidirectional interactions are referred to as thymic crosstalk. The tumor necrosis factor receptor superfamily (TNFRSF) member, receptor activator of nuclear factor kappa-B (RANK) and its cognate ligand RANKL have emerged as key players of the crosstalk between TECs and thymocytes. RANKL, mainly provided by positively selected $CD4^+$ thymocytes and a subset of group 3 innate lymphoid cells, controls mTEC proliferation/differentiation and TEC regeneration. In this review, I discuss recent advances that have unraveled the high heterogeneity of TECs and the implication of the RANK-RANKL signaling axis in TEC differentiation and regeneration. Targeting this cell-signaling pathway opens novel therapeutic perspectives to recover TEC function and T-cell production.

Keywords: bone marrow transplantation, central tolerance, receptor activator of nuclear factor kappa-B, thymic crosstalk, thymic epithelial cells, thymic regeneration

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INTRODUCTION

The thymus supports the generation of distinct T-cell subsets such as conventional $CD4^+$ and $CD8^+$ T cells, $Foxp3^+$ regulatory T cells, γδ T cells, and invariant natural killer T cells (iNKT). The development of these different T-cell subsets depends on stromal niches composed of thymic epithelial cells (TECs). TECs control T-cell development from the entry of T-cell progenitors to the egress of mature T cells. According to their anatomical localization and functional properties, TECs are subdivided into two main populations: cortical TECs (cTECs) and medullary TECs (mTECs). cTECs support the initial stages of T-cell development, including T-cell progenitor homing, T-cell lineage commitment, the expansion of immature thymocytes, death by neglect of thymocytes that do not recognize peptide-MHC complexes and positive selection of thymocytes into $CD4^+$ and $CD8^+$ T cells. By contrast, mTECs control

late stages of T-cell development, mainly the induction of self-tolerance characterized by the clonal deletion of autoreactive thymocytes and $CD4^+$ thymocyte diversion into the $Foxp3^+$ regulatory T-cell lineage. Conversely, thymocytes control TEC expansion and differentiation. These bidirectional interactions between thymocytes and TECs are termed thymic crosstalk (1–3).

TEC HETEROGENEITY IN MOUSE AND HUMAN

Historically, cTECs and mTECs were identified by histology using distinct markers such as cytokeratin 8 for cTECs and cytokeratin-5 and -14 for mTECs (4). TEC identification by flow cytometry on enzymatically-disaggregated thymus has greatly aided in studying TEC heterogeneity and functionality. TECs are non-hematopoietic cells, which express the Epithelial Cell Adhesion Molecule (EpCAM), and are generally identified as $CD45^-EpCAM1^+$. TECs can be further segregated into cTECs and mTECs based on the detection of Ly51 and reactivity to the lectin Ulex Europaeus Agglutinin 1 (UEA-1), respectively. cTECs and mTECs have distinct phenotypic and functional properties. Recent advances based on single-cell transcriptomic analyses have highlighted that TECs constitute a more diverse and dynamic population than previously thought.

FEATURES OF CORTICAL TECs

cTECs express several molecules that govern the initial stages of T-cell development. They express CXCL12 and CCL25 chemokines that guide the homing of T-cell progenitors into the thymus (5, 6). cTECs also express the NOTCH ligand Delta-like 4 (DLL4), which induces the engagement of progenitors into the T-cell lineage (7, 8). Moreover, they express IL-7 and stem cell factor (SCF) cytokines that promote the survival and proliferation of immature thymocytes (9). They are equipped with protein degradation machineries important for the positive selection of $CD4^+$ thymocytes such as the lysosomal endopeptidase cathepsin L (encoded by *Ctsl*) and the thymus-specific serine protease TSSP (encoded by *Prss16*) that contributes to MHC class II-associated self-peptide generation (10). They also express the thymoproteasome subunit β5t (encoded by *Psmb11*), which produces MHC class I-associated self-peptides required for the positive selection of $CD8^+$ thymocytes (11).

cTECs are heterogeneous based on the expression level of MHCII, CD40, DLL4, and IL-7. Intriguingly, a cTEC subset specific of the perinatal thymus termed perinatal cTECs has been identified by single-cell transcriptomics (12). These cells, representing one-third of all TECs at 1 week of age, are highly proliferative and express synaptogyrin 1 (*Syngr1*) and G protein-coupled estrogen receptor 1 (*Gper1*) in addition to classical cTEC markers. Furthermore, by enveloping many viable double-positive (DP) thymocytes, a fraction of cTECs can form multi-cellular complexes called thymic nurse cells (TNCs) (13). TNCs likely provide a microenvironment favorable to secondary TCRα rearrangements in long-lived DP thymocytes, thereby optimizing TCR repertoire selection (14). Although TNCs remain poorly characterized, they exhibit a distinct gene expression profile characterized by high expression of CXCL12 and TSSP. TNCs thus constitute a cTEC subpopulation with distinct morphological and functional properties. Given that cTECs ensure multiple functions such as i) lymphoid progenitor homing, ii) T-cell lineage commitment, iii) immature thymocyte expansion, and iv) positive selection of thymocytes, it is likely that cTECs contain discrete functional subsets. Further investigations are required to clarify cTEC heterogeneity. Their development is regulated by signals provided by developing thymocytes. Human CD3ϵ transgenic mice (tgϵ26 mice), in which T-cell development is blocked at the early DN1 stage, have a disorganized cortex with cTECs arrested at the $CD40^-MHCII^{lo}$ stage (15, 16). However, the transplantation of tgϵ26 recipients with bone marrow cells from *Rag2*$^{-/-}$ mice, exhibiting a subsequent block at the DN3 stage, restores the cortical organization (4, 17). Furthermore, cTECs with a $CD40^+MHCII^{hi}$ phenotype develop in the thymus of *Rag1*$^{-/-}$ mice (16). Thus, cTEC development requires signals from thymocytes beyond the DN1 stage. Nevertheless, the cell-signaling pathways responsible for their development remain to be determined.

FEATURES OF MEDULLARY TECs

Compared to cTECs, mTECs are better characterized, likely because they are more abundant. mTECs have the unique ability to express up to 85%–90% of the genome and virtually all protein-coding genes (18). This promiscuous gene expression program is induced by the autoimmune regulator (Aire) and the transcription factor Fez family zinc finger 2 (Fezf2) (18, 19). mTECs contain two main subsets identified on MHCII and CD80 cell surface expression levels: $MHCII^{lo}CD80^{lo}$ ($mTEC^{lo}$) and $MHCII^{hi}CD80^{hi}$ ($mTEC^{hi}$) (20). These two subsets are heterogeneous based on distinct markers and functional properties. $mTEC^{lo}$ contain $mTEC^{hi}$ precursors expressing alpha-6 integrin (*Itga6*) and Sca1 (*Ly6a*) (21–23). They also comprise $CCL21^+$ mTECs implicated in the migration of positively-selected thymocytes into the medulla (24). Cell fate mapping studies have identified that $mTEC^{lo}$ contain post-Aire cells characterized by the loss of Aire protein and low surface levels of MHCII and CD80 molecules (25–27). Another subset of terminally differentiated mTECs closely resembling the gut chemosensory epithelial tuft cells are also present in $mTEC^{lo}$ (28, 29). These cells express the doublecortin-like kinase 1 (Dclk1) marker and the transcription factor Pou2f3. Thus, the $mTEC^{lo}$ compartment is particularly heterogeneous, containing not only $mTEC^{hi}$ precursors but also $CCL21^+$, post-Aire and tuft-like mTECs. The $mTEC^{hi}$ compartment is also diverse, containing $Aire^-Fezf2^+$ and $Aire^+Fezf2^+$ subsets.

Single-cell transcriptomic analyses have identified dozens of TEC subsets, including perinatal cTECs, mature cTECs, mTEC progenitors, $Aire^+$, post-Aire and tuft-like mTECs (12, 28, 30). Among them, two other minor subsets termed neuronal and structural TECs have been identified based on their expression signatures associated with neurotransmitters and extracellular matrix such as collagens and proteoglycans (12). Further investigations are required to define their anatomical localization

and function. Interestingly, a subset of proliferating mTECs expresses substantial levels of *Aire*, suggesting that it corresponds to a maturational stage just before Aire$^+$ mature mTECs (12, 30).

In humans, cTECs and mTECs are defined as EpCAM^{int}CDR2^{hi} and EpCAM^{hi}CDR2$^-$, respectively (31). AIRE and FEZF2 are also expressed in human mTECs, indicating a conserved mechanism for the regulation of tissue-restricted self-antigens (19, 32, 33). Recent single-cell transcriptomic analyses across the lifespan showed a largely conserved TEC heterogeneity in humans (34). cTECs are more abundant during early fetal development, then a population with cTEC and mTEC properties appears in the late fetal and pediatric human thymus and lastly mTECs are dominants. Interestingly, two rare TEC subsets expressing *MYOD1* and *NEUROD1* genes that resemble myoid and neuroendocrine cells, respectively, were also identified. Although these subsets are preferentially located in the medulla, their respective function remains to be studied.

RANK-RANKL AXIS IN MTEC EXPANSION AND DIFFERENTIATION

The tumor necrosis factor receptor superfamily (TNFRSF) member, receptor activator of nuclear factor kappa-B (RANK; encoded by *Tnfrsf11a*) and its cognate ligand RANKL (encoded by *Tnfsf11*) play a privileged role in mTEC expansion and differentiation. During embryonic development, RANK gradually increases and is expressed by Aire$^+$ mTEC precursors (35). In the adult, RANK is expressed by subsets that reside within mTEC^{lo} and mTEC^{hi}, including CCL21$^+$ and Aire$^+$ cells (36). Importantly, the RANK-RANKL axis activates the classical and non-classical NF-κB signaling pathways that control the development of Aire$^+$ mTECs (37). In the embryonic thymus of RANK- or RANKL-deficient mice, Aire$^+$ mTECs are absent, indicating that this axis governs the emergence of Aire$^+$ mTECs (37, 38). At this stage, RANKL is provided by CD4$^+$CD3$^-$ lymphoid tissue inducer (LTi) cells and invariant Vγ5$^+$ dendritic epidermal T cells (DETC) (39). Nevertheless, other hematopoietic cells might be implicated since few Aire$^+$ mTECs are still detected in the embryonic thymus of mice lacking both LTi cells and DETC. In the postnatal thymus, the absence of RANK or RANKL leads to a partial reduction in Aire$^+$ mTECs, showing that other signal(s) are involved in mTEC differentiation after birth (37, 40). Although *Cd40*$^{-/-}$ and *Cd40lg*$^{-/-}$ mice show subtle defects in Aire$^+$ mTECs, these cells are further decreased in *Tnfrsf11a*$^{-/-}$ × *Cd40*$^{-/-}$ double-deficient mice compared to *Tnfrsf11a*$^{-/-}$ mice, showing that RANK and CD40 cooperate to induce mTEC differentiation after birth (37). In the postnatal thymus, whereas CD40L is exclusively provided by CD4$^+$ thymocytes, RANKL is higher in CD4$^+$ than in CD8$^+$ thymocytes and detected in iNKT cells (40–42) **(Figure 1A)**. The contribution of LTi, DETC and iNKT cells in the adult might be limited due to their paucity compared to the large numbers of CD4$^+$ thymocytes. This assumption is corroborated by the fact that mice deficient in CD4$^+$ thymocytes have a dramatic reduction in Aire$^+$ mTECs and an underdeveloped medulla (41, 43).

RANKL is primarily synthesized as a membrane-bound trimeric complex that can be cleaved into its soluble form by proteases (44). A recent study showed that mice lacking soluble RANKL have normal numbers of Aire$^+$ mTECs, indicating that membrane-bound rather than soluble RANKL induces their differentiation (45). Accordingly, RANKL and CD40L signals are delivered by CD4$^+$ thymocytes in the context of antigen-specific TCR/MHCII-mediated interactions with mTECs (41, 43, 46). This is well illustrated in Rip-mOVAxOTII-*Rag2*$^{-/-}$ mice, in which the Rip-mOVA transgene drives the expression of membrane-bound OVA in mTECs allowing high affinity interactions with OVA-specific OTII CD4$^+$ thymocytes. Aire$^+$ mTECs develop in these mice in contrast to OTII-*Rag2*$^{-/-}$ mice. RANKL in CD4$^+$ thymocytes is likely regulated by TGFβRII signaling (47). Mice lacking TGFβRII in αβ thymocytes at the early DP stage (*Cd4*-cre x *Tgfbr2*$^{fl/fl}$ mice) have reduced RANKL levels in Helios$^+$ autoreactive CD4$^+$ thymocytes. Conversely, the stimulation of purified autoreactive CD4$^+$ thymocytes with TGF-β increases RANKL expression. This upregulation is prevented by MAPK pathway inhibitors, indicating that TGFβRII signaling induces RANKL by its SMAD4/TRIM33-independent pathway. Similarly, TGF-β stimulation was shown to increase RANKL in TCR-activated T-cell hybridoma (48).

RANK signaling is regulated by the soluble decoy receptor for RANKL, osteoprotegerin (OPG; encoded by *Tnfrsf11b*), which inhibits RANKL interaction with its receptor RANK. OPG deficiency leads to an increased mTEC cellularity resulting in enlarged medulla with an enrichment in Aire$^+$ mTECs (49). Mice harboring a *Tnfrsf11b* deletion in mTECs have increased numbers of total and Aire$^+$ mTECs, similarly to *Tnfrsf11b*$^{-/-}$ mice (50). Thus, OPG produced locally by mTECs rather than serum OPG regulates mTEC cellularity and differentiation. RANK activates *Aire* expression by the NF-κB signaling because *Aire* contains in its upstream coding region a highly conserved noncoding sequence 1 (CNS1) with two NF-κB binding sites (51, 52). CNS1-deficient mice consequently lack *Aire* expression in mTECs and show many characteristics of *Aire*$^{-/-}$ mice including reduced Aire-dependent tissue-restricted self-antigens. Noteworthy, the RANK-RANKL axis does not only induce *Aire* by itself but also controls mTEC cellularity and differentiation. In addition to Aire$^+$ mTECs, *Tnfsf11*$^{-/-}$ mice show reduced numbers of mTEC^{lo} and mTEC^{hi} (37). Conversely, *Tnfrsf11b*$^{-/-}$ mice have increased numbers of CCL21$^-$ and CCL21$^+$ mTEC^{lo} and Aire$^-$ and Aire$^+$ mTEC^{hi} (36, 49). Accordingly, the stimulation of 2-deoxyguanosine-treated thymic lobes with RANKL show increased mTEC cellularity including Aire$^+$ mTEC^{hi}, which is further augmented by the addition of CD40L protein (43, 53). Furthermore, *in vivo* anti-RANKL blockade results in a severe depletion of around 80% of mTECs with a substantial loss of mTEC^{lo} and Aire$^+$ mTEC^{hi} (49). In addition to control Aire$^+$ mTECs, RANK signaling therefore regulates the overall mTEC cellularity.

In humans, scRNA-seq data indicate that RANK is expressed by Aire$^+$ mTECs (34). Interestingly, the stimulation of primary human mTECs with RANKL leads to the upregulation of *AIRE* mRNA, suggesting a conserved role for RANK signaling (54). Given the implication of RANK-RANKL axis in bone resorption, a monoclonal antibody specific of human soluble and membrane-bound RANKL, Denosumab, has been developed to inhibit osteoclast development and activity. Denosumab is now used in

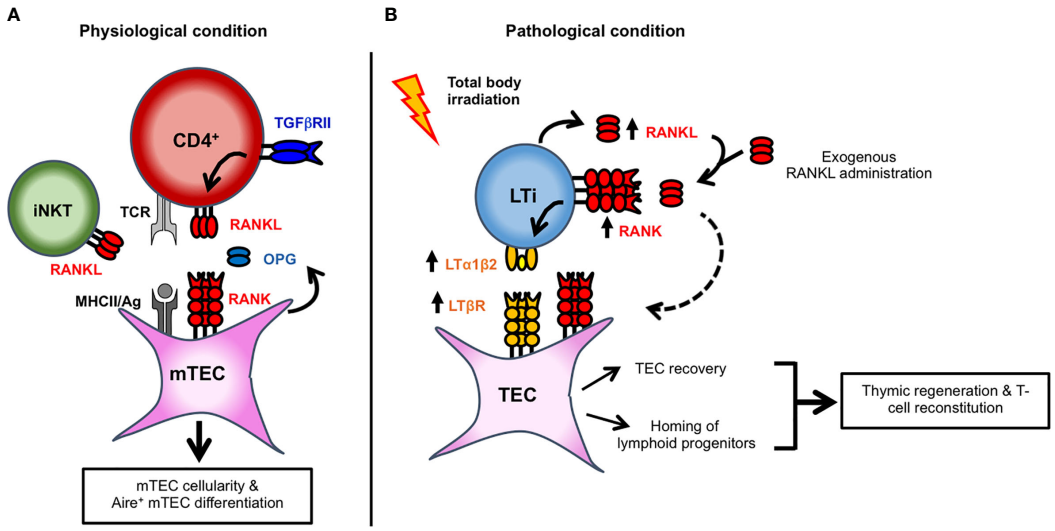

FIGURE 1 | Key cellular actors implicated in RANK-RANKL signaling axis and in mTEC development and thymic regeneration. **(A)** TGFβRII signaling upregulates the expression of RANKL in autoreactive CD4$^+$ thymocytes. RANKL, expressed by autoreactive CD4$^+$ thymocytes and iNKT cells, controls mTEC cellularity and Aire$^+$ mTEC differentiation. By binding to RANKL as a decoy receptor, OPG produced by mTECs inhibits RANK signaling and thereby regulates mTEC development. **(B)** Upon total body irradiation, radio-resistant LTi cells and CD4$^+$ thymocytes upregulate RANKL expression. LTi cells also upregulate RANK receptor. RANKL upregulation or exogenous RANKL administration induces the heterocomplex LTα1β2 at the cell surface of LTi. RANKL could also stimulate RANK signaling in TECs (dashed arrow). TECs also upregulate the corresponding LTβR receptor after total body irradiation. In turn, LTα1β2-LTβR axis activation induces TEC regeneration by promoting their proliferation and survival. Furthermore, by inducing the expression of chemokines and adhesion molecules, this axis also favors the thymus homing of circulating T-cell progenitors.

therapy to treat osteoporosis, primary bone tumors and bone metastases (55, 56). Nevertheless, considering the importance of RANK-RANKL axis in Aire$^+$ mTEC differentiation, it remains to be defined whether this treatment could affect central tolerance and increase the risk of autoimmunity.

SENSIBILITY OF TECs TO MYELOABLATIVE CONDITIONING REGIMEN

Myeloablative treatments such as radiation and chemotherapy deplete hematopoietic cells and in particular DP thymocytes that are extremely sensitive. These treatments also impair the recruitment of circulating T-cell progenitors and induce damages on TECs (**Figure 2**). Consequently, the generation of newly produced naïve T cells is reduced. Since TECs dictate the size of stromal niches, TEC injury contributes in a delayed T-cell reconstitution upon bone marrow (BMT) or hematopoietic stem transplantation (HSCT). In humans, allogeneic HSCT survivors are immunodeficient in T cells for at least 1 year, a period of high susceptibility to opportunistic infections, autoimmunity or tumor relapse, increasing the risk of morbidity and mortality (57, 58). Although innate cells and antibodies may limit viral infections, cytotoxic CD8$^+$ T cells and helper CD4$^+$ T cells are essential in viral clearance and the prevention of recurrent infections. T-cell recovery thus protects from lethality after BMT or HSCT. Importantly, T-cell immunity relies on the regeneration of the thymus and its capacity to produce naïve T cells. Total body irradiation (TBI) leads rapidly in a profound reduction of the cortex due to the loss of DP thymocytes and a substantial decrease of the medulla (59). Both cTECs and mTECs are radiosensitive (60, 61). Among mTECs, Aire$^+$ mature mTECs are lost upon TBI and treatment with the chemotherapy agent cyclophosphamide or the immunosuppressant cyclosporine A, used to prevent allograft rejection (61, 62). However, the effects of such treatments on the recently identified dozens of TEC subsets remain to be investigated. Remarkably, the injured thymic tissue retains potent regenerative capacity. Targeting the pathways implicated in endogenous TEC regeneration is expected to improve thymic-dependent T-cell recovery. Potential strategies based on keratinocyte growth factor (KGF), IL-22 or Bone Morphogenic Protein 4 (BMP4) have been reviewed in (58, 63, 64). Strategies based on FOXN1 protein or cDNA administration also improve TEC regeneration both in the context of HSCT and aging (65, 66). A novel role for the RANK-RANKL axis in TEC regeneration and T-cell recovery is highlighted below.

RANK-RANKL AXIS IN TEC REGENERATION

RANKL is upregulated in radio-resistant LTi cells and CD4$^+$ thymocytes during the early phase of thymic regeneration after total body irradiation (TBI) (61, 67, 68). Although LTi cells are rare in the thymus, they express a higher level of RANKL than CD4$^+$ thymocytes after TBI (61). Interestingly, the administration of a neutralizing anti-RANKL antibody impairs TEC regeneration,

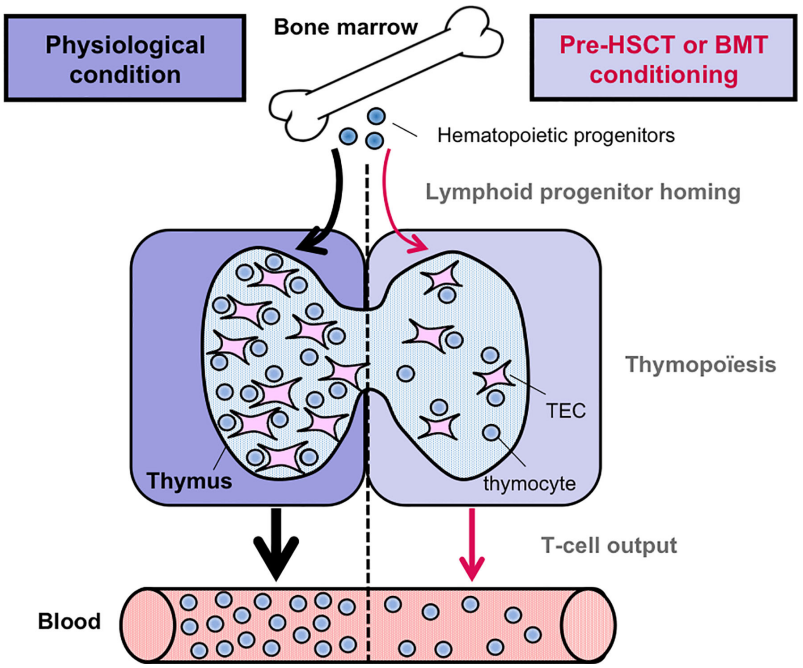

FIGURE 2 | Pre-transplantation conditioning regimen alters thymic-dependent T-cell production. In contrast to the physiological condition, the homing ability of circulating T-cell progenitors is reduced after pre-HSCT or BMT conditioning regimen. Furthermore, T-cell production is also reduced, notably due to TEC damages induced by myeloablative regimens. Consequently, the output of newly generated naïve T cells is diminished.

emphasizing an important role for RANKL in endogenous TEC recovery. Conversely, RANKL protein administration increases TEC numbers at a level close to unirradiated mice. RANKL enhances cTEC and mTEC numbers, including Aire^{+} mTEC^{hi} and TEPC-enriched cells, likely by stimulating their proliferation and survival. These observations are in agreement with a previous study indicating that RANKL increases *in vitro* the proliferation of cortical and medullary TEC cell lines (69). Of clinical relevance, RANKL administration upon BMT boosts not only the regeneration of several TEC subsets but also increases T-cell progenitor homing **(Figure 1B)** (61). This latter effect could be explained by an enhanced cellularity of endothelial cells upon RANKL administration although further investigations are required. Consequently, this treatment ameliorates *de novo* thymopoiesis and peripheral T-cell reconstitution. Noteworthy, a single course of RANKL after BMT boosts thymic regeneration at least during 2 months, indicative of a lasting effect. This therapeutic strategy is also efficient in aged individuals in whom T-cell recovery upon BMT is less efficient and delayed (70). Age-related thymic involution results in a disrupted thymic architecture with a reduced TEC cellularity, which alters T-cell production (71). RANKL treatment could be thus of special interest to the elderly, although further studies are required.

Mechanistically, RANKL upregulates another TNF family ligand, lymphotoxin α (LTα; encoded by *Lta*), expressed as a membrane anchored LTα1β2 heterocomplex, in LTi of recipient origin **(Figure 1B)** (61). Conversely, the RANK-Fc antagonist fully blocks LTα1β2 upregulation. Noteworthy RANKL also induces LTα1β2 expression in LTi cells during lymph node formation (72). Likewise RANKL, LTα is upregulated during the early phase of thymic regeneration. Since CD4^{+} thymocytes upregulate RANKL and since LTi cells express both RANK and its ligand, RANK signaling may be triggered in LTi in an autocrine and paracrine manner. Given that LTi cells upregulate RANKL, LTα1β2, IL-22, IL-23R, and RORγt after thymic injury (61, 68), these cells are likely in a quiescent stage at steady state and activated after irradiation to repair the injured thymic tissue. Accordingly, the depletion of ILC3, comprising LTi cells, in an experimental model of graft-versus-host disease (GVHD) results in impaired thymic regeneration (73). Interestingly, LTβR is also upregulated in cTECs, mTECs, and TEPC-enriched cells after TBI, suggesting that the LTα1β2-LTβR axis is implicated in TEC regeneration (61). At steady state, *Lta*$^{-/-}$ mice show normal numbers of TEC subsets. In contrast, cTECs, mTECs including Aire^{+} mTEC^{hi} and TEPC-enriched cells are substantially reduced in these mice upon BMT. These observations indicate that the mechanisms implicated in TEC regeneration are distinct from those used at steady state. Furthermore, these mice show reduced numbers of early T-cell progenitors (ETPs) because LTα controls the homing capacity of circulating T-cell progenitors by regulating the expression of CCL19 and CCL21 in TECs and ICAM-1, VCAM-1, and P-selectin in endothelial cells, all implicated in T-cell progenitor homing (61, 74). Similarly, *Ltbr*$^{-/-}$ mice have an altered recruitment of T-cell progenitors after sublethal TBI (75). In agreement with defective TEC regeneration and T-cell progenitor homing, BM-transplanted *Lta*$^{-/-}$ mice have impaired thymic and peripheral T-cell reconstitution. These beneficial effects induced by RANKL depend on LTα since they are essentially lost when RANKL is

administered in *Lta*$^{-/-}$ recipients. RANKL administration thus constitutes a novel therapeutic strategy to improve T-cell function recovery after thymic injury. Interestingly, RANK and LTβR expression is conserved in the human thymus, opening potential therapeutic perspectives (34). Besides applications linked to myeloablative conditioning regimen, these *in vivo* findings open new avenues to treat patients whose thymus has been severely damaged by aging, viral infections, or malnutrition.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

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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, β5t, 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 β5t^{+} embryonic bipotent progenitors, adult β5t^{+} 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 β5t^{+} bipotent progenitors, they develop into mTEC-restricted SSEA-1^{+} CLD3/4^{hi} progenitors after losing β5t 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 β5t 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 β5t$^+$ or β5t$^-$ progenitors independently (38). This proposal contradicts the argument put forth by Ohigashi et al. (23) that β5t$^+$ and β5t$^-$ 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 (0 g) conditions during spaceflight, which was partially mitigated by exposure to 1 g 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
Baren-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

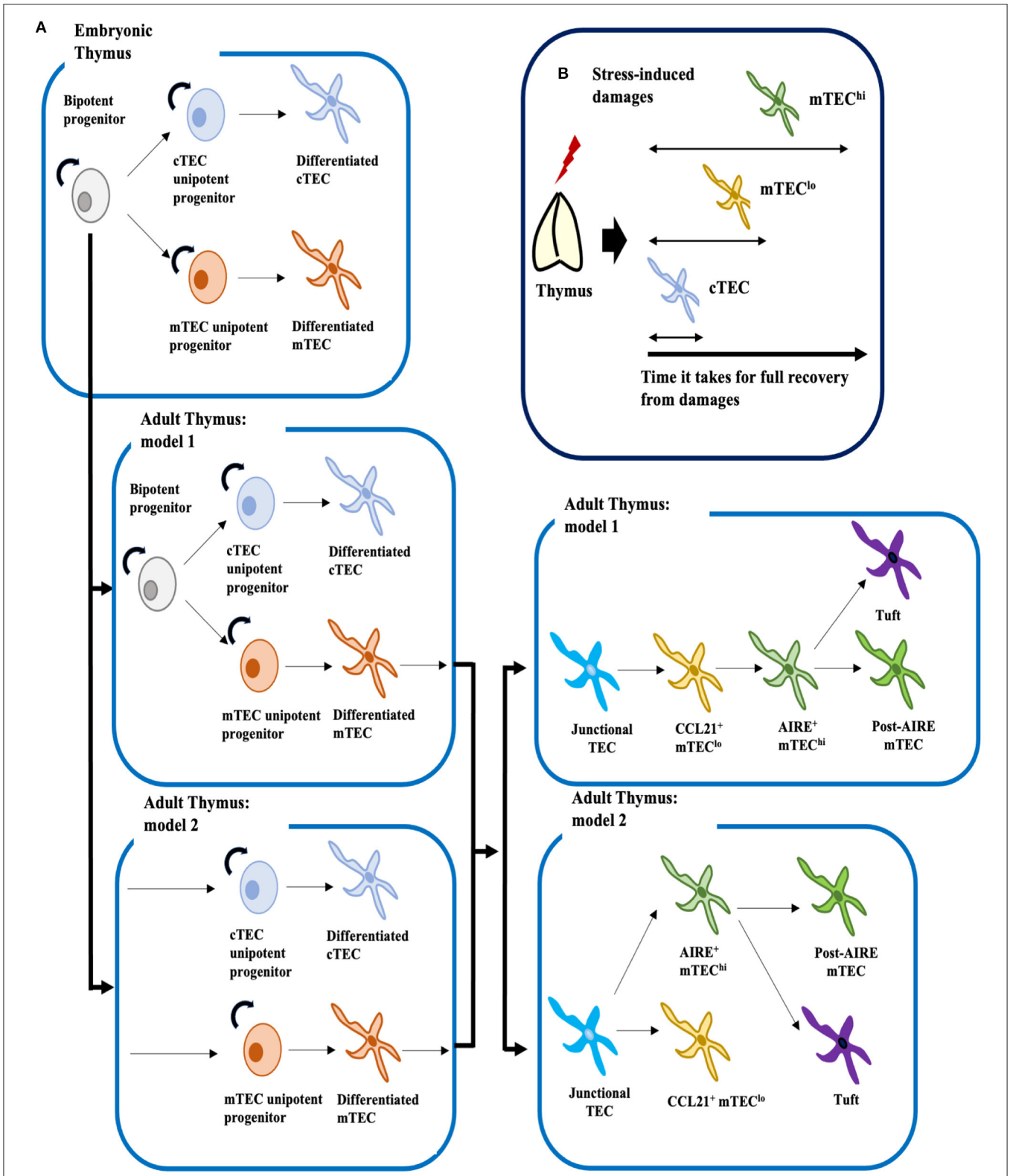

FIGURE 1 | Proposed mechanistic models for development of TECs under homeostatic conditions, and cellular mechanisms of TEC recovery after insults. **(A)** Embryonic bipotent progenitors in the thymus can generate both mature cTECs and mTECs. In the adult thymus, two models for development of cTECs and 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 necessary 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 β5t^{+} 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.

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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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REVIEW
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Non-Epithelial Stromal Cells in Thymus Development and Function

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The thymus supports T-cell development *via* specialized microenvironments that ensure a diverse, functional and self-tolerant T-cell population. These microenvironments are classically defined as distinct cortex and medulla regions that each contain specialized subsets of stromal cells. Extensive research on thymic epithelial cells (TEC) within the cortex and medulla has defined their essential roles during T-cell development. Significantly, there are additional non-epithelial stromal cells (NES) that exist alongside TEC within thymic microenvironments, including multiple subsets of mesenchymal and endothelial cells. In contrast to our current understanding of TEC biology, the developmental origins, lineage relationships, and functional properties, of NES remain poorly understood. However, experimental evidence suggests these cells are important for thymus function by either directly influencing T-cell development, or by indirectly regulating TEC development and/or function. Here, we focus attention on the contribution of NES to thymic microenvironments, including their phenotypic identification and functional classification, and explore their impact on thymus function.

Keywords: thymus, thymocyte development, mesenchyme cells, endothelial cell, lymphoid tissue development

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INTRODUCTION

The production of a diverse αβ-T-cell pool is vital to establishing and maintaining a functional adaptive immune system. While adult lymphoid progenitor cells are produced by the bone marrow, their lineage commitment and development into mature T-cells is dependent on their migration into the thymus, where essential interactions with heterogeneous thymic stromal cells take place (1). Arguably, the most recognized and well-studied stromal populations in thymus biology are thymic epithelial cells (TEC) within the cortical and medullary areas, which are defined by their anatomical separation and function. Cortical epithelial cells (cTEC) support the earliest thymocyte progenitor populations, guiding them through the cortex, directing them to a T-cell lineage fate and ensuring their functional qualities as self-MHC restricted cells through the process of positive selection (2). Medullary epithelial cells (mTEC), in conjunction with dendritic cells, then take over control of developing thymocytes. Through the process of producing and presenting a diverse array of self-antigens, mTEC drive single positive thymocytes either down conventional or $Foxp3^+$ regulatory thymocyte lineages (3). In addition, mTEC screen thymocytes for their expression of high affinity αβ-TCRs, with negative selection limiting the release of these reactive T-cells into the peripheral pool (4). In addition to intrathymic selection, and during this medullary residency period, $CD4^+$ and $CD8^+$ single positive (CD4SP, CD8SP) thymocytes acquire the ability to proliferate in response to TCR stimulation, and undergo licensing for cytokine production, prior to exiting the thymus to join

the peripheral T-cell pool as Recent Thymus Emigrants (RTE) (5, 6). While many studies have demonstrated the importance of cTEC and mTEC during T-cell development, thymic stromal microenvironments also contain heterogenous non-epithelial stromal (NES) populations in a similar manner to that seen in peripheral lymphoid tissues. Broadly separated into mesenchymal and endothelial cells, NES have been implicated in thymus organogenesis, thymocyte development, tolerance induction and development/maintenance of epithelial stroma. As with TEC, our understanding of NES has been improved through the phenotypic identification of new subpopulations, such studies on thymic mesenchyme and endothelium have provided new and important insight into their complexity and functional importance. In this review, we cover how the non-epithelial compartment of thymic stroma represent essential cell populations in regulating thymus function.

THYMIC MESENCHYME

Mesenchyme in Early Thymus Development and Function

Organogenesis and Origin

In early experiments, physical separation of mesenchymal and epithelial stroma from embryonic murine thymus resulted in defective thymus development when epithelium was cultured *in vitro* in the absence of mesenchyme (7). These findings were confirmed in later studies in which removal of mesenchyme from embryonic day 12 murine thymic lobes impaired thymus growth *in vitro* (8). Furthermore, *in ovo* surgical ablation of cephalic neural-crest mesenchyme resulted in significantly reduced thymic size in fertile Arbor Acre chick embryos (9). Collectively, these studies provided clear evidence that the presence of mesenchymal stroma is key during the earliest stages of thymus development, when the endodermal-derived TEC rudiment is enveloped within and colonized by peri-thymic neural crest (NC)-derived mesenchyme. Moreover, studies by Jiang et al. (10) utilized $Wnt1^{Cre}$ mediated fate mapping models to directly demonstrate the association of NC-derived mesenchymal cells with the embryonic murine thymus. Interestingly, although this study, and another by Yamazaki et al. (11) using a myelin protein zero fate mapping model, suggested that NC-derived mesenchymal cells could be scarcely detected in the postnatal thymus, subsequent studies using $Wnt1^{Cre}$ based models provided evidence that NC-derived mesenchyme persists in adult thymic microenvironments (12, 13). Collectively, these findings in mammalian systems are consistent with the studies by Bockman and Kirby (9) using avian models that specifically pointed towards the importance of cells of neural crest origin in early thymus development.

With regard to the mechanisms of thymic mesenchyme function, their production of a number of growth factors has been shown to influence TEC populations. For example, Revest et al. discovered that fibroblast growth factor 7 (FGF7) and FGF10 produced by thymic mesenchyme and their receptor FGF-R2IIIb expressed by TEC were also essential for normal TEC development (14). In the absence of FGF-R2IIIb, thymus development appeared arrested around embryonic day 12.5, and the absence of either FGF-R2IIIb or FGF10 resulted in hypoplastic thymi and reduced TEC proliferation (14). In later studies, enzymatic removal of thymic mesenchyme was shown to reduce TEC proliferation (15), which could be restored by the addition of exogenous FGF7 and/or FGF10 *in vitro*. Interestingly, this study also found that while mesenchyme and FGF7/10 are essential for TEC proliferation, they are disposable for TEC differentiation, with embryonic TEC acquiring cTEC and mTEC phenotypes in the absence of mesenchyme and importantly these epithelial cells are functionally mature (15). Indeed, transplantation of embryonic day 12 thymus lobes stripped of thymus mesenchyme remained small, but retained the ability to support a complete program of T-cell development (16). Such findings suggest that an important property of thymic mesenchyme is to induce the proliferation of developing TEC in order to provide increasing numbers of intrathymic niches to support efficient T-cell development. In the absence of such mesenchyme-derived signals, niche availability remains limited and results in diminished T-cell production. In addition, epidermal growth factor (EGF) has been suggested to be a product potentially produced by thymic mesenchyme to support TEC proliferation and thymus lobule formation, as EGF can replace thymic mesenchyme in supporting TEC in cultures (17). Similarly, insulin-like growth factor (IGF) has been shown to be a mesenchyme product and IGF has been shown to regulate TEC development, but it has not been directly shown that mesenchymal-specific loss of either IGF or EGF leads to TEC defects (16–18). Thus, thymic mesenchymal cells play an important role in quantitatively regulating thymus function. While several mesenchymal growth factors have been implicated in this process (e.g., FGF7/10), further studies are required to examine the distinct functional properties, and cellular origins, of these molecules. Relevant to this, expression of the transcription factor MafB by thymic mesenchyme was shown to regulate production of Wnt3, Wnt11, and BMP4 which regulate TEC function, including the production of chemokines required for normal progenitor homing to the thymus (19).

Finally, as well as influencing the functional properties of TEC, mesenchyme has also been shown to influence the anatomic positioning of the thymus during its development. Studies of $Pax3^{Sp/Sp}$ mice, which carry a point mutation in Pax3 eliminating protein function, revealed that separation of the thymus from the pharynx, migration of the thymus to its position at the mediastinum and distribution of the thymus: parathyroid domains are dependent on the presence of thymic mesenchyme (17, 20). The thymus of these mice was significantly increased in size whereas the parathyroid was reduced and there was a delay in the separation of the two as a result of dysfunctional boundary formation between the thymus and parathyroid domains (20). Interestingly, evidence also suggests that a bi-directional relationship may exist between neural-crest derived mesenchyme and endoderm, where epithelial-derived FGF8 may regulate mesenchymal populations, where FGF8 hypomorphs demonstrate ectopic thymus positioning and

thymic hypoplasia possibly as a result of impaired mesenchymal expression of FGF10 (21).

Mesenchyme and T-Cell Development

Using the reaggregate thymic organ culture (RTOC) technique, where defined thymocyte and stromal subsets can be reassembled into 3-dimensional structures, studies provided evidence that specific stages of thymocyte development are also dependent on the presence of thymic mesenchyme (22, 23). For example, culture of $CD4^{-}CD8^{-}$ (DN) T-cell precursors in the presence of TEC alone resulted in an absence of further T-cell development. In contrast, the addition of mesenchymal cells resulted in development to the $CD4^{+}CD8^{+}$ double positive (DP) and CD4SP and CD8SP stages (23). Importantly, TEC alone were able to support the development of $CD4^{+}CD8^{+}$ thymocytes to the CD4SP and CD8SP stages, indicating that a stage-specific requirement for mesenchyme operates at the DN-DP but not DP-SP transition. Later, further analysis revealed that the importance of thymic mesenchyme was specific to supporting the development of $CD25^{+}CD44^{+}$ DN2 thymocytes, with independence from mesenchyme occurring from the $CD25^{+}CD44^{-}$ DN3 stage (22). How thymic mesenchyme influences specific stages of T-cell precursor development remain unclear. However, it is interesting to note that this requirement can be met by both mesenchyme cells of non-thymic origin (eg embryonic lung) or fibroblast cell lines (eg NIH-3T3) (23). Moreover, and as demonstrated for bone marrow stroma (24), mesenchymal cells that regulate lymphoid progenitor development are effective presenters of the key cytokine IL7 *via* their production of extracellular matrix components (22), suggesting a possible mechanism for their involvement in early T-cell development. Importantly however, it is important to note that studies on the regulation of T-cell development by thymic mesenchyme are based largely on the requirements for fetal lymphoid progenitors, and often involve analysis of T-cell development using *in vitro* culture. As such, further studies are required to assess whether similar requirements operate in both the fetal thymus *in vivo*, and whether DN T-cell precursors in the adult thymus are similarly controlled by mesenchyme.

Mesenchyme in the Adult Thymus

Characterization

While initial studies suggested that NC-derived thymic mesenchyme does not persist beyond the embryonic thymus (10, 11), further studies utilizing NC specific $Wnt1^{Cre}$ and $Sox1^{Cre}$ mouse models (crossed to Rosa26 reporter mice) enabled identification of NC-derived mesenchymal cells within adult thymus (12, 13). Here, thymic mesenchyme is located in multiple places. It helps contribute to the structure of the thymus lobe making up the capsule around the outside of the thymus (25, 26) and thymic mesenchyme has been reported in the cortex (13), medulla (26) and as well as around blood vessels both dispersed throughout the thymus, and at the corticomedullary junction (CMJ) (27–29). In addition, Komada et al. demonstrated that while NC-derived mesenchyme contributes to pervivascular cells, mesoderm-derived cells contribute to endothelial compartments (30). Thus, mesenchymal cells are present in the adult thymus at multiple sites where distinct stages of T-cell development take place. In addition to anatomic location, various phenotypic markers have been used to identify thymic mesenchymal cells, and different studies have used various marker combinations to define thymic mesenchymal stroma and subsequent subpopulations. Currently, a clear consensus on the panel of mesenchymal markers to be used is lacking, and for markers that are used, there is often overlap in cell populations that are studied. For example, commonly used markers of thymic mesenchyme include PDGFRα, PDGFRβ, Ly51, Podoplanin (Pdpn, gp38) MTS-15, ERTR7, and FSP1 (13, 16, 25, 27, 31–33). Pdpn is a notable marker, as it is frequently used to identify thymic mesenchyme and often stained alongside CD31 to distinguish between endothelium and mesenchymal cells, an approach also used in peripheral lymph nodes to identify the fibroblastic reticular, lymphatic endothelial and blood endothelial cell subsets (29, 34). Interestingly, in addition to being utilized as a phenotypic marker of mesenchymal populations, Pdpn may potentially play functional roles in the thymus. For example, Pdpn has been proposed to contribute to CCL21 localization in thymic microenvironments and contribute to the development of regulatory T cells in young mice (35). It is important to note that while Pdpn and CD31 expression can highlight some similarities in the populations of non-epithelial stroma, unlike peripheral secondary lymphoid tissues where a clear lymphatic stromal population is present, the presence/absence of lymphatics within the thymus has been difficult to resolve. Previous confocal analysis of the thymus to detect expression of VEGFR3 and LYVE-1 suggested the presence of lymphatic vessels (25). Using $Lyve\text{-}1^{Cre}$–mediated fate mapping, or dual analysis of CD31/Pdpn expression by flow cytometry, lymphatics were extremely rare and difficult to observe (29). Use of $Lyve1^{Cre}$ to target any potential lymphatic population within the thymus is complicated by the observation that this approach labels a significant proportion of thymocytes when crossed to a reporter model (36).

Recent phenotypic analysis of thymic mesenchyme from Sitnik et al. revealed $Pdpn^{+}$ and $Pdpn^{-}$ populations within $PDGFR\beta^{+}$ thymic mesenchyme in the adult thymus (27). The inclusion of the additional markers CD34 and Itgα7 was further used to identify these two subsets as $Pdpn^{+}CD34^{+}$ adventitial mesenchyme cells and $Pdpn^{-}Itg\alpha7^{+}$ thymic pericytes, where adventitial mesenchyme surrounds pericytes which in turn surround blood endothelium of vessels (27, 28). A recent advancement has shown that the marker Dipeptidyl-peptidase 4 (DPP4) can be used to distinguish between capsule fibroblasts (capFb, which are $DPP4^{+}$) and medullary fibroblasts (mFb, which are $DPP4^{-}$), further characterizing the different mesenchymal populations present in the adult thymus (26). How these $DPP4^{+}$ mesenchymal subsets relate to $CD34^{+}$ adventitial and $Itg\alpha7^{+}$ pericytes described previously is not clear. Thus, follow up studies are important to examine how various definitions of mesenchyme populations correlate with

each other, in order to provide a clearer stratification of subset heterogeneity that would allow effective analysis of thymic mesenchyme function in the adult thymus.

Functional Roles of Adult Thymic Mesenchyme

Regulation of Thymic Epithelial Cells

While mesenchymal cells regulate the proliferation of embryonic TEC (14, 16), studies demonstrate that thymic mesenchyme can also be a negative regulator of TEC expansion during embryogenesis and that this property is maintained in the adult thymus (32). Thus, thymic mesenchyme was identified as a major intrathymic source of retinoic acid (RA) which was found to restrict TEC proliferation, in particular within the cTEC compartment (32). Interestingly, these same cells that produce RA may also have the ability to produce growth factors such as FGF7/10 that have a positive impact on TEC proliferation during thymus organogenesis and perhaps have a continued role in the adult thymus (14–16). Thus, thymic mesenchyme has the ability to both positively and negatively regulate the TEC compartment in the developing and adult steady-state thymus. Interestingly, thymic mesenchymal cells from adult thymus have also been shown to be capable of sphere-formation, a property initially thought to define TEC stem cells. Thus, Sheridan et al. (37) showed that thymosphere-forming cell (TSFC) represent long-lived cells of mesenchymal origin which also possess progenitor-like capacities, and are able to give rise to adipocytes (37). While, the functional properties of sphere-forming mesenchymal cells require further examination, evidence of a role for thymic mesenchyme in regulating TEC subsets was reported in studies where mesenchyme-specific deletion of LTβR was achieved using Twist-2^{Cre} mice (26). Here, deletion of LTβR on thymic mesenchyme resulted in a number of intrathymic changes, including reduced mTEC numbers in the adult thymus (26). Whether this is indicative of mechanism where thymic mesenchyme regulates TEC in the steady state adult thymus, or rather relates to the role of mesenchyme regulating TEC during thymus organogenesis requires further examination. Similarly, thymic fibroblasts defined by Fibroblast specific protein 1 (FSP1) expression appear to be an important regulator of TEC populations (33). Indeed, $FSP1^+$ mesenchyme produce IL-6 and FGF7, and the absence of $FSP1^+$ mesenchyme results in a significantly smaller thymus size and reduced TEC numbers (33). Interestingly, as well as being a useful phenotypic marker, FSP1 itself may be an important molecular mediators of adult thymus mesenchyme function, as addition of FSP1 to TEC in culture significantly increases their proliferation and expression of CD80 and AIRE (33).

Collectively, the studies above highlight how thymic mesenchymal stroma supports the TEC compartment in the adult thymus at steady state. Additionally, some findings also implicate thymic mesenchyme in supporting TEC and thymus recovery following damage. For example, Sun et al. found that deleting $FSP1^+$ thymic mesenchyme resulted in significantly delayed regeneration in a cyclophosphamide-induced thymic involution model (33). Gray et al. also found that upon cyclophosphamide-induced thymic involution, MTS-15^+ thymic fibroblasts expanded during regeneration and produced FGF7, FGF10 and IL-6 to promote TEC proliferation and increase T-cell production (31). Interestingly Sun et al. found that $FSP1^+$ mesenchyme was distinct from MTS-15^+ fibroblasts thus demonstrating some conservation of function between the two mesenchymal populations (31, 33). These are distinct from MTS-15 expressing thymic mesenchyme as shown by FACs but also by confocal showing these cells were localized in different areas of the thymus (33). Finally, expression of CD248 (endosialin), a protein linked to remodeling of tissues, is induced within PDGFRα$^+$ thymic mesenchyme during infection (38). In a Salmonella infection model, CD248-deficient mice exhibited reduced thymus size and poor thymus regeneration, indicating a specific but poorly understood role for CD248 expression by mesenchyme in controlling the recovery of thymus function following acute thymus atrophy (38).

Thymus Emigration

The microanatomical positioning of adventitial cells and pericytes around blood vessels in adult thymus, suggest a possible role in regulating the egress of mature thymocytes from the thymus following their intrathymic development. The sphingosine-1-phosphate (S1P) pathway has been shown to be a key axis for the egress of mature thymocytes (29, 39, 40), with a complex interplay of multiple cellular compartments, including thymic mesenchymal cells being involved in this pathway. Regulation of the S1P pathway centers on maintaining a low gradient of S1P within the thymic parenchyma and restricting availability to points of exit, *via* the production of S1P *via* kinases and transporters, or the degradation of S1P (which can be by reversible and non-reversible means) (41). The sphingosine kinases SPHK1 and SPHK2 are essential regulators of S1P production, where deletion of *Sphk1* in NC-derived thymic mesenchyme, using conditional Wnt1^{Cre} mediated deletion, disrupts thymocyte egress (29). Mature SP in the thymus of these mice had elevated levels of S1PR1 expression that is consistent with reduced exposure to available S1P. These findings were accompanied by an intrathymic accumulation of mature SP thymocytes, a phenotype highly indicative of an egress defect (29). Mesenchyme can also influence the S1P gradient through S1P lyase, an enzyme which irreversible cleaves S1P and thus in the context of egress acts to maintain low intrathymic S1P levels and ensure migration of mature thymocytes towards high concentrations of S1P present in blood (39). Consistent with this role, it has been shown that S1P lyase expression is focused around blood vessels where it is expressed by thymic endothelial cells as well as thymic mesenchyme (39). The importance of S1P lyase in regulating egress was highlighted by the use of the S1P lyase inhibitor 2-Acetyl-4-tetrahydroxybutylimidazole (THI), which causes an intrathymic accumulation of mature SP thymocytes and reduced T-cell output (39, 42). Based on the location of its expression and its mode of action the authors suggest that S1P lyase degrades S1P in the PVS irreversibly to create a low S1P environment which again acts to promote the egress of mature thymocytes (39).

In addition to the S1P pathway, mesenchyme regulates thymic egress through an additional pathway that includes the chemokine receptor CCR7, a pathway previously shown to play an important role in neonatal thymus egress (43, 44). We showed that in the absence of both CCL21 and CCL19, thymic egress of conventional SP thymocytes is impeded, with this requirement specifically mapping to a non-redundant role of CCL21 but not CCL19 (28). In the adult thymus it is known that CCL21 is produced by an mTEC subset, as most recently shown through the use of $CCL21^{TdTom}$ reporter mice (45). Interestingly, analysis of CCL21 protein distribution in the neonatal thymus revealed this was concentrated around blood vessels at the corticomedullary junction (28). Further analysis revealed that expression of heparin sulfate by $CD34^{+}$ adventitial cells, and to a slightly lesser extent expression by $Itg\alpha7^{+}$ pericytes, regulated CCL21 presentation by these mesenchymal populations found at this site (28). Thus, while CCL21 is a product of mTEC, its presentation is dependent on thymic mesenchymal populations for neonatal thymic egress, highlighting the combined action of epithelium and mesenchyme for regulation of a key process in thymocyte development. It is important to note that the role of CCL21 in egress is limited to the neonatal window, as adult CCR7-deficient mice show no thymic egress defect (44, 46). Thus, thymic mesenchymal regulation of thymocyte egress is temporal and limited to the neonatal window and thus the earliest waves of αβ thymocyte egress (28). The precise differential impact of CCL21 on thymocyte emigration at neonatal and adult stages remains unclear. Interestingly however, previous studies have suggested potential differences in the expression of the S1P receptor S1P1 in neonatal versus adult SP thymocytes (47). Evidence from this study demonstrated that neonatal thymocyte egress can be blocked using FTY720 treatment, indicating that the S1P-axis is functional in the window when S1P1 expression is low and that CCL21 also regulates thymus egress, raising the interesting notion that CCL21 may act to compensate for or facilitate reduced S1P- axis activity in the perinatal period, with this requirement being superseded by increased S1P1 expression at adult stages.

Additional studies have further proposed that expression of the lymphotoxin β receptor (LTβR) by specific thymic stromal compartments, including mesenchyme, may regulate thymocyte egress in the adult thymus. Germline LTβR deletion leads to an intrathymic accumulation of mature thymocytes and reduced recent thymic emigrants (RTE) being indicative of a thymic egress defect (48, 49). In a $Twist\text{-}2^{Cre}$ x $Ltbr^{Flox}$ mouse model, where LTβR deletion is restricted to thymic mesenchyme, Nitta et al. additionally noted an intrathymic accumulation of mature SP thymocytes suggesting that the egress defect seen in germline LTβR-deficient mice could potentially include a requirement for

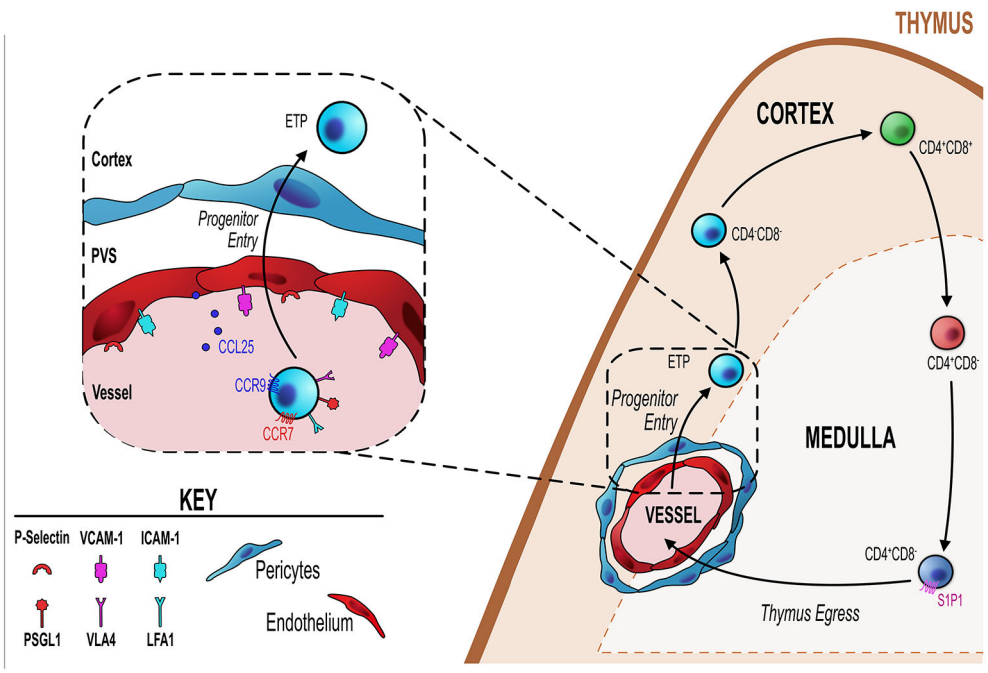

FIGURE 1 | Endothelial cells regulate T-cell progenitor entry. Initial steps in thymocyte development involve homing to, and colonizing of, the thymus by bone marrow-derived blood-borne progenitors *via* blood vessels at the corticomedullary junction (orange dotted line). Entry of these progenitors is controlled by thymic endothelial cells (EC) which regulate this process through a number of different mechanisms detailed in the inset figure (black dotted line/box). ECs express P-selectin, the ligand for which (P-selectin glycoprotein ligand 1, PSGL-1) is expressed by lymphoid progenitor cells. P-selectin-PSGL1 interactions are essential in slowing down progenitor movement, allowing them to interact with ICAM-1 and VCAM-1 expressed by ECs. In adult thymus, CCL25 (ligand for CCR9) which is produced by both TEC and ECs has also been shown to be essential for progenitor homing to the thymus. Additionally, CCR7 is involved in thymus colonization, although its precise ligand requirements (CCL19 and/or CCL21) is not known.

LTβR on thymic mesenchyme, however increased intrathymic numbers of SP thymocytes could be a consequence of reduced negative-selection observed in these mice (26). In other models aimed at deletion of LTβR from mesenchyme ($Wnt1^{Cre2}$x$LTBR^{Flox}$), this SP thymocyte accumulation and emigration defect was not observed. While the reasons for this difference are not clear, it may be that while both $Wnt1^{Cre2}$ and $Twist2^{Cre}$ are active in thymic mesenchyme, the exact subsets that are targeted differ. It will be important to formally define the types of thymic mesenchyme that are targeted in specific Cre expressing mouse strains in order to gain a clearer picture of the role of individual mesenchyme subsets controlling T-cell development.

Thymic Tolerance

In line with the abundance of mesenchymal stroma within medullary areas, these cells have also been implicated in the regulation of tolerance induction of developing thymocytes. Interestingly this finding was perhaps born from attempts to understand how signaling *via* LTβR within TEC microenvironments influenced thymocyte development. First described by Boehm et al., 2003, mice deficient for LTβR exhibited a clear reduction in mTEC numbers, and severely disrupted medullary organization (48). Instead of characteristic large medullary areas surrounded by cortex, thymus from LTBR-KO mice had an increased frequency of smaller medullas dispersed throughout the organ (48, 50). These mice also displayed intrathymic accumulations of mature SP thymocytes indicating disrupted thymocyte egress, as well as autoimmune symptoms including autoantibodies and lymphocytic infiltrates into tissues (48, 50, 51).

Through the use of a panel of stromal-cell specific Cre mice crossed to $LT\beta R^{Flox}$ mice, we examined the contribution of epithelium, mesenchyme and endothelium to regulation of LTβR-dependent tolerance induction in the thymus (50). Deletion of *Ltbr* on TEC recapitulated the mTEC phenotype of $Ltbr^{-/-}$ mice, but despite this, there was no autoimmune or T-cell egress defect in these mice (50, 51). *De novo* Treg generation was found to be independent of LTβR signaling and instead it appeared that dendritic cells were also disrupted in the LTβR-germline KO thymus (50). Screening of different stromal cell specific *Ltbr* deletion models revealed that only when *Ltbr* is deleted on $Wnt1^{Cre2}$-expressing thymic mesenchyme ($LT\beta R^{Mes}$), identified as $CD45^{-}EpCAM\text{-}1^{-}CD31^{-}Pdpn^{+}$ by flow cytometry, was the DC-defect of $Ltbr^{-/-}$ mice replicated and this pathway was proposed as the mechanism by which LTβR signaling regulates thymic tolerance (50). Further analysis of DC kinetics revealed an essential role for CCR7 expression by DC and CCL21 production by thymus for development of $Sirp\alpha^{-}$ conventional DC type 1 (cDC1), a population significantly reduced in $Ltbr^{-/-}$ and $LT\beta R^{Mes}$ mice (52). Thus, it is interesting to speculate whether the role of CCL21 presentation by thymic mesenchyme is LTβR-dependent and the possible mechanism that causes the tolerance breakdown in $Ltbr^{-/-}$ thymus. Interestingly it has been shown that thymic DC can regulate thymocyte egress *via* S1Plyase (53). However, $LT\beta R^{Mes}$ mice, utilizing the $Wnt1^{Cre2}$ promotor, do not exhibit the egress defect seen in $Ltbr^{-/-}$ mice, suggesting that the LTβR-dependent regulation of thymic DC is not essential for thymocyte egress (50, 51).

Using $Twist2^{Cre}LT\beta R^{Flox}$ mice Nitta et al. showed that despite no observed DC defect, there were increased auto-antibodies and cellular infiltrates and this correlated with reduced numbers of $Pdpn^{+}$ thymic mesenchyme which lacked mFb-specific genes including specific TRAs (26). They suggest that expression of MHC Class I, in conjunction with evidence of protein handover/pickup by thymic DC, work to support central tolerance induction during thymocyte development and thus thymic mesenchyme has a significantly more direct role in thymus tolerance *via* self-Ag production rather than regulating DCs (26). Interestingly, this pathway would appear to mirror that proposed for regulation of peripheral tolerance by non-epithelial fibroblastic reticular stromal cells in secondary lymphoid tissues (54). The striking difference here between the two mesenchyme-specific models for deleting *Ltbr* is that while there is a clear DC defect in $Wnt1^{Cre2}LT\beta R^{Flox}$ mice used in the Cosway et al., study there is no egress defect, whereas in the $Twist2^{Cre}LT\beta R^{Flox}$ of the Nitta et al., study the reverse is true (26, 50). As the Cre-lines used in each study appear faithful it remains difficult to fully elucidate the exact role that LTβR and thymic mesenchyme is playing in regulating thymic tolerance or thymic egress. A follow up of Nitta et al. study to understand how their data of an Ag presentation role of mFbs fits into the landscape of $CD34^{+}$ adventitial and $Itg\alpha7^{+}$ mesenchyme, and what further gene expression differences exist within these mesenchyme populations would be very interesting and useful to further our understanding of the heterogenous populations and roles that thymic mesenchyme in the adult thymus carry out.

THYMIC ENDOTHELIUM

Regulation of Thymocyte Development

Thymus Colonization by Lymphoid Progenitors

The thymus does not contain a pool of self-renewing hematopoietic stem cells and so relies on remote colonization of bone-marrow derived progenitor cells from the blood into the thymus. Thus, thymic endothelial cells play an essential role in interacting with the T-cell progenitors in the blood to ensure site-specific extravasation of progenitor cells into the thymus for the ongoing production of T-cells (55) (**Figure 1**). During development, thymus vascularization is evident around embryonic day 15.5 (E15) (56), with $CD31^{+}$ endothelial cells abundant in thymus sections at E15.5, but not at earlier time points (56–58). The dense 3-D vascular network present at this stage was shown using an elegant approach in which intravenous microinjections of resin into embryos was performed, with subsequent digestion of tissues to leave a resin cast which was then imaged using scanning electron microscopy (56). It is interesting to note that T-cell progenitors colonize the thymus at earlier time points prior to thymus vascularization. At earlier stages (<E15) T-cell progenitors have to migrate through the perithymic mesenchymal layer that invests the early epithelial

rudiment at this early stage in thymus organogenesis to enter epithelial microenvironments (56, 59, 60). During this period the CCR9 ligand CCL25 produced by Foxn-1–dependent thymic primordium and the CCR7 ligand CCL21 produced by Gcm2-dependent parathyroid, are essential in progenitor colonization of the early thymus (56, 61, 62). CXCR4 also aids colonization of the embryonic thymus, but the precise intrathymic cellular source is not clear (63). Critically, the requirement for CCR7 and CCR9-dependent thymus colonization is maintained into adulthood, where CCR7/CCR9 double deficient mice (*Ccr7*$^{-/-}$*Ccr9*$^{-/-}$) demonstrate reduced thymus colonizing cells, albeit such reductions are potentially made up for by subsequent compensatory expansion of thymocytes (64).

A key endothelial mechanism that regulates T-cell progenitor entry into the thymus is the interaction between endothelial expressed P-selectin and P-selectin glycoprotein ligand 1 (PSGL-1)–bearing progenitor (65–67). PSGL1-deficient thymi have reduced numbers of ETP due to the reduced ability of progenitor cells to enter the thymus (66). Parabiotic or adoptive transfer experiments revealed clear evidence for the essential role of P-selectin:PSGL1 interactions for progenitor homing to the thymus (66, 67). Consistent with this, treating cells with anti-P-selectin neutralizing antibody significantly impaired progenitor homing to the thymus (67). This P-selectin/PSGL-1 interaction slows down progenitors allowing them to interact with ICAM-1 and VCAM-1, whose expression by the endothelium is also key in the process of progenitor homing and entry into the thymus (67). In a related study, Gossens et al. found that in addition to P-selectin, endothelial production of CCL25 is also be a key regulator of T-cell progenitor homing to the adult thymus (65). They found that mouse models with T-cell progenitor intrinsic entry defects were significantly more receptive to the homing and entry of WT T-cells progenitors (65). This increased receptivity correlated with increased P-selectin and CCL25 expression by thymic endothelial cells, suggesting both are involved in T-cell progenitor homing and colonization (65). Interestingly this same study revealed that endothelial expression of P-selectin and CCL25 is the mechanism by which endothelial cells enforce the temporal, gated, nature of thymus colonization, wherein the thymus goes through periods of receptiveness and non-receptiveness to colonization by new T-cell progenitors (55, 65, 68). This phenomenon may be part of a process by which progenitor niche availability within the thymus directly feeds back and acts on the ability of progenitors to enter the thymus or not (65, 69). Gossens et al. showed that progenitors act on the endothelium to limit the expression/production of P-selectin and CCL25, when the intrathymic population of progenitors is

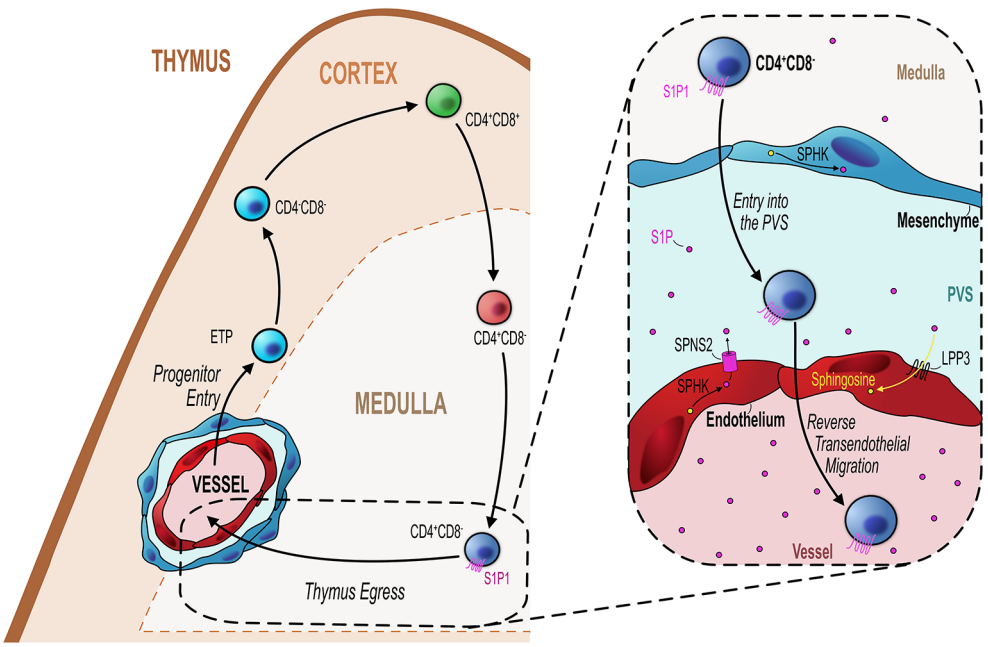

FIGURE 2 | Endothelial cells regulate mature thymocyte egress. Similar to progenitor entry into the thymus, egress of mature thymocytes that have completed their intrathymic development occurs *via* blood vessels at the corticomedullary junction (orange hashed line). Mature CD4$^+$CD8$^-$ (SP4) or CD4$^-$CD8$^+$ (SP8) thymocytes which express the S1P receptor S1P1 migrate from the medulla to the CMJ to begin their exit from the thymus. As detailed in the inset figure (black dotted line/box), mature SP thymocytes first enter the perivascular space (PVS) and then cross the endothelium into the blood *via* reverse transendothelial migration, with both processes being S1P-dependent. Thymic ECs play an essential role in this process by maintaining the S1P gradient within the thymic parenchyma/PVS. ECs can increase S1P levels through their expression of the S1P transporter Spinster homolog 2 (SPNS2) which transports S1P synthesized through the phosphorylation of sphingosine by sphingosine kinase (SPHK). Conversely, ECs reduce S1P levels through expression of lipid phosphate phosphatase 3 (LPP3) which dephosphorylates S1P to sphingosine or by irreversibly degrading S1P *via* S1P lyase. These mechanisms maintain the export of mature thymocytes essential to contribute to the peripheral T-cell pool.

reduced and thus niche availability is increased, endothelial cells are signaled to increased P-selectin and CCL25 (65). This then drives increased progenitor entry, which fills the intrathymic niches signaling endothelial cells to reduce their production of P-selectin/CCL25 and the cycle continues (65). In line with this, P-selectin and CCL25 expression levels correlated with phases of thymic T-cell progenitor receptivity and non-receptivity (65). Additionally, in mouse models which have a significant T-cell progenitor reduction in the thymus and subsequently have increased niche availability, these mice showed increased expression of P-selectin and CCL25 and also increased receptiveness to peripherally induced WT T-cell progenitors (65). Therefore, not only do thymic endothelial cells regulate the entry of progenitor cells by their expression of key molecules such ICAM-1, VCAM-1, P-selectin and CCL25, but they act as gatekeepers regulating the phases of entry and no-entry to new progenitor cells by adjusting the amount and periodicity of their expression of CCL25/P-selectin (65). The mechanism that regulates these changes in expression of key genes is not clear. However, an interesting study highlighted a key role for the transcriptional regulator early growth response gene 1 (Egr1) as part of a negative feedback loop in this system. Here, Schnell et al. showed that Egr1-deficient mice had increased thymocyte number as a direct result of increased T-cell progenitor homing and interestingly Egr1 was expressed by DN thymocytes (70). Correlating with increased progenitor homing, they found that endothelial cells of Egr1-deficient thymus had increased P-selectin expression, suggesting that Egr1 mediates a feedback mechanism whereby the number of DN thymocytes controls the entry of new T-cell progenitors by regulating P-selectin expression by the thymic endothelium (70).

When T-cell progenitors enter the thymus *via* blood vessels, they do so *via* large venules found at the corticomedullary junction (CMJ), after which they migrate through the cortex towards the subcapsular zone (SCZ) to begin the step-wise development program within the thymus (71, 72). It has been shown that a subset of endothelial cells appears enriched at the CMJ and such cells play key roles in regulating T-cell progenitor entry. Shi et al. describe 3 subpopulations of $CD31^+$ thymic endothelial cells based on their expression of Ly6C and P-selectin (73). Thymic portal endothelial cells (TPEC) were identified as $Ly6C^-$P-$selectin^+$ and were found to be enriched in vessels at the CMJ, with ~60% of $CD31^+$ vessels at the CMJ found to contain TPEC (73). Importantly, TPEC are selectively reduced in mice lacking LTβR either in all cells, or in models were LTβR is deleted specifically on endothelial cells using a endothelial-specific Cre mouse model ($Tie2^{Cre}$) crossed with $LT\beta R^{Flox}$ mice, to generate $LT\beta R^{Endo}$ mice (49, 73). Loss of TPEC in these mice is accompanied with a significant reduction in the number of early T-cell progenitors (ETP) within the thymus, indicating a T-cell progenitor homing/colonization defect (73, 74). Despite reduced ETP in the thymus of LTβR-deficient mice, thymocyte development is rescued by compensatory expansion of DN3 thymocytes which may expand due to the increased niche availability (69, 74). Interestingly, levels of ICAM-1 and VCAM-1 were noticeably reduced in thymic endothelial cells from LTβR-deficient mice, a finding consistent with a previous study showing that inhibition of ICAM-1 or VCAM-1 reduces thymus homing of progenitor cells (67, 74). The localization of TPEC at the CMJ and reduced ETP strong supports the proposed role of these cells in regulating progenitor entry (73, 74). Interestingly, in addition to a requirement for LTβR in the development of TPEC, absence of αβ-T-cells (as seen in $TCR\alpha^{-/-}$ mice) also significantly reduced the TPEC population, suggesting that provision of LTβR ligands by SP thymocytes may act as part of a crosstalk mechanism between thymocytes and endothelial cells to regulate development of the latter (73).

Thymocyte Development

In addition to regulating the entry of lymphoid progenitor cells, thymic endothelial cells have also been shown to regulate the development of downstream thymocyte populations. For example, Kit ligand (KitL) is a key regulator of early thymocyte proliferation and differentiation (75), with membrane-bound KitL (mKitL) being expressed by both cTEC and vascular endothelial cells found within the cortex (76). Deletion of mKitL specifically on endothelial cells (using $Tie2^{Cre}PDGFR\beta^{Cre}$ endothelial specific mice) resulted in a significant reduction in the frequencies of both ETP and DN1 thymocytes (76). In thymus sections, while DN1 thymocytes are in close proximity to mKitL producing endothelial cells (76), endothelial-derived mKitL was dispensable for T-cell development, suggesting the involvement of additional mKitL producing cells, such as cTEC, in control of ETP/DN1 progenitors (76). Interestingly it has been found that this mKitL-c-Kit interaction is bi-directional, with mKitL signaling leading to increased endothelial cell proliferation and thus endothelial mKitL is essential in both ETP and endothelial cell regulation (77).

In addition to regulating the thymic entry, endothelial cells also play a key role in regulating the egress of mature thymocytes from the thymus into the blood (**Figure 2**). As discussed previously, the sphingosine-1-phosphate (S1P) pathway is an essential mechanism regulating egress of mature thymocytes from the adult thymus (78). Maintenance of localized intrathymic levels of S1P is important to ensure optimal egress and as such this is regulated by the balance of production or inhibition of S1P by particular cell types in the thymus. Endothelial cells have the capacity to achieve this by their expression of particular enzymes or transport molecules allowing them to influence the S1P gradient. For example, S1P lyase is one particular regulator of this process, and as well as being expressed by thymic mesenchymal cells, endothelial-specific expression of S1P lyase is also essential for egress (39, 42). Furthermore, endothelial cells express lipid phosphate phosphatase 3 (LPP3), a class of S1P-degrading enzyme which dephosphorylates S1P (79). Much like S1P lyase, its role in thymocyte egress is to help maintain low levels of S1P, but unlike S1P lyase this process is not irreversible, as LPP3 works to dephosphorylate S1P reverting it to sphingosine which is inactive to S1P1 (42, 79, 80). By deleting LPP3 in various cell types in the thymus, Breart et al. demonstrated the essential role of this enzyme in regulating thymocyte egress (79). Cell specific deletion of *Lpp3* through use of VE-$Cadherin^{Cre}$ to target endothelial cells resulted in the intrathymic accumulation of mature SP thymocytes suggesting a block in thymocyte egress (79). Interestingly deletion of *Lpp3* in TEC using $K14^{Cre}$ also caused an intrathymic accumulation,

suggesting that both populations play non-redundant roles in regulating thymocyte egress *via* manipulating/maintaining the S1P gradient (79).

The expression of these two enzymes are examples of how endothelial cells can downmodulate levels of S1P. However, endothelial cells may also directly contribute to S1P availability by virtue of their expression of the S1P transporter spinster homolog 2 (*Spns2*) (81). Deletion of *Spsn2* in endothelial cells using a $Tie2^{Cre}$ crossed to $Spsn2^{Flox}$ mouse model resulted in the intrathymic accumulation of SP thymocytes and reduced T-cell numbers in the periphery, indicating a severe T-cell egress defect (81). Follow up studies to understand these mechanisms in light of the more recent endothelial population definitions would significantly aid our understanding of how endothelial cells regulate egress *via* the S1P pathway. For instance, the localization of TPEC at the CMJ would make them an attractive candidate to regulate T-cell egress *via* these mechanisms. Perhaps in line with this, in the $LT\beta R^{Endo}$ mouse model which have a T-cell progenitor homing defect, there is also clear indication that thymic egress is also LTβR-endothelial dependent (49). Thus, $LT\beta R^{Endo}$ mice exhibit an intrathymic accumulation of mature thymocytes at a similar magnitude as seen in the germline LTβR-deficient mouse thymus (48, 51). As stated previously, these mice lack TPEC, suggesting these cells regulate both entry and exit of T-cell populations in an LTβR dependent manner (51, 73, 74). Interestingly, this endothelial-regulated stage of egress was restricted to the PVS-entry step, the penultimate step in the process of cells leaving the thymus (29, 39, 72, 82). Moreover, short-term FTY720 treatment prevents entry of mature T-cell in the PVS which may suggest that this process is S1P dependent; however; the exact mechanisms by which endothelial cells regulate thymus egress in an LTβR-dependent process remains unclear.

It has also been suggested that a thymus:blood barrier exists in the thymus (83). However, this idea is at odds with the fact that many of the vessels within the thymus function to allow the trafficking of progenitors and mature T-cells into and out of the thymus. Heterogeneity amongst thymic blood vessels indicates that different vessels have different permeabilities and provides evidence of how a thymus:blood barrier may be present while also serving as entry and exit points of the thymus. It has been shown that all cortical vessels express Cld5 but only half of the blood vessels in the medulla and CMJ express Cld5 (84). Thymus sections of mice injected intravenously with a biotin tracer revealed that the biotin tracer leaked into the medulla *via* Cld^{-} vessels whereas little penetration was found around $Cld5^{+}$ vessels (84). The importance of Cld5 expression by cortical vasculature in maintaining their impermeable status was highlighted in experiments where Cld5 deletion led to cortical vessels becoming leaky (84). It is likely the leaky nature of medullary/CMJ vessels aids entry/exit of trafficking cells, consistent with these vessels being located at/near the CMJ. This is supported by findings of accumulations of mature thymocytes within the PVS of $Cld5^{-}$ vessels and also by IV injection of tetramethylrhodamine-conjugated S1P which was found to leak into the medulla in thymus sections of injected mice likely contributing to the S1P-gradient required for thymus egress (84).

TEC Regulation by Thymic Endothelium

Endothelial cells have also been reported to regulate TEC in the steady-state and during thymus regeneration. For example, Choi et al. showed that *via* $Tie2^{Cre}$ mediated deletion of plexinD1 on endothelial cells, thymus lobes displayed reorganization of vasculature, with a significant increase of vessels in the subcapsular zone and a corresponding disruption of medulla localization, where medullary areas were found at the subcapsular zone and fused with the capsule (85). Thus, plxnd1 expression by thymic endothelial cells influences medullary formation within the thymus (85). Upon damage *via* total body sublethal irradiation, endothelial cells upregulate their production of bone morphogenetic protein 4 (BMP4) which in turn upregulates Foxn-1 expression in TEC, leading to increased expression of downstream Foxn-1 targets such as delta-like ligand 4 (DLL4) which are involved in TEC development, maintenance and regeneration (86). This process is essential for optimal thymus regeneration as blocking BMP using a pan BMP inhibitor significantly impairs thymus repair (86). Interestingly, thymic endothelial cell regulation of TEC and thymus recovery may have therapeutic implications. Indeed, intravenous administration of thymic endothelial cells that were expanded *in vitro* (exEC) significantly improved thymus regeneration upon damage with exEC reported to home to the thymus and potentially exert this effect intrathymically (86). Further therapeutic potential was established by a follow up study which found that administering zinc increased the thymus regeneration and TEC proliferation (87). Zinc supplementation simultaneously increased endothelial cell numbers and induced increased expression of BMP4 to significantly promote thymic reconstitution (87). Interestingly, the zinc receptor GPR39 is expressed at steady-state, but expression is increased upon damage suggesting endothelial cells increase their receptiveness to zinc to enhance their regeneration function (87).

Thymic Endothelium Regulation by TEC

In a similar fashion to bi-directional cellular crosstalk reported to occur between thymic epithelium and mesenchyme, and thymic epithelium and thymocytes, crosstalk may also operate between epithelial and endothelial compartments. Vascular endothelial growth factor (VEGF) is a key regulator of vascular development and has been shown to be key in the thymus endothelial development during neonatal development but is largely dispensable in adults (88). Thymic epithelial cells, while being influenced by thymic endothelial cells during their development, maintenance and regeneration as described above, may themselves have a reciprocal role in regulating endothelial cells. Muller et al. showed that by targeting deletion of VEGF-A to thymic epithelial, thymus vasculature was significantly altered. Deleting VEGF-A on TEC resulted in hypovascularization and disruption of the normal vascular distribution within the thymus, highlighting VEGF-A as an epithelial growth factor which is required for normal thymus vasculature development (89). Inhibition of VEGF in neonatal

mice also significantly reduces the frequency of endothelial cells within the thymus (90, 91). Additionally, loss of Foxn-1 expression in Foxn-$1^{\Delta/\Delta}$ embryonic mice (homozygous for hypomorphic Foxn-1^{Δ} allele; model with less severe phenotype than foxn-1^{null} mice) causing significantly reduced VEGF expression which resulted in significant changes to the vasculature within the thymus including reduced vessel integrity and disorganized patterning/distribution (92). These data are interesting as they emphasize how epithelial cells support the development of the vasculature and other studies demonstrate that normal vascular development is essential for the support of thymic epithelial cells, highlighting a level of cross-talk between the two populations to ensure the optimal development of each compartment.

NON-EPITHELIAL STROMA AND AGE-RELATED THYMIC INVOLUTION

The thymus undergoes drastic age-associated involution which impacts immune function (93, 94). With age, the thymus becomes smaller, T-cell output is diminished and stromal microenvironments change (93, 95, 96). With regards to the latter, a considerable amount of aged thymic space is composed of adipose tissue, which occurs alongside a reduction in the area and organization of the TEC compartment due to cTEC and mTEC loss (97, 98). These impaired cTEC and mTEC compartments are associated with significantly reduced thymocyte development and output in aged thymus (97). In contrast, how thymic NES populations change with age is less well documented, and so little is known about how changes in these cell types may relate to declining age-related thymus function. Relevant to this, fibroblasts represent an increasing stromal population within the aging thymus (95). While the reasons for this are not fully clear, it has been suggested that it may occur as a result of increased epithelial-mesenchyme transitioning (EMT) occurring during aging, which causes increased fibroblasts, which may then also transition further into adipocytes (99). Consistent with this, thymic mesenchymal cells possess the ability to differentiate into adipocytes from thymosphere-forming cells (37).

Interestingly, it has also been shown that adipocytes accumulate in the PVS within the aged thymus (100). While the potential functional significance of this is not clear, it raises interesting questions, including how might increased adipocyte frequency at this site influence thymus entry and/or emigration? As the PVS is a site of mature thymocyte egress, alterations in these regions may contribute to any reduced thymic output already caused by thymocyte development defects linked to reduced/altered TEC. In addition, it has been shown that thymocyte development is supported by NES, for example mKitL production by endothelium, or IL-7 presentation by mesenchymal ECM components (22, 76). Thus, age-related alterations in NES may impair thymopoiesis by limiting these processes. Interestingly, IL-7 is reduced with age and may contribute to reduced thymopoiesis seen with aging (101). Also, while IL-7 is a TEC product (102) it may be interesting to consider whether changes in thymic mesenchyme in an aged thymus result in less effective presentation of this cytokine, as compared to their counterparts in a younger thymus. In sum, it is important that as our understanding of the characteristics and functions of the NES compartment continues to expand in the steady state thymus, it should also be applied to investigations of disease and aging.

CONCLUDING REMARKS

The non-autonomous nature of intrathymic T-cell development, and the subsequent requirement for thymocyte-extrinsic signals from thymic stromal cells, is well established. Of the diverse stromal cell types that contribute to cortical and medullary microenvironments, thymic epithelial cells (TEC) have been the predominant focus of study over many years. This is likely due at least in part to their fundamental importance in shaping the functional properties of αβ-T-cells produced in the thymus. Indeed, TEC populations control MHC restriction and determine the specificity of the T-cell receptor repertoire by both positive and negative selection events. In addition to the importance of TEC biology, non-epithelial stromal cells (NES) have also been a focus of study, but to a significantly lesser extent. However, NES such as mesenchyme and endothelium have been implicated in key aspects of thymus function. For mesenchyme, their influence on T-cell development can operate by multiple mechanisms. Thus, mesenchyme-derived signals can either target developing thymocytes directly (22, 23), or indirectly by regulating TEC function (14, 16, 32). This fits well with the nature of the thymus epithelial-mesenchymal organ and highlights the need for further study of NES-TEC and NES-thymocyte interactions. Relevant to this, the recent identification of newly defined mesenchyme subsets within NES should facilitate a better understanding of their importance.

For endothelial populations within NES, growing evidence supports their importance in controlling cellular trafficking into and out of the thymus. As the efficacy of these processes can act as rate-limiting steps in T-cell production (51, 65, 67, 74, 76, 79), again a better understanding of the endothelial subsets that contribute to intrathymic vessels for both thymus entry and exit is an important goal of future research. Finally, that endothelial cells also play a role in the regeneration of the thymus following damage (86) opens a new chapter in the relevance of these cells in controlling thymus function in health and disease.

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Thymic Epithelial Cell-Derived IL-15 and IL-15 Receptor α Chain Foster Local Environment for Type 1 Innate Like T Cell Development

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Expression of tissue-restricted antigens (TRAs) in thymic epithelial cells (TECs) ensures negative selection of highly self-reactive T cells to establish central tolerance. Whether some of these TRAs could exert their canonical biological functions to shape thymic environment to regulate T cell development is unclear. Analyses of publicly available databases have revealed expression of transcripts at various levels of many cytokines and cytokine receptors such as IL-15, IL-15Rα, IL-13, and IL-23a in both human and mouse TECs. Ablation of either IL-15 or IL-15Rα in TECs selectively impairs type 1 innate like T cell, such as *i*NKT1 and γδT1 cell, development in the thymus, indicating that TECs not only serve as an important source of IL-15 but also trans-present IL-15 to ensure type 1 innate like T cell development. Because type 1 innate like T cells are proinflammatory, our data suggest the possibility that TEC may intrinsically control thymic inflammatory innate like T cells to influence thymic environment.

Keywords: IL-15, IL-15Rα, thymic epithelial cells, iNKT cells, γδT cells, type 1 innate like T cells

How innate like T cell such as iNKT cell and γδT cell development is regulated and the role of thymic epithelial cells (TECs) in their development is not fully understood. We analyzed publicly available databases and have found that transcripts of many cytokines and cytokine receptors are expressed in both human and mouse TECs. We demonstrated that TEC-derived IL-15 and IL-15Rα play important and selective roles for type 1 innate like T cell, such as *i*NKT1 and γδT1 cell, development in the thymus. As iNKT1 cells are proinflammatory and contribute to adipogenesis, our data suggest the possibility that TEC may intrinsically control thymic inflammatory innate like T cells to influence thymic environment.

INTRODUCTION

Two lineages of T cells, the αβT cell and γδT cell lineages that express distinct TCR receptor αβ chains and γδ chains, are generated in the thymus. αβT cells develop sequentially from the $CD4^-CD8^-$ double negative (DN) stage, the $CD4^+CD8^+$ double positive (DP) stage, and to the $TCR\alpha\beta^+CD4^+CD8^-$ or $TCR\alpha\beta^+CD4^-CD8^+$ single positive (SP) stage. Several αβT cells sublineages, including conventional $CD4^+$ and $CD8^+$ αβT cells, regulatory T cells,

invariant natural killer T (*i*NKT) cells, and mucosal associate invariant T (MAIT) cells, with both distinct and common phenotypic and functional properties are evolved within the thymus (1–4). DN thymocytes can be sequentially defined into early T cell progenitors (ETP, $Lin^-cKit^+CD44^+CD25^-$), $CD44^+CD25^+$ DN2, $CD44^-CD25^+$ DN3, and $CD44^-CD25^-$ DN4 stages. At the DN2 and DN3 stages, γδT cells are generated after productively expressing functional γδ TCRs (5). In contrast to conventional αβT cells, *i*NKT cells, MAIT cells, and γδT cells can complete their differentiation into effector cells in the thymus, which appears to be regulated by thymic environment (6–11). These effector lineages include the type 1 sublineage (*i*NKT1/MAIT1/γδT1) that express T-bet and IFNγ, the type 2 sublineage (*i*NKT2/MAIT2/γδT2) that express Gata3 and IL-4, and the type 3 sublineage (*i*NKT17/MAIT17/γδT17) that express RORγt and IL-17A (8, 9, 12–19). While naïve T cells require several days to differentiate to effector cells, these innate like T cells can be activated quickly and are able to rapidly produce a variety of cytokines in response to agonistic stimuli to shape both innate and adaptive immunity.

In addition to crucial roles of TCR signals for both αβT and γδT cell development, local environment plays important roles in these innate like T cell maturation and differentiation to effector lineages. IL-15 is critical for development of *i*NKT cells, especially, for the $NK1.1^+CD44^+$ stage 3 and IFNγ-producing T-bet^+ *i*NKT1 cells (20–23). Similarly, γδT cell effector lineages are also controlled by local cytokines. IFNγ-producing γδT1 cells are severely decreased in pLNs in IL-15 or IL-15Rα deficient mice. IL-15 induces γδT1 cell proliferation and survival via upregulating Bcl-xL and Mcl-1 (24, 25). An important feature of IL-15 signaling is that IL-15Rα serves as a high affinity IL-15-binding protein to *trans*-present IL-15 to the IL-15Rβ/γc complex on neighboring cells (26–30). IL-15Rα mediated *trans*-presentation of IL-15 promotes NK cells and CD8 T cell homeostasis (26–30). Interestingly, IL-15Rα deficiency causes severe impairment of stage 3 *i*NKT1 cell development (6, 7). Although it has been reported that radiation-resistant cells in the thymus provide IL-15 and *trans*-present IL-15 via IL-15Rα to promote *i*NKT cell development (6, 7), the exact cellular source of IL-15 and the cell type(s) that *trans*-present IL-15 via IL-15Rα have been unclear as the thymus contains many cell types including radiation resistant non-hematopoietic cells and some hematopoietic cells that could also be radiation resistant.

Thymic epithelial cells (TECs) are crucial for thymopoiesis and thymus function to generate a vast repertoire of T cells that are able to perform immune defenses but are also self-tolerated. Cortical TECs (cTECs) and medullary TECs (mTECs) localize in discrete regions in the thymus and perform different function (31–33). cTECs are mainly responsible for positive selection of developing thymocytes expressing functional TCRs capable of recognition of self-peptide/MHC complexes (34–37). mTECs ensure highly self-reactive T cells are ablated to establish central tolerance via presentation of promiscuously expressed tissue restricted antigens (TRAs) controlled by Aire and Fezf2 (34, 36, 38–41). In this report, we analyzed publicly available databases and revealed that TECs indeed express a variety of cytokine and cytokine receptors at various levels. We demonstrated further that ablation of either IL-15 or IL-15Rα in TECs selectively impaired development and/or homeostasis of *i*NKT1 and γδT1 cells in the thymus, indicating that TECs not only serve as an important source of IL-15 but also trans-present IL-15 to ensure type 1 innate like T cell development. Our data suggest that possibility that TEC may intrinsically control thymic inflammatory innate like T cells, which may in turn influence thymic environment.

RESULTS

Expression of a Variety of Cytokines and Cytokine Receptors Including IL-15/IL-15Rα by mTECs

To determine the expression of cytokines and cytokine receptors in mTECs, we searched the publicly available Skyline RNAseq database from The Immunological Genome Project (Immgen.org) for mRNA levels in mTEC. mRNAs of many cytokines and their receptors could be detected in mTECs at various levels (**Figures 1A,B**). For cytokines, *Il7* is expressed at high levels and *Il23a* is expressed close to high levels (**Figure 1A**); *Csf1, IL12a, Il15, Il27, Tgfb2, Tgfb3, Tnf, Tnfsf9, and Tnfsf10* are expressed at intermediate levels; Many other cytokines such as *Il10, Il12b, il17c, Il1b, Il4, Il33,* and several Tnf superfamily members are expressed at low levels; several other cytokines such as *Ifng*, *Il17a*, *Il17d* and *Tgfb1*were expressed at very low or trace levels. For cytokine receptors, *Csf2rb, Ifngr2*, *Il11ra1, Il13ra1*, *Il1rn*, *Il2rg,* and *Il4ra* are expressed at high levels, whereas most cytokine receptors including *Il15ra* are expressed at intermediate levels and a few of cytokine receptors such as *Il22ra2, Csf3r,* and *Il17rd* were expressed between low and trace levels. Compared with different types of immune cells and other stromal cells, mTECs were among the highest expressers of mRNAs for multiple cytokines and cytokine receptors such as *Il7*, *Il10*, *Il11ra1*, *Il13*, *Il15*, *Il15ra, Il17c, Il20rb, Il23a*, *Il27*, *Tnfsf4*, *Tnfsf9*, and *Tnfsf15* (**Figure 1C**). Thus, mTECs express mRNAs of many cytokines and cytokine receptors at various levels.

Expression of Discrete Cytokines in Murine TEC Subsets

Recently, murine TECs have been defined into 5 subsets based on single cell RNA sequencing analysis (42–48). To further investigate expression of cytokines and their receptors in TEC subsets, we analyzed scRNAseq data of TECs generated by the Ido Amit group, which had sequenced more TECs than other reports (42). Using the Seurat package approach (49), we could define TECs from 4 to 6 week old mice into 10 populations (**Figure 2A**). Populations 3, 4, and 8 are $Psmb11^+$ and represent cTECs; populations 2 and 9 are $Krt14^+$ and represent mTEC-I; populations 1, 6, and 7 are $Aire^+$ and $Fezf2^+$ and represent mTEC-II; population 5 is enriched with *Il25, Pou2f3*, and *Dclk1* and represents mTEC-IV or Tuft cells; population 0 is the most abundant population that expresses the highest levels of multiple molecules such as *H2-ab1, Psmb11, Krt14, Aire, Fezf2*, and *Dclk1* as well as cytokines and cytokine receptors, although at low frequencies. This population may represent mTEC-III

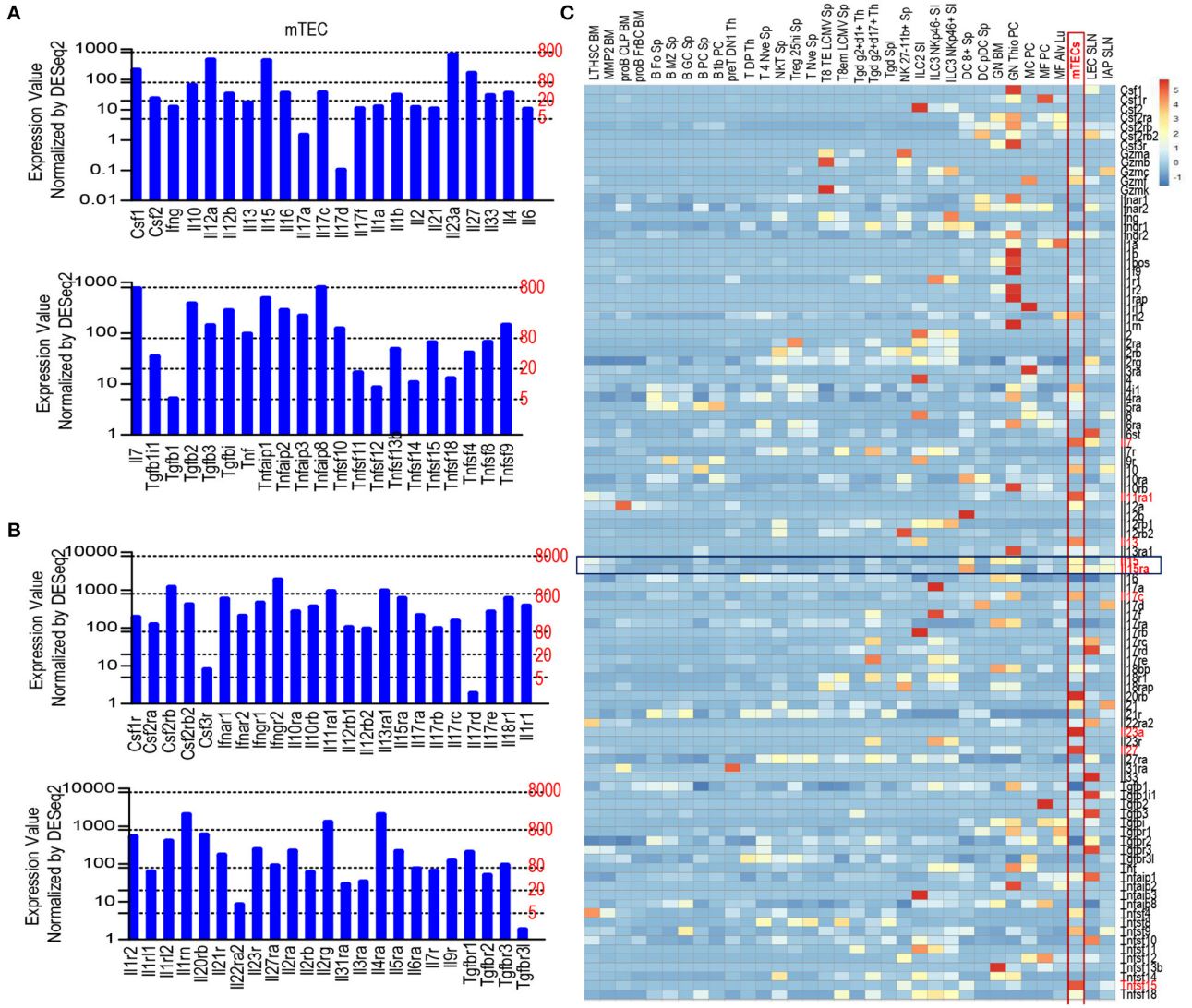

FIGURE 1 | Expression of various cytokines and cytokine receptors in mTECs. **(A)** mRNA levels of cytokines in mTECs. Expression levels: 0–5, Trace; 2–20, very low; 20–80, low; 80–800, intermediate; 800–8,000, high according to Immgen.org. **(B)** mRNA levels of cytokine receptors in mTECs. **(C)** Heatmap showing relative mRNA levels of cytokine and cytokine receptor among mTECs, immune cells, and other stromal cells. Data shown are compiled from the RNAseq data from Immgen.org.

(**Figure 2B**). Interestingly, *Aire*$^{+}$/*Fezf2*$^{+}$ populations 1, 6, and 7 (mTEC-II) also contain high levels and/or frequencies of cytokines/cytokine receptor mRNAs such as *Il13, Il23a, Il27*, and *Tnf*. In addition to *Il25*, mTEC-IV also is the highest *Il10* expresser. Although cTECs (populations 3, 4, and 8) contain highest frequencies of *Il7*$^{+}$ cells, populations 1, 2, and 9 (mTEC-I/III) contain cells expressing higher levels of *Il7* than cTECs. *Il15* is expressed at high frequencies in population 1 and its levels appear higher in mTEC populations than cTEC populations, which is consistent with the detection of IL-15 reporter expression in the medulla in the mouse thymus (50). *Il15ra* is expressed at higher frequencies in populations 1 and 2 of mTECs and populations 3 and 4 of cTECs. However, the expression levels in these mTECs appear higher than in cTECs. Overall, *Aire*/*Fezf2*$^{+}$ mTECs appear to express multiple cytokines at levels higher than cTECs while cTECs express higher levels of *Il7* than mTECs.

Expression of Cytokines and Cytokine Receptors in Human TEC Subsets

Similar to murine TECs, a recent report has found human TECs could also be defined into multiple populations based scRNAseq transcriptomic analysis (51). Human TECs also contain TEC-I – IV populations that mimic their murine counterparts. In addition, human TECs also contain MYOD1- and MYOG-expression myoid TEC-myo and NEUROD1- and NEURODG1- expressing TEC-neuro populations (**Figure 3A**)

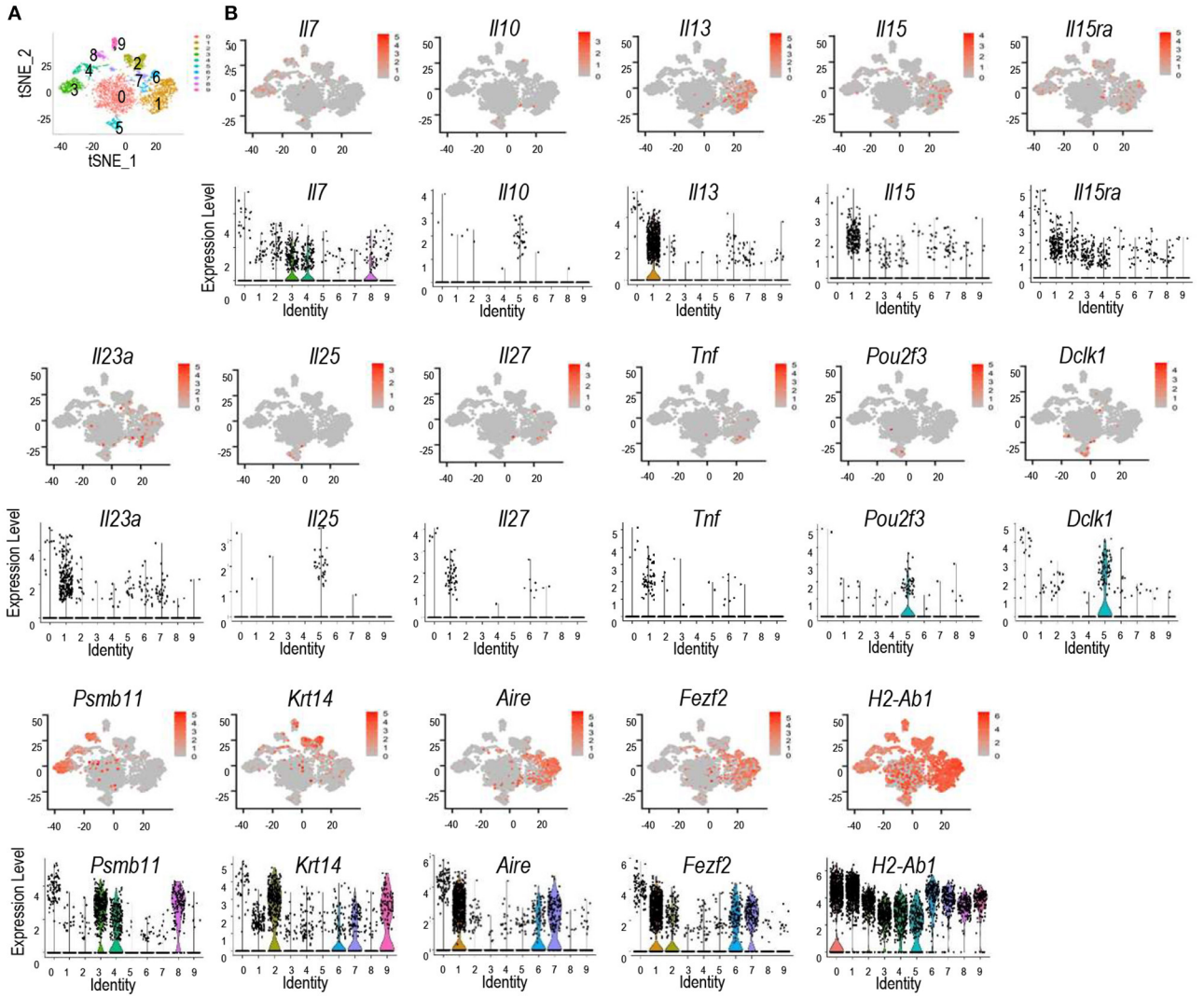

FIGURE 2 | Discrete and promiscuous expression of cytokines and cytokine receptors in murine TEC subsets. scRNAseq data of TECs from 4 to 6 weeks old mice were analyzed. **(A)** tSNE plots showing TEC populations. **(B)** tSNE plots (top panels) and violin plots (bottom panels) showing distribution of cytokine/cytokine receptor expressing cells in different TEC populations. Data shown are generated from the scRNAseq data from Bornstein et al. (42).

(51). We searched the Human Fetal Thymic Epithelium Gene Expression Web Portal (https://developmentcellatlas.ncl.ac.uk/datasets/HCA_thymus/human_epi/) for cytokines and cytokine receptors and revealed that human TECs also express many cytokine mRNAs at various levels (**Figure 3B**). *IL15*, *IL15RA*, *IL11RA*, *IL13RA1*, *IL1R1*, *IL23A*, *IL32*, *IL34*, *TGF1B1*, *TNF*, and *CSF1* are noticeably expressed at intermediate or high levels. Thus, similar to murine TECs, human TECs also expressed various cytokine/cytokine receptors at the mRNA levels.

TEC-Derived IL-15 Promoted *i*NKT1 Development

Thymic *i*NKT cells are defined into 0–3 stages based on differential expression of CD24, CD44, and NK1.1. IL-15/IL-15R signal promoted the development of T-bet$^+$ *i*NKT1 cells, which occupy most of the CD44$^+$NK1.1$^+$ stage 3 *i*NKT cells (6, 7, 20–23). To investigate whether IL-15 expressed on TECs may exert biologic consequence besides serving as a TRA, we generated and analyzed TEC-specific IL-15 deficient, *Il15*$^{f/f}$-*Foxn1Cre* mice. *Foxn1Cre* mice direct Cre expression starting on embryonic day 11.5 in TECs and ablate gene in both mTECs and cTECs (52). Compared with WT control mice, *Il15*$^{f/f}$-*Foxn1Cre* mice did not show obvious alterations in thymocyte development (**Figure 4A**). However, their thymic *i*NKT cells, which were CD1d-Tetramer loaded with PBS-57 positive (CD1d-Tet$^+$) and TCRβ$^+$, showed 42.8 and 50.4% decreases of both percentages and numbers, respectively (**Figures 4B,C**). Within *i*NKT cells, CD24$^+$CD44$^-$ stage 0 and CD24$^-$CD44$^-$ stage 1 *i*NKT cells were not altered; CD24$^-$CD44$^+$NK1.1$^-$ stage 2 *i*NKT cell percentages were not changed but numbers were

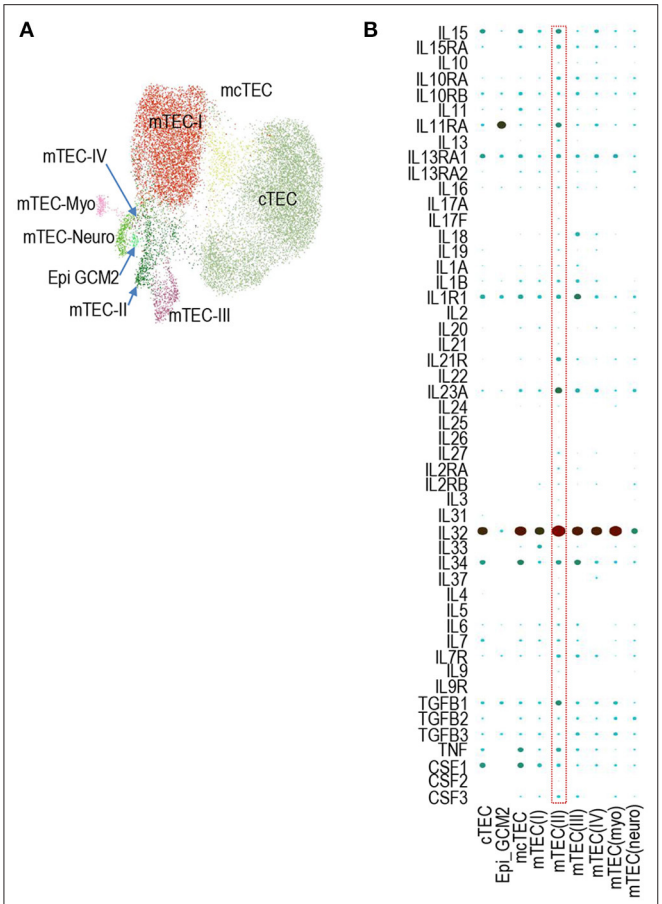

FIGURE 3 | Expression of cytokines and cytokine receptors in human TEC subsets. **(A)** UMAP presentation of human fetal TEC clusters adapted from Jong-Eun Park et al. (51). **(B)** Dot-plot showing mRNA levels of indicated cytokine/cytokine receptors in the nine human TEC clusters from scRNAseq analysis. The size and color of the dot represent the percentage of cells within a cluster expressing the mRNA and the average expression level across all cells within a cluster. Light green and dark red represent low and high levels, respectively.

decreased 54.8%; $CD24^{-}CD44^{+}NK1.1^{+}$ stage 3 *i*NKT cells were decreased in both percentages (51.1%) and more severely in numbers (74.4%) (**Figures 4D,E**). Moreover, $T\text{-bet}^{+}ROR\gamma t^{-}$ *i*NKT1 cells were decreased in both percentages (31.8%) and, more severely, in numbers (66.2%). In contrast, $T\text{-bet}^{-}ROR\gamma t^{+}$ *i*NKT17 cell percentages were not decreased, although numbers of these cells were moderately decreased (54.9%). In contrast, $T\text{-bet}^{-}ROR\gamma t^{-}Gata3^{+}$ *i*NKT2 cells were not altered in either percentages or numbers (**Figures 4F,G**). Thus, TEC-derived IL-15 is important for *i*NKT1 but not *i*NKT2 differentiation and/or homeostasis. Additionally, TEC-derived IL-15 also exerts a weak role for *i*NKT17 cell differentiation/homeostasis.

IL-15Rα Expressed in TECs Selectively Promoted *i*NKT1 Cell Development

IL-15Rα can *trans*-present IL-15 to IL-15R to trigger IL-15R signaling (26, 27). It has been reported that radiation-resistant thymic stromal cells may trans-present IL-15 to promote stage 3 and *i*NKT1 cell development via enhancing Bcl-2 mediated survival. The data were generated in lethally irradiated $IL15R\alpha^{-/-}$ mice reconstituted with WT bone marrow cells (6, 7). However, these studies did not distinguish the role of TECs, other stromal cells, and radiation-resistant tissue resident macrophages or lymphoid tissue inducer cells. To investigate whether IL-15Rα expressed on TECs has biological consequences, we analyzed TEC-specific IL-15Rα deficient, *Il15ra*$^{f/f}$*-Foxn1Cre* mice. Thymocyte development was not grossly affected in *Il15ra*$^{f/f}$*-Foxn1Cre* mice (**Figure 5A**). However, *Il15ra*$^{f/f}$*-Foxn1Cre* mice displayed 62.7 and 66.4% decreases of thymic *i*NKT cell percentages and numbers, respectively (**Figures 5B,C**). Within *i*NKT cells, percentages of stage 0, 1, and 2 cells were increased 2.1, 1.5, and 1.5-fold, respectively. However, their numbers were not significantly changed (**Figures 5D,E**). Stage 3 *i*NKT cells were decreased in both percentages (19.5%) and numbers (72.8%). Furthermore, $T\text{-bet}^{+}ROR\gamma t^{-}$ *i*NKT1 cells but not $T\text{-bet}^{-}ROR\gamma t^{+}$ *i*NKT17 or $T\text{-bet}^{-}ROR\gamma t^{-}GATA3^{+}$ *i*NKT2 cells were severely decreased in *Il15ra*$^{f/f}$*-Foxn1Cre* thymus (**Figures 5F,G**). Thus, IL-15Rα on TECs played an important and selective role for *i*NKT1 but not for *i*NKT2/17 differentiation or early *i*NKT cell development.

IL-15 and IL-15Rα Expression in TECs Selectively Promoted γδT1 but Not γδT17 Cell Development

γδT cells are another innate like T cell lineage that differentiate to effector lineages in the thymus. γδT cells also contain $T\text{-bet}^{+}$ IFNγ-producing γδT1 and $ROR\gamma t^{+}$ IL-17A-producing γδT17 lineages (53–55). γδT1 cells express CD122, the IL-2/15Rβ chain, and IL-15R signal is also important for γδT1 cell differentiation as well as γδT cell homeostasis and migration (20, 56–61). In *Il15*$^{f/f}$*-Foxn1Cre* thymus, γδT cell percentages and numbers were not obviously different from controls (**Figures 6A,B**). However, $T\text{-bet}^{+}ROR\gamma t^{-}$ γδT1 cells but not $T\text{-bet}^{-}ROR\gamma t^{+}$ γδT17 cells were decreased 54.8% in percentages and 57.7% numbers (**Figures 6C,D**), indicating that TEC-derived IL-15 plays an important role for γδT1 cell development/homeostasis in the thymus.

Similarly, IL-15Rα deficiency in TECs in *Il15ra*$^{f/f}$*-Foxn1Cre* mice did not obviously affect total γδT cell percentages or numbers (**Figures 6E,F**). However, γδT1 but not γδT17 cells in the thymus were decreased 69.1% in percentages and 70.4% numbers (**Figures 6G,H**). Thus, IL-15Rα on TECs also selectively promoted γδT1 cell differentiation but appeared dispensable for γδT17 differentiation.

DISCUSSION

It has been long appreciated that TECs control local environment to shape both conventional and innate like T cell development. We analyzed publicly available RNAseq and scRNAseq data and found that TECs, especially mTECs, express mRNAs for numerous cytokines and cytokine receptors such as *Il13*, *Il23a*, *Il15*, and *Il27* as well as *Il15ra* in mouse and/or human.

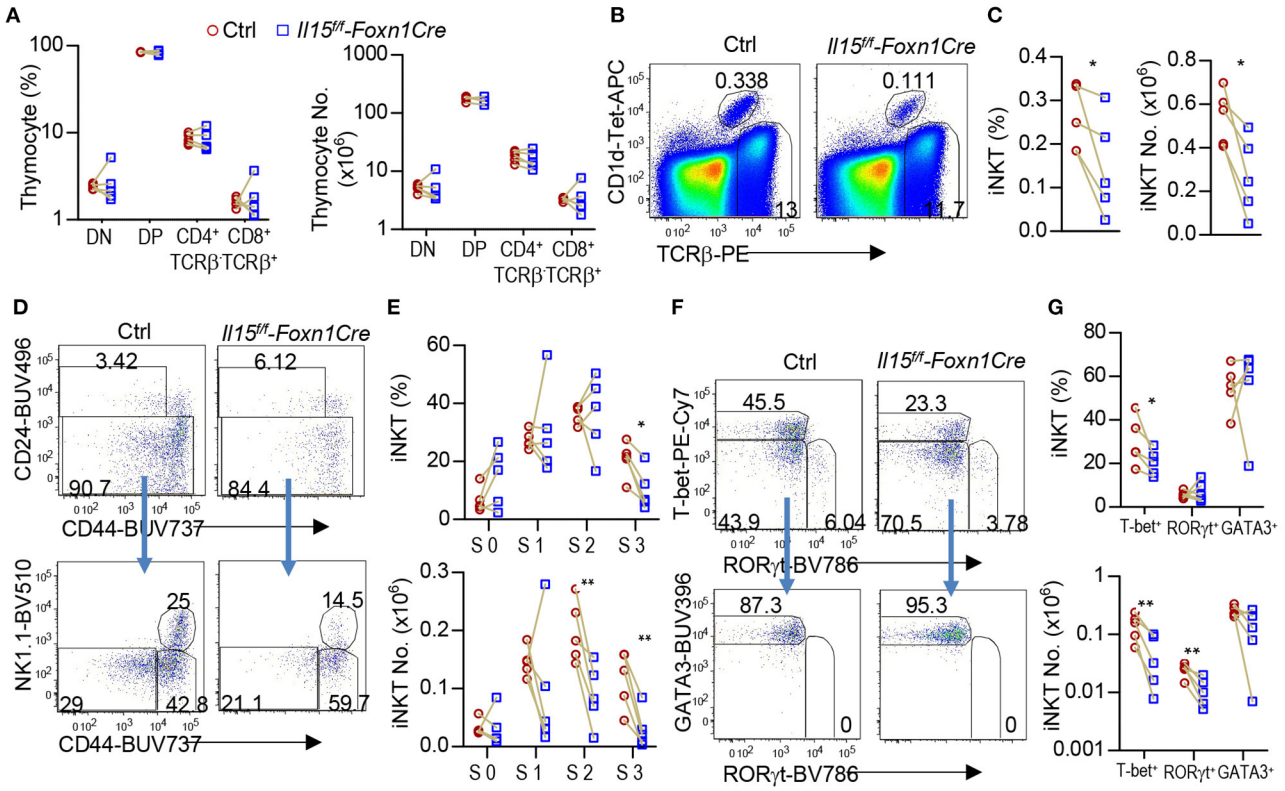

FIGURE 4 | Impairment of *i*NKT1 development/homeostasis in TEC-specific IL-15 deficient mice. Thymocytes from 2 to 3 weeks old *Il15*$^{f/f}$*-Foxn1Cre* and WT (*Il15*$^{+/+}$*-Foxn1Cre* or *Il15*$^{f/f}$) control mice were stained with fluorescently labeled PBS-57-loaded CD1d-Tetramer (CD1d-Tet), antibodies for TCRβ, CD4, CD8, other indicated molecules, and lineage markers (CD11b, B220, Ter119, CD11c, F4/80) as well as a fixable Live/Dead stain. **(A)** Scatter graph represents percentages and numbers of CD4$^-$CD8$^-$ double negative (DN), CD4$^+$CD8$^+$ double positive (DP), and TCRβ$^+$ CD4$^+$CD8$^-$ or CD4$^-$CD8$^+$ single positive (SP) mature T cells. **(B)** Representative FACS plots showing TCRβ and CD1d-Tet staining of live gated Lin$^-$ thymocytes. **(C)** Scatter plots of *i*NKT cell percentages and numbers. **(D)** Representative FACS plots showing CD24 vs. CD44 staining of total *i*NKT cells and CD44 vs. NK1.1 staining of CD24$^-$ *i*NKT cells. **(E)** Scatter graphs of percentages and numbers of stage 0–3 *i*NKT cells. **(F)** Representative FACS plots showing T-bet vs. RORγt staining of CD24$^-$ *i*NKT cells and GATA3 vs. RORγt staining of CD24$^-$ T-bet$^-$RORγt$^-$ *i*NKT cells. **(G)** Scatter graphs of percentages and numbers of *i*NKT1/2/17 cells. Data shown are representative of or pooled from five experiments. Connection lines indicate sex-matched littermates. $^*p < 0.05$; $^{**}p < 0.01$ determined by two-tail pairwise Student *t*-test.

Some cytokines and cytokine receptors including IL-15 and IL-15Rα are single chain molecules. It is conceivable that these molecules could be expressed as biologically functional molecules in TECs if they are properly processed inside these cells. While multiple previous studies have found radio-resistant cell derived IL-15 and/or IL-15Rα or have suggested that mTEC-derived IL-15 and/or IL-15Rα are important for iNKT cell, especially iNKT1 cell, development, no TEC-specific ablation of these molecules have been reported (6, 7, 62). We examined how TEC-specific IL-15 or IL-15Rα deficiency affects T cell, especially innate like T cell, development. We found that ablation of either IL-15 or IL-15Rα in TECs causes significant impairment of *i*NKT1 and γδT1 cell development in the thymus. Our data reveal that TECs not only serve as an indispensable source of IL-15 but also *trans*-present IL-15 for proper type 1 innate T cell development. At present, we do not known whether expression of various cytokine and cytokine receptors in TECs is dependent on Aire or Fezf2 and whether they function in TECs as TRAs to ensure T cell central tolerance. Nevertheless, our observations, together with those that mTEC-IV-derived IL-25 promotes *i*NKT2 development in the thymus (42, 43), suggest the possibility that some cytokines and cytokine receptors expressed in TECs may function both as TRAs and biologically active molecules that can exert their canonical biological functions in the thymus to shape local thymic environment to regulate T cell, particularly innate like T cell, development. Further studies are needed to examine whether TEC-specific ablation of IL-15 and IL-15Rα leads to escape the negative selection of T cells reactive to these molecules.

Of note, TEC-deficiency of IL-15 or IL-15Rα does not completely abolish type 1 innate like T cell development. It is possible other cell types such as dendritic cells and macrophages in the thymus may play partially redundant roles with TECs. Interestingly, TEC-specific IL-15 deficiency weakly reduced *i*NKT17 numbers in the thymus. This observation is consistent with previous reports that injection of IL-15/IL-15Rα complex induced expansion of both thymic

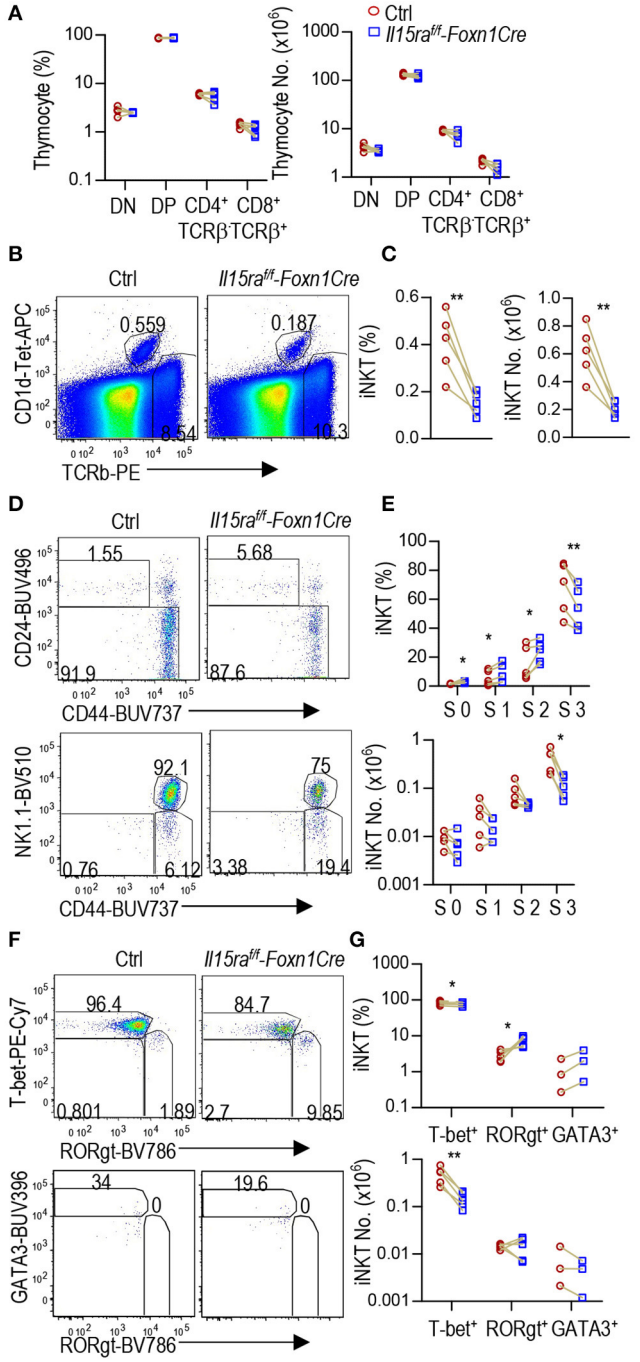

FIGURE 5 | Selective defects in *i*NKT1 but not *i*NKT2/17 cell differentiation in *Il15ra*$^{f/f}$*-Foxn1Cre* mice. Thymocytes from 6 to 8 weeks old *Il15ra*$^{f/f}$*-Foxn1Cre* and WT (*Il15ra*$^{+/+}$*-Foxn1Cre* or *Il15ra*$^{f/f}$) control mice were analyzed similarly as **Figure 4**. **(A)** Scatter graph represents percentages and numbers of DN, DP, and TCRβ^{+} CD4^{+}CD8^{-} or CD4^{-}CD8^{+} SP mature T cells. **(B)** Representative FACS plots showing TCRβ and CD1d-Tet staining of live gated Lin^{-} thymocytes. **(C)** Scatter plots of *i*NKT cell percentages and numbers. **(D)** Representative FACS plots showing CD24 vs. CD44 staining of total *i*NKT cells and CD44 vs. NK1.1 staining of CD24^{-} *i*NKT cells. **(E)** Scatter graphs of percentages and numbers of stage 0–3 *i*NKT cells. **(F)** Representative FACS plots showing T-bet vs. RORγt

(Continued)

FIGURE 5 | staining of CD24^{-} *i*NKT cells and GATA3 vs. RORγt staining of CD24^{-} T-bet^{-}RORγt^{-} *i*NKT cells. The gating of GATA3^{+} *i*NKT cells is based on its levels in T-bet^{+} *i*NKT cells. **(G)** Scatter graphs of percentages and numbers of *i*NKT1/2/17 cells. Data shown are representative of or pooled from three to five experiments. Connection lines indicate sex-matched littermates. $^{*}p < 0.05$; $^{**}p < 0.01$ determined by two-tail pairwise Student *t*-test.

iNKT1 and iNKT17 cells in mice (62, 63). Thus, TEC-derived IL-15 also plays an important role for *i*NKT17 cell development. Of note, our study does not distinguish the role of mTEC and cTEC derived IL-15/IL-15Rα for iNKT1 and γdT1 development as Foxn1Cre ablates genes in both mTECs and cTECs. However, IL-15 appears to be expressed mainly in mTECs and IL-15Rα is expressed at higher levels in mTECs than cTECs (**Figure 2**). Additionally, it has been found that mTECs are critical for iNKT1 cell development and induction of IL15R signaling by injecting IL-15/IL-15Rα complex into micer is able to overcome mTEC deficiency to promote iNKT1 development (62, 63). Similarly, γdT cells differentiate into effector lineages in the medulla (64). Together, these observations support that mTECs provide critical source of IL-15 for iNKT1 and γδT1 cell development.

Although mRNAs encoding many cytokines and cytokine receptors are expressed in TECs, some of them are biologically active only after complex with other molecules. For example, IL-12 and IL-23 that are heterodimers of an IL-12B (IL-12p40) subunit and the IL-12A (IL-12p35) subunit or the IL-23A (IL-23p19) subunit, respectively. Simultaneous expression of both subunits in the same cells would be required for formation of a functional protein. It is intriguing that expression levels among cytokines and cytokine receptors varies drastically in TECs. *Il23a* is expressed at the highest levels in mTECs. Whether such high levels of expression ensure full deletion of IL-23A reactive T cells, increase the chance of coexpression with IL-12B in some TECs, or IL-23A itself has biological activity in TECs remain to be explored.

The ability of TECs to produce cytokines and *trans*-presentation of cytokine(s) to shape thymic environment to control innate like T cell effector lineage differentiation/homeostasis in the thymus could have important implications for thymus biology. Despite the importance of the thymus for T cell generation, it undergoes involution or atrophy with advancing age. Thymic involution may contribute to the decline of immune functions, increased infection-induced mortality and morbidity, and autoimmune diseases in the elderly population (65–67). Although many extrinsic factors that can modulate the course of thymic involution have been identified, none is able to prevent or stop thymic involution. It has been noted that age-associated thymic involution is associated with accumulation of fatty tissue and inhibition of adipogenesis delays thymic involution. Interestingly, adipogenesis is promoted by local inflammation that is negatively controlled by *i*NKT2 and M2 macrophages

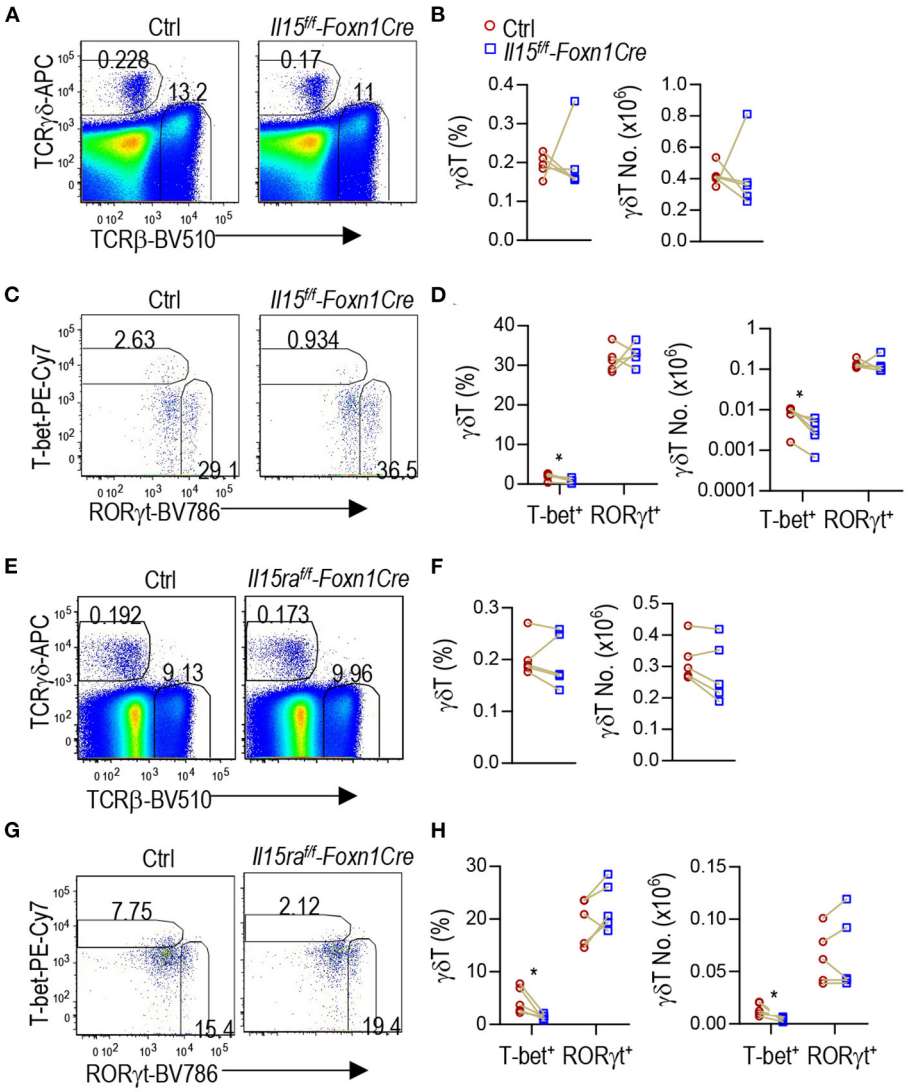

FIGURE 6 | Selective defects in γδT1 but not γδT17 cell differentiation in TEC-specific IL-15 or IL-15Rα deficient mice. **(A–D)** Thymocytes from 2 to 3 weeks old *Il15*$^{f/f}$*-Foxn1Cre* and WT (*Il15*$^{+/+}$*-Foxn1Cre* or *Il15*$^{f/f}$) control mice were labeled with fluorescently tagged antibodies as well as a fixable Live/Dead stain. **(A)** Representative FACS plots showing TCRβ and TCRγδ staining of live gated thymocytes. **(B)** Scatter graphs showing γδT cell percentages and numbers. **(C)** Representative FACS plots showing T-bet vs. RORγt in γδT cells. **(D)** Scatter graphs showing percentages and numbers of γδT1/17 lineages. Data shown are representative of or pooled from five experiments. Connection lines indicate sex-matched littermates. *$p < 0.05$ determined by two-tail pairwise Student *t*-test. **(E–H)** Thymocytes from 6 to 8 weeks old *Il15ra*$^{f/f}$*-Foxn1Cre* and WT (*Il15ra*$^{+/+}$*-Foxn1Cre* or *Il15ra*$^{f/f}$) control mice were labeled with fluorescently tagged antibodies as well as a fixable Live/Dead stain. **(E)** Representative FACS plots showing TCRβ and TCRγδ staining of live gated thymocytes. **(F)** Scatter graphs showing γδT cell percentages and numbers. **(G)** Representative FACS plots showing T-bet vs. RORγt in γδT cells. T-bet$^+$ γdT cell gating is based on its levels in TCRβ$^+$CD44$^-$CD122$^-$ cells (**Supplementary Figure 1B**). **(H)** Scatter graphs showing percentages and numbers of γδT1/17 lineages. Data shown are representative of or pooled from five experiments. Connection lines indicate sex-matched littermates. *$p < 0.05$ determined by two-tail pairwise Student *t*-test.

but positively controlled by IFNγ and M1 macrophages (68–70). Given the ability of TEC sublineages to control type 1 and type 2 innate like T cell differentiation and *i*NKT cells can in turn regulate mTECs and thymic dendritic cells (63, 71), it is possible that thymic involution is an intrinsically programmed process encarved in and triggered by TECs (particularly mTECs) via shaping local thymic environment and presence of innate like T cell effector lineages in the thymus. A hypothesis warrants further investigation.

MATERIALS AND METHODS

Mice

Il15ra$^{f/f}$ mice (28) and *Il15*$^{f/f}$ mice (72) were kindly provided by Drs. Kimberly Schluns and Averil Ma and Drs. Nan-Shih Liao and Shirley Luckhart, were bred with B6(Cg)-*Foxn1*$^{tm3(cre)Nrm}$/J (*Foxn1Cre*) mice (52) that were kindly provided by Dr. Nancy Manley, to generate *Il15ra*$^{f/f-}$*Foxn1Cre* and *Il15*$^{f/f-}$*Foxn1Cre* mice as well as *Il15ra*$^{f/f}$, *Il15*$^{f/f}$, and *WT-Foxn1Cre* control mice.

Mice were maintained in a pathogen free facility. All mouse experiments were performed following a protocol approved by the Institutional Animal Care and Use Committee of Duke University.

Flow Cytometry and Antibodies

Thymocytes cells were prepared according to published protocols (73, 74). Cells were stained for surface markers with appropriate fluorochrome-conjugated antibodies and tetramers in PBS containing 2% FBS on ice for 30 min followed by intracellular staining of transcription factors using the eBioscience Foxp3 Staining Buffer Set according to the manufacturer's protocols. PE- or APC-labeled PBS-57-loaded CD1d-Tetramers (CD1d-Tet) were provided by the NIH Tetramer Core Facility. Fluorochrome-conjugated anti-TCRβ (clone H57-597), NK1.1 (clone PK136), CD44 (clone IM7), CD24 (clone M1/69), CD11b (clone M170), CD11c (clone N418), F4/80 (clone BM8), B220 (clone RA3-6B2), TER119/Erythroid Cells (clone TER-119), CD4 (GK1.5), CD8a (53-6.7), T-bet (4B10), TCRγδ (clone GL3), CD3 (clone 145-2C11), CD45 (clone 30-F11), CD27 (clone LG.3A10) were purchased from Biolegend; GATA3 (L50-823), RORγt (Q31-378) were purchased from BD Biosciences. Cell death was identified using the Live/Dead™ Fixable Violet Dead Cell Stain (Thermo Fisher Scientific). Data were collected using a BD LSRFortessa™ cytometer (BD Biosciences). Data were analyzed using the FlowJo Version 9.2 software (Tree Star).

Expression of Cytokines and Cytokine Receptors From the Immunological Genome Project

Skyline RNAseq database from the Immunological Genome Project (Immgen.org) was searched for mRNA levels of indicated cytokines and cytokine receptors. In the Immunological Genome Project, 34 immune cell types from male and female mice were profiled by RNA-seq. Expression of mRNA was normalized for each cell types with the Z-score method. To visualize the different values among different cell types, the data for each cell were plotted as a heatmap using the pheatmap program (75).

Analyses of Murine TEC scRNAseq Data

Raw counts of scRNAseq data of TECs from 4 to 6 weeks old mice reported by Bornstein et al. (42) were downloaded from GEO Database under the accession number GSE103967. scRNAseq data were pre-processed using the Seurat package (version 3.1.1) (49) in R (version 3.5.3). Genes expressed in fewer than 3 cells and cells with no more than 50 detected genes were filtered out. Filtered datasets were normalized the gene expression measurements for each cell by the total expression multiplied with a scale factor of 10,000 by default, followed by log-transformation of the results using the global-scaling normalization method, LogNormalize. The technical noise and/or biological sources of variation were mitigated via ScaleData function to improve downstream dimensionality reduction and clustering. Highly variable genes were screened with Find Variable Features function for downstream analysis. Principle component analysis (PCA) were performed on the scaled data using the RunPCA function. Significant PCs were identified as those with a strong enrichment of low *p*-value genes based on the Jackstraw algorithm. For cell clustering, k-nearest neighbors were calculated and the SNN graphs were constructed using Find Neighbors. Top 20 PCs were selected for analysis using Find Clusters. Cells within the graph-based clusters determined above were co-localized for visualization on the tSNE plot via RunTSNE and TSNEPlot. Find All Markers were applied to find markers that define clusters via differential expression. Feature Plot was applied to visualize individual gene expression on a tSNE plot. VlnPlot was applied to show expression probability distributions across clusters.

Analyses of Human TEC scRNAseq Data

Expression of cytokines and cytokine receptors in human TECs was searched online based on scRNAseq analyses (https://developmentcellatlas.ncl.ac.uk/datasets/HCA_thymus/human_epi/) (51). Data were presented as a bubble plot with bubble size representing percentages of TECs expressing individual molecules and bubble color representing expression levels.

Statistical Analysis

Data shown represent means ± SEMs and were analyzed with the two-tailed pairwise Student *t*-test using the Prism 5/GraphPad software for statistical differences. Each pair of mice represents sex-matched littermates and is indicated by a connecting line between test and control mice. *P*-values < 0.05 were considered significant ($^{*}p < 0.05$, $^{**}p < 0.01$).

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Institutional Animal Care and Use Committee of Duke University.

AUTHOR CONTRIBUTIONS

HT and LL designed and performed experiments, analyzed data, and participated manuscript preparation. N-SL, KS, and SL provided critical reagents and participated in manuscript preparation. X-PZ conceived the project, designed experiments, and wrote the manuscript. JS participated in manuscript preparation. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.623280/full#supplementary-material

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Metabolic Regulation of Thymic Epithelial Cell Function

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The thymus is the primary site of T lymphocyte development, where mutually inductive signaling between lymphoid progenitors and thymic stromal cells directs the progenitors along a well-characterized program of differentiation. Although thymic stromal cells, including thymic epithelial cells (TECs) are critical for the development of T cell-mediated immunity, many aspects of their basic biology have been difficult to resolve because they represent a small fraction of thymus cellularity, and because their isolation requires enzymatic digestion that induces broad physiological changes. These obstacles are especially relevant to the study of metabolic regulation of cell function, since isolation procedures necessarily disrupt metabolic homeostasis. In contrast to the well-characterized relationships between metabolism and intracellular signaling in T cell function during an immune response, metabolic regulation of thymic stromal cell function represents an emerging area of study. Here, we review recent advances in three distinct, but interconnected areas: regulation of mTOR signaling, reactive oxygen species (ROS), and autophagy, with respect to their roles in the establishment and maintenance of the thymic stromal microenvironment.

Keywords: thymus, thymic stromal cells, mTOR, tolerance, autophagy

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INTRODUCTION

Appropriate tissue function requires integration of intra- and extracellular signals that govern cellular division, migration, and growth, as well as the regulation of organelle size, macromolecule synthesis, and gene expression. Efficiently carrying out such functions requires a balance of catabolic activity required for energy generation, and anabolic activity required for biogenesis. The mechanisms by which signal transduction pathways downstream of growth factor signaling regulate metabolism to influence cellular energy and redox status are well-characterized (1), and it is now clear that the metabolic pathways employed in a given cell feedback to signal transduction pathways. For instance, metabolite-mediated and ROS-mediated modification of proteins involved in signal transduction may alter their activity, and *de novo* intracellular signaling transduction can be initiated by mitochondrial ROS production (2). These types of metabolic signaling influence fundamental cellular decisions such as quiescence vs. activity (3–5), and stem cell self-renewal vs. differentiation (6–8).

The role of metabolic pathways as regulators of cellular function has become an area of increasing interest over the last decades, and T cells have been a major area of focus (3, 9). Metabolic control of processes such as activation downstream of TCR engagement, and effector functions such as IFNg production have been shown to be mediated by mitochondrial ROS and the glycolytic enzyme GAPDH, respectively, in T cells [reviewed in (3)]. In contrast, relatively little is known

about metabolic regulation of thymic stromal cell function. Understanding metabolic regulation of stromal cells is important as a basic feature of their biology, but is particularly relevant for thymic stromal cells for several reasons. First, metabolic function and dysfunction are closely linked to aging (6, 10), and the stromal cells of the thymus are the primary targets of what could be considered among the first hallmarks of aging, thymic atrophy [reviewed in (11)]. Second, integration of metabolic information is critical in controlling cell size and morphology (7, 12), which are uniquely and dynamically regulated in thymic stromal cells (13), directly governing the niches available for T cell generation (14). Moreover, autophagy, in addition to its ubiquitous roles in energy homeostasis and repair of oxidative damage to organelle, also plays an additional role in thymic stromal cells by generating peptide antigens for presentation critical for T cell selection and tolerance induction (15). In this review, we will consider the integration of three aspects of metabolic regulation: mTOR signaling, the redox status of the cell, and autophagy, in the steady-state function and age-associated dysfunction of thymic stromal cells.

mTOR SIGNALING IN TEC

The mechanistic target of rapamycin, mTOR, plays an integral role in cell growth and proliferation in response to a wide array of environmental cues. mTOR is a serine/threonine protein kinase belonging to the PI3K-related protein Kinases (PIKK) family (16, 17) and is the main catalytic subunit in two distinct complexes named mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). These complexes integrate environmental cues and result in both distinct and common cellular outcomes, with significant crosstalk between mTORC1 and mTORC2 signaling pathways. mTORC1 responds to inputs such as energy status, nutrients, growth factors, oxygen and stress, and promotes biosynthetic pathways. It also inhibits autophagy and other catabolic process. mTORC2 is thought to be activated primarily by growth factor signaling, and, like mTORC1, also promotes anabolic metabolism, proliferation, and survival. In addition, mTORC2 signaling regulates cytoskeletal reorganization, with impacts on cell motility (17).

mTOR signaling appears to be a critical regulator of thymus function, as demonstrated by the pronounced thymic atrophy caused by high dose rapamycin administration (18–20). Rapamycin-induced atrophy has been associated with arrested thymocyte proliferation (18), consistent with its well-characterized immunosuppressive properties (21). However, recent studies have revealed that mTOR signaling is also a critical regulator of thymic stromal cell function.

Liang and colleagues explored the role of mTOR signaling in thymic epithelial cells (TECs) using a tissue-specific knockout mouse model (19). In this system, the mTOR protein is ablated in TEC, resulting in disruption of both mTORC1 and mTORC2 signaling. This genetic ablation resulted in decreases in the number of medullary TECs (mTECs) and cortical TECs (cTECs) during fetal development and at 2 weeks after birth, as well as a reduction in the frequency and number of MTS24$^+$ progenitors. Knockout mice also showed decreased proliferation and increased autophagy in TEC, as well as dysregulated T cell development (19). These effects may be explained by disruptions of either mTORC1 or mTORC2.

A pair of recent studies addressed the role of each mTOR complex independently. In mice in which mTORC1 was selectively inhibited in TEC, Wang et al. found that total thymus cellularity, cTEC, and mTEC number decreased. TEC in knockout mice also showed decreased proliferation, and glucose uptake, but TEC survival was not affected. These results are consistent with a role for mTORC1 signaling in TEC proliferation and early growth of the thymus. The effect on cell number was most substantial in mTEC, such that the cTEC frequency in knockout mice was significantly higher than in wildtype mice. The frequency of MHCII high mature cTEC and mTEC were decreased in knockout mice up to ∼3 weeks of age, after which the frequency was the same as in wildtype mice, consistent with a role for mTORC1 signaling in the establishment and maturation of the TEC compartment in growth phases (22).

In mice in which mTORC2 was selectively inhibited, total thymus cellularity and TEC cell number were likewise decreased (23). In contrast to mTORC1 deficiency, cTEC and mTEC ratios were not altered in mTORC2 deficient mice, because the average number of both cTEC and mTEC declined (although the cTEC declines were not statistically significant), indicating a potential additional role for mTORC2 signaling in cTEC as well as mTEC. Consistent with this, maturation of cTEC, as indicated by high MHCII, expression was diminished. Although cTEC appeared to be less affected in the knockout mice relative to mTEC, T cell numbers were decreased beginning at the earliest (cortical) stages, consistent with decreased cTEC function (23).

A role for mTOR signaling in cTEC is also supported by our recent confocal imaging study, in which we found a unique cTEC morphology. cTEC morphology is characterized by projections that comprise extensive labyrinths creating compartments within each cTEC that contained up to approximately 100–150 lymphoid cells per cTEC. The overall shape of cTECs was generally similar to a compressed ovoid and they were aligned radially with respect to the capsule (13). In aged mice, cTEC processes collapsed, and this loss of cell size occurred in the absence of changes in cell number, resulting in increased cTEC density. During thymus regeneration cTECs partially recovered their processes and labyrinth morphology, but did not proliferate extensively (13). Thus, the size and shape of cTEC are critical for maintaining overall thymus cellularity with age, as well as the regeneration induced by castration. In order to understand the mechanisms regulating cTEC morphology and size, we mined our transcriptional database to find Reactome pathways (reactome.org) associated with cell signaling that were significantly enriched in cortical stromal cells. We found that 3 of the top 4 most significantly enriched pathways were related to mTOR signaling (13), which, as described above, is well-recognized as a regulator of cell and tissue size via effects on metabolism and cytoskeletal organization (16, 24). When we looked in more detail at changes in mTOR pathway enrichment in cortical stromal cells during aging and regeneration, we found that mTOR signaling pathway enrichment declined with age

and increased dramatically in the early stages of regeneration, before falling again as regeneration wanes (13). The expression patterns of key mTOR pathway components likewise support the notion that mTOR signaling in cortical stromal declines with age, and is activated during regeneration. Notably, we find upregulation of the mTORC1 regulator Tsc1, consistent with preferential signaling through the mTORC2 pathway important for cytoskeletal remodeling [reviewed in (12)].

We also investigated potential sources of soluble ligand capable of activating the mTOR pathway. These were either absent or not changed during aging and regeneration in cortical stromal cells, making autocrine signaling unlikely (13). Such soluble ligands could be endocrine-derived, however, the cortex of the thymus (but not the medulla) is immune-privileged and separated by a relatively impermeable blood-thymus barrier (25). These observations indicate that intrathymic paracrine signaling may account for the mTOR activation seen in young mice and during regeneration. We found that several ligands, most notably known TEC regulators IGF1 (26) and FGF21 (27), were both diminished with age, and dynamically upregulated in medullary stromal cells during regeneration, presumably as a response to systemic signals induced by castration.

Together, the literature indicates a critical role for mTOR signaling in regulating TEC development, proliferation, size, and function. mTORC1 activity may be particularly critical for early growth phases of the thymus in ontogeny and during regeneration, when anabolic metabolism is required for generation of macromolecules for cell growth and division. mTORC2 activity may be more important during maintenance phases in TEC, when catabolic metabolic process such as autophagy are important for TEC function. As discussed below, extensive crosstalk between mTOR signaling, ROS, and autophagy has been described in diverse model systems, and this integration optimizes cellular responses.

REDOX REGULATION OF TEC FUNCTION

Reactive oxygen species (ROS) are generated as byproducts of cellular respiration (28), and may therefore be regulated by mTOR-mediated increases in metabolism (6, 29–31). ROS can also be generated by oxidative enzymes, detoxified by antioxidant enzymes, and when present at moderate levels, function as critical signaling molecules (32), including as important regulators of T cell receptor signaling (3). ROS are critical modulators of stem cell activity, including in intestinal epithelium (33) and bone marrow (34), when within moderate concentration ranges (6). At high levels, ROS can cause oxidative damage to cellular proteins, lipids, DNA, and other macromolecules (35), and oxidative damage has long been considered to be a primary cause of aging (36).

Several lines of evidence point to an unusual redox environment within thymic stromal cells. First, thymic stromal cells, notably cTEC, are continuously exposed to developing T cells undergoing especially high rates of cell division (37, 38). As a result, the stromal cells, unlike lymphoid cells which quickly exit the cell cycle and emigrate (39), will persist in a state of exposure to the cell-permeable products of high metabolic rates and cell division such as ROS (2), including H_2O_2, and may therefore experience particularly high ROS levels. Indeed, a similar scenario has been demonstrated in the bone marrow, where Cx43-depenent channels facilitate transfer of ROS from proliferating hematopoietic stem cells to adjacent bone marrow stromal cells, a function critical for hematopoietic regeneration (40). In addition, studies have shown that thymic stromal cells, especially those in the cortex, express conspicuously low levels of the H_2O_2-quenching enzyme, catalase (41). As a result, TECs are especially vulnerable to oxidative DNA damage, which accumulates in TEC at significantly higher levels than that found in thymic lymphocytes in mice (41) and humans (42). This oxidative damage is a major contributor to age-associated thymic atrophy, which is delayed by dietary or genetic complementation of catalase activity (41).

Given the cellular damage incurred as a result of oxidative stress, as well as the impact of oxidative damage on thymus size, it is somewhat surprising that catalase expression is found at such low levels in thymic stromal cells, and suggests a selective advantage for a highly oxidative environment within this population. Positive regulators of catalase expression include FOXO transcription factors, which are inhibited by AKT signaling downstream of many growth factors, including those that promote mTOR activation, such as IGF (28, 43, 44). In this way, the same ligands that activate mTOR in TEC, may also inhibit expression of catalase and other antioxidant enzymes and promote ROS production. Conversely, the unfolded protein response (UPR), which can be initiated by ROS (45), can negatively regulate mTORC1 activity (17). In the sections below, we consider the interactions between high levels of ROS, mTOR activity, and autophagy in the regulation of critical TEC functions in the steady state thymus.

AUTOPHAGY IN TEC

Autophagy is the process through which cellular components are degraded and shuttled to the lysosome in order to produce new building blocks during times of nutrient deprivation (46). In the immune system, autophagy is also considered an important regulator of inflammation and antigen presentation (47). Autophagy in thymic stromal cells is essential for presentation of self-antigens, positive and negative selection, and induction of central tolerance (15). Transplantation of autophagy deficient (Atg5 KO) thymi into athymic hosts results in aberrant T cell selection and profound autoimmune disease due to loss of central tolerance in the defective transplanted thymic microenvironment (15), and this result has been corroborated by studies using varying models of autophagy deficiency in TEC (48).

As mentioned above, cTECs partially recover the age-associated loss of their processes and labyrinth morphology during regeneration (13). These results indicate that cTEC morphology regulates overall thymus size, and is also likely to affect the cell surface area available for antigen presentation necessary for proper T cell selection. The observation that thymus size is regulated by cTEC morphology suggests a novel mechanism by which autophagy may regulate thymus function, in addition to the known roles in generation of self-peptide and antigen presentation. Autophagy has an emerging role in establishing and maintaining cellular morphology in a

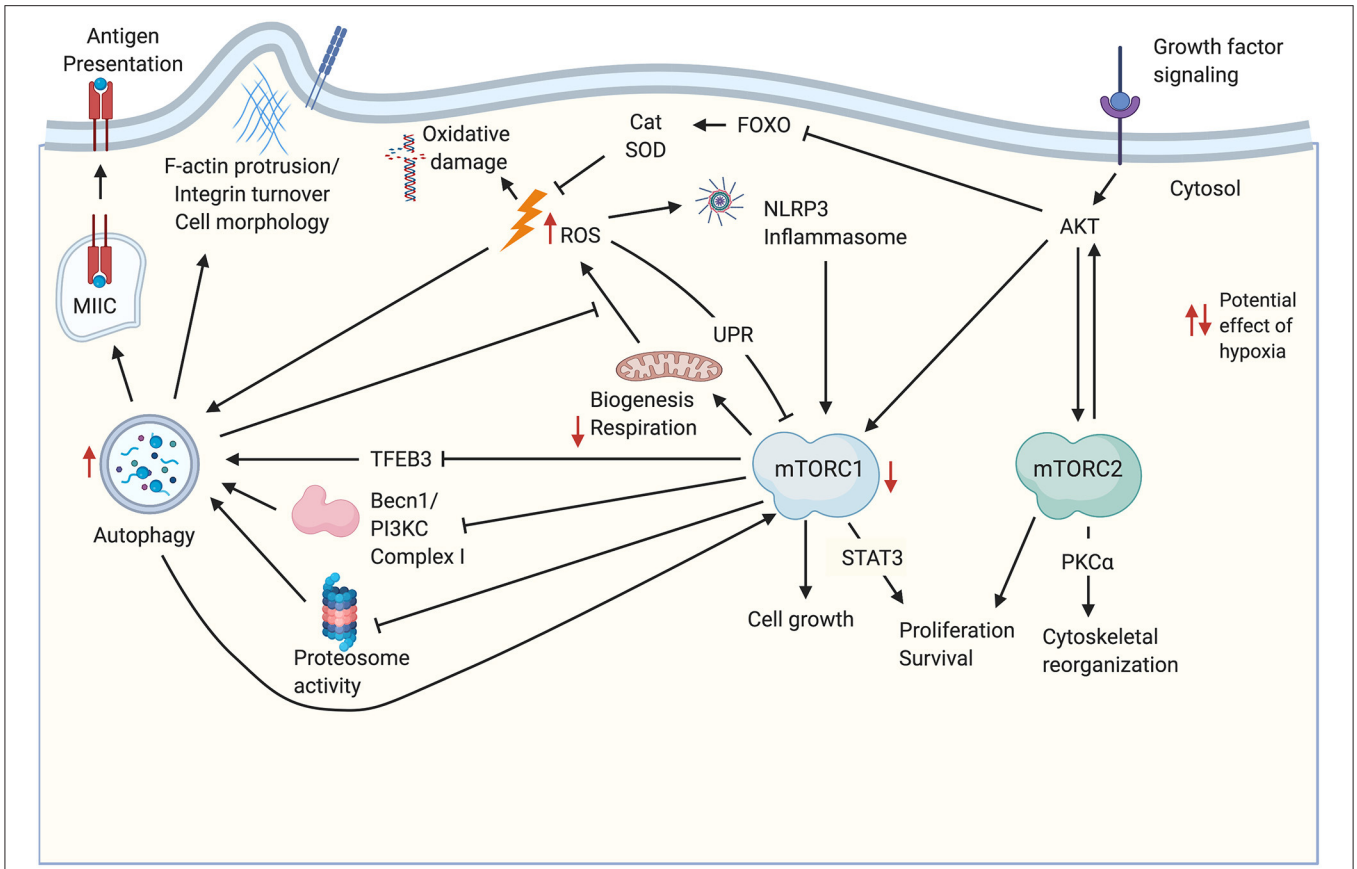

FIGURE 1 | Potential cross-talk and co-regulation of mTOR signaling, ROS, and autophagy in TEC. Growth factor signaling induces AKT activation upstream of mTORC1/mTORC2 complexes. Both complexes promote cell proliferation and survival. mTORC1 signaling drives biogenesis including production of mitochondria required for oxidative phosphorylation. Increased metabolism leads to increased byproducts of metabolism including ROS, which can damage DNA and other macromolecules, including mitochondrial damage that exacerbates ROS production. ROS production is also promoted through the AKT-mediated inhibition of FOXO transcription factors that regulate antioxidant enzymes like Cat and SOD. ROS can feedback to inhibit mTORC1 activity via the UPR, and can activate the NLRP3 inflammasome. ROS promote autophagy, which also mitigates mitochondrial damage by increasing turnover of damaged organelles. Autophagy also promotes antigen processing and presentation in TEC, and may influence cell morphology, for instance, through turnover of integrins. Autophagy is mitigated by mTORC1 signaling via decreases in TFEB-mediated transcription of autophagy genes, as well as inhibition of proteosome activity and autophagosome assembly. mTORC2 phosphorylates several PKC family members, including PKCα, which regulate cell morphology and size through the actin cytoskeleton. Potential impacts of hypoxia are indicated by red arrows. Created with BioRender.com. Becn1-PI3KC Complex, Beclin 1- phosphatidylinositol 3-kinase complex; Cat, catalase; Foxo, class O of forkhead box transcription factors; MIIC, MHC class II-containing compartment; PKCα, Protein Kinase Cα; SOD, superoxide dismutase; STAT3, signal transducer and activator of transcription 3; ROS, reactive oxygen species; TFEB3, transcription factor EB; UPR, unfolded protein response.

number of systems including macrophages in flies and mice (49), HeLa cells (50), and mouse mammary tumor models (51), where autophagy has generally been shown to promote cell spreading by promoting extension of F-actin protrusions, and turnover of integrins and focal adhesions, respectively. Together, these observations suggest that autophagy may regulate cellular projections that are critical for cTEC function via generation of extensive niches which may regulate antigen presentation and thymus size.

In TEC, unlike most other cells, autophagy is active constitutively, rather than being starvation-induced (15, 46). This is somewhat predictable for functions such as antigen presentation or maintenance of cell morphology, which are continuously required. However, the mechanisms regulating this constitutive activation of autophagy have not been identified in TEC. In other biological systems, ROS are known to induce autophagy [(2) and reviewed in (52)], with established roles during physiological stress [i.e., oxidation/activation of Atg4 during starvation (53)] and in disease [i.e., cardiac ischemia (54)]. This suggests that the constitutively high levels of ROS established by low catalase expression in TEC, especially cTEC, may promote the high basal levels of autophagy critical for their function.

CROSSTALK BETWEEN MTOR, ROS, AND AUTOPHAGY

Significant crosstalk occurs between the metabolic pathways described above, and the balance between them may be critical for thymic stromal cell function and maintenance. Some relevant potential interactions are highlighted in **Figure 1**. For instance,

upon stimulation by an mTOR-stimulating ligand such as IGF1, growth factor receptor signaling initiates a kinase cascade that activates AKT (17). AKT activation leads to stimulation of the mTORC1 and mTORC2 complexes (17, 55), as well as inhibition of antioxidant activity via downregulation of FOXO-mediated transcription of enzymes like catalase and SOD (44), which would be expected to cause an increase in ROS. The consequences of increased ROS may include inhibition of mTORC1 through the UPR (17), stimulation of the NLRP3 inflammasome (56), and increased autophagy flux, for instance via activation of Atg4 (53). Increased ROS-mediated autophagy may in turn mitigate some ROS-induced cellular damage by increased turnover of damaged mitochondria (1), and may also promote self-antigen presentation required for T cell tolerance induction (15). This autophagic activity may in turn be antagonized by mTORC1 (12). In addition to the effects on antioxidant enzyme expression, activation of mTORC1 by AKT may also increase ROS by increasing metabolic flux as described above (6), however, mitochondrial biogenesis downstream of mTORC1 activation may balance this effect by producing healthy mitochondria to replace those that are damaged and may be a source of ROS (17). Notably, both ROS-induced oxidative damage (41) and NLRP3 inflammasome signaling (57) promote TEC damage during thymic atrophy.

The interaction of mTOR signaling, ROS, and autophagy should also be considered within the context of hypoxia. The thymus is hypoxic under physiological conditions (58, 59), and in fact hypoxia appears to promote thymocyte survival and development (58). This is consistent with studies showing that long-term repopulating hematopoietic stem cells are largely concentrated in hypoxic regions of the bone marrow (60). Little is known regarding the effect of hypoxia on TEC biology. Although stabilization of HIF1a represents a primary signaling pathway downstream of mTORC1 signaling under normoxic conditions (61), hypoxia also inhibits mTORC1 activity (62). This represents one way in which the downstream outcome and balance of mTORC1/mTORC2 signaling may be unique in hypoxic TEC, relative to other populations. In TEC, physiologically hypoxic conditions may generally inhibit mTORC1 signaling, while HIF1a stabilization, and therefore downstream signaling, is maintained by hypoxic conditions directly, independent of mTORC1. Another way the hypoxic steady state conditions in the thymus may affect the balance of mTORC signaling outcomes is by diminishing TCA cycle flux and downstream ETC flux [reviewed in (63)]. Increases in ROS mediated by low levels of O_2 available as an electron acceptor (63) may influence ROS-mediated impacts on mTOR signaling. Hypoxia also promotes autophagy (64), which may allow for higher levels of autophagic flux in TEC under conditions favoring mTORC1 activity relative to cells under normoxic conditions. Potential impacts of hypoxia on mTOR activity, ROS, and autophagy are indicated in **Figure 1**.

On balance, the available data support a role for mTORC1 activation in promoting TEC proliferation during thymus growth (13, 22). Activation of mTORC2 would be expected to promote lipogenesis required for cell growth, and for cytoskeletal organization (16) that may be important for maintaining TEC morphology, consistent with published reports (13, 23). Given the physiological importance of mTOR signaling, ROS, and autophagy in TEC, as well as the highly interactive nature of these metabolic pathways, further studies will be required to unravel the mechanisms that regulate the balance of catabolic and anabolic processes in TEC. Such studies will be most informative when done in a physiological setting, *in situ*. The genetic and imaging tools required for assessing the morphology of individual TEC, autophagy flux, as well as selective ablation or promotion of the individual pathway components are emerging. For instance, by randomly activating expression of one of four potential fluorophores, Confetti mice (65) allow identification of individual cell morphology, including TEC morphology *in situ*. Beclin 1 knock-in mice (66) allow independent manipulation of autophagy flux, which can be visualized using RFP-GFP-LC3 fusion mouse models (67). mTORC1 and mTORC2 signaling pathways can be independently disrupted using floxed Raptor (68) and Rictor (69) alleles, respectively. ROS can be independently manipulated by overexpression or ablation of antioxidant genes such as Catalase (70). Studies exploiting models such as these may allow more comprehensive understanding of the basic biology of stromal cells in the steady state thymus, and facilitate the design of informed strategies for delaying and reversing age-associate thymus dysfunction.

AUTHOR CONTRIBUTIONS

MS, NJ, and AG researched, wrote, and edited the manuscript. All authors contributed to the article and approved the submitted version.

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OPINION
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How Many Thymic Epithelial Cells Are Necessary for a Proper Maturation of Thymocytes?

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Keywords: thymic epithelial cells, thymocyte education, Eph, ephrin, regulatory T-cells

INTRODUCTION

The central tolerance to self-antigens is achieved in the thymus through a complex process in which developing thymocytes (T) sequentially interact with thymic epithelial cells (TECs) in a 3D network histologically organized in a cortex and a medulla (1). This T-TEC crosstalk is assumed to be essential for performing the named thymocyte education (2).

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IS T-CELL MATURATION POSSIBLE IN AN ALTERED THYMIC EPITHELIAL MICROENVIRONMENT?

There are examples in the literature showing that profound alterations of the thymic epithelial organization, which course with total or partial absence of T-TEC interactions mediated by distinct experimental conditions, do no result in important changes in the phenotype of T-cell subsets or in a breakage of central tolerance.

Revest et al. (3) demonstrated that FGF signaling was essential for TEC proliferation and that its lack coursed with low numbers of TECs that presumably would established reduced T-TEC interactions. However, in these conditions, phenotypical intrathymic lymphoid cell differentiation occurred normally, producing both DP and SP thymocytes. Authors remarked that even a low number of TECs would establish sufficient T-TEC interactions for supporting, at least phenotypical, lymphoid cell maturation. In a similar way, mice deficient in p63, a homolog of the p53 tumor suppressor gene involved in the morphogenesis and maintenance of thymic epithelium and epidermis, exhibit hypoplasia with increased proportions of apoptotic cells but normal T-cell development (4).

The Kremen (Krm) family of proteins that interact with the secreted Wnt regulator Dickkopf (Dkk) is considered a specific inhibitor of the canonical Wnt signaling pathway (5, 6). The loss of Krm1, that results in an excessive canonical Wnt signaling, induces profound alterations of the thymic architecture including incomplete separation of cortex and medulla, big epithelial free areas (EFAs), increased numbers of immature $K5^{+}K8^{+}$ TECs and loss of cell processes in cortical (c) TECs, that undoubtedly affect the T-TEC crosstalk. However, the lack of Krm1 gene had no effects on the numbers and proportions of thymocytes (7). Accordingly, authors speculated that the histological organization of the thymus might be less relevant for the T-cell maturation than the mere occurrence of different TEC subsets. On the other hand, the presence of small groups of TECs properly organized from a histological view and expressing key molecules involved in the functional crosstalk between TECs and thymocytes would be sufficient to allow the thymocyte differentiation in mutant thymuses (3, 7). In addition, Benz and colleagues (8) used other experimental approach to demonstrate that in CCR9-deficient thymuses DN2 and DN3 thymocytes did not migrate and

occupy the subcapsular cortex but developed normally, questioning the relevance of thymic niches that could just optimize the T-cell maturation rather than being a mandatory requisite for achieving the process. More recently, Cosway et al. (9) reported maintenance of T-cell tolerance in mice with a specific deletion of lymphotoxin β receptor (LTβR) gene in TECs that exhibited profound alterations of thymic medulla. In this case, dendritic cells (DCs) rather than medullary TECs appeared to be supporting the negative selection (10).

THE CONDITION OF EphB-DEFICIENT THYMUSES

In the last years, we have analyzed the role of tyrosine kinase receptors of the family Eph (Erythropoietin-producing hepatocyte) and their ligands ephrins (Eph receptor-interacting proteins) in the biology of the thymus. Eph-ephrin signaling participates in numerous processes in the immune and other tissues through the modulation of cell attachment, detachment, migration, proliferation, differentiation and cell fate (11). Our results, which have been summarized several times (12–14), confirm a role for EphB2, EphB3, ephrin-B1, and ephrin-B2 in the development and homeostasis of thymic epithelium. In these studies, as in those previously reported, we demonstrated that altered thymic epithelial network, which partially blocked the T-TEC interactions, does not course irrefutably with remarkable changes in the functional maturation of thymocytes, including both positive and negative selection (13).

On the other hand, the absence of these molecules clearly affected the T-TEC interactions. The mere morphological analysis of EphB-deficient thymuses suggested difficulties for establishing T-TEC contacts: scattered at random medullary epithelial islets, existence of large EFAs, retracted and disappeared epithelial cell processes (12). On the other hand, lymphoid progenitor cells differentiate in grafted EphB-deficient thymic lobes (15) and *in vitro* treatment of thymic reaggregates (RTOCs) with anti-EphB2 or anti-EphB3 specific antibodies shortened the TEC processes (13). The named thymic nurse cells (TNC) are cell complexes consisting of a single cTEC that homes 7–50 thymocytes and constitute specialized thymic niches for T-cell maturation (16). TNCs derived from EphB-deficient thymuses exhibit significantly reduced numbers of the most frequent ones containing 6–10 thymocytes (13). In fact, Eph and ephrin modulate both kinetic of cell conjugated established between DP thymocytes and isolated TECs and the formation of functional immunological synapsis (17, 18). Together all these results justify that the lack of Eph and ephrin signals alters the cellular positioning making it impossible that, as reported in other tissues, for thymocytes and TECs to intermingle and interact.

In agreement with all these data, we recently analyzed the T-cell populations of EphB2- or EphB3-deficient mice observing no phenotypical changes in the proportions of T-cell subsets phenotypically defined by specific cell markers (13, 19). Previously, we reported delayed maturation of DN ($CD4^-CD8^-$) cells but no variations in the proportions of both DP ($CD4^+CD8^+$) and SP thymocytes ($CD4^+CD8^-$ and $CD4^-CD8^+$) (20) and an analysis on the EphB-deficient TCR repertoire only found increased percentages of $V\beta3^+CD4^+$ cells in thymus and lymph nodes (14). In periphery, no changes occurred in the proportions of mutant Th1, Th2, and Th17 as compared to the values of WT cell populations, and only regulatory T-cells (Treg) of the inguinal lymph nodes, but not of spleen or thymus, showed significantly higher values as compared to WT ones (13, 19). Remarkably, the evaluation of the frequencies of positive and negative selected thymocytes of EphB-deficient thymuses did not exhibit significant differences with respect to WT proportions (13, 19). Nevertheless, as demonstrated in other experimental models (9), a role for thymic DCs in the maintenance of T-cell tolerance in all these mutants cannot be discarded.

CHANGES IN THE NUMBER OF TECs AFFECT BOTH THYMIC GROWTH AND THYMOCYTE DIFFERENTIATION

In order to test the hypothesis that reduced T-TEC crosstalk would support thymocyte differentiation, as others had pointed out (21, 22), we recently analyzed by flow cytometry the T-cell maturation in RTOCs containing different numbers of E14.5 thymic stromal cells (TSCs) ($1–0.085 \times 10^6$) 1 month after grafting under the kidney capsule of $FoxN1^{-/-}$ mice (19). Reaggregates were performed from E14.5 WT thymic lobes receiving for 7 days 2′-dGuo that eliminate any dividing thymic cell. At 1 month, RTOCs established with $1–0.5 \times 10^6$ stromal cells grew *in vivo* around 5–7 times, but those containing lower numbers of TSC yielded significantly less thymic cells. In addition, the RTOCs established with low numbers of TSCs, mainly those containing 0.085×10^6 TSCs, exhibited significantly reduced proportions of DP ($CD4^+CD8^+$) cells and increased percentages of $CD4^+$ thymocytes. In addition, there were higher frequencies of $TCR\alpha\beta^{hi}$ thymocytes, including both $TCR\alpha\beta^{hi}CD4^+$ and $TCR\alpha\beta^{hi}CD8^+$ cells, but no differences in the percentages of total positively selected thymocytes ($TCR\alpha\beta^{hi}CD69^+$) were seen, although the frequencies of total thymic Treg cells increased (19).

CONCLUSIONS AND FURTHER RESEARCH

In summary, these results support our proposal that reduced, but not total, disappearance of T-TEC crosstalk, due to alterations in the formed thymic epithelial network formed by low numbers of TECs may be enough for supporting T-cell differentiation; below those levels a normal T lymphopoiesis seems to be impossible. Nevertheless, functional studies and the determination of the condition of the negative selection in these RTOCs, not analyzed in these preliminary studies, are necessary to accomplish these results. However, it seems obvious that to establish the minimal numbers of thymocytes and TECs to obtain functional immuno-competent cells may represent a promising tool to modulate central tolerance and to avoid autoimmunity.

AUTHOR CONTRIBUTIONS

SM-H, JG-C, and AGZ: investigation and manuscript writing. AGZ: funding acquisition. All authors accept the published version of the manuscript.

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SOCS3 Expression by Thymic Stromal Cells Is Required for Normal T Cell Development

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The suppressor of cytokine signaling 3 (SOCS3) is a major regulator of immune responses and inflammation as it negatively regulates cytokine signaling. Here, the role of SOCS3 in thymic T cell formation was studied in *Socs3*$^{fl/fl}$ *Actin-creER* mice (Δ*socs3)* with a tamoxifen inducible and ubiquitous *Socs3* deficiency. Δ*socs3* thymi showed a 90% loss of cellularity and altered cortico-medullary organization. Thymocyte differentiation and proliferation was impaired at the early double negative (CD4-CD8-) cell stage and apoptosis was increased during the double positive (CD4+CD8+) cell stage, resulting in the reduction of recent thymic emigrants in peripheral organs. Using bone marrow chimeras, transplanting thymic organoids and using mice deficient of SOCS3 in thymocytes we found that expression in thymic stromal cells rather than in thymocytes was critical for T cell development. We found that SOCS3 in thymic epithelial cells (TECs) binds to the E3 ubiquitin ligase TRIM 21 and that *Trim21*$^{-/-}$ mice showed increased thymic cellularity. Δ*socs3* TECs showed alterations in the expression of genes involved in positive and negative selection and lympho-stromal interactions. SOCS3-dependent signal inhibition of the common gp130 subunit of the IL-6 receptor family was redundant for T cell formation. Together, SOCS3 expression in thymic stroma cells is critical for T cell development and for maintenance of thymus architecture.

Keywords: SOCS3, thymus, T cells, thymic epithelial cell, TRIM21

INTRODUCTION

The function of the thymus is to generate T lymphocytes that express T cell receptors with sufficient diversity to combat different microorganisms and tumors, while eradicating potentially autoreactive T cells. The thymus is histologically structured into discrete peripheral cortical and central medullary regions. These regions contain distinct stromal cell populations where thymic epithelial cells (TECs) are the main cell type, as well as immature T cells referred as thymocytes at defined stages of maturation. Diverse subsets of TECs in the cortex (cTECs) and medulla (mTECs) provide signals required for the survival and differentiation of thymocytes. The stepwise progression of thymocyte development requires their migration through these thymic regions, where interactions with cTEC and mTEC subsets take place (1).

Thymocytes enter the thymus as CD4-CD8- double negative (DN) progenitors. DN cells are subdivided into 4 sequential stages (DN1-DN4), based on the expression of CD44 and CD25. TCRγ and TCRδ rearrangements are completed at the DN3 stage when TCRβ rearrangement start. Progression beyond the DN3 stage requires expression of TCRβ, and TCRα chain rearrangement follows. Thymocytes expressing TCRαβ upregulate CD4 and CD8 [entering the double positive (DP) stage] and are further selected by cTECs to become CD4 or CD8 single positive (SP) cells binding either MHC-I or MHC-II molecules with their TCR (1, 2). After further negative selection in the medulla, SP thymocytes leave the thymus as functional mature T cells (3).

Cytokines are essential for the coordination of the stepwise T cell development in the thymus. Some cytokines, such as IL-7, are produced by TECs, support the proliferation and survival of thymocytes (4–6). Also, cytokines produced by thymocytes stimulate proliferation and differentiation of TECs (7–9).

A tight control of cytokine release and responses to cytokines is required for the correct development of T cells. The suppressor of cytokine signaling-3 (SOCS3) hampers signaling in response to the IL-6 family of cytokines. SOCS3 binds to the common gp130 subunit of the IL-6 receptor family impairing STAT3 activation (10). SOCS3 also regulates responses to cytokines, growth factors, and hormones that are independent of gp130 [i.e., IL-12R, granulocyte-colony stimulation factor (G-CSF), leptin, insulin] (11). SOCS3 is a central regulator of immunity and of the differentiation of diverse lymphoid and myeloid populations (12). Of importance, SOCS3 has been also been shown to regulate B cell lymphopoiesis, granulopoiesis, and erythropoiesis (13–15).

STAT3-mediated signaling has been demonstrated to contribute to optimal development of mTECs (but not cTECs) (16, 17). Comparatively little is known about the function of SOCS3 during thymic T cell development. Studies so far suggest that SOCS3 has a limited role during early thymopoiesis *in vitro* (18, 19).

Given the importance of SOCS3 in regulating different stages in T cell and the importance of the thymus in T cell maturation and homeostasis, the role of SOCS3 in T cell differentiation in the thymus was analyzed in this study. Since the genetic deletion of SOCS3 leads to mid-gestational embryonic lethality (13, 20), *Socs3*$^{fl/fl}$ *actin-creER* mice (Δ*socs3*) showing an inducible and tissue-broad deletion of *Socs3* were used in this study. Our results show a critical role of SOCS3 in T cell formation in the thymus and in the maintenance of thymic cellularity and architecture, mediated by the regulation of thymic stromal functions.

MATERIALS AND METHODS

Mice

The animals were housed according to directives and guidelines of the Swedish Board of Agriculture, the Swedish Animal Protection Agency, and the Karolinska Institute (djurskyddslagen 1988:534; djurskyddsförordningen 1988:539; djurskyddsmyndigheten DFS 2004:4). The study was performed under approval of the Stockholm North Ethical Committee on Animal Experiments permit number N397/13 and N3506/17.

Mice were housed at the Dept. of Microbiology, Tumor and Cell Biology the Astrid Fagreus and the Wallenberg Laboratories, Karolinska Institutet, Stockholm, Sweden, under specific pathogen-free conditions.

Mice containing loxP-flanked socs3 alleles have been described before (21). To allow temporal control of Cre activity, mice transgenic for a fusion between Cre and a mutated ligand-binding domain of the estrogen receptor (CreERT2) under the control of the β-actin promoter (CAGGCre-ER^{TM}) (22) were crossed with *Socs3*$^{fl/fl}$ mice (21) are referred as Δ*socs3* mice.

For a lymphoid-specific deletion *Socs3*$^{fl/fl}$ were bred with *lck cre* (23) and *cd4 cre* transgenic animals (24). *Gp130*$^{F/F}$ mice with an aminoacid substitution within gp130 abrogating the SOCS3 binding site have been described before (25). *Trim21*$^{-/-}$ mice were generated by homologous recombination as previously described (26).

The C57BL/6 congenic strain carrying the differential pan leukocyte marker CD45.1 was used in bone marrow radiation chimeric mice studies.

Flow Cytometry

Single cell suspensions from spleen, lymph node (LN) and thymus were obtained by mechanical disruption, straining over a 40-μm nylon mesh and lysis of erythrocytes. Cells were counted and surface stained with respective antibodies: anti-CD3 (clone: 17A2), anti-CD4 (GK1.5), anti-CD8 (53-6.7), anti-CD44 (IM7), anti-CD62L (MEL-14), anti-CD127 (A7R34), anti-CD24 (M1/69), anti-Qa-2 (695H1-9-9), anti-βTCR (H57-597), anti-γδTCR (GL3) all from eBioscience), and anti-CD25 (7D4) from BD Pharmingen). For analysis of thymic cell populations a dump channel with markers of lineage positive cells including CD11b (monocytes), Ter19 (erythrocytes), Ly6G (neutrophils), CD19/B220 (B cells), NK1.1 (NK cells) was included.

For characterization of TECs, a previously described thymic stromal cell isolation procedure was used (27). Thymi were dissected from freshly killed mice and trimmed of fat and connective tissue. Small cuts into the capsules were made with a pair of fine scissors and the thymi were gently agitated in 50 ml of RPMI-1640 with a magnetic stirrer at 4°C for 30 min to remove the majority of thymocytes. The resulting thymic fragments were transferred into 10 ml of fresh RPMI-1640 and dispersed further to free more thymocytes. The thymic fragments were then incubated in 5 ml of 0.125% (w/v) collagenase D with 0.1% (w/v) DNAse I (both from Boehringer Mannheim, Germany) in RPMI-1640 at 37°C for 15 min, with gentle agitation. Enzyme mixtures with isolated cells were removed after fragments had settled, then replaced with fresh mixture for further incubation. Gentle mechanical agitation was performed with a 3-ml syringe and 26G needle to break up aggregates remaining in final digestions. After 2 digestions, cells were centrifuged, resuspended in 5 mM EDTA in PBS+1% FCS+0.02% (w/v) NaN_3 (EDTA/FACS buffer) and allowed to incubate for 10 min at 4°C to disrupt rosettes. Cells were then passed through 100-μm mesh to remove clumps, and cells were labeled with antibodies as described above.

Single cell suspension was stained with CD45 (clone 30-F11, eBioscience), EpCAM (clone G8.8, eBioscience), Ly51 (clone

6C3, BD Pharmingen), UEA-1 (Vector Laboratories), CD80 and MHCII antibodies.

Apoptosis was determined by Annexin-V binding according to supplier's protocol (BD Pharmingen). Data were acquired in LSRII flow cytometer (BD) and analyzed using FlowJo software (Tree star).

Thymus Transplantation

The survival surgery was performed under sterile conditions after intra-peritoneal administration of the anesthetics, ketamine (100 mg/kg) and xylazine (10 mg/ kg) to CD45.1+ mice as described (28). A small dorsolateral incision was made to expose the kidney and a small hole was made in the kidney capsule. One fifth of a thymic lobe from 1 week old WT or Δ*socs3* (CD45.2+) donors were placed under the kidney capsule and the incision was closed with sterile sutures. One month after the transplantation, recipient mice were treated with Tm for 5 days. The graft dissected for flow cytometric analysis 7 days after the last Tm dose. The grafted thymus was analyzed for CD45.1 (derived from recipients) and CD45.2 (carried over from grafted thymus) cells.

BrdU Incorporation

WT and Δ*socs3* mice were injected intraperitoneally with 5-bromo-2-deoxyuridine (BrdU; Sigma; 0.1 mg/g) and were sacrificed 4 or 72 h after injection. Mice were sacrificed 10 days after Tm administration. For FACS analysis, single-cell suspensions were prepared from the thymi of BrdU pulse-labeled mice. Thymocytes were incubated with CD4, CD8, IL-7R, αβ, and γδ TCR antibodies followed by BrdU staining using the FITC BrdU Flow Kit (BD Pharmingen).

TEC Sorting

Thymic stroma were separated after enzyme digestion as described above. Then $CD45^{neg}$ cells were negatively selected using MACS magnetic beads labeled with anti-CD45 antibodies following instructions from the manufacturer. Cells were further labeled with anti-CD45 and anti-EpCAM antibodies and selected EpCAM+ cells sorted using a FACSAria™ Fusion device.

Overexpression of SOCS3

Transfection of CMV-driven SOCS3 EGFP expressing constructs, empty vector control and GFP-expressing plasmid was performed with lipofectamine following the indications of the manufacturer. In brief OP9-DL1 cells in 50–60% confluent in 100 mm dishes, were washed and incubated in 1.5 ml serum free OptiMEM and transfected with 14 μg plasmid and 5 μl.

Lipofectamine 3000, for 8 h 37°C. Cells then were washed and incubated in 11 ml OptiMEM + 10% fetal calf serum, mercaptoethanol for 12 h 37°C. Cells were then washed and incubated with OptiMEM 10% FCS at 32°C for 24 h. Then, cells were lysed for subsequent WB or IP studies. The efficiency of transfection was also analyzed by FACS.

Immunoprecipitation

Transfected and control OP9-DL1 cells 1.5×10^7 were resuspended in lysis buffer (120 mM NaCl, 50 mM Tris pH 8.0, 0.5% NP-40 and protease inhibitor cocktail p8340, Sigma), incubated for 1 h and then centrifuged at 14,000 × g for 10 min. Supernatants (1 mg protein/ ml) were incubated with 1 μg mouse anti-Myc (clone 9E10, Santa Cruz Biotechnology) or isotype antibodies overnight at 4°C. Samples were incubated then with Protein-G Agarose (Santa Cruz Biotechnologies) 4 h at 4°C. The samples were then washed 3 times in PBS 0.1% Tween and frozen for subsequent LC/MS-MS analysis or resuspended in 20 ul Laemli buffer, boiled for 5 min for Western blot.

Western Blot

Soluble protein concentration from OP9-DL1 cells was quantified by DC™ Protein Assay Kit (5000111; Bio-Rad). Thirty microgram of total protein were then mixed with 4x Laemmli buffer containing 8% SDS and β-mercaptoethanol followed by heat-denaturation for 5 min and cooled 15 min RT. Immunoprecipitated proteins or lysed proteins were separated by electrophoresis (Invitrogen NuPAGE electrophoresis system) through 4–12% Bis-Tris gradient gels with MOPS running buffer and transfered to a nitrocellulose membrane. The NC membranes were then blocked in PBS 5% BSA 0.1% Tween and incubated with primary mouse anti-Myc tag (Santa Cruz), rabbit anti-TRIM-21 or mouse anti-GAPDH antibodies (In vitrogen, H 06737 and 6C5) overnight at 4°C. NC membranes were then washed and incubated with HRP-conjugated anti-rabbit or anti-mouse IgGs at RT for 1 h. The membranes were developed using enhanced chemiluminescence (ECL, GE Health Care).

Mass Spectrometry

On-bead reduction, alkylation and digestion (trypsin, sequencing grade modified, Pierce) was performed followed by SP3 peptide clean-up of the resulting supernatant (29). Each sample was separated using a Thermo Scientific Dionex nano LC-system in a 3 h 5–40% ACN gradient coupled to Thermo Scientific High Field QExactive. The software Proteome Discoverer vs. 1.4 including Sequest-Percolator for improved identification was used to search the mouse Uniprot database for protein identification, limited to a false discovery rate of 1%.

Microarray Analysis

Total RNA was isolated from WT and Δ*socs3* TECs using the RNeasy Total RNA Isolation Kit (Qiagen) and cRNA was prepared as described. Briefly, cDNA was specifically transcribed from 500 ng of mRNA using a Poly-T nucleotide primer containing a T7 RNA polymerase promoter (Geneworks). Biotinylated, antisense target cRNA was subsequently synthesized by *in vitro* transcription using the BioArray High Yield RNA Transcript Labeling kit (Enzo Diagnostics). Fifteen micrograms of biotin-labeled target cRNA was then fragmented and used to prepare a hybridization mixture, which included probe array controls and blocking agents. Hybridization, washing and scanning were performed according to the manufacturers recommendations. Absolute levels of expression of genes were determined and scaled to 150 using algorithms in MicroArray Analysis suite 5.0 (MAS5) software (Affimetrix). The signal value represents the level of expression of a transcript and was expressed as a $\log_2$ ratio. In-depth analyses and clustering of data were conducted using GeneSpring software (Silicon Genetics).

Normalization was performed using a per chip 50th percentile normalization and per gene median normalization method. Genes that were consistently absent or below the noise level were excluded from analysis.

To identify genes with statistically significant differences between WT and Δ*socs3* TECs a *p*-value cut-off of 0.05 and the Benjamini and Hochberg false discovery rate as multiple testing correction were performed. The Student-Newman-Keuls *post-hoc* test was used to identify the specific groups in which significant differential expression occurred. Genes that showed a change of 2-fold or greater were considered differentially expressed. The IPA and WebGEstalt softwares were used to identify pathways and gene sets based on common functional features that are differentially expressed in Δ*socs3* TECs. The raw and processed data has been deposited in the GEO public repository with the series accession number GSE165216.

Histopathology

Thymi, lungs and livers from Δ*socs3* and WT mice were processed for histological analysis. In brief, organs were fixed in 4% buffered paraformaldehyde for 24 h and paraffin-embedded. Eight micrometer sections were obtained, paraffin-removed and dehydrated before staining with hematoxylin and eosin (Sigma-Aldrich).

Immunohistochemistry

Thymi from Δ*socs3* and WT mice were processed for histological analysis. In brief, tissues for immunofluorescence were embedded in OCT and frozen immediately in liquid nitrogen. Sections (8 μm) were fixed for 10 min in 4% PFA, washed in PBS and blocked in 2% BSA, 10% goat serum, 0.1% Tween-20 and 0.1% NaN_3 in PBS.

Thymic sections were incubated with rabbit polyclonal antibodies to K5 and rat anti-K8 antibodies. Subsequently, sections were incubated with primary and fluorochrome-labeled secondary antibodies. After DAPI staining, sections were mounted with mounting gel (Invitrogen), and images of stained sections were captured using a Leica fluorescence microscope.

Bone Marrow Radiation Chimeric Mice

Recipient WT and Δ*socs3* mice were irradiated 2x with 550 rad and received 5×10^6 BM cell from either WT or Δ*socs3* mice. In some experiments CD45.1 congenic C57Bl/6 mice were used as bone marrow donors for irradiated WT or Δ*socs3* mice. Mice were kept for 3 weeks on antibiotics after transplantation (Tribrissen in drinking water). Tm was administered either 15 d before the BM transplantation or 15 d before sacrifice. Chimeric mice were sacrificed 70 days after the transplantation.

Real Time-PCR

Transcripts were quantified by real time PCR as previously described (30). *Hprt* was used as a control gene to calculate the ΔC_t values for independent triplicate samples. The relative amounts of *socs3*/*hprt* transcripts was calculated using the $2^{-(\Delta\Delta Ct)}$ method. These values were then used to calculate the relative expression of cytokine mRNA Din treated or untreated cells and tissues.

Statistical Analysis

Statistical analysis was performed using Graphpad Prism version 8. The *p*-values were calculated by two-tailed, unpaired Student's *t*-test or by one-way ANOVA analysis with a Kelch correction.

For the microarray analysis, a *p*-value cut-off of 0.05 and the Benjamini and Hochberg false discovery rate as multiple testing correction were performed to identify genes with statistically significant differences between WT and Δ*socs3* TECs. The Student-Newman-Keuls *post-hoc* test was used to identify the specific groups in which significant differential expression occurred. Genes that showed a change of 2-fold or greater were considered differentially expressed.

RESULTS

SOCS3 Is Required for Maintenance of Thymus Structure and Thymocyte Differentiation

In order to study the role of SOCS3 in the production of T cells in the thymus, Δ*socs3* mice were treated with tamoxifen (Tm) for 5 days. Seven days after the last Tm dose mutant and $Socs3^{fl/fl}$ (WT) thymi were compared. The cellularity of Tm-treated Δ*socs3* thymi was severely reduced. No reduction of thymic cellularity was observed in Tm-untreated Δ*socs3* control mice (**Figures 1A,B**). To exclude an off-target effect of Cre expression on thymocytes, $Socs3^{fl/+}$ *actin creER* and $Socs3^{fl/+}$ mice were generated. These animals showed similar thymic cellularity before and after Tm treatment (**Figures 1C,D**). Δ*socs3* mice showed no weight loss, spleen or lymph node enlargement, diahrrea and piloerection as compared to WT controls. Moreover, histopathological analysis of livers and lungs showed no signs of localized or disseminated inflammatory lesions (**Supplementary Figure 1A**).

Δ*socs3* mice showed an increased frequency of lineage negative DN cells among thymocytes (**Figures 1E,F**). Within the DN compartment, the frequency of Δ*socs3* DN3 cells was increased suggesting a block in thymocyte maturation at this stage of thymocyte development (**Figures 1J,K**). The frequency of γδ- and β-TCR expressing DN Δ*socs3* thymocytes was reduced compared to controls, which is line with this (**Figures 1L,M**).

The percentage of DP cells in Δ*socs3* thymi was dramatically reduced (**Figures 1E,G**), while the frequency of CD4 and CD8 SP cells were increased as compared to Tm-untreated Δ*socs3*, and to Tm-treated WT mice (**Figures 1H,I**). The numbers of all Δ*socs3* thymocyte populations (with exception of CD8 SP) and DN subpopulations were lower than controls (**Supplementary Figures 1B–G**).

Thus, SOCS3 plays a major role in thymocyte differentiation and in the maintenance of thymic cellularity.

SOCS3 Expression by Thymic Stroma Cells Is Central for Thymus Maintenance and T Cell Differentiation

In order to determine whether SOCS3 expression in bone marrow (BM)-derived cells or non-hematopoietic cells is involved in the T cell differentiation in the thymus, BM

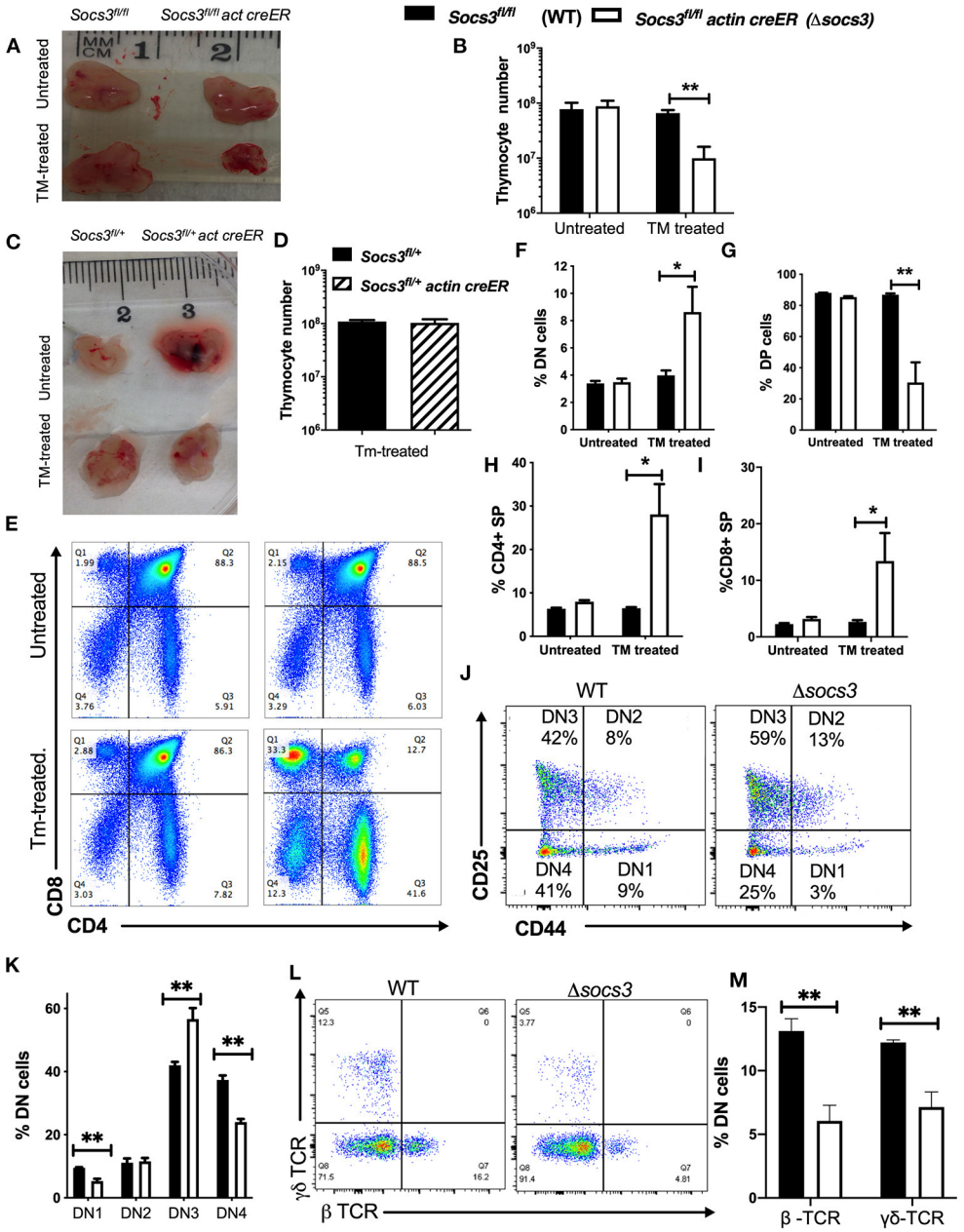

FIGURE 1 | SOCS3 is required for maintenance of thymus structure and thymocyte differentiation. **(A)** Representative image and **(B)** mean total thymic cell numbers from Δ*socs3* and WT mice untreated or 7 days after the last dose of Tm. **(C)** Photograph from *Socs3*$^{fl/+}$ and *Socs3*$^{fl/+}$ *actin creER* thymi 7 days after Tm or left untreated. **(D)** Mean thymocyte numbers ± SEM from *Socs3*$^{fl/+}$ and *Socs3*$^{fl/+}$ *actin creER* mice 10 days after Tm treatment. Differences between groups are significant at $^{*}p \leq 0.05$, $^{**}p \leq 0.01$ ANOVA with Welch correction. **(E)** Representative dot plots and mean frequencies of lineage negative **(F)** DN, **(G)** DP, **(H)** CD4, and **(I)** CD8 SP thymocytes ± SEM in Δ*socs3* and WT mice (6 per group) treated or not with Tm. Four independent experiments were performed. These experiments were repeated more than 5 times with similar results. Differences between groups are significant at $^{*}p \leq 0.05$, $^{**}p \leq 0.01$ ANOVA with Welch correction. **(J)** Representative plot and **(K)** mean of frequency of DN1-DN4 Δ*socs3* and WT subpopulations ± SEM ($n = 5$ per group) defined by CD44 and CD25 expression. Differences between groups are significant at $^{**}p \leq 0.01$ unpaired Student's *t*-test. **(L)** Representative dot plots and **(M)** mean frequency ± SEM **(M)** of γδ and βTCR+ cells within the DN thymocyte population in Δ*socs3* and WT mice ($n = 6$ per group). Differences between groups are significant at $^{**}p \leq 0.01$ unpaired Student's *t*-test.

radiation chimeras were generated and sacrificed 7 days after Tm-treatment completion (**Figure 2A**). The thymocyte numbers were reduced in Δ*socs3* recipient mice as compared to WT recipients. Instead, thymocyte levels in WT → WT and Δ*socs3*→ WT were similar (**Figure 2B**), suggesting a role for SOCS3 in non-hematopoietic cells. In addition, Δ*socs3*→ Δ*socs3* mice showed lower numbers of thymocytes as compared to WT→ Δ*socs3* mice (**Figure 2B**).

The Δ*socs3* recipient mice contained lower percentages of DP, and higher of DN, CD4, and CD8 SP cells than WT recipients

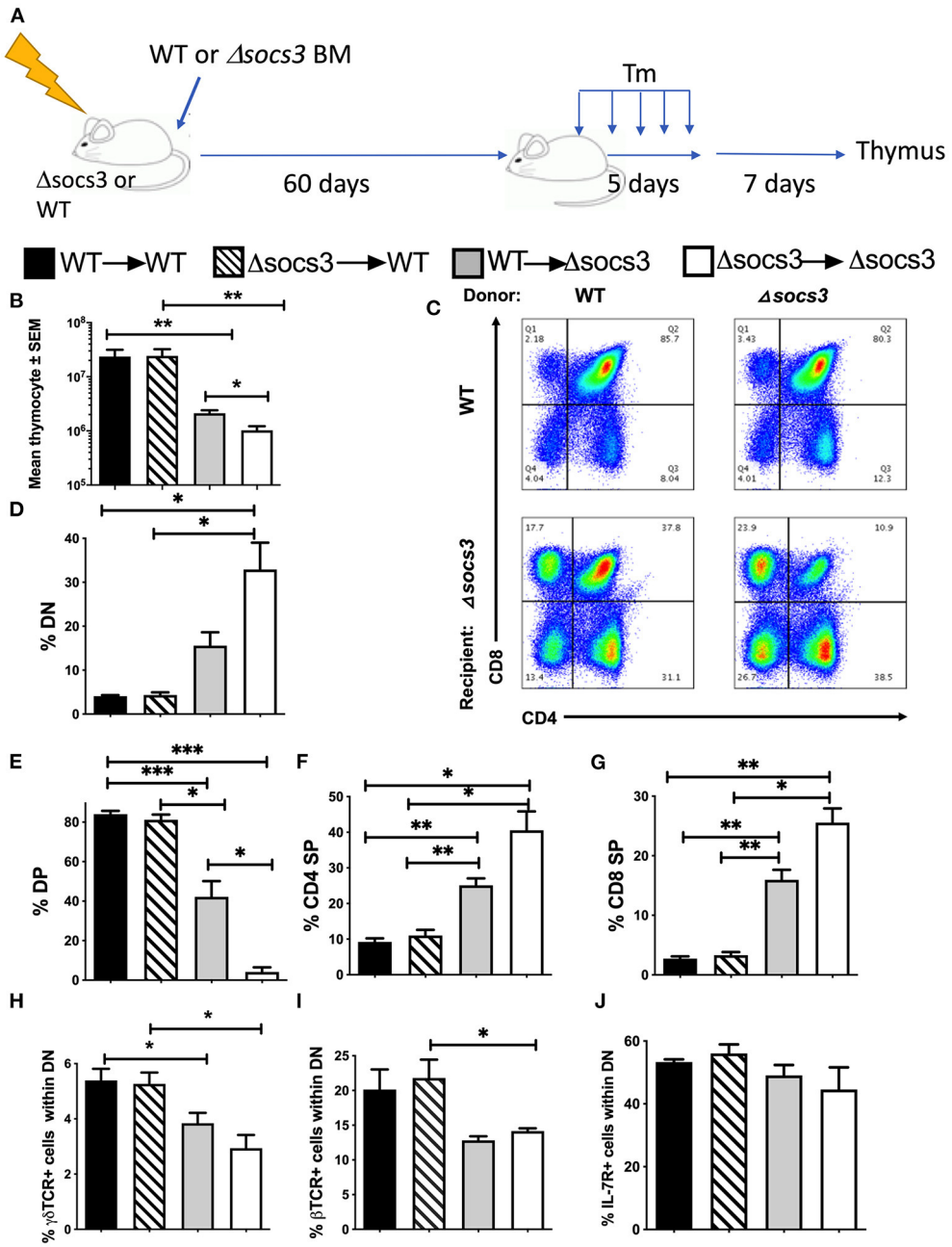

FIGURE 2 | SOCS3 expression by non-hematopoietic cells is central for thymus maintenance and T cell differentiation. **(A)** Radiation bone marrow (BM) chimeras were generated using WT and Δ*socs3* mice as recipients or donors. Sixty days after transplantation, mice were treated with Tm and sacrificed 7 days after the last dose. **(B)** The mean thymic cell numbers ± SEM is depicted ($n \geq 5$ per group). Differences between groups are significant at $^{*}p \leq 0.05$, $^{**}p \leq 0.01$ and $^{***}p \leq 0.01$ Welch one way ANOVA. **(C)** Representative dot plots and **(D–G)** mean frequencies of DN, DP, CD4 SP, and CD8 SP thymocytes ± SEM in BM chimeric Δ*socs3* and WT mice are depicted ($n \geq 5$ per group). Differences between groups are significant at $^{*}p \leq 0.05$, $^{**}p \leq 0.01$ Welch one-way ANOVA. **(H,I)** The mean frequency of **(H)** γδ and **(I)** β TCR+ cells within DN ± SEM BM chimeric mice is depicted. Differences between groups are significant at $^{*}p \leq 0.05$ Welch one-way ANOVA. **(J)** The mean % IL-7R+ cells within DN population ± SEM in BM chimeras of Δ*socs3* and WT is shown.

(**Figures 2C–G**). The percentages of DN, CD4, and CD8 SP were higher and DP cells were lower in Δ*socs3*→ Δ*socs3* compared to WT→ Δ*socs3* thymi suggesting a redundant role for hematopoietic SOCS3 expression in the control of thymic T cell development. The frequencies of thymocyte subpopulations in WT→ WT and Δ*socs3*→ WT mice were similar (**Figures 2C–G**). These results indicate that SOCS3 in non-hematopoietic cells is required to maintain T cell formation in the

thymus. We observed lower numbers of DPs in Δ*socs3* than in WT recipients, while differences in other thymocyte populations were not statistically significant (**Supplementary Figures 2A–D**).

The percentage of γδ and αβTCR+ in DN thymocytes from Δ*socs3* recipient chimeric mice was reduced as compared to those of WT recipients (**Figures 2H,I**). Binding of IL-7 to its receptor (IL-7R) plays a non-redundant role in T cell development, by promoting the survival and proliferation of DN progenitors and of SPs cells during the positive selection (31). We observed that the frequency of IL-7R+ DN from Δ*socs3* or WT recipient mice was similar (**Figure 2J**). The frequencies of IL7R+ CD4 and CD8 SPs from Δ*socs3* recipients were higher than those of WT controls. The percentages of IL-7R+ CD4 and CD8 SPs from Δ*socs3*→ Δ*socs3* were higher than those from WT→ Δ*socs3* mice, while that of IL-7R+ CD4 and CD8 SP from Δ*socs3*→ WT and WT→ WT mice were similar, suggesting that SOCS3 in non-hematopoietic cells regulates IL-7R expression during late stages of thymocyte development (**Supplementary Figures 2E–G**).

A sequence of events within the SP stage is necessary before T cell export occurs: Maturation of SP thymocytes is associated with downregulation of CD24 and upregulation of Qa-2 molecules (3). $CD24^{low}Qa2^{high}$ SPs thymocytes proliferate when triggered through the TCR prior to export to the periphery (32). CD4 and CD8 SPs from Δ*socs3* thymi contained higher Qa2 (**Supplementary Figures 3A–D**) and lower CD24 levels (**Supplementary Figures 3E–H**) than controls. Thus, Δ*socs3* SP cells display a higher maturation level than WT controls.

T regs cells that develop in, and emerge from, the thymus maintain self-tolerance and prevention of autoimmune disorders. We found that the frequency of FOXP3+ cells within Δ*socs3* and WT CD4 SPs was similar, suggesting that SOCS3 does not preferentially regulate the development of Tregs in the thymus (**Supplementary Figure 3I**).

Next, we investigated whether the expression SOCS3 in thymic stromal cells regulates thymic T cell development. For this purpose, WT or Δ*socs3* CD45.2 thymic fragments were implanted under the kidney capsule of CD45.1 mice. Four weeks after implantation mice were treated with Tm for 5 days. The engraftment was resected 7 days after Tm treatment, and the presence of CD45.1+ thymocytes in the kidney organoid evaluated (**Figure 3A**, **Supplementary Figures 4A,B**). The frequencies of CD45.1+ thymocyte DN, DP, SP, and subpopulations in the WT graft were similar to that of the endogenous thymus of recipient mice, indicating a functional T cell development in the ectopic thymus (**Supplementary Figures 4C,D**), in agreement with previous studies (33). The Δ*socs3* graft instead showed a diminished frequency of DP and increased DN and CD4+ and CD8+ SP levels as compared with those in the WT graft or the endogenous thymus (**Figures 3B–D**).

To confirm that SOCS3 in thymocytes plays a minor role if any in thymic T cell development, thymocyte populations in $Socs3^{fl/fl}$ *lck cre* and $Socs3^{fl/fl}$ *cd4 cre* mice were analyzed. Lck is expressed by the early DN (34) while CD4 will be first expressed at the late DN to DP stage of thymocyte development. $Socs3^{+/+}$*lck cre* (*lck cre*) were used as controls for $Socs3^{fl/fl}$*lck cre* thymi since lck cre expression alters thymic T cell development (35). The numbers of thymocytes in $Socs3^{fl/fl}$*lck cre* and *lck cre* mice were similar (**Figure 3E**). The frequency of thymocyte DN, DP, and SP subpopulations, the levels of IL-7R in DN and the frequency of β- and γδ TCR+ cells in $Socs3^{fl/fl}$ *lck cre* and control DN were similar (**Figures 3F–K**). In agreement with these results, the frequencies of thymocyte DN, DP and SP subpopulations in $Socs3^{fl/fl}$ *cd4 cre* and *cd4 cre* animals were also similar (**Figures 3L–N**). The percentages of βTCR+ and γδTCR+ $Socs3^{fl/fl}$ *cd4 cre* and $Socs3^{+/+}$*cd4 cre* DN thymocytes were also comparable (**Supplementary Figures 4E,F**). Altogether, SOCS3 in thymic stroma is critical for T cell formation in thymus.

Decreased Thymocyte Proliferation, Differentiation, and Increased Frequency of Apoptotic Thymocytes in Δsocs3 Mice

The proliferation and differentiation of Δ*socs3* and WT thymocytes was investigated after a single BrdU pulse performed 7 days after Tm administration. The frequency of BrdU+ WT thymocytes was increased from 4 to 72 h after the administration of the nucleoside showing proliferation of labeled cells (**Figure 4B**). The frequency of BrdU+ thymocytes at 72 h after administration was lower in Δ*socs3* than in WT controls (**Figures 4A,B**).

We then analyzed BrdU incorporation within the different thymocyte subpopulations (**Supplementary Figure 3G**). The frequency of BrdU+ DN measured 4 h after the pulse was lower in Δ*socs3* than in control thymocytes (**Figure 4C**), suggesting a lower DNA synthesis in the absence of SOCS3. A tendency toward a reduction of the proportion of BrdU+ DN was observed when comparing 4–72 h time points after BrdU pulse in WT thymocytes, while the opposite was observed for Δ*socs3* thymocytes (**Figure 4C**). This further supports a block in the Δ*socs3* thymocyte differentiation at the DN stage. The frequencies of BrdU+ DP Δ*socs3* and WT thymocytes increased from 4 to 72 h after BrdU administration (**Figure 4D**). The percentage of BrdU+ CD4+SPs augmented from 4 to 72 h after the nucleoside administration (**Figure 4E**). The fraction of BrdU+ Δ*socs3* CD4 SPs at 72 h after administration was lower than that of WT controls (**Figure 4E**).

To study whether the thymus hypoplasia in Δ*socs3* mice was due to increased cell death, the proportion of Annexin-V+ thymocytes was measured. We found higher frequencies of Annexin-V+ cells in the Δ*socs3* thymocytes as compared to WT controls (**Figure 4F**). The percentage of Annexin-V+ DN and particularly that of DP from Δ*socs3* thymi was increased as compared to WT controls (**Figures 4G,H**). On the other hand, the Annexin V labeling in CD4 and CD8 SP thymocytes from Δ*socs3* and WT mice was similar (**Figures 4I,J**). Thus, SOCS3 deficiency in the thymus results in a reduced proliferation, blocked differentiation and increased apoptosis of the thymocyte populations.

Abnormal Thymus Architecture in Δsocs3 Mice

Since the defect of thymocyte maturation in Δ*socs3* mice was assigned to the thymic stroma, the levels of

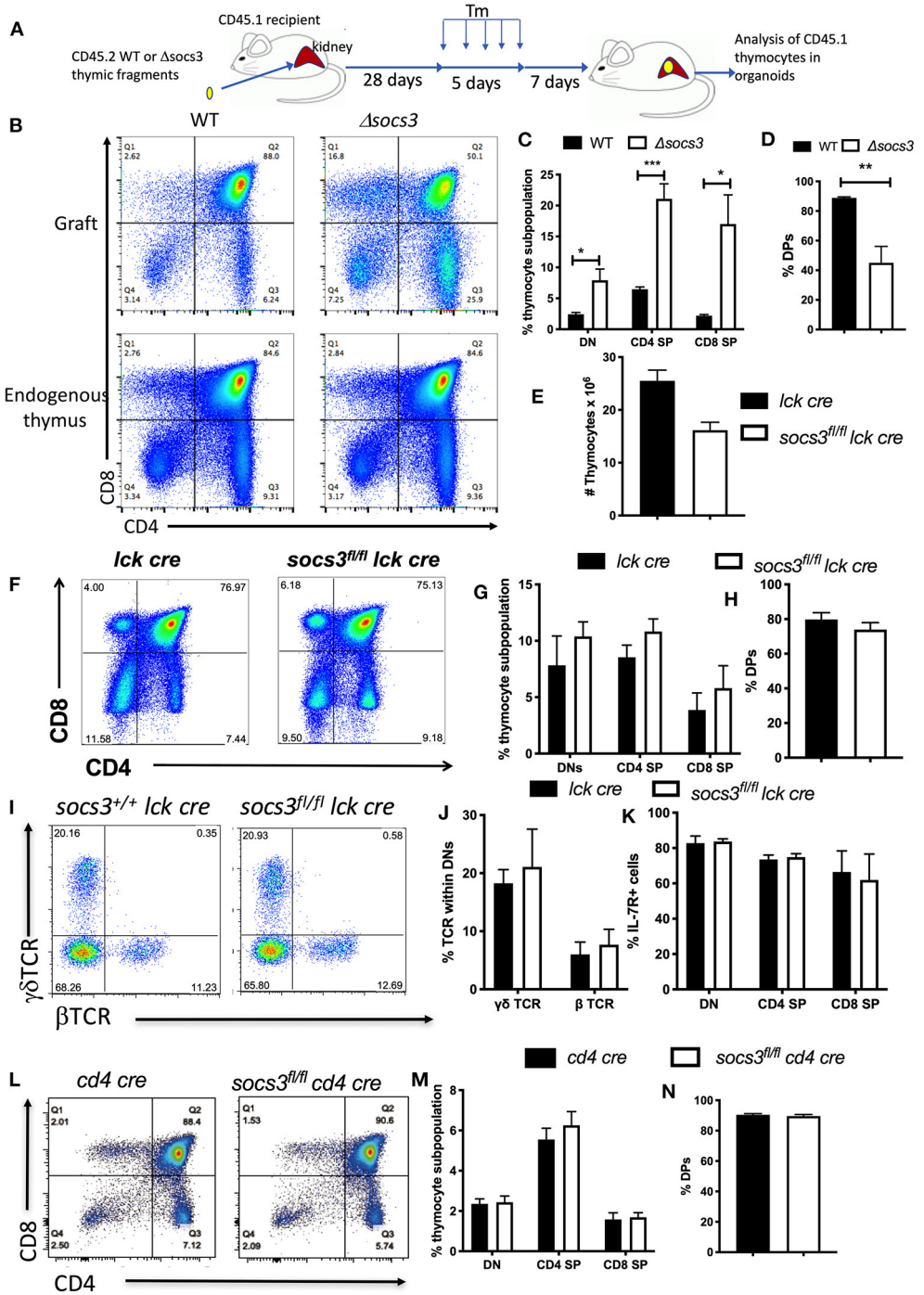

FIGURE 3 | SOCS3 expression in thymic stroma but not in thymocytes is required for T cell maturation in the thymus. **(A)** Thymic fragments from 1 to 3 days old WT and Δ*socs3* mice were transplanted into the kidney capsule of CD45.1+ mice. Four weeks after the implantation mice were treated with Tm daily for 5 days. Seven days after the last Tm dose mice were sacrificed and the thymic organoid grafts and the "endogenous" thymus were explanted for analysis. **(B)** Representative dot plots shown compare the thymocytes from mice grafted with CD45.2 WT and Δ*socs3* thymic fragments and the endogenous thymus (gated on CD45.1+ recipient cells), 7 days after the last Tm dose. **(C,D)** The mean frequencies of CD45.1+ DN, DP, CD4 SP, and CD8 SP ± SEM in the thymic grafts obtained as indicated above are shown. Differences between groups are significant at $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$ (unpaired Student's *t*-test). These experiments were performed 3 times. **(E)** The mean number of *Socs3*$^{fl/fl}$ *lck cre* and *Socs3*$^{+/+}$ *lck cre* thymocytes ± SEM (at least 6 per group) are depicted. **(F)** Representative dot plots and **(G,H)** the mean frequencies of *Socs3*$^{fl/fl}$ *lck cre* and *lck cre* DN, DP, CD4 SP, and CD8 SP ± SEM are shown. **(I)** Representative dot plots and **(J)** mean frequencies ± SEM of *Socs3*$^{fl/}$ *lck cre* and *Socs3*$^{+/+}$*lck cre* γδ and β TCR DN cells ± SEM are depicted. **(K)** The frequency of IL-7R+ *Socs3*$^{fl/fl}$ *lck cre* and *Socs3*$^{+/+}$ *lck cre* thymocyte populations ± SEM mice are shown. **(L)** Representative dot plots and **(M,N)** the mean frequencies of DN, DP, CD4, and CD8 SP thymocytes ± SEM in *Socs3fl/fl cd4 cre* and *Socs3*$^{+/+}$ *cd4 cre* mice are shown.

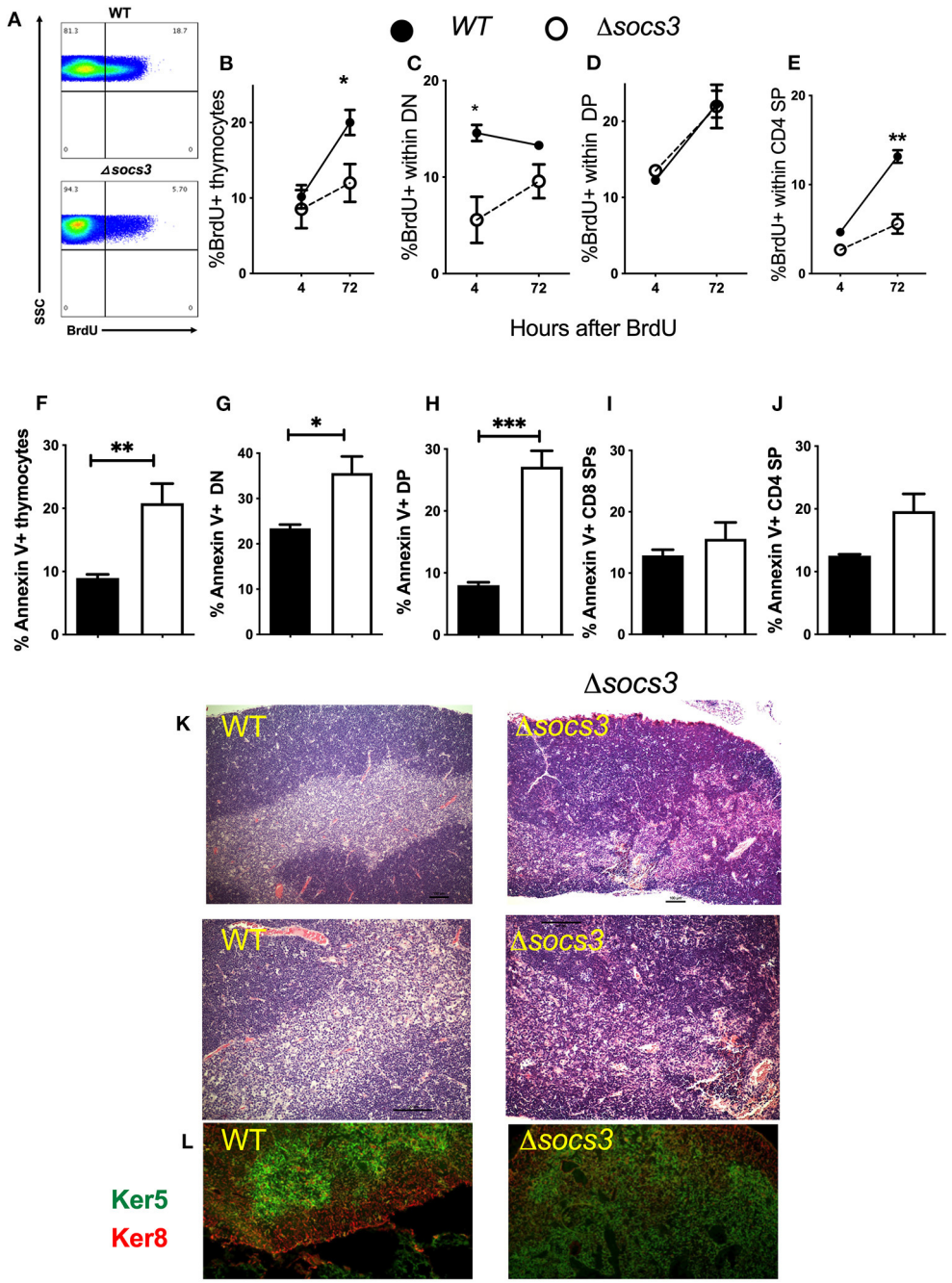

FIGURE 4 | Decreased proliferation, differentiation and increased frequency of apoptosis in Δsocs3 thymocytes. **(A)** A representative histogram shows BrdU+ thymocytes from Δ*socs3* and WT mice measured 72 h after BrdU administration. **(B)** The mean frequency of BrdU+ thymocytes ± SEM from WT and Δ*socs3* mice ($n = 5$ animals per groups) 4 and 72 h after BrdU inoculation are depicted. The mean frequency ± SEM of BrdU+ cells within **(C)** DN, **(D)** DP, and **(E)** CD4 SP Δ*socs3* and WT thymocytes at 4 and 72 h after BrdU administration is shown. The mean frequencies of Annexin V+ **(F)** total thymocytes and **(G)** DN, **(H)** DP, **(I)** CD4 SP, and **(J)** CD8 SP subpopulations ± SEM in Δ*socs3* and WT mice (at least 5 per group) are shown. Differences between WT and Δ*socs3* thymocytes are significant at $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$, two-way ANOVA and unpaired Student's *t*-test. **(K)** The haematoxylin and eosin staining depicting the organization of thymi from WT as compared to Δ*socs3* mice. A representative micrograph from tissues from 5 mice per group analyzed is shown. Scale bar: 100 μm. **(L)** Double immunolabelling of keratin 5 and keratin 8 targeting the thymic cortex and medulla, respectively, in WT and Δ*socs3* mice is shown.

cTECs and mTECs in Δ*socs3* thymi were then compared. We found that the frequencies of TECs (defined as CD45-EpCAM+ cells) were higher in Δ*socs3* than in WT thymi (**Supplementary Figures 5A,B**). The total numbers of TECs in Δ*socs3* and control thymi were similar, and the increased TEC frequencies in Δ*socs3*

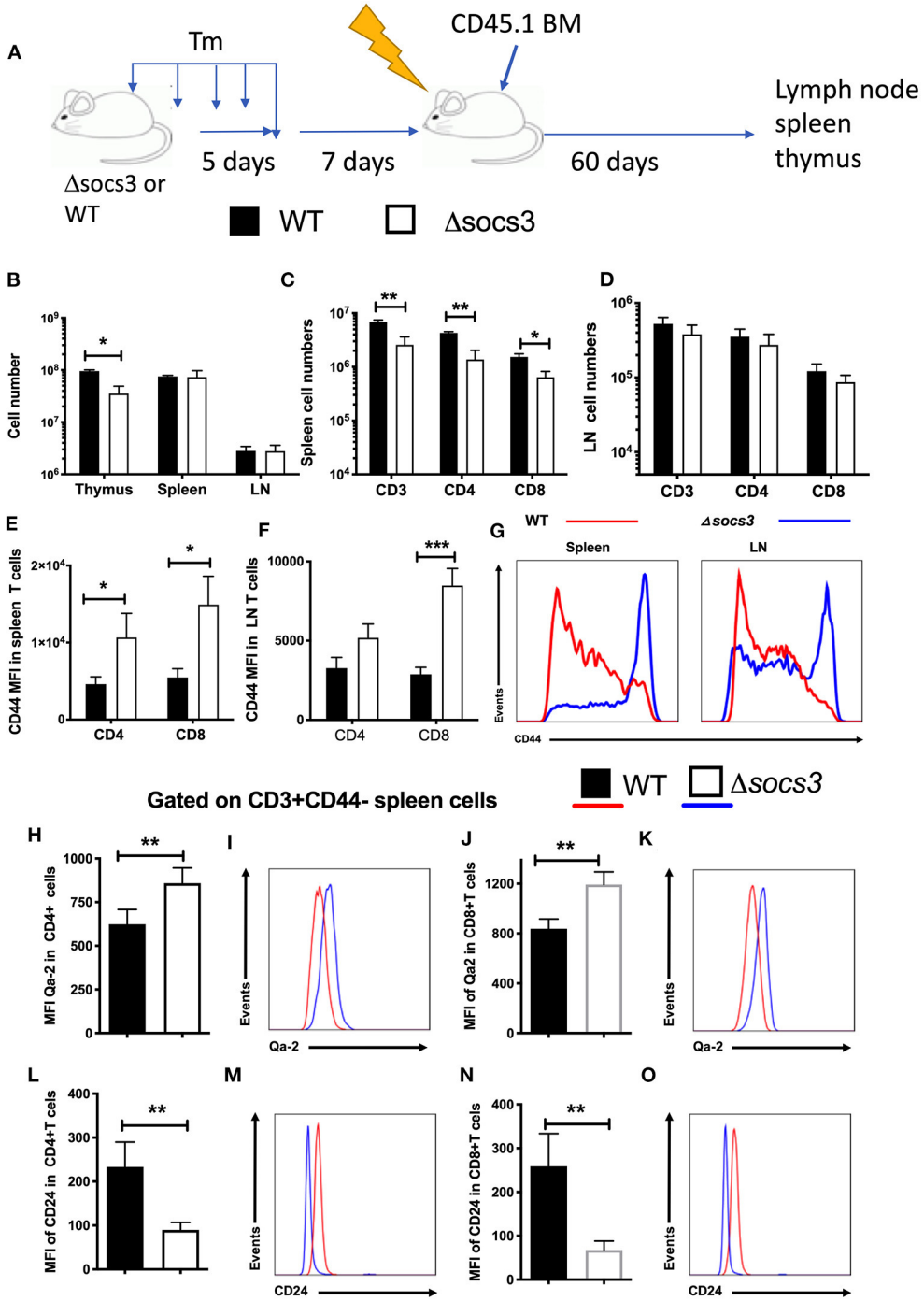

FIGURE 5 | Non-hematopoietic SOCS3 expression regulates the frequency recent thymic emigrants and of naïve peripheral T cells. **(A)** WT or Δ*socs3* mice treated with Tm and 7 days after the last dose of Tm were irradiated and transplanted with CD45.1+ WT bone marrow cells. Mice were sacrificed 60 days after BM transplantation. **(B)** The mean number of thymus, spleen and lymph node cells ± SEM of transplanted mice are depicted ($n = 5$ per group). The mean number ± SEM of CD45.1+ and CD3 gated CD4, CD8, and γδ T cells in **(C)** spleens and **(D)** lymph nodes of radiation chimeric mice treated as described above is shown ($n = 5$ per group). The mean MFI of CD44 in CD45.1 gated CD4 or CD8 T cells in spleens **(E)** and lymph nodes **(F)** is shown. **(G)** A representative histogram of CD44 in CD8 T cells in lymph node or spleen cells from Δ*socs3* and WT mice. The mean MFI and representative histograms of Qa-2 **(H–K)** and CD24 **(L–O)** expression in naïve CD4 **(H,I,L,M)** and CD8 **(J,K,N,O)** splenic T cells of WT and Δ*socs3* mice 7 days after Tm treatment are depicted. Differences between groups are significant at $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$, unpaired Student's *t*-test.

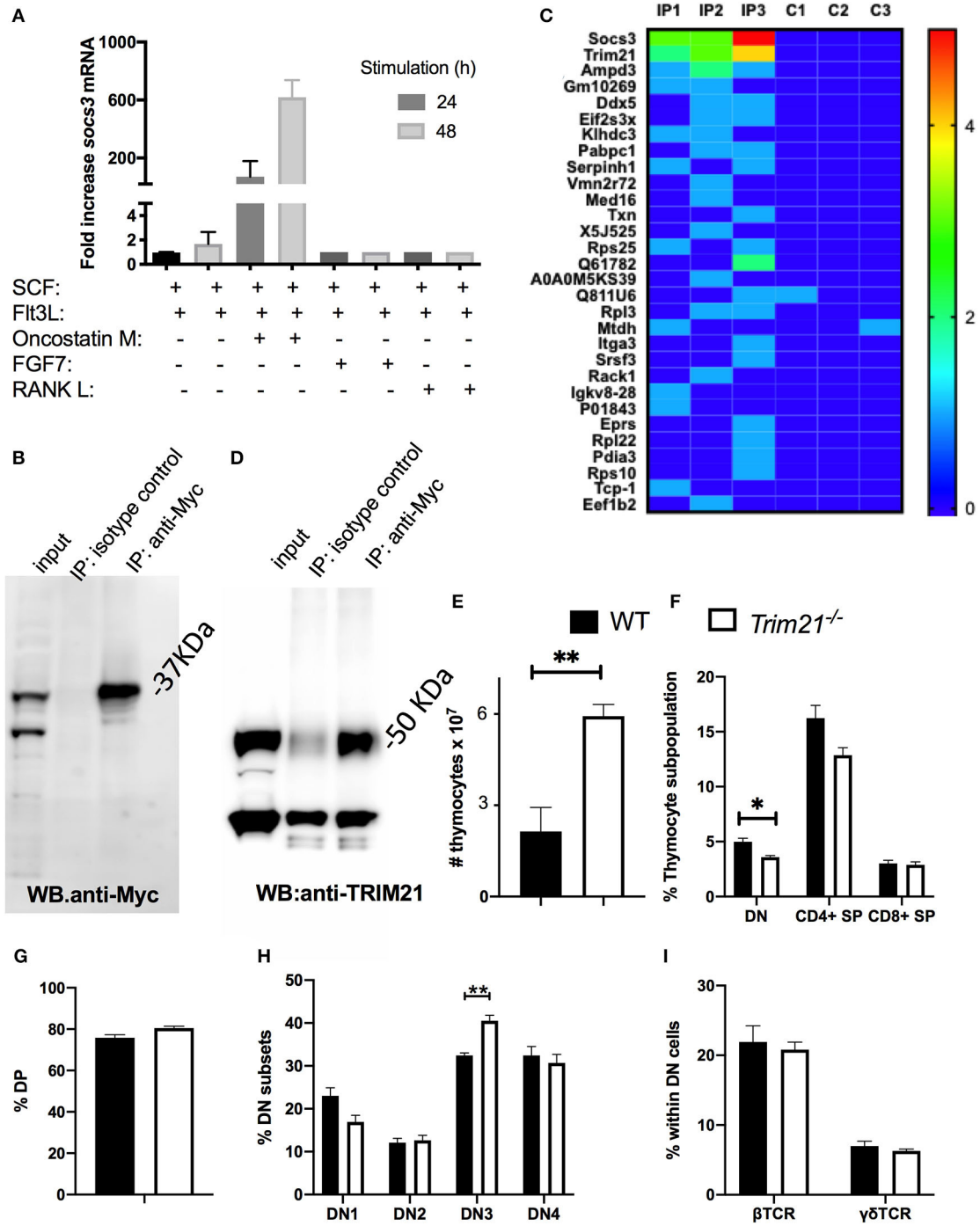

FIGURE 6 | SOCS3 in thymic epithelial cells binds to TRIM21. **(A)** OP9-DL1 cells were incubated with either SCF, Flt3L, OMS, FGF7, or RANK. Twenty four and forty eight hour after stimulation, the total mRNA was extracted and *Socs3* and *Hprt* mRNA levels measured by real time RT-PCR. The mean relative fold induction of *Socs3*/ *Hprt* mRNA ratio in stimulated and unstimulated cells are depicted. **(B)** OP9-DL1 cells were transfected with an Myc-SOCS3 plasmid. The western blot of transfected cell lysates before and after immunoprecipitation with anti-myc or an isotype controls is shown. **(C)** Heat map of ranking proteins identified after tandem mass spectrometry in immunoprecipitates from *socs3*-transfected OP9-DL1 cells with anti-myc or isotype controls. Three replicates per condition were analyzed. Ranking was calculated with an algorithm weighing the number of replicates in which the protein was detected in the IP and in the control group, the number of peptide spectral matching the protein, the number of unique peptides and the intensity (the area under the curve). **(D)** Anti-SOCS3 or anti-isotype immunoprecipitates were analyzed by immunoblot using anti-TRIM21. **(E)** The mean number of WT and *Trim21*$^{-/-}$ thymocytes ± SEM are depicted ($n \geq 5$ per group). Differences between groups are significative (**$p \leq 0.01$ unpaired Student's *t*-test). **(F,G)** The mean frequency of thymic DN, DP, and SP subpopulations ± SEM in *Trim21*$^{-/-}$and WT thymi ($n = 5$ per group). **(H)** The mean of frequency ± SEM of DN1-DN4 subpopulations (defined by CD44 and CD25 expression) in *trim21*$^{-/-}$and WT thymi (n≥5 per group). **(I)** The mean % of β and γδ TCR+ cells within the DN thymocytes of WT and *trim21*$^{-/-}$ mice ($n \geq 5$ per group). Differences between groups are significant at **$p \leq 0.01$ unpaired Student's *t*-test.

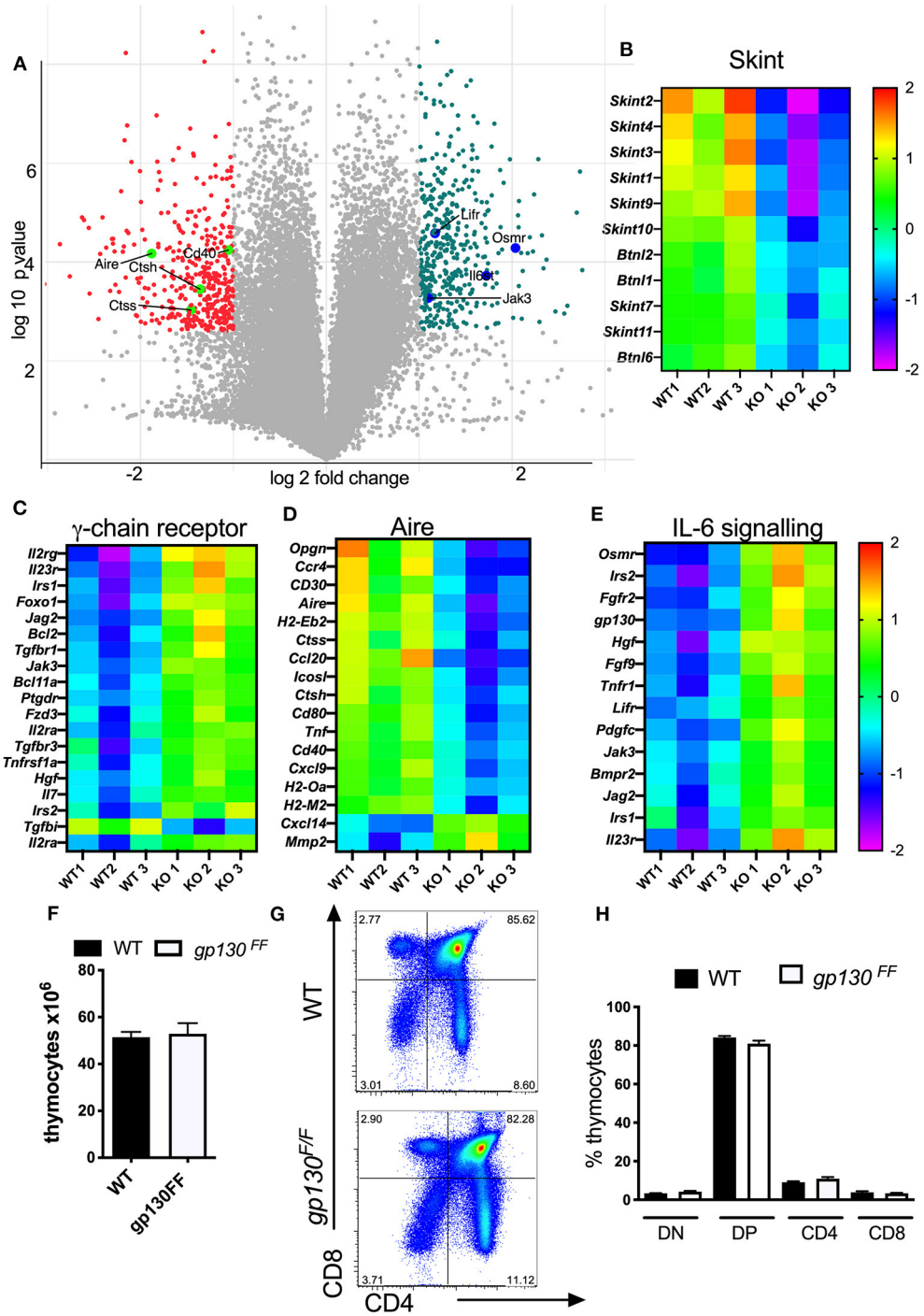

FIGURE 7 | Δsocs3 TECs show diminished expression of genes involved in T-cell selection and increased levels of those from the IL-6 family. **(A)** Volcano plot of the gene expression comparing the $\log_2$ fold change (FC) differences and the statistical significance ($\log_{10}$ probability) of transcript levels from three independent Δ*socs3* and WT TECs samples. Highly dysregulated in absence of SOCS3 are labeled in pink. The Student-Newman-Keuls *post-hoc* test was used to identify the specific groups in which significant differential expression occurred. The heat maps of **(B)** skint, **(C)** common γ-chain receptor **(D)** aire, and **(E)** IL-6 signaling pathways representing transcript levels in mutant and control TECs are depicted. Data were normalized for each gene by mean subtraction of $\log_2$ transformed values. Gene names are shown adjacent to heat maps. For all genes included in the heat maps, differences between WT and Δ*socs3* TECS are statistically significant. **(F)** The mean thymocyte number, **(G)** a representative dot plot and **(H)** the frequencies of DN, DP, CD4, and CD8 SP thymocytes ± SEM in *Gp130*$^{F/F}$ and WT mice ($n = 5$ per group).

thymi are explained by the diminished Δ*socs3* thymocytes numbers (**Supplementary Figure 5C**).

Δ*socs3* and WT thymi showed similar numbers and frequencies of cTECs and mTECs (distinguished by UEA-1 binding and Ly51 expression) (**Supplementary Figures 5D,E**). cTECs with high levels of MHCII and CD40 and mTECs with high CD80 and MHCII levels are considered functionally mature (1). The MHC-II expression level was lower in Δ*socs3* mTECs compared to WT controls, while similar MHCII levels were measured in mutant and WT cTECs (**Supplementary Figures 5F–H**).

Hematoxylin and eosin (H&E)-stained sections revealed densely packed cells in the Δ*socs3* thymi. Whereas, cortex and medulla are distinguishable in WT thymi, the cortico-medullary junction in Δ*socs3* thymi is imprecise (**Figure 4K**). Keratin-5 (K5) and keratin-8 (K8) are specific markers for mTECs and cTECs, respectively. IHC staining with K5 and K8 revealed that the cTEC and mTEC subsets were present in Δ*socs3* thymi, however the fraction of area with an overlapping staining of K5 and K8 were more extensive in Δ*socs3* compared to WT mice (**Figure 4L**).

Stromal SOCS3 Regulates Numbers and Frequencies of Peripheral Naive T Cell Subpopulations and Their Activation

Then, whether SOCS3 deletion in stromal cells regulated the levels of naïve T cells in the secondary lymphoid organs was further investigated. For this purpose, Δ*socs3* or WT CD45.2 mice were first treated with Tm. Ten days after Tm treatment, mice were irradiated and transplanted with CD45.1+ BM cells (**Figure 5A**). The levels of CD45.1+ T cells in the lymph nodes and spleens were studied 60 days after transplantation. The thymus of transplanted Δ*socs3* mice showed a reduced cellularity as compared to that of WT controls (**Figure 5B**), albeit differences were lower compared to those analyzed 10 days after Tm administration. The thymocyte subpopulations numbers in Δ*socs3* and WT chimeric recipients was similar (**Supplementary Figure 6A**). The spleen and lymph node cell numbers in transplanted Δ*socs3* and WT mice were similar (**Figure 5B**). The levels of CD45.1+ CD4 and CD8 T cells in spleens (but not in lymph nodes) of Δ*socs3* recipient mice were diminished as compared to WT controls (**Figures 5C,D**). In spleens and lymph nodes of Δ*socs3* recipient mice, the expression of CD44 in CD4 and CD8 T cells was enhanced compared to controls (**Figures 5E–G**). This indicates that lymphoid organs of BM-transplanted Δ*socs3* mice contain lower frequencies of naïve T cells compared to those of WT controls when analyzed 70 days after Tm treatment.

T cell differentiation of naïve T cells continues post-thymically, with progressive maturation of both surface phenotype and immune functions. Recent thymic emigrants lose CD24 and gain of Qa2 and CD45RB expression upon transition to mature naïve T cells (36, 37). CD4 and CD8 SP thymocytes expressed lower levels of Qa-2 (**Supplementary Figures 6B,C**) and higher levels of CD24 (**Supplementary Figures 6D,E**) than spleen or LN CD44 neg T cells, in coincidence with previous results (36). The expression level of Qa-2 was higher in naïve CD4+ or CD8+ spleen cells from Δ*socs3* mice 7 and 14 days after Tm treatment compared to those in WT spleens (**Figures 5H–K**). Instead, naïve spleen T cells from Δ*socs3* mice showed lower levels of CD24 (**Figures 5L–O**). Thus both, the frequency of peripheral naïve T cells and the levels of recent thymic emigrants within this population are regulated by SOCS3 expression.

SOCS3 in Thymic Epithelial Cells Binds to the E3-Ubiquitin Ligase TRIM21

We next studied the binding partners of SOCS3 in TECs. We observed that OP9 epithelial cells expressing the Notch ligand-Delta like 1 (OP9-DL1) cultured in conditions that enable thymopoiesis (with SCF and Flt3l) (38) express high levels of SOCS3 when OSM, (but not RANK-L or FGF7) was added into the culture (**Figure 6A**) and therefore choose these cells for our investigation.

OP9-DL1 cells were transfected with SOCS3-Myc expressing plasmids. The expression of SOCS3-Myc was immunoprecipitated (IP) from lysates of transfected cells (**Figure 6B**) and the IP proteins were tryptically digested and analyzed by liquid chromatography-mass spectrometry. Proteins were ranked with regards to their presence in replicates and the spectral counting. Three proteins were found in all 3 replicate target IP's of transfected cells and 6 proteins were identified in 2 out of 3 of the target IP's but not in the negative control.

One of the 3 proteins present in all replicates was SOCS3, and other was TRIM21 (**Figure 6C**). SOCS proteins act as substrate adapters for ubiquitination and proteosomal degradation of different receptors (11). Tripartite motif (TRIM) proteins, including TRIM21, have been implicated in multiple cellular functions that rely on their E3-ubiquitin ligase activity (39). Other members of the TRIM family have been shown to interact with SOCS proteins (40, 41). We validated the presence of TRIM21 in the SOCS3 IP using anti-TRIM21 antibodies in the WB (**Figure 6D**). Then, whether TRIM21 could play a role in T cell formation in the thymus was studied. Contrary to Δ*socs3*, thymi from *trim21*$^{-/-}$ mice showed an increased number of thymocytes (**Figure 6E**) and reduced frequency of DN cells (**Figure 6F**), while the frequencies of other thymocytes populations in WT and *Trim21*$^{-/-}$ were similar (**Figures 6F–H**). The frequency of γδ and β TCR+ DN cells in a WT and *Trim21*$^{-/-}$ mice was also similar (**Figure 6I**).

Reduced Central T Cell Tolerance Transcript Levels and Increased Expression of IL-6 Cytokine Family Regulated Genes in Δsocs3 TECs

The genome wide transcriptome of Δ*socs3* and WT CD45-EpCAM+ TECs was then compared. Enriched TEC populations were negatively selected with CD45 magnetic beads and subsequently sorted as EpCAM+ cells. The whole genome transcriptome of three independent samples per group was determined. Among 9,392 transcripts expressed above threshold levels in both groups, 703 were ≥ 2-fold and significantly

differently ($p < 0.05$) expressed, 367 higher and 336 lower in WT than in Δ*socs3* WT TECs (**Figure 7A**). A GO analysis indicated several families with different transcript levels (**Supplementary Table 1**). Expression levels of 8 out of 11 transcripts of the Skint- and 3/ 6 of the related butyrophylin-like gene family were increased in WT TECs (**Figure 7B**). Genes within cytokine receptor interaction pathways were increased while others from the same GO were reduced in WT TECs (**Supplementary Tables 2A–D**). The relative levels of several genes stimulated via the common cytokine receptor γ-chain (IL-$2R_{\gamma c}$) signaling pathway were decreased in WT compared to Δ*socs3* TECs (**Figure 7C**).

The expression level of genes involved in T cell selection (such as *Aire, CD40, CD80*); several genes from the MHC-II locus and *Cathepsin m* and *-l* (involved in antigen presentation); chemokines and chemokine receptors as well as members of the TNF-receptor family involved in thymocyte development were all higher in WT than in Δ*socs3* TECs (**Figure 7D**).

The SOCS3 regulation of STAT3 activation by the gp130 receptor signaling controls inflammatory responses via the IL-6 receptor family (42). In line with this, several genes related to IL-6 signaling or regulated by SOCS3 such as *Gp130, Osmr, Lifr, Irs1, Irs2,* and *Lifr* were reduced in WT TECs (**Figure 7E**). *Socs3* mRNA levels were significantly reduced in Δ*socs3* TECs as compared to controls. We then asked if SOCS3 binding to gp130 was critical for T cell generation in the thymus. Cells from $Gp130^{F/F}$ mice, harboring a mutation that ablates SOCS3 binding to gp130, show exaggerated STAT3 responses (25). We found that the thymus cellularity, the frequency and numbers of different thymocyte subpopulations in $Gp130^{F/F}$ and WT controls were similar (**Figures 7F–H**). Thus, SOCS3 control of gp130 signaling is redundant for the differentiation of T cells in the thymus.

DISCUSSION

We here show that SOCS3 is critical in the regulation of T cell formation in the thymus and for maintenance of thymus architecture by using mice with a Tm-inducible *Socs3* gene deletion. Off-targets effects of cre and Tm treatment previously reported by others and us were carefully ruled out (35, 43, 44).

The thymus is highly sensitive to acute stress, and malnutrition, pregnancy, infection, autoimmune diseases and cancer might result in the reduction in thymus size (45). Our data demonstrate an important role for SOCS3 in thymus homeostasis under physiological conditions.

Mice lacking SOCS3 in the skin developed exacerbated chronic inflammation (46, 47). Δ*socs3* mice showed neither clinical signs nor inflammatory histopathology in other organs. This might be explained by the fact that we recorded thymic changes at an early time point, 7–10 days, after the induction of *Socs3* gene deletion. In comparison, mice lacking SOCS3 in hematopoietic cells or in keratin-5-expressing epithelial cells that showed inflammatory disease at 3 or 4 months of age (14, 46). In line with our observation, mice deficient in SOCS3 in neurons (48), glia (48), endothelial cells (49), smooth muscle cells (50), gut epithelial cells (51), myeloid cells (21) or B cells (52) showed no spontaneous autoimmune or inflammatory disease.

Δ*socs3* mice displayed a striking reduction in the numbers of all thymocyte subpopulations. The reduced proliferation of Δ*socs3* DN and the increased frequency of DN3 cells indicate that SOCS3 regulates early stages of T cell development in the thymus. This is further evidenced by very low frequency and the dramatically increased level of apoptosis of remaining Δ*socs3* DP cells. The BrdU labeling also revealed the impaired differentiation to of thymocytes.

The lower frequencies of naïve $CD44^{low}$ T cells in secondary lymphoid organs of Δ*socs3* mice suggest a lymphopenia-triggered homeostatic proliferation of naïve T cells that may acquire a memory phenotype even in the absence of antigenic stimulus (53). The reduced CD24 and increased Qa-2 levels in Δ*socs3* SPs and in peripheral CD44 negative T cells suggest the accumulation of pre-recent thymic emigrants (RTE) in the thymus and RTE in secondary lymphoid organs. This might compensate for a dysfunctional T cell formation in the Δ*socs3* thymus: pre-RTEs display a selective survival advantage over other thymocyte populations and both pre-RTEs and RTEs are essential for the establishment and maintenance of a self-tolerant and a diverse and functional T cell repertoire (54, 55).

The thymic involution was recapitulated in BM chimeric mice in which recipient cells were SOCS3-deficient. Moreover, thymus transplantation experiments showed that SOCS3 in thymic stroma cells is required during T cell formation, while the role of SOCS3 in thymocytes or in hematopoitic cells in T cell development was minor and redundant and it could only be observed in chimeric mouse when Δ*socs3* mice were used as recipients.

Whereas, the numbers of cTECs and mTECs in the mutant thymus remained unaltered, the thymus structure was altered in Δ*socs3* thymi. A clear demarcation of medulla and cortex was absent, and an aberrant co-localization of cTECs and mTECs was observed in Δ*socs3* thymi.

Here we show that SOCS3 binds to TRIM21 in OP9-DL-1 epithelial cells. SOCS and TRIM proteins target the receptor complex for ubiquitination and proteasome- degradation, acting as substrate adaptors (56). SOCS3 in TECs could promote TRIM21 degradation or TRIM21 might target SOCS3 for degradation thus impairing the JAK kinase inhibition. The increased numbers of thymocytes and reduced DN population frequencies are dissimilar to the features of Δ*socs3* thymi and suggest that TRIM21 might reduce SOCS3 stability in TECs, a possibility that remains to be confirmed. Another member of the family, TRIM8 has been shown to interact with SOCS1 decreasing its stability and levels (40). TRIM8 interacted also with PIAS3 and Hsp90β regulating STAT3 activation (57, 58). TRIM21 is expressed in the thymus (26), and the alterations in frequencies and numbers of thymocyte subpopulations can reflect an altered T cell development in the thymus, which might contribute to the autoimmune phenotype of $Trim21^{-/-}$ mice (26).

SOCS3 binds to gp130 hampering the response to cytokines of the IL-6 family (10). Transgenic animals overexpressing IL-6 cytokine family members LIF, IL-6 or OSM show thymus involution, and similar results were observed after administration of recombinant cytokines (59, 60). These cytokines present in the thymic microenvironment, are produced by TECs, increase with age and have been associated to thymic atrophy (60). Gp130

is expressed ubiquitously on thymocytes (61) and on thymic epithelium (62) and thymic atrophy caused by these cytokines was reverted by gp130 neutralization. On the other hand, gp130 was shown to be required for proper thymic formation (63), and deficiency of OSM or IL-6 resulted in thymic hypoplasia, altered medullary structure and autoreactivity (64–66). Thus, the IL-6R family of cytokines in physiological states protects the thymic structure but might suppress thymic functions at high concentrations. In our hands, $Gp130^{F/F}$ mice showed no differences in thymocyte populations numbers and frequencies, indicating that SOCS3 signaling through gp130 is redundant in SOCS3-mediated thymus maintenance. This is in agreement with previous data showing that thymi from $Gp130^{F/F}$ and WT controls have similar cellularity (47). Yet, *Lifr* and *Osmr* transcripts were increased in Δ*socs3* TECs indicating that a more indirect role of SOCS3 increasing these or transcripts for *Irs1, Irs2, Tgfr1, Hgf* and *Igf1* all previously suggested to be involved in thymic formation or maintenance.

Skint and butyrophilin are members of the butyrophilin-like subfamily of B7-related proteins that, similar to MHC or CD1, modulate T cell functions and are considered to be co-stimulatory molecules (67). Butyrophilins-like molecules are expressed by TECs and regulate thymic T cell selection, particularly that of γδ T cells (68, 69). We observed a remarkable reduction in the levels of several transcripts of the butyrophilin family in Δ*socs3* TECs. This suggests that SOCS3 in TECs control T cell maturation in the thymus by regulating the levels of butyrophilin-like proteins.

The alterations in Δ*socs3* thymi resemble the age-associated changes in thymopoiesis, in which defects within the thymic stromal niche result in impaired T cell development. Several studies have demonstrated that with age, the thymic microenvironment undergoes structural, phenotypical, and architectural changes (70), including the down regulation of MHC-II (71), which we observed in Δ*socs3* mTECs. While our data indicate that SOCS3 affects thymocyte maturation already at DN stage, a role of the molecule at later stages of thymic maturation cannot be ruled out. The autoimmune regulator AIRE transcription factor, the cell surface receptors CD40 and RANK mediate mTECs development and central tolerance, whereas LT-β receptor is required for mTEC differentiation and expression of adhesion molecules needed for proper localization of T cell precursors. AIRE+ $mTEC^{hi}$ subsets are further subdivided based on osteoprotegerin (OPG) expression. OPG regulates the cellularity of mTECs and the size of the medullary region in the thymus, by attenuating the RANK-mediated mTECs proliferation (7). In line with this, the levels of *Aire*, the members of the TNFR superfamily *Opg, Cd40, Rank,* and *CD30*, chemokines like *Ccl20* and transcripts coding for MHC molecules controlling T cell selection in the thymus were all diminished in Δ*socs3* TECs (72).

In summary, SOCS3 expression by the thymic stroma, but not in thymocytes is required for the maintenance of the thymic architecture and the correct localization and maturation of cTECs and mTECs. SOCS3 inhibits the expression of a number of genes in TECS (including *Irs1, Irs2, Il23r,* or *Lepr*) that regulate thymocyte differentiation and promotes several genes involved in central tolerance. In absence of SOCS3 in the thymic stroma, thymocyte proliferation and differentiation are hampered. Δ*Socs3* thymocytes accumulate at the DN stage where generation of both β- and γδ TCR DN cell frequencies are reduced. The diminished frequency and survival of DP cells may lead to a deficient differentiation of SPs cells observed. Consequently, the production of recent thymic emigrants and the frequency of naïve T cells are reduced.

Altogether, we here show that SOCS3 plays a central role in maintaining the maturation and morphology and tissue distribution of TECs. Our results indicate that SOCS3 through this role provides niches for thymocyte maturation, and consequently is required for proper thymocyte development and naïve T cell export.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: https://www.ncbi.nlm.nih.gov/geo/, GSE165216.

ETHICS STATEMENT

The animal study was reviewed and approved by Stockholms North Region Animal Research Ethic Committee.

AUTHOR CONTRIBUTIONS

BC and MR: conceptualization. YG, RL, CH, JB, CH-Z, AY, FZ, BC, AE, and MW-H: investigation. YG, JB, AY, BC, MK, MW-H, and MV: formal analysis. MR: wrote the manuscript. All authors revised the manuscript and approved the final version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.642173/full#supplementary-material

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REVIEW
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The Role of Proteasomes in the Thymus

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The thymus provides a microenvironment that supports the generation and selection of T cells. Cortical thymic epithelial cells (cTECs) and medullary thymic epithelial cells (mTECs) are essential components of the thymic microenvironment and present MHC-associated self-antigens to developing thymocytes for the generation of immunocompetent and self-tolerant T cells. Proteasomes are multicomponent protease complexes that degrade ubiquitinated proteins and produce peptides that are destined to be associated with MHC class I molecules. cTECs specifically express thymoproteasomes that are essential for optimal positive selection of $CD8^+$ T cells, whereas mTECs, which contribute to the establishment of self-tolerance in T cells, express immunoproteasomes. Immunoproteasomes are also detectable in dendritic cells and developing thymocytes, additionally contributing to T cell development in the thymus. In this review, we summarize the functions of proteasomes expressed in the thymus, focusing on recent findings pertaining to the functions of the thymoproteasomes and the immunoproteasomes.

Keywords: thymus, TEC, thymoproteasome, immunoproteasome, thymic selection

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INTRODUCTION

Proteasomes degrade ubiquitinated proteins into peptide fragments in the cytoplasm, and these fragments are transported to the endoplasmic reticulum (ER) by TAP transporter. In the ER, the peptides are trimmed by aminopeptidases, loaded onto major histocompatibility complex class I (MHC-I) molecules, and transported to the cell surface for antigen recognition by T cells. Peptide fragments loaded onto MHC-I molecules are typically 8–10 amino acids in length and contain hydrophobic or basic residues at the carboxyl terminus.

The proteasome is a 26S protein complex comprised of a 20S enzymatic core particle located between two 19S regulator particles. The 20s core particle is a multi-catalytic protease complex made up of 28 subunits arranged in a cylindrical structure. The subunits of the proteasome are arranged into four heteroheptameric rings: two outer rings comprised of α subunits α1–α7 and two inner rings consisting of β subunits β1–β7. Within the proteasome, three subunits β1, β2, and β5 are responsible for the proteolytic activity. The chymotrypsin-like activity mediated by the β5 subunit enables the production of peptides enriched with hydrophobic C-terminal residues for high-affinity association with the peptide-binding groove of MHC-I molecules (1–3).

The proteolytic subunits of the proteasomes are diverse and vary among the constitutive proteasome (β1, β2, and β5), the immunoproteasome (β1i, β2i, and β5i), and the thymoproteasome (β1i, β2i, and β5t). These different catalytic subunits provide unique endopeptidase activity that

alters the repertoire of degraded peptides generated by each proteasome (**Figure 1**). The immunoproteasome is abundant in interferon-γ-stimulated cells and constitutively expressed in various hematopoietic cells, including dendritic cells (DCs) and developing thymocytes (4). In the thymic medulla, medullary thymic epithelial cells (mTECs), which contribute to the establishment of self-tolerance in T cells, also express the immunoproteasome (5). In contrast, the thymoproteasome is exclusively and constitutively expressed in the cortex of the thymus by cortical thymic epithelial cells (cTECs) and is essential for optimal positive selection of $CD8^+$ T cells (6–8).

In this review, we will discuss the functions of proteasomes expressed in the thymus, focusing on recent findings pertaining to the functions of immunoproteasomes and thymoproteasomes.

THYMOPROTEASOME IN THE THYMUS FOR CD8 T CELL GENERATION

The thymoproteasome contains a specific component β5t encoded by *Psmb11*, which is exclusively expressed by cTECs (6). Recent studies have identified that the cTEC-specific expression of β5t is directly regulated by Foxn1 (9, 10), a transcription factor important for the development of the thymic epithelium (11). It has been reported that β5t has altered proteolytic activity that leads to the preferential cleavage of proteins at hydrophilic peptide residues, reduced chymotrypsin-like activity, and reduced enzyme kinetics compared with β5, which probably result in the generation of a unique set of TCR ligands bound to MHC-I molecules on cTECs (6, 12). The β5t-containing proteasome produces peptides with not only different amino acid residues but also different quantities from those generated by other proteasomes, and the combination of these quantitative and qualitative differences may lead to the presentation of unique, or "private", MHC-I-associated peptides (13). The inability to form peptide-MHC-I complexes with certain peptides may contribute to differential peptide display in the cortical and medullary regions of the thymus.

An *in vivo* function of the thymoproteasome was shown by analyses of β5t-deficient mice. β5t-deficient mice exhibited the selective reduction of $CD4^-CD8^+$ (CD8SP) thymocytes to approximately 25% of the normal number, whereas the cellularity of $CD4^+CD8^-$ (CD4SP) thymocytes was unaffected in these mice (6). The reduction of CD8SP thymocytes in β5t-deficient mice was primarily due to the decrease of $CD69^+TCR\beta^+$ $CD4^+CD8^+$ (DP) thymocytes, which are post-selection cortical thymocytes, whereas the loss of pro-apoptotic protein Bim, which is required for the negative selection of T cells, increased $CD69^+TCR\beta^+$ DP thymocytes in β5t-deficient thymus similar to

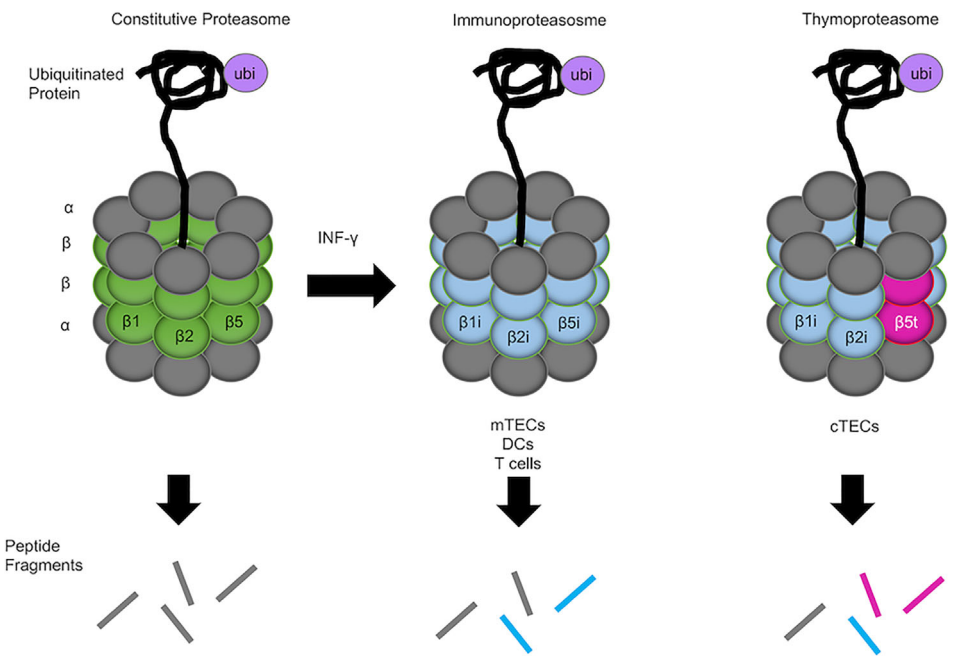

FIGURE 1 | Constitutive proteasome, immunoproteasome, and thymoproteasome. 20s enzymatic cores have altered proteolytic activity and generated unique peptide fragments. The constitutive proteasome is ubiquitously expressed in the body and contains β1, β2, and β5 catalytic subunits (left). mTECs, DCs, and T cells in the thymus express the immunoproteasome, in which β1i, β2i, and β5i catalytic subunits are preferentially incorporated into the 20S core proteasome in lieu of β1, β2, and β5 subunits (middle). In cTECs in the thymus, a unique catalytic subunit β5t is specifically expressed instead of β5 or β5i and incorporated into the 20S core proteasome together with β1i and β2i, forming the thymoproteasome (right). Different catalytic subunits in different proteasomes provide different endopeptidase activity that alters the degraded peptides generated by the proteasomes (represented by different colored lines).

that in β5t-sufficient thymus (8). These findings highlight the specific role of the thymoproteasome in the positive selection of $CD8^+$ T cells, rather than the contribution of negative selection in the thymoproteasome-dependent generation of CD8SP thymocytes. In addition to being reduced in cell number, $CD8^+$ T cells generated in β5t-deficient mice were altered in TCR repertoire, such as the differential usage of TCRVβ and TCRVα in $CD8^+$ T cells but not in $CD4^+$ T cells, and different TCR-transgenic $CD8^+$ T cells were differentially susceptible to the loss of β5t in the thymus (7). Furthermore, $CD8^+$ T cells generated in β5t-deficient thymus exhibited aberrant TCR responsiveness and immune response to infection (14). Therefore, these findings indicate that the thymoproteasome is required for the optimal positive selection of $CD8^+$ T cells with functional TCR repertoire and determines the antigen responsiveness of mature $CD8^+$ T cells in the periphery.

In addition to the analyses of β5t-deficient mice, the role of the thymoproteasome restricted in cTECs was examined by the analysis of β5t-transgenic mice in β5i-deficient background ($\beta5i^{-/-}$β5t-Tg) in which the thymoproteasome was aberrantly expressed in antigen-presenting cells, including mTECs and DCs. Unlike β5t-deficient mice, $\beta5i^{-/-}$β5t-Tg mice exhibited unaffected cellularity of $TCR\beta^{low}CD69^{-/+}CD103^+$ DP thymocytes, which are enriched with $CD8^+$ lineage cells after positive selection, due to the retained expression of β5t in cTECs (15). On the other hand, the population of $TCR\beta^{high}CD69^+CD103^+$ DP thymocytes, which include post-selected $CD8^+$ lineage cells, was lost in $\beta5i^{-/-}$β5t-Tg mice, possibly resulting from enhanced negative selection (15). These findings are in agreement with the concept that a switch in self-peptides displayed by the cortex and the medulla may be important for the thymoproteasome-dependent development of $CD8^+$ T cells in the thymus (16). Furthermore, the extra-thymic expression of β5t induced autoreactive responsiveness of $CD8^+$ T cells that underwent thymoproteasome-dependent positive selection in the thymus (15). Therefore, cTEC-specific thymoproteasomes are important not only for the optimal generation of $CD8^+$ T cells in the thymus but also for the prevention of aberrant T cell responses in the periphery. However, the systemic β5t-Tg mice were metabolically aberrant and showed weight loss due to the systemic reduction of chymotrypsin-like proteasomal activity (17). The study also showed that MHC-I expression was reduced in those $\beta5i^{-/-}$β5t-Tg mice (15). Thus, the aberrant thymocyte development in their $\beta5i^{-/-}$β5t-Tg mice may not be simply due to the loss of the MHC-I-associated peptide switching but possibly results from the combination of multiple abnormalities, including systemic aberrancy in metabolism, in those transgenic mice.

A recent study that employed RNA sequencing analysis identified that β5t regulated 850 cTEC-specific genes and exhibited a pervasive effect on CD4SP and CD8SP thymocyte development *via* the regulation of gene expression in cTECs (18). However, these results could not be reproduced in an additional study that found only minor differences in gene expression profiles between β5-sufficient cTECs and β5t-deficient cTECs through the combination of quantitative RT-PCR analysis and RNA sequencing analysis, and no significant effects on the development of CD4SP thymocytes (19). The contradictory results between these two studies may be due to differences in the genetic background of mice and the purity of isolated cTECs for transcriptomic analysis. It is also important to note that the former analysis lacked the verification of the modest differences in transcriptomic abundance detected in RNA sequencing analysis, by employing additional analysis, such as quantitative RT-PCR analysis. Furthermore, proteomic analysis revealed that β5t-deficient mice had reduced 20S core particle components (β1, β1i, β2, and β2i) except β5i and β5, which were elevated potentially to compensate for the loss of β5t, whereas neither chymotrypsin activity nor degradation of ubiquitinated proteins was apparently altered by the β5t deficiency (19). These results suggest that β5t may not pervasively regulate gene expression in cTECs, but is critical for maintaining normal proteasomal subunit composition in cTECs.

IMMUNOPROTEASOME IN THE THYMUS FOR ANTIGEN PROCESSING AND BEYOND

The importance of the immunoproteasome in antigen presentation was examined by generating mice that completely lacked the immunoproteasome by the deletion of catalytic components: β1i-encoding *Psmb9*, β2i-encoding *Psmb10*, and β5i-encoding *Psmb8*. The defects detected in immunoproteasome-deficient mice resembled those detected in β1i-, β2i- or β5i-deficient mice. The number of CD8SP thymocytes in immunoproteasome-deficient mice decreased to approximately 50% of that in control mice (20), similar to the reduction detected in β1i-deficient mice (21). The expression of surface MHC-I molecules was reduced in the thymus and secondary lymphoid organs of immunoproteasome-deficient mice, similar to the reduced expression of surface MHC-I molecules in β5i-deficient mice (20, 22). The reduction of MHC-I expression was due to the reduced exportation of the peptide-MHC complex, not the instability of surface MHC-I molecules (20). Mass spectrometry analysis of peptides associated with MHC-I in splenocytes from immunoproteasome-deficient mice and control mice revealed that the MHC-I-associated peptide repertoire was altered by immunoproteasome deficiency (20). Altered generation of MHC-I-associated peptides in DCs was also detectable in β2i/β5i double-deficient mice (23). Although the repertoire of MHC-I-associated peptides in the thymus has not been examined, these results suggest that the generation of MHC-I-associated self-peptides in the thymus is also altered in immunoproteasome-deficient mice. As β1i and β2i are also components of the thymoproteasome, peptide generation in cTECs and thymic selection of cortical thymocytes may also be affected in immunoproteasome-deficient mice lacking β1i and/or β2i.

It has been reported that the number of polyclonal CD8SP thymocytes is not severely affected in β5i single-deficient mice (15, 22). However, one study disclosed that the generation of

$CD8^+$ T cells specific for viral glycoprotein $GP_{118-125}$ was impaired in β5i-deficient mice, probably due to the altered thymic selection by the β5i deficiency in thymic antigen-presenting cells (24). Furthermore, the generation of naturally occurring self-peptide $CP\alpha1_{92-99}$, which likely contributes to the positive selection of ovalbumin (OVA)-specific OT-I T cells (25), is immunoproteasome-dependent, and β5i deficiency led to reduced generation of OT-I T cells in the thymus of OT-I-TCR transgenic mice (26). Therefore, β5i in the immunoproteasome within the thymus is involved in the generation of $CD8^+$ T cells that express certain TCR specificities. It is possible that the deficiency in β1i more severely affects the generation of polyclonal $CD8^+$ T cells (20, 21) than the deficiency in β5i (15, 22).

The role of immunoproteasomes beyond peptide processing in the thymus has been uncovered in several studies. mTECs exhibited promiscuous gene expression and synthesized more proteins than other thymic cells, which may require the alleviation of proteotoxic stress in these cells (27). The immunoproteasome deficiency caused by the genetic ablation of β2i-encoding *Psmb10* and β5i-encoding *Psmb8* in mice resulted in the selective reduction of mTEC cellularity and impaired mTEC regeneration due to the short half-lives of these cells and the exhaustion of their progenitors (27). Therefore, the immunoproteasome plays an important role in the homeostasis of mTECs.

An adoptive transfer of T cells isolated from mice deficient in immunoproteasome subunit β1i, β2i, or β5i exhibited decreased proliferation and increased apoptosis in virus-infected wild-type mice (28). Experiments in mixed bone marrow chimera mice showed that the altered homeostatic proliferation of T cells in secondary lymphoid organs was due to T cell-intrinsic deficiency in β2i (29). These studies suggest that the immunoproteasome is an intrinsic factor for the proliferation and survival of T cells, although the detailed mechanisms have yet to be elucidated. The immunoproteasome also regulates the activation of T cells. The specific inhibition of β5i reduced the production of IL-2 and INF-γ, as well as the expression of CD69 in T cells stimulated with anti-CD3 and anti-CD28 antibodies (30, 31). Furthermore, the phosphorylation of ERK, a molecule involved in the TCR signaling pathway, was reduced in activated T cells as a result of selective inhibition of β5i (31). Thus, the immunoproteasome plays a role in the regulation of TCR signaling.

Several studies that used non-immune cells have shown the involvement of the immunoproteasome in cellular senescence, aging, and longevity (32–34). Regarding cellular senescence, it has been reported that the TCR-mediated induction of the proteasome is impaired in senescence-associated PD-1^+ $CD44^{high}$ $CD4^+$ T cells, and vice versa, the senescence phenotype is induced by proteasome inhibition (35). Although $CD4^+$ T cells express both the constitutive proteasome and the immunoproteasome, these results indicate the involvement of proteasomes, including the immunoproteasome, in the senescence of T cells. However, whether the impaired induction of proteasomes by TCR stimulation is the cause or effect of T cell senescence remains unknown.

THE THYMUS LACKING IMMUNOPROTEASOMES AND THYMOPROTEASOMES

Three types of proteasomes are detected in the thymus: the constitutive proteasome, the immunoproteasome, and the thymoproteasome. Among major antigen-presenting cells in the thymus, cTECs in the cortex specifically express the thymoproteasome, whereas antigen-presenting cells in the medulla, such as mTECs and DCs, predominantly express the immunoproteasome. In one study, mice that were engineered to lack all components of those tissue-specific proteasomes were examined to address the function of the tissue-specific proteasomes in the thymus (16). In that study, mice deficient in β1i, β2i, β5i, and β5t (4KO mice) were generated. The mice were viable and bred normally, whereas their proteasomes were limited to constitutive proteasomes. Most strikingly, the 4KO mice had 90% fewer $CD8^+$ T cells than control mice; this was substantially greater than the 75% reduction of $CD8^+$ T cells seen in β5t-deficient mice (specifically deficient in thymoproteasomes) and the 50% reduction of $CD8^+$ T cells seen in β1i/β2i/β5i-triple-deficient mice (deficient in immunoproteasomes) (16). There was no defect in the development of $CD4^+$ T cells in the 4KO mice. Further evidence was provided to show that in the thymus of the 4KO mice, cortical thymocytes that undergo positive selection toward $CD8^+$ T cells were detected. However, the 4KO mice had 68% fewer mature $CD4^+CD8^+CD69^+TCR\beta^{hi}CCR7^{hi}$ cells than wild-type mice, suggesting the occurrence of a developmental block downstream of positive selection. To assess the effect of tissue-specific proteasomes on negative selection, 4KO irradiated hosts were reconstituted with bone marrow from either wild-type or Bim-KO mice. Interestingly, the number of mature $CD8^+$ thymocytes in the 4KO hosts reconstituted with Bim-KO bone marrow cells increased by 22.7-fold ± 8.8-fold than that in the 4KO hosts reconstituted with wild-type bone marrow cells. In contrast, the number of mature $CD8^+$ thymocytes was only 5.0-fold ± 2.3-fold abundant in wild-type hosts reconstituted with Bim-KO bone marrow cells relative to that in wild-type hosts reconstituted with wild-type bone marrow cells. These data suggest that mature $CD8^+$ thymocytes are lost due to increased events of apoptosis-dependent negative selection in the absence of tissue-specific proteasomes, which can be rescued by the loss of Bim (16). Based on these results, it was proposed that the restricted expression of tissue-specific proteasomes in the thymus, including cTEC-specific expression of the thymoproteasome and mTEC/DC-abundant expression of the immunoproteasome, facilitated a "peptide switch" between positive selection-inducing self-peptides presented by cTECs and negative selection-inducing self-peptides presented by mTECs and DCs. Such a switch in self-peptides may create a window for positively selected thymocytes to escape from subsequent negative selection. The difference in self-peptides displayed in the cortex and the medulla may be essential for the development of CD8+ T cells.

HUMAN DISEASE AND GENETIC VARIATIONS IN IMMUNOPROTEASOMES AND THYMOPROTEASOMES

The involvement of the immunoproteasome in human diseases has been reviewed by others (4, 36). It has been demonstrated that a single polymorphism in β1i or β5i is associated with risk of cancers and mutations in β5i lead to autoinflammatory syndromes. The alteration of immunoproteasome activity is also associated with the development of neurodegenerative diseases, such as Alzheimer disease and Huntington disease. Regarding the thymoproteasome, it has been shown that patients with Down syndrome have decreased expression of β5t, which may contribute to elevated mortality due to increased susceptibility to various cancers and infections (37). In addition, it has been shown that a single nucleotide polymorphism that changes the 49th amino acid from glycine to serine (G49S) in the β5t protein is associated with Sjögren's syndrome, an autoimmune disease that affects exocrine glands, specifically the lacrimal glands and the salivary glands (38). On the other hand, another study reported that the G49S variation had little association with severe human diseases, including cancer, hepatitis, and tuberculosis (39). The G49S variation impaired the post-translational processing of β5t protein in both mouse and human cells, whereas the homozygous G49S variation in mice reduced thymoproteasome expression in cTECs and decreased CD8$^+$ T cell production in the thymus (39). Although the number of CD8$^+$ T cells in homozygous human individuals has not been assessed, these findings suggest that G49S variation may affect the production of CD8$^+$ T cells in the human thymus. A long-term and large-scale cohort study is expected to further deepen our understanding of the role of the thymoproteasome in human health.

CONCLUSIONS AND PERSPECTIVES

Since the discovery of the thymoproteasome by Murata, et al. in 2007, its functional significance and differential function from the immunoproteasome have been an interesting subject of studies. In the thymus, the processing of self-peptides that facilitate positive and negative selection events in developing thymocytes is presumably a major function of the thymoproteasome in cTECs and the immunoproteasome in mTECs and DCs. A study using fibroblasts with ectopic expression of the thymoproteasome and the immunoproteasome identified MHC-I-associated peptides generated by these proteasomes and showed a substantial difference in repertoire between thymoproteasome-dependent and immunoproteasome-dependent peptides (12). The difference in peptide generation between the thymoproteasome and the immunoproteasome was also shown in an experiment using a human lymphoblastoid cell line (13). Despite these findings, thymic selection-inducing self-peptides generated in thymic epithelium have not been identified yet. Therefore, biochemical analysis of MHC-I-associated peptides presented by cTECs and mTECs is required to improve our understanding of the difference between the thymoproteasome and the immunoproteasome, and contribute to the development of novel strategies to boost thymic selection for immunological disorders.

The explanation of the principle behind the differential expression of proteasomes between cortical and medullary thymic microenvironments remains controversial. As mentioned in this review, peptide switching between positive selection-inducing self-peptides presented by cTECs and negative selection-inducing self-peptides presented by mTECs and DCs may create a window to escape from the negative selection of positively selected thymocytes (15, 16). Alternatively, but not mutually exclusively, structural motifs in self-peptides generated by the thymoproteasome in cTECs may be advantageous for the optimal induction of positive selection. Indeed, we recently found that the thymoproteasome shapes the TCR repertoire directly in cortical thymocytes independent of the thymic medulla and independent of negative selection, indicating that the thymoproteasome hardwires the TCR repertoire of CD8$^+$ T cells with cortical positive selection independent of negative selection (40). Additional studies are necessary to verify the nature of tissue-specific proteasomes in the thymus, which would ultimately improve current understanding of thymic selection in the thymus.

AUTHOR CONTRIBUTIONS

MF, YT, and IO wrote and edited the manuscript. All authors contributed to the article and approved the submitted version.

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High-Oxygen Submersion Fetal Thymus Organ Cultures Enable FOXN1-Dependent and -Independent Support of T Lymphopoiesis

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T cell development is effectively supported in fetal thymus organ cultures (FTOCs), which places thymus lobes atop an air-liquid interface (ALI) culture system. The direct exposure to air is critical for its success, as fetal thymus lobes placed in low oxygen submersion (LOS)-FTOCs fail to support thymocyte development. However, submersion cultures performed in the presence of high concentration of ambient oxygen (60~80%) allow for normal thymocyte development, but the underlying mechanism for this rescue has remained elusive. Here, we show that FOXN1 expression in thymic epithelial cells (TECs) from LOS-FTOCs was greatly reduced compared to conventional ALI-FTOCs. Consequently, the expression of important FOXN1 target genes, including *Dll4* and *Ccl25*, in TECs was extinguished. The loss of DLL4 and CCL25 interrupted thymocyte differentiation and led to $CD4^+CD8^+$ cells exiting the lobes, respectively. High oxygen submersion (HOS)-FTOCs restored the expression of FOXN1 and its target genes, as well as maintained high levels of MHCII expression in TECs. In addition, HOS-FTOCs promoted the self-renewal of $CD4^-CD8^-CD44^-CD25^+$ cells, allowing for the continuous generation of later stage thymocytes. Forced FOXN1 expression in TECs rescued thymocyte developmental progression, but not cellularity, in LOS-FTOCs. Given that oxidative stress has been reported to accelerate the onset of age-associated thymic involution, we postulate that regulation of FOXN1 by oxygen and antioxidants may underpin this biological process.

Keywords: FOXN1, Delta-like 4, T cell development, FTOC, oxygen, thymus epithelial cells

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INTRODUCTION

T cell development is an intricate process that requires the thymus microenvironment, especially thymic epithelial cells (TECs), which provide signals to promote progenitor cells to differentiate towards the T-lineage (1). FOXN1 is an indispensable master transcriptional regulator for the development and differentiation of TECs. FOXN1 also regulates the expression of many TEC genes that are essential to induce and support T cell development within the thymus (2). For example, *Dll4* is a direct transcriptional target of FOXN1 and DLL4-mediated Notch signaling is absolutely

required for thymocyte development (2–4). CCL25 and CXCL12, two chemokines whose expression are regulated by FOXN1 (2), are known to facilitate homing of thymic seeding progenitors (TSPs) to thymus, and intrathymic trafficking and differentiation of developing thymocytes (5).

Differentiation of progenitor cells with multi-lineage potential towards the T-lineage is conventionally classified into four stages based on CD4 and CD8 expression. The earliest thymocytes do not express either CD4 or CD8, termed double negative (DN) cells, which first give rise to $CD8^+$ immature single positive (ISP) cells and then $CD4^+CD8^+$ double positive (DP) cells. After successful selection events, DP cells differentiate into either CD4 or CD8 single positive (SP) T cells. DN cells can be further classified based on the expression of CD44 and CD25, as $CD44^+CD25^-$ DN1, $CD44^+CD25^+$ DN2, $CD44^-CD25^+$ DN3, and $CD44^-CD25^-$ DN4 stages.

The first method to culture mouse fetal thymuses employed an air-liquid interface (ALI) system (6). This ALI culture system was later developed into the currently widely used fetal thymus organ culture (FTOC), in which thymus lobes from embryonic gestation day 12 to 15 (E12 ~ E15) are placed onto a Nuclepore filter that rests on top of a surgical Gelfoam sponge soaked with culture medium (7). The direct exposure to air was critical for its success, as low oxygen submersion (LOS) of fetal thymus lobes in culture medium failed to effectively support thymocyte differentiation compared to ALI-FTOCs (8–10). On the other hand, placing fetal thymus lobes in high oxygen (~70% O_2) submersion (HOS)-FTOCs allowed for T cell development, and rescued both cellularity and differentiation progression towards conventional αβ T-lineage cells (10). However, how increased oxygen availability promoted αβ T cell development in submersion cultures remains unknown.

Here, we found that FOXN1 levels, and consequently the expression of its direct transcriptional targets, such as *Dll4* and *Ccl25*, are rapidly reduced in TECs from LOS-FTOCs, as compared to ALI-FTOCs, and could be partially restored by HOS-FTOCs. Additionally, restoring FOXN1 protein levels by forced expression in LOS cultures resulted in the emergence of TCRβ-expressing $CD4^+$ and $CD8^+$ SP cells without affecting total cellularity. Furthermore, we observed that increased oxygen availability could promote self-renewal of DN3 cells to augment the cellularity of submersion FTOCs. Taken together, expression of FOXN1 appears to be regulated by oxidative states that dictate thymic epithelial cell function and hence T cell development.

MATERIALS AND METHODS

Mice

All mice were bred and housed in the Comparative Research Facility at Sunnybrook Research Institute under specific pathogen-free conditions. All animal procedures were approved by Sunnybrook Research Institute Animal Care Committee and performed in accordance with the committee's ethical standards.

R26Foxn1 mouse strain was a kind gift from Dr. Clare Blackburn (University of Edinburgh, UK), and it differs from the previously reported R26Foxn1ER strain (11) only in that a non-fused FOXN1 protein, rather than fused $FOXN1ER^{T2}$ protein, is expressed when upstream *loxp/STOP/loxp* sequence is removed by Cre recombinase. $Foxn1^{ex9cre}$ [B6(Cg)-$Foxn1^{tm3(cre)Nrm}$/J, stock #: 018448], R26-$CreER^{T2}$ [B6.129-$Gt(ROSA)26Sor^{tm1(cre/ERT2)Tyj}$/J, Stock #: 008463], RAG2 deficient [B6(Cg)-$Rag2^{tm1.1Cgn}$/J, stock #: 008449], Vav-iCre (B6.Cg-$Commd10^{Tg(Vav1\text{-}icre)A2Kio}$/J, stock # 008610), ROSA26-rtTA-IRES-EGFP [B6.Cg-$Gt(ROSA)26Sor^{tm1(rtTA,EGFP)Nagy}$/J, stock #: 005670] strains were all purchased from The Jackson Laboratory (12–16). Vav-iCre mice were bred to ROSA26-rtTA-IRES-EGFP mice to generate mice whose thymocytes express EGFP. Timed-pregnant CD1 females were purchased from Charles River Laboratories. For in-house timed-mating, female and male mice were housed together overnight and then separated next morning. Thymus lobes from embryos 14 days after separation, considered as E14.5, were used for all experiments.

Air-Liquid Interface (ALI)- and Submersion FTOC

To set up conventional ALI-FTOC, individual thymus lobes were place on a Whatman® Nuclepore™ Track-Etched membrane (Cat. # WHA110409 from Sigma-Aldrich) that was placed on top of SURGIFORM® absorbable gelatin sponge (Cat. # 1974 from Ethicon) in wells with 1.5 ml of DMEM containing 10% fetal bovine serum in a 12-well plate. For submersion FTOC, individual thymus lobes were placed in wells with 0.2 ml of culture medium in non-tissue culture treated 96-well plates (Falcon® 351177), which avoids stromal cells from attaching and spreading onto plate surface, in order to maintain thymus lobe architecture. The 96-well plates were either placed inside of a cell culture incubator with 5% CO_2 balanced with air, as low oxygen submersion (LOS) culture, or sealed inside of a plastic bag filled with 70% O_2, 5% CO_2, and 25% of N_2, as high oxygen submersion (HOS) culture. For LOS culture with tamoxifen, 4-OHT was added to the cultures at a final concentration of 5 nM. Unless indicated otherwise, fetal thymus lobes from the same timed-pregnant females were used for different experiments so that each culture in any assay represented an independent biological replicate.

Flow Cytometric Analysis

To examine the developmental stages of thymocytes, cells outside of lobes were collected directly by transferring the culture medium with cells to a 1.5-ml microcentrifuge tube, and cells inside of lobes were released by squeezing lobes against 70 μm nylon mesh with a syringe plunger. Live cells were counted using hemocytometer and stained with the following panel of antibodies: AF700/CD45 (clone 30-F11, BioLegend), PE-Cy7/CD8a (clone 53-6.7, BioLegend), APC/CD4 (clone GK1.5, in-house conjugated), APC-Cy7/CD25 (clone PC61, BioLegend), PerCp-Cy5.5/CD44 (clone IM7, BioLegend), BV510/CD3 (clone 145-2C11, BioLegend), FITC/TCRβ (clone H57-597, in-house conjugated), and PE/γδTCR (clone GL3, BD

Biosciences). FITC/CD11b (clone M1/70, BioLegend) and FITC-CD19 (clone MB19-1, BioLegend) were included in the analysis of *Rag2*-deficient thymocytes. Data were acquired using BD-LSR II and analyzed with FlowJo.

To measure thymocyte proliferation and apoptosis, cultures were incubated with 20 μM of EdU (ThermoFisher Scientific) at 37°C for an hour before harvest. Cells inside of the lobes from one ALI-FTOC culture, 3 HOS cultures, 4 LOS, or 4 iFoxN1-LOS cultures were pooled together. After Fc block and surface staining with AF700/CD45 (clone 30-F11, BioLegend), PE-Cy7/CD8a (clone 53-6.7, BioLegend), APC/CD4 (clone GK1.5, in-house conjugated), APC-Cy7/CD25 (clone PC61, BioLegend), PerCp-Cy5.5/CD44 (clone IM7, BioLegend), and PE/γδTCR (clone GL3, BD Biosciences), each sample was split into two halves, with one half incubated with CellEvent Caspase 3/7 Green Reagent (ThermoFisher Scientific) at 37°C for 30 min to detect apoptotic cells and the other half continuing with EdU staining by Click-iT Plus Edu AF488 Flow Cytometry Assay kit (ThermoFisher Scientific) after fixable live/dead eFluor 450 staining (ThermoFisher Scientific) to detect cells at S phase of a cell cycle.

To estimate TEC numbers and subtypes, cultures were enzymatically disassociated using 1X TrypLE (ThermoFisher Scientific) at 37°C for 20 min followed by pipetting (~10 times) until lobes were fully disassociated. Live cells were counted using hemocytometer and, after Fc block, were stained with PerCp-Cy5.5/CD45 (clone 30-F11, eBioscience), PE-Cy7/CD326 (clone G8.8, BioLegend), PE/Ly-51 (clone 6C3, BioLegend), AF647/CD205 (clone NLDC-145, BioLegend), APC-Cy7/Streptavidin/Biotinylated UEA-1 (BD Bioscence), and AF700/MHCII (I-A/I-E, clone M5/114.15.2, BioLegend). TEC cellularity was estimated by multiplying the numbers of total live cells to the percentage of $CD45^{-}EpCam^{+}$ cells among single live cells from each culture.

To measure the expression levels of DLL4, MHCII, and FOXN1 in TECs, cells were enzymatically disassociated in the same way as above followed by staining with APC/DLL4 [clone YW152F, Genentech (17), in-house conjugated], or APC-Cy7/MHCII (I-A/I-E, clone M5/114.15.2, BioLegend), together with AF700/CD45 (clone 30-F11 from BD Biosciences) and PE/CD326 (clone G8.8, eBioscience). For FOXN1 intracellular staining, samples pre-stained with APC/DLL4 were incubated with fixable live/dead eFluor 450 (ThermoFisher Scientific) on ice for 30 min, fixed with 4% paraformaldehyde at room temperature for 10 min, permeabilized with pre-chilled (−20°C) methanol for 5 min, then incubated with a mouse monoclonal anti-FOXN1 antibody (18), a kind gift from Dr. Hans-Reimer Rodewald (dkfz, Heidelberg, Germany), for 30 min followed by incubation with PE-Cy7-conjugated anti-mouse IgG2b secondary antibody (clone RMG2b-1, Biolegend). For flow cytometric analysis of *Dll4* and *Foxn1* mRNA levels in TECs, PrimeFlow™ RNA assay kit and type I mouse *Dll4* or *Foxn1* target probe (ThermoFisher Scientific) was used.

Quantitative PCR (qPCR)

Individual cultures were lysed in 0.5 ml of Trizol (ThermoFisher Scientific) for RNA preparation and cDNA was then synthesized using LunaScript RT SuperMix kit (New England Biolabs). Expression levels of *Il7*, *Scf*, and *Ccl25* were quantified by qPCR using Luna Universal qPCR kit in a QuantStudio™ 5 PCR system (ThermoFisher Scientific) and keratin 8 (*Krt8*) was used as reference gene to normalize the epithelial content within the inputs. Data were analyzed with DA2 App (ThermoFisher Scientific).

Statistical Analysis

Statistical analysis as indicated in figure legends were performed using GraphPad Prism 9 graphing and statistics software. Depending on the data distribution, a log-transformation was applied for the analysis of data shown using log scales. Not significant (ns) $p > 0.05$; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$.

RESULTS

FOXN1 Expression in TECs Is Regulated by Oxygen Availability

To address the effect of low oxygen submersion (LOS)-FTOCs on TEC function, we first examined whether the expression of the key Notch ligand, *Dll4*, is altered, as previously shown in thymic stromal monolayer cell cultures (19). After a 2-day period in LOS culture conditions, *Dll4* expression in TECs was lost, while in high oxygen (70% O_2) submersion (HOS) culture *Dll4* expression in a significant fraction of TECs was largely maintained (**Figure 1A** and **Supplementary Figure 1A**). Since *Dll4* is a direct transcriptional target of FOXN1 (2), we investigated whether the changes in *Dll4* expression levels were due to alternations in FOXN1 levels. Flow cytometric analysis revealed that while FOXN1 protein levels in TECs from LOS-FTOCs were lower than that from ALI-FTOCs, increasing oxygen availability in HOS cultures restored FOXN1 protein levels in TECs after a 2-day culture period (**Figure 1B**). This modulation of *Foxn1* expression by oxygen availability occurred at transcription level (**Supplementary Figure 1B**). However, the difference in FOXN1 protein levels between HOS and LOS conditions became less apparent when cultures were extended to day 4, at which point FOXN1 protein levels in TECs from HOS-FTOCs decreased. Cell surface expression of DLL4 in TECs from day 4 HOS cultures was also reduced but still detectable. To address whether the reduction of FOXN1 expression, and its target genes, between days 2 and 4 in HOS cultures may be due to the growth of developing thymocytes, which would restrict oxygen diffusion inside lobes and/or directly affect FOXN1 levels *via* thymocyte-stroma crosstalk (18), we performed cultures with fetal thymuses from *Rag2*-deficient mice. Of note, FOXN1 and DLL4 expression in TECs from $Rag2^{-/-}$ HOS-FTOCs retained higher levels than those from corresponding LOS-FTOCs throughout 9 days of culture (**Supplementary Figure 1C**), suggesting that a potential mechanism for HOS cultures to support T cell development is the maintenance of both FOXN1 and DLL4 expression.

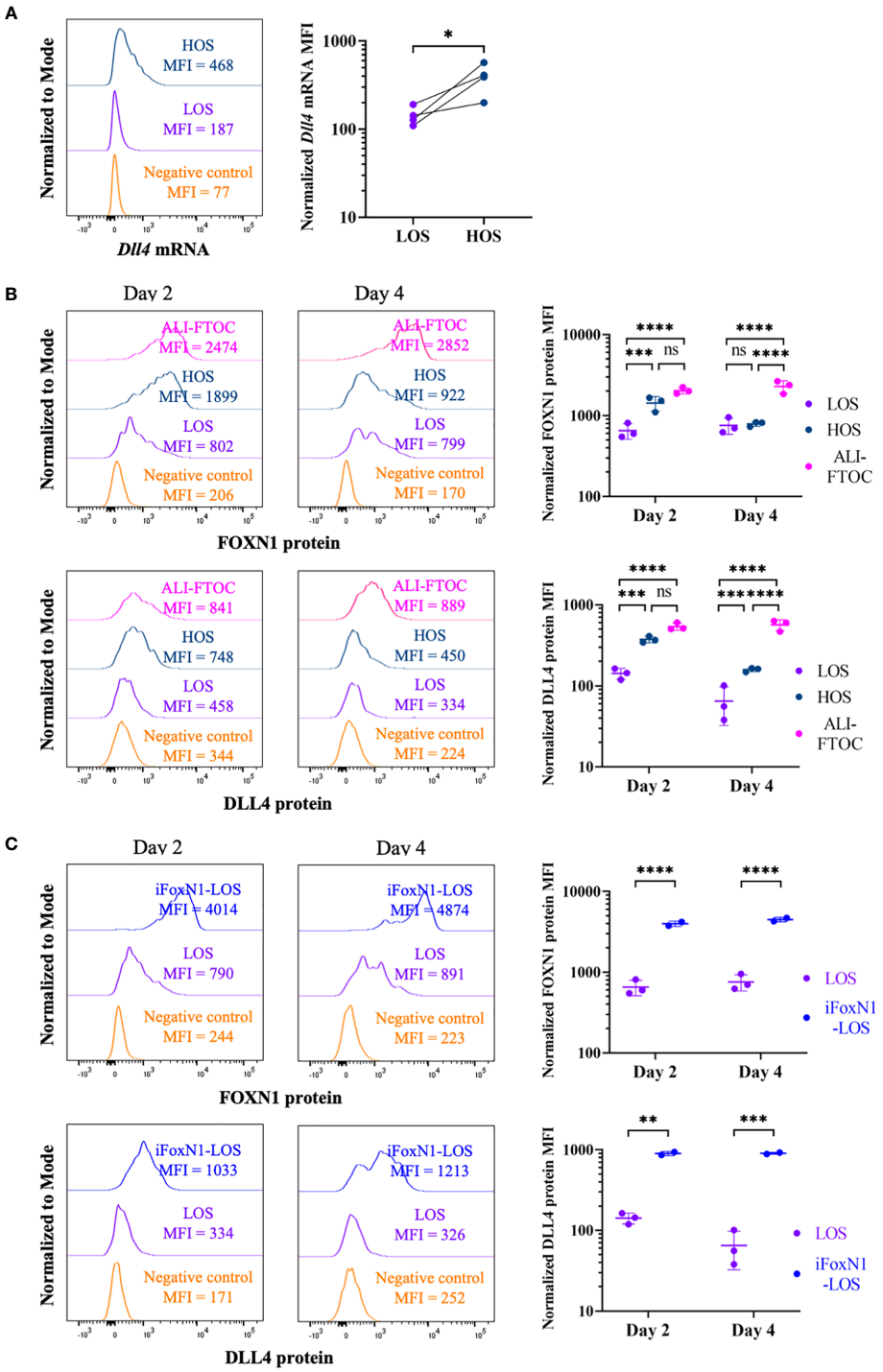

FIGURE 1 | Regulation of *FoxN1* and *Dll4* expression by oxygen availability. **(A)** The left panel shows representative histogram overlays of *Dll4* transcript levels using PrimeFlow RNA assay and the right panel shows scatter dot plots and statistical analysis (ratio paired Student's t-test) of the mean fluorescence intensity (MFI) of *Dll4* transcript, as indicated. **(B)** Flow cytometric analysis of intracellular FOXN1 and cell surface DLL4 expression in TECs from LOS-, HOS-, and ALI-FTOC, as indicated. Representative histogram overlays and statistical analysis (two-way ANOVA with *post-hoc* Tukey's test) are shown for FOXN1 (top panels) and DLL4 (bottom panels) protein expression in TECs from day 2 and day 4 cultures. **(C)** Flow cytometric analysis of intracellular FOXN1 and cell surface DLL4 expression in TECs from LOS and iFoxN1-LOS cultures, as indicated. Samples were pre-gated on $CD45^{-}EpCam^{+}$ cells except for negative control, which were $CD45^{+}$ cells. Numbers of biologically independent trials are indicated. ns, not significant; $p > 0.05$; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$.

Forced FOXN1 Expression Rescues Thymocyte Development, but Not Cellularity, in LOS Cultures

To investigate whether FOXN1 mediates the rescue of thymocyte development by increased oxygen availability in HOS, we took advantage of the R26Foxn1 transgenic mice developed by Dr. Blackburn's group, in which a mouse *Foxn1* expression cassette, preceded by a *loxp-STOP-loxp* element, was inserted into the *Rosa 26* locus. When this strain was bred to Foxn1^{ex9cre} mice, developed by Dr. Manley's group, all TECs from the resulting E14.5 thymus lobes that had expressed endogenous *Foxn1* maintained high levels of FOXN1 protein, and its target DLL4, even when cultured as LOS-FTOCs (**Figure 1C**, iFoxN1-LOS). These results show that ectopic expression of FOXN1 in LOS cultures can rescue DLL4 expression. Although exposing thymus lobes from E14.5 embryos of R26Foxn1^{creERT2} mice to tamoxifen could also induce FOXN1 expression (**Supplementary Figure 1D**, LOS-4OHT), this approach raised the concern that exposing thymocytes to tamoxifen and potential FOXN1 induction in non-TEC cells might have undesired side effects. Therefore, we withheld exposure to tamoxifen to lobes from E14.5 embryos of R26Foxn1^{creERT2} mice, which were used as control cultures.

Consistent with previous studies (8–10), after 8 days of culture, few DP cells and TCRβ-expressing CD4SP or CD8SP cells emerged from LOS-FTOCs, as compared to their presence in HOS-FTOCs (**Figure 2A**). An 8-fold increase in total cellularity was obtained from HOS-FTOCs, including 230- and 40-fold increase in CD4SP and CD8SP cells, respectively, as compared to control LOS-FTOC (**Figure 2B**). Notably, DP cells and TCRβ-expressing CD4SP and CD8SP cells developed in iFoxN1-LOS-FTOCs (**Figure 2A**). Although there was no difference in the total cellularity between LOS and iFoxN1-LOS cultures, iFoxN1-LOS-FTOCs produced 16- and 6-fold more CD4SP and CD8SP cells, respectively, than LOS-FTOCs (**Figure 2B**), demonstrating that enforced FOXN1 expression in TECs could rescue T lymphopoiesis in LOS cultures, but yielding much lower total cellularity than HOS-FTOCs. We tested whether iFoxN1 lobes in HOS cultures would be affected by enforced

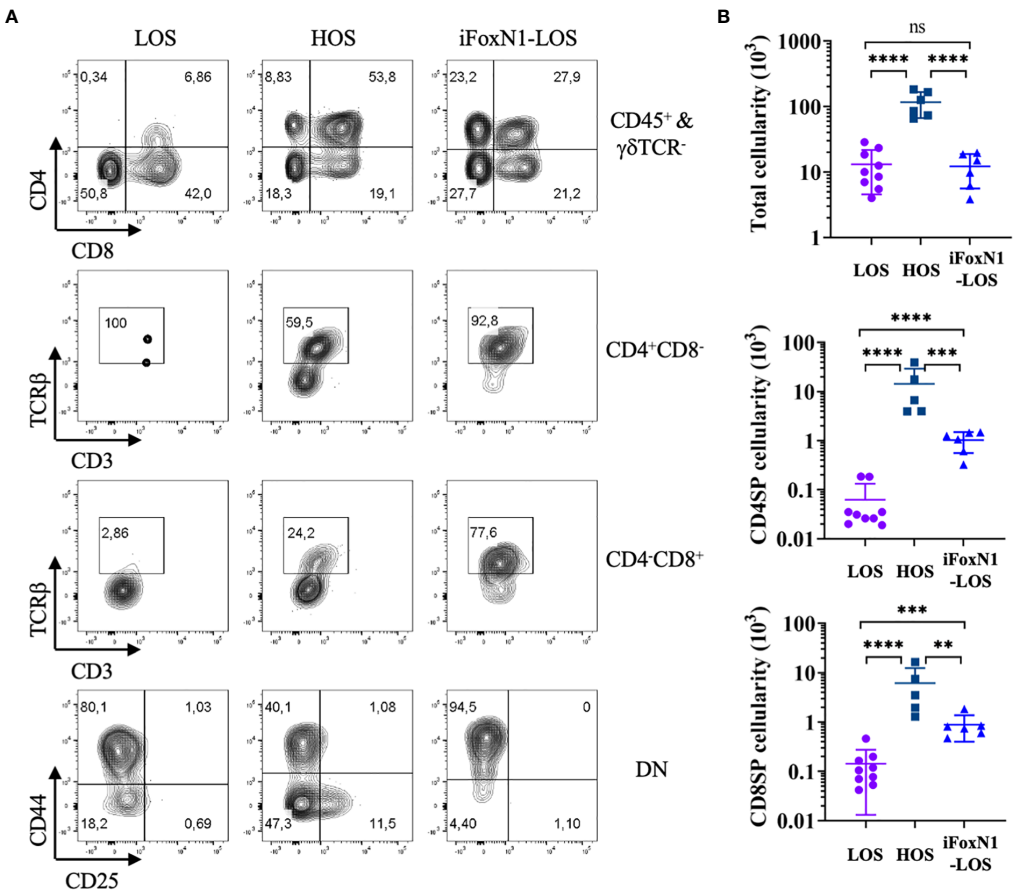

FIGURE 2 | Generation of αβ T cells in HOS- and iFoxN1-LOS-FTOC. **(A)** Flow cytometric analysis of thymocytes from 8-day LOS, HOS, and iFoxN1-LOS cultures, as indicated. The top panels display CD4 and CD8 expression of CD45$^+$γδTCR$^-$ gated cells. The middle two panels display TCRβ and CD3 expression of CD4$^+$CD8$^-$ gated cells, and CD4$^-$CD8$^+$ gated cells, respectively. The bottom panels display CD44 and CD25 expression of CD4$^-$CD8$^-$ DN cells. **(B)** Scatter dot plots and statistical analysis (one-way ANOVA with *post-hoc* Tukey's test) of total (top), CD4SP (middle), and CD8SP (bottom) cell numbers from 8-day cultures, as indicated. ns, not significant; $p > 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$.

FOXN1 expression and found HOS cultures showed increased total cellularity and the numbers of CD4SP and CD8SP. In addition, DP cells remained as the dominant subpopulation, the same as the HOS cultures of lobes without forced FOXN1 expression. Therefore, the significant lower cellularity from iFoxN1-LOS compared to HOS cultures was not due to very high levels of FOXN1 proteins and its target genes in TECs (**Supplementary Figure 2**).

Oxygen Availability Influences TEC Cellularity and Thymopoietic Activity

TECs provide necessary cues to promote thymocyte survival and proliferation in addition to guide their commitment and differentiation towards the T-lineage. The notable difference in total cellularity between FTOCs from HOS and LOS conditions, despite the rescue in thymocyte differentiation afforded by the expression of FOXN1, prompted us to investigate whether oxygen availability modulates TEC cellularity and differentiation. We recovered significantly less TECs from LOS than HOS cultures after 8 days regardless of FOXN1 protein levels (**Supplementary Figure 3A**). While no significant difference in the numbers of recovered TECs between 4-day and 8-day cultures, it was clear that there was a significant, ~5-fold, reduction in the numbers of TECs that could be recovered during the first 4 day of culture from lobes without forced FOXN1 expression (**Supplementary Figures 3A, B**), suggesting a loss of TECs in LOS cultures or that LOS culture condition made it difficult to recover TECs for flow cytometric analysis. Furthermore, HOS cultures had a higher thymopietic index, the ratio of thymocytes to TECs (20), indicating TECs from HOS cultures were functionally more capable of supporting thymocyte survival and/or proliferation (**Supplementary Figure 3A**). No consistent difference in the expression of common subtype markers was observed between LOS and HOS cultures although cultures with forced FOXN1 expression showed higher level of CD205, which is a direct FOXN1 target gene (**Supplementary Figures 3A, C**) (2).

To test whether the failure of TECs to support thymocyte growth and differentiation in LOS cultures is reversible, we switched FTOCs from LOS to HOS condition after 8 days of culture and concomitantly added fetal liver lineage-negative $Sca1^+Kit^+$ (LSK) cells. The cultures were analyzed 16 days after the switch, with HOS condition showing robust cellularity and differentiation potential, as evidenced by the presence of DP cells, in stark contrast to cultures that remained as LOS conditions (**Supplementary Figure 4**). These findings demonstrate that defects of TECs in LOS cultures is reversible.

IL-7 and SCF Levels Are Not Affected by Oxygen Availability

The difference in thymopoietic activity between TECs from HOS and LOS cultures urged us to examine two important lymphopoietic cytokines, IL-7, and SCF, whose deficiency leads to lower thymic cellularity in knockout mice (21–25). qPCR analysis revealed no difference in *Il7* transcript levels between HOS and LOS culture conditions (**Supplementary Figure 5**). In addition, no significant difference was noted in *Scf* transcript levels between HOS and LOS FTOCs. This suggests that it is unlikely that increased oxygen availability in HOS led to higher cellularity through elevating cytokine levels.

Increased Oxygen Availability in HOS Cultures Promotes Initial Rapid Growth

We next examine the temporal kinetics of thymocyte cellularity and differentiation during early period of submersion cultures. Since cells were found present outside of the lobes (**Figure 3A**), we analyzed cells inside and outside of lobes separately, with the consideration that cells outside of the thymus might develop differently due to a lack of direct support from TECs. There was negligible cell growth within the first two days of LOS cultures as the total numbers of cells, including cells both outside and inside of lobes, were slightly lower than the numbers of cells ($1.5–2.0 \times 10^4$) of the starting E14.5 thymus lobes (**Figure 3B**), consistent with a previous report (9). Of note, a large fraction of cells in LOS cultures migrated out of lobes during the first two days (**Figure 3C**). In contrast, HOS cultures promoted thymocyte growth from the start of culture, yielding a ~3-fold increase in cellularity compared to LOS-FTOCs on day 1, increasing to 4-fold by day 2 (**Figure 3B**). In addition, most cells in HOS cultures remained inside of lobes (**Figure 3C**), which would ensure direct support from TECs. Further examination of thymocyte proliferation and apoptosis rates revealed that increased proliferation, rather than decreased apoptosis, was the driving force behind the initial rapid increase in total cellularity in HOS cultures (**Supplementary Figure 6**).

Notwithstanding the stark differences in total cellularity and cell location, all three cultures showed similar initial developmental progression (**Figure 3D**), with appearance of $CD8^+$ ISP on day 1 and DP cells on day 2. The seemingly low percentage of DP cells in HOS on day 2 was due to relatively higher numbers of DN and $CD8^+$ ISP, as the number of DP was comparable to, if not higher than, the other two cultures (**Supplementary Figure 7**). The LOS-FTOCs showed an increase in the frequency of DP cells up to day 4, which then collapsed by day 5 of culture (**Figure 3B**). This was in contrast to HOS and iFoxN1-LOS cultures, which maintained high percentages of DPs, and, in the case of HOS-FTOCs, higher DP cellularity, by day 5 of culture.

Enhanced Self-Renewal of DN3 Cells in HOS-FTOC

Notably, the numbers of DP cells inside both LOS and iFoxN1-LOS cultures peaked on day 3 and then declined (**Supplementary Figure 7**), consistent with a single wave of DP cells being generated. In contrast, the numbers of DP cells inside HOS-FTOCs remained relatively constant after day 3, even until day 8 of culture, suggesting a continuous generation of DP cells. These findings were consistent with the notable persistence of DN3 cells seen in HOS-FTOCs, but not in LOS cultures (**Figures 2A**, **3D**).

To test whether increased oxygen availability could promote the expansion and/or self-renewal of DN3 cells in HOS-FTOCs, we

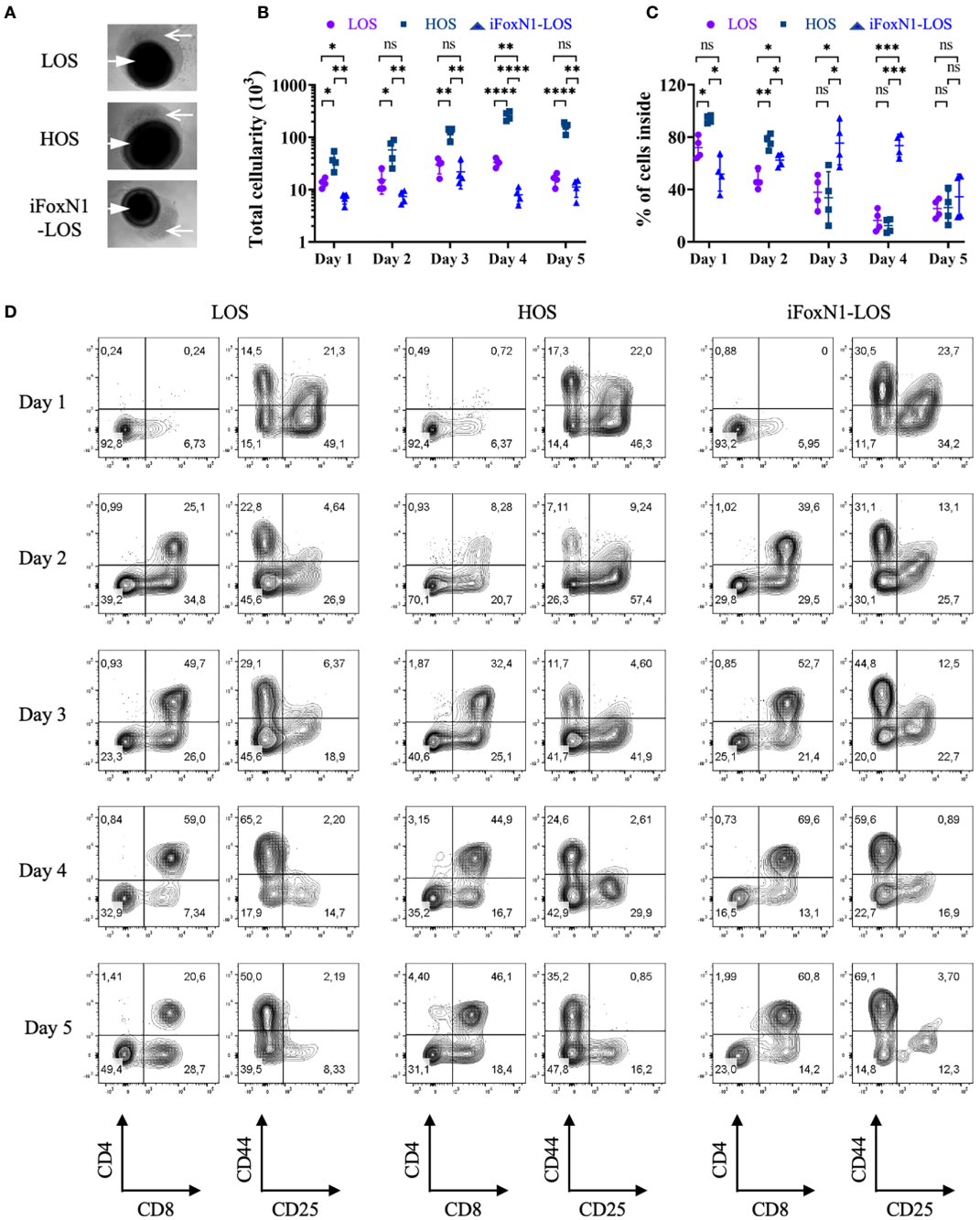

FIGURE 3 | Temporal analysis of T cells development from LOS, HOS, and iFoxN1-LOS cultures. **(A)** Phase-contrast images of submersion cultures on day 2 of culture are shown, with closed arrow pointing to thymus lobes and open arrow indicating cells outside of lobes. **(B)** Scatter dot plots and statistical analysis (two-way ANOVA with *post-hoc* Tukey's test) of total cell numbers, including cells both inside and outside of lobes, of individual culture conditions on each day within the first 5 days of culture. **(C)** Scatter dot plots and statistical analysis (two-way ANOVA with *post-hoc* Tukey's test) of the percentage (%) of cells inside of lobes, as indicated. **(D)** Flow cytometric analysis of thymocytes inside of lobes from LOS, HOS, and iFoxN1-LOS cultures, as indicated. For each culture condition, the left contour plots show CD4 and CD8 expression of $CD45^+\gamma\delta TCR^-$ gated cells and the right panels display CD44 and CD25 expression of $CD45^+\gamma\delta TCR^-CD4^-CD8^-$ gated cells. ns, not significant; $p > 0.05$; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$.

cultured E14.5 *Rag2*$^{-/-}$ thymus lobes in submersion cultures. As shown in **Figure 4**, by day 5 of culture, the number and frequency of DN3 cells are severely reduced in LOS as compared to HOS FTOCs. By day 10 of culture, there were few, if any, DN3 cells left in LOS, while HOS cultures contained ~40% DN3 cells, suggesting that DN3 cellularity is maintained under HOS conditions.

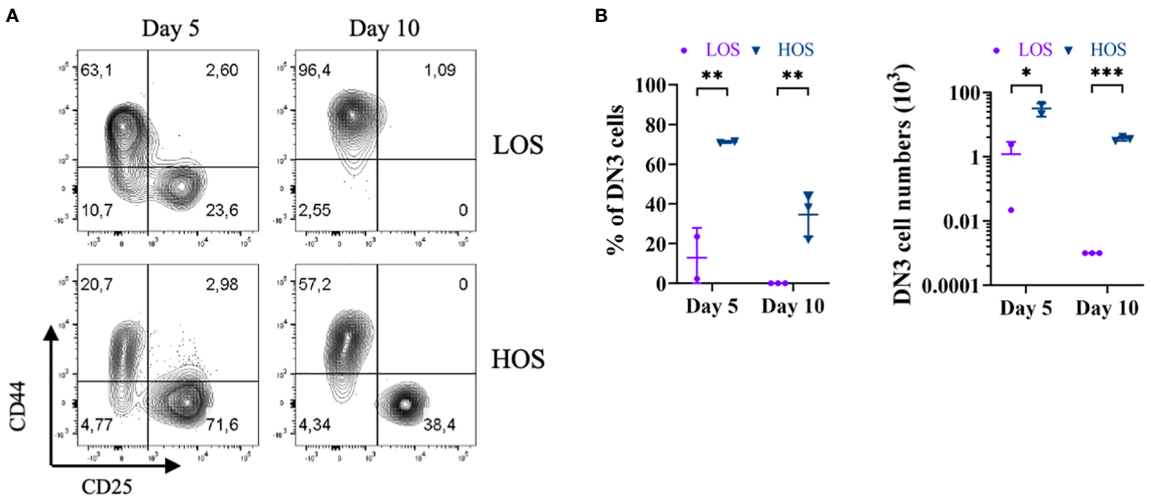

FIGURE 4 | Increased oxygen availability promotes DN3 self-renewal in HOS. **(A)** Flow cytometric analysis of *Rag2*-deficient thymocytes from 5- and 10-day LOS and HOS cultures, as indicated. Cells shown were pre-gated as $CD45^+CD11b^-CD19^-CD4^-CD8^-$. **(B)** Scatter dot plots and statistical analysis (two-way ANOVA with *post-hoc* Sidak's test) of percentage (%) of DN3 cells (left), and numbers of DN3 cells (right), as indicated. DN3 cells were not detected in LOS cultures on day 10, as such were indicated and analyzed as one cell to enable log-transformation. *p < 0.05; **p < 0.01; ***p < 0.001.

Failure to Retain DP cells Inside and Down-Regulation of MHCII in LOS Cultures

Consistent with previous work (8–10), $TCR\beta^+$ CD4SP and CD8SP cells were not generated in LOS-FTOC (**Figure 2A**). Hence, the DP cells present early on in LOS cultures (**Figure 3D**) somehow fail to give rise SP cells, while iFoxN1-LOS-FTOCs allowed for the generation of $TCR\beta^+$ SP cells (**Figure 2A**). One outstanding difference between LOS and iFoxN1-LOS cultures was the location of DP cells; while most DP cells in iFoxN1-LOS remained inside of the lobes, most DP cells in LOS cultures were found outside of the lobes, where they would not likely receive support to undergo positive selection (**Supplementary Figure 7**).

To test whether LOS-FTOCs are less capable of attracting/maintaining DP cells inside, thymus lobes free of unattached cells were transferred to new wells together with sorted GFP^+ DP or DN2/DN3 cells, distinguishing GFP^- endogenous from GFP^+ exogenous thymocytes. Cells outside or inside of the lobes were collected, counted and analyzed separately. Both GFP^- and GFP^+ cells could be detected inside and outside of the lobes, with GFP^- cells outside of the lobes being endogenous cells that emigrated within the 1-day culture period (**Figure 5A**). It was clear that more GFP^+ DP cells were attracted into iFoxN1-LOS than LOS lobes. As a result, more exogenous GFP^+ DP cells were present inside the lobes of iFoxN1-LOS than LOS cultures (**Figure 5B**). In contrast, a significant fraction (~70%) of endogenous GFP^- DP cells emigrated from LOS-FTOCs, while iFoxN1-LOS cultures maintained most (~80%) of endogenous GFP^- DP cells inside of the lobes (**Figure 5C**). In addition, iFoxN1-LOS-FTOCs seemed to attract/maintain all subpopulations inside of the lobes better than LOS cultures (**Figure 5C**). To address a potential molecular mechanism responsible for the difference in attracting/maintaining DP cells inside iFoxN1-LOS, as opposed to LOS-FTOCs, we examined the expression of *Ccl25*, a direct FOXN1 target gene (2) and a chemoattractant of $CCR9^+$ DP cells. Our analysis revealed that after a 2-day culture, *Ccl25* transcript levels were higher in HOS and iFoxN1-LOS-FTOCs compared to LOS cultures, and remained higher in iFoxN1-LOS-FTOCs after a 4-day of culture (**Figure 5D**).

Additionally, LOS cultures, not only failed to keep DP cells from emigrating, but also showed lower levels of MHCII expression in TECs compared to HOS-FTOCs (**Figure 5E** and **Supplementary Figure 3C**). The expression of MHCII on TECs was found to be dependent on oxygen availability and independent of FOXN1, as control HOS conditions maintained high levels of MHCII on day 4, when FOXN1 levels were down-regulated (**Figure 1**). In addition, about half of the TECs from iFoxN1-LOS cultures expressed very low levels of MHCII. The low levels of MHCII in half of the TECs from iFoxN1-LOS likely affected the selection of CD4SP cells.

DISCUSSION

Access to adequate oxygen levels is required for T cell development within the thymic microenvironment, which is achieved by the vascularization of thymus at about E15.5 *in vivo* (26), or by direct exposure to air in ALI-FTOCs *in vitro* (7). Sufficient oxygen supply provides more than energy necessary for developing thymocytes to proliferate and survive, as immature thymocytes could still grow and respond to cytokine stimuli, but failed to develop into mature αβ T cells, when fetal thymus lobes were cultured as submersion FTOCs (8–10), an unquestionable hypoxic environment due to limitations in

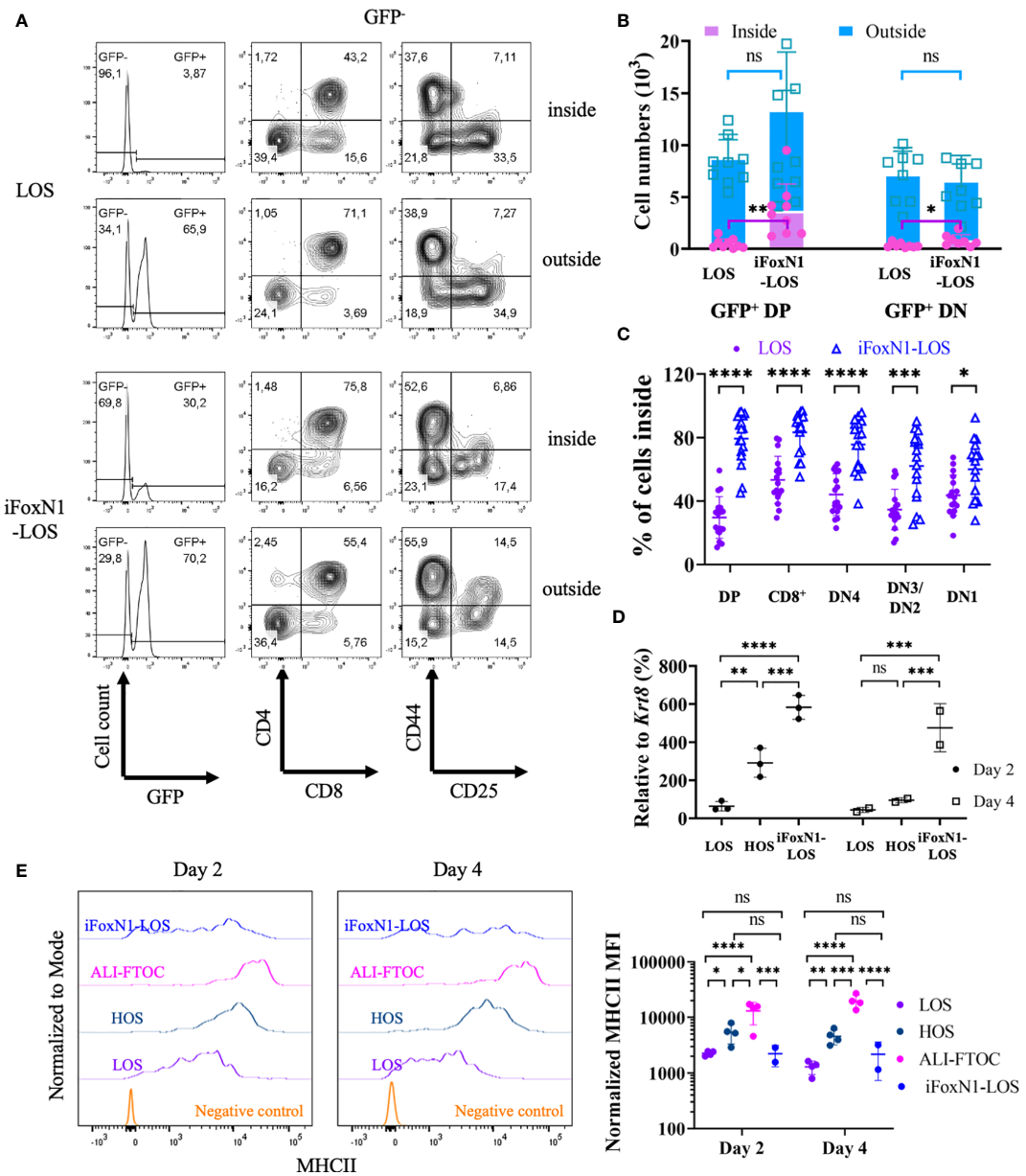

FIGURE 5 | Migration of CD4$^+$CD8$^+$ cells in LOS cultures. **(A)** Flow cytometric analysis of cells inside and outside of lobes from LOS- and iFoxN1-LOS-FTOCs. Sorted GFP-expressing CD4$^+$CD8$^+$ cells were added to LOS-FTOCs and all cells were analyzed after 1-day of culture. The left panels show histograms of GFP expression from CD45$^+$γδTCR$^-$ gated cells. The middle and right panels show CD4 and CD8 expression on CD45$^+$γδTCR$^-$GFP$^-$ cells, and CD44 and CD25 expression on CD45$^+$γδTCR$^-$GFP$^-$CD4$^-$CD8$^-$ cells, respectively. **(B)** Stacked column graphs and statistical analysis (unpaired Student's t-test) of numbers of added GFP$^+$ CD4$^+$CD8$^+$ (DP), or CD4$^-$CD8$^-$ DN2/DN3 (DN) cells, inside and outside of lobes after 1-day of culture, as indicated. **(C)** Scatter dot plots and statistical analysis (unpaired Student's t-test) of percentage (%) of endogenous (GFP$^-$) cells in different subsets that remained inside of the lobes after 1-day of culture, as indicated. **(D)** Scatter dot plots and statistical analysis (two-way ANOVA with *post-hoc* Tukey's test) of *Ccl25* transcript levels relative to keratin 8 (*Krt8*) quantified by RT-qPCR, as indicated. **(E)** Flow cytometric analysis of MHCII expression on TECs from LOS-, HOS-, ALI-, and iFoxN1-LOS-FTOCs. Representative histogram overlays and statistical analysis (two-way ANOVA with *post-hoc* Tukey's test) of MHCII levels on day 2 and day 4 of cultures are shown, as indicated. Samples were pre-gated on CD45$^-$EpCam$^+$ cells except for negative control, which were CD45$^+$ cells. ns, not significant; $p > 0.05$; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$.

oxygen diffusion (27). Increasing oxygen concentration in the air surrounding submersion FTOCs from 20% ambient to 60–80% was shown to alleviate the hypoxic environment and allowed for normal thymocyte development, although the molecular mechanism(s) remained elusive (10). Here, we show that increased oxygen availability in HOS cultures enabled intrathymic T lymphopoiesis through both FOXN1-dependent and -independent processes.

We found that access to oxygen was required for TECs to maintain FOXN1 expression levels and the expression of FOXN1 target genes, such as *Ccl25* and *Dll4* (2). We addressed the biological significance of maintaining high expression levels of CCL25 in submersion FTOCs, and showed that low levels of CCL25 in LOS-FTOC allowed for DP cells to emigrate from the lobes. Consequently, DP cells would lose MHC-mediated support from TECs to undergo positive selection. In HOS-FTOC, *Ccl25* expression was initially maintained, but then decreased as FOXN1 levels wane at later time points, with a subsequent migration of DP cells out of the lobes. Nevertheless, there was always a considerable number of DP cells remaining inside the HOS lobes, ensuring that these cells would be able to undergo positive selection.

On the other hand, the relevance of DLL4 during early T cell development cannot be overstated (3, 4). However, previous works (8–10), as well as this study, made use of E14.5 fetal thymuses, and, by the time DLL4 protein expression is extinguished from the cell surface of TECs in LOS-FTOCs, the majority of thymocytes are already committed to the T cell lineage, and either undergoing or already passed β-selection, two critical events that depend on Notch signaling (28, 29). Nonetheless, the continuous presence of DLL4 on TECs in HOS-FTOC likely supported a more persistent generation of DP cells, which were still the dominant subset on day 8. Consistent with this notion, previous work showed that the earlier the fetal thymus lobes are harvested, the fewer DP cells observed after 7 days of LOS cultures (9). Thus, the more severe developmental block seen with earlier fetal thymuses reflects the importance of maintaining DLL4 levels in FTOCs to enable developing thymocytes to survive β-selection.

There are potentially at least two FOXN1-independent mechanisms by which adequate oxygen supply is required to support T lymphopoiesis in submersion FTOCs: maintaining high expression levels of MHCII on TECs; and promoting self-renewal of DN3 cells. The FOXN1-independent regulation of MHCII expression by oxygen and low levels of MHCII on about half of TECs from iFoxN1-LOS-FTOCs are consistent with the previous finding that MHCII is not a direct FOXN1 target gene (2). However, it leaves open the question as to how MHCII levels remained high on the other half of TECs in iFoxN1-LOS cultures after 4 days.

Limited self-renewal of progenitor thymocytes in the absence of new TSPs has been observed *in vivo* (30, 31), and attributed to the transit amplifying DN2/DN3 subsets. It was proposed that losing competition for the DN3 niche to newly generated DN3 cells leads to the cessation of self-renewal of pre-existing DN3 cells (32), but the underlying molecular mechanism(s) has remained elusive. Submersion FTOCs might provide an excellent system to study this question as input of new TSPs can be tightly controlled. We have demonstrated that self-renewal of *Rag2*$^{-/-}$ DN3 cells takes place in HOS, but not in LOS cultures. Loss of DLL4 expression likely contributed to the lack of self-renewal by *Rag2*$^{-/-}$ DN3 cells in LOS cultures, as our previous work showed that Notch signaling promotes the survival of *Rag2*$^{-/-}$ DN3 cells in OP9-DL co-culture system (29). The use of the OP9-DL cell system also shows that T cell development *per se* can take place in LOS conditions, further emphasizing that reducing oxygen availability intrinsically affects TEC rather thymocyte functionality. However, whether and what other factor(s), in addition to DLL4, constitutes the intrathymic DN3 niche and how pre-existing DN3 cells lose their competitive edge remain open questions, which could be investigated using submersion FTOC system.

Adaption to hypoxia is usually accompanied with changes in the expression of hundreds of genes that are involved in a plethora of cellular activities. Therefore, it is unlikely that *Foxn1* and its target genes are the only ones whose levels were affected in LOS cultures. Nonetheless, rescue of T cell differentiation by genetically forced *Foxn1* expression demonstrates that it is one of the critical genes that mediate the regulation of T cell development by oxygen availability. Further study is needed to unveil the molecular mechanism underlying its transcriptional regulation by oxygen availability. HIF1 is the most-studied transcription factor that responds to oxygen tension to maintain cellular homeostasis under hypoxia. ChIP-Seq data demonstrated that HIF1 functions primarily as a transcription activator, as HIF1 did not bind to genes that were downregulated by HIF1 (33). Although HIF1 has been reported to directly suppress the transcription of at least two genes in nucleus pulposus cells, the stability and transcription activity of HIF1α in these cells is oddly not regulated by oxemic state (34, 35). Therefore, if HIF1 is involved in the downregulation of *Foxn1* in LOS cultures, then its effect is likely transduced by one of its target genes, rather than directly.

The physiological significance of regulating FOXN1 expression by oxygen availability remains an open question. It is noteworthy to relate the potential role of increased oxidative stress in TEC function to the findings showing that treatment with antioxidants can delay the onset of age-related thymus involution (36). The effect of antioxidants on the onset of age-related thymus involution was attributed to alleviating oxidative damage in TECs. Our observations, such as the reversibility of TEC function from LOS cultures, make us to speculate whether the effect of antioxidants on thymic involution is at least partially mediated by enabling the survival of TECs under high oxygen stress, which is required for the maintenance of FOXN1 expression. This view is also consistent with the effect of forced expression of FOXN1 in transgenic mice that appeared to rejuvenate an aged thymus (11).

In conclusion, our findings revealed that increased oxygen availability in HOS cultures restores the expressions of FOXN1 and its target genes, as well as FOXN1-independent MHCII expression, which together safeguard the normal developmental progression of conventional αβ T cells. In addition, HOS cultures promote the survival and expansion of DN3 cells, which secures a persistent supply of progenitors for β-selection and subsequent positive selection. Working together, these mechanisms enable high-oxygen support of robust T lymphopoiesis in submersion FTOCs. In addition, our results warrant further investigations on the physiological significance of FOXN1 regulation by oxygen availability and its underlying

mechanisms, as well as mechanisms governing self-renewal of DN3 cells using submersion FTOCs.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Sunnybrook Research Institute Animal Care Committee.

AUTHOR CONTRIBUTIONS

JH designed and performed all the experiments, analyzed the data, and wrote the manuscript. JCZ-P conceived the project, analyzed the data, and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.652665/full#supplementary-material

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Post-Aire Medullary Thymic Epithelial Cells and Hassall's Corpuscles as Inducers of Tonic Pro-Inflammatory Microenvironment

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While there is convincing evidence on the role of Aire-positive medullary thymic epithelial cells (mTEC) in the induction of central tolerance, the nature and function of post-Aire mTECs and Hassall's corpuscles have remained enigmatic. Here we summarize the existing data on these late stages of mTEC differentiation with special focus on their potential to contribute to central tolerance induction by triggering the unique pro-inflammatory microenvironment in the thymus. In order to complement the existing evidence that has been obtained from mouse models, we performed proteomic analysis on microdissected samples from human thymic medullary areas at different differentiation stages. The analysis confirms that at the post-Aire stages, the mTECs lose their nuclei but maintain machinery required for translation and exocytosis and also upregulate proteins specific to keratinocyte differentiation and cornification. In addition, at the late stages of differentiation, the human mTECs display a distinct pro-inflammatory signature, including upregulation of the potent endogenous TLR4 agonist S100A8/S100A9. Collectively, the study suggests a novel mechanism by which the post-Aire mTECs and Hassall's corpuscles contribute to the thymic microenvironment with potential cues on the induction of central tolerance.

Keywords: Hassall's corpuscles, medullary thymic epithelial cells, AIRE, thymus, central tolerance, S100A8, S100A9, TLR4 – Toll-like receptor 4

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INTRODUCTION

The thymus is a primary immune organ required for T cell development. The maturation process of the developing T cells, the thymocytes, involves somatic recombinations to randomly generate functional T cell receptors that in principle can recognize all possible antigenic determinants [reviewed in (1)]. Thus, in order to avoid the escape of potentially harmful, self-reactive T cell clones, these clones need to be either eliminated physically or changed functionally before their exit from the thymus. These processes, collectively known as central tolerance induction, take place in the thymic medulla and comprise negative selection and thymic regulatory T cell (Treg) induction. Both negative selection and Treg differentiation are believed to rely on T-cell receptor (TCR)-

derived signal strength (2), which in turn depends on the availability and affinity of self-peptides in the thymus and is regulated by a range of co-stimulatory molecules expressed on antigen presenting cells (APC) (1, 2). Therefore, the development of efficient and self-tolerant T-cells depends on complex interactions between a range of different thymic cell types and is shaped by the local microenvironment (3).

As opposed to all other organs, a specific feature of the thymic microenvironment is a constitutive low-grade expression of proinflammatory mediators, inflammatory cytokines and chemokines even under physiological conditions (4–6) (covered in detail below). There is accumulating evidence that this low-grade inflammatory signaling may play a role in thymocyte development. Indeed, the recent data suggest that the tonic inflammation can affect the final stages of single positive T cell development as well as thymic Treg generation *via* mobilization of thymic dendritic cells (7, 8). Hence, the tonic pro-inflammatory microenvironment in the thymus has the potential to affect central tolerance induction and to shape the resulting repertoire of peripheral T cells and Tregs. The cellular and molecular mechanisms leading to this unique phenomenon, however, are not fully understood. Below we will summarize the current knowledge together with some novel evidence that these inflammatory signals are provided by the medullary thymic epithelial cells (mTECs) at the very late stages of their differentiation.

CENTRAL TOLERANCE INDUCTION AND mTEC DIFFERENTIATION

Although the developing thymocytes comprise the majority of the cellular mass of the thymus, their proper development is directed by the non-hematopoietic thymic stroma including the thymic epithelial cells and fibroblasts as well as by the non-thymocyte hematopoietic compartment including the APCs, i.e. the dendritic cells, B cells and macrophages (3). In regard to the induction of central tolerance, a central role is played by the mTECs that have a unique property to express a huge variety of different genes and proteins including the ones whose expression is otherwise restricted to a certain peripheral cell or tissue type (9). This ectopic gene expression is largely controlled by a transcriptional regulator Aire and is critical for the induction of central tolerance to these self-proteins either by directing the self-reactive thymocyte clones to apoptosis (i.e. negative selection) or directing them toward the Treg lineage (10–12). Accordingly, mutations in Aire result in a defect in central tolerance in humans as well as in mice and rats affecting both of these arms of central tolerance (10, 13, 14). In addition to the well-characterized role in ectopic antigen expression, Aire has been proposed to control a number of other functions including regulation of thymic chemokines (15–17) and mTEC maturation (18). Regardless of the precise mechanism, however, the end-result of Aire-deficiency is a defect in central tolerance, which may precipitate in autoimmunity.

Due to the central role of Aire expressing mTECs in thymic tolerance induction, there has been a lot of interest in the Aire+ mTEC lineage differentiation and mTEC differentiation in general [reviewed in (19)]. It is now widely accepted that at least during fetal development both the cortical as well as the medullary epithelial cells are derived from a single bipotent progenitor (20, 21), while the existence of the bipotent progenitor in the adult thymus has remained controversial. After receiving a signal yet to be identified, some of the progenitors are directed toward the mTEC lineage and upregulate the proliferation marker Ki67 (22) to become more populous and can still give rise to different mature mTEC lineages. Recent advances in single-cell transcriptomics in mice (22–26) and humans (27) have highlighted the heterogeneity of these functionally diverse thymic cells and although minor differences exist between the results, most of the respective studies agree that in mice the mature mTECs can be divided into four subpopulations: 1) mTEC I, characterized by the dependency of lymphotoxin (LT)β signaling, high expression of CCL21 and lack of MHCII expression (28, 29); 2) mTEC II, characterized by RANK-dependency and high expression of Aire, MHCII and thousands of tissue-restricted genes (30, 31); 3) mTEC III, known as post-Aire cells or corneocyte-like mTECs, which express low/mid MHCII and whose gene expression profile resembles late-stage keratinocytes/corneocytes (see below); and 4) mTEC IV, known as thymic tuft cells, which express IL-25 and whose gene expression profile resembles intestinal tuft cells (23, 24). Single-cell analysis of the human thymus confirmed these four main mTEC subpopulations but added mTEC-myo and mTEC-neuro as two additional subpopulations present in humans but not in mice (32).

THE AIRE+ mTECS, POST-AIRE mTECS AND HASSALL'S CORPUSCLES

As it is at the Aire+ (mTEC II) stage, where the mTECs express thousands of self-antigens, MHCII and co-stimulatory molecules CD80 and CD86, this population in particular has been profoundly studied as a central player in central tolerance induction. As these cells represent a functionally mature post-mitotic cell population they were, until quite recently, also considered the endpoint of the Aire+ lineage existence. However, several fate-mapping and single cell transcriptomics approaches have identified that the differentiation of mTECs extends beyond the Aire+ differentiation stage to become the mTEC III (25, 31, 33–35). At this post-Aire stage, the cells down-regulate Aire together with most of the Aire-dependent proteins and lose accordingly their ability to express a broad range of ectopic genes. In addition, these cells downregulate the machinery required for direct antigen presentation including MHCII and the co-stimulatory molecules (31, 34) and depend from this point on the APC-mediated cross-presentation to present the expressed proteome to the developing thymocytes. At the same time, the post-Aire cells become enriched for proteins classically associated with end-stage keratinocytes, such as involucrin (Ivl) (36), Lekti (37), and a variety of different keratins (31), obtaining a corneocyte-like phenotype.

In addition to the conventional mTECs described above, the thymic medulla contains unique structures called Hassall's corpuscles (HCs). These structures, firstly characterized in 1846 by Arthur Hill Hassall (38) in human thymi have, after their initial description, been found in several other mammals (39) but as well as in bird and fish species (40, 41). In humans, the HCs appear as a concentric merged cluster of unnucleated cells with a typical diameter of 20-100 μm and are present in large quantities already by the 28th week of prenatal development (42). As the size and numbers of HCs shrink together with age-related thymic involution (43) and thymic hyperplasia in myasthenia gravis or lymphomas are characterized by increased numbers of HCs (44, 45), their abundance seems to be related with thymic activity. As the size of HCs, on the other hand, correlates with the size of the thymus (39, 46), they are relatively hard to detect in smaller rodents such as mice and usually require antigen-specific immunostainings to visualize (18). Although the function and nature of the HCs have been studied and speculated since their discovery, it has been established only quite recently that these unique structures represent the final differentiation stage of the Aire+ lineage (19). The evidence comes from studies showing retention of Aire reporters in the HCs once the Aire itself has been already down-regulated (34), from studies showing further upregulation of corneocyte-associated proteins in the HCs (18, 34), and are indirectly also reinforced by the fact that the HCs are nearly missing in the Aire KO mouse (18). Further support comes from human studies showing that in thymomas the presence of HCs is restricted to the subtypes with Aire expression (47) and that in patients with Down syndrome, the presence of the third copy of the chromosome 21 (where the AIRE gene is located) results in increased numbers of Aire positive cells together with enlarged Hassall's corpuscles (48).

Collectively, the current data corroborate the conclusion that following the Aire+ (mTEC II) stage, the differentiation to post-Aire mTECs (mTEC III) and further to the HCs represent the final steps of the Aire+ lineage. Regarding the function of the post-Aire stages, however, the corresponding data is rather scarce. Because of their natural location in the thymic medulla, i.e. the site of central tolerance induction, most of the proposed functions have been related to their specific expression-pattern of self-proteins. For example, post-Aire cells/HCs have been reported to express keratinocyte-restricted and pemphigus related autoantigens Dsg-1 and Dsg-3 (34, 49, 50) but also proinsulin (51), an autoantigen in type 1 diabetes.

TONIC INFLAMMATORY SIGNALING, LATE-STAGE mTECS, AND CENTRAL TOLERANCE INDUCTION

Inflammation, characterized by increased production of inflammatory cytokines and chemokines, is a biological response to harmful stimuli such as pathogens and tissue damage. In this sense, the thymus seems to be a unique organ with constitutive production of pro-inflammatory mediators even under physiological conditions (4). A steady-state expression of type 1 interferons (IFN) has been shown in mouse mTECs and dendritic cells using an IFNβ reporter (6) and the secretion of type 1 IFN has been demonstrated in normal human thymi without any pathological stimulus (5). Although the precise upstream stimuli and functional consequences of this phenomenon are still largely unknown, there is increasing evidence that the post-Aire cells and HCs may play a role behind this specific feature of the thymic microenvironment, and that this in turn may modulate the induction of central tolerance. Thus, HCs have been shown to express thymic stromal lymphopoietin (TSLP) (52), which in turn is known for its capability to convert the conventional T cells to Tregs (52, 53) and can accordingly potentially promote the thymic Treg induction. Also, a recent study connected the senescence-like phenotype of HCs to IFNα production from thymic DCs and suggested that the lack of the low-grade inflammatory signaling results in impaired development of single-positive thymocytes (7). Another recent study showed the importance of Toll-like receptors and the resulting MyD88 signaling in mTECs for the expression of several inflammatory cytokines, DC recruitment and Treg induction (8). The evidence supporting the role of post-Aire mTECs and HCs in the induction of the pro-inflammatory microenvironment comes from the Aire KO mouse which, on the one hand, lacks post-Aire cell populations (18) but on the other hand, has reduced expression of several constitutively expressed inflammatory mediators (7) as well as functional defects both in negative selection and Treg induction (10, 11).

THE PROTEOME OF HUMAN LATE-STAGE mTECS AND HCS DISPLAYS A PRO-INFLAMMATORY SIGNATURE

Therefore, there is an increasing amount of evidence that at least in mice the post-Aire cells and HCs may contribute to the tonic inflammatory signaling in the thymic medulla, which in turn may play a role in the induction of central tolerance. We aimed to complement these findings with data obtained from human thymi and chose to analyze the proteomic pattern during mTEC maturation, as HCs are known to lose their nuclei and related transcriptional machinery. Accordingly, we microdissected three distinct morphological thymic areas (see **Figure S1**): 1) thymic medulla (labeled as mTECs throughout the proteomics section) 2) the epithelial layer immediately surrounding the HCs and characterized by flattened nuclei (labeled as late mTECs) and 3) the HCs to characterize the changes in the mTEC proteome during three consecutive differentiation stages. The mTEC population is likely to include all different mTECs but the mTEC III (i.e. non-mTEC III) whereas the easily distinguishable late-mTECs and HCs correspond to mTEC III and HCs, respectively. The dissection was performed from three thymi of patients (one year 2 months, one year 7 months, and two years 7 months old) undergoing cardiac surgery. The collected samples were analyzed by nano-LC/MS/MS and the raw data processed by MaxQuant, followed by the differential

analysis of the detected proteins and pathway analysis for the changed protein groups (see supplementary materials for detailed description). In addition, we compared this data obtained from thymi to the one obtained from three consecutive differentiation stages of human epidermal keratinocytes collected from healthy grown-up individuals: 1) stratum basale 2) stratum spinosum and 3) stratum granulosum + stratum corneum, in order to detect parallels in mTEC vs keratinocyte late-stage differentiation.

We were able to detect 1095 unique proteins in mTECs, 1026 in late mTECs and 880 in the HCs. Samples from all thymic areas were markedly enriched for keratins comprising between 5-7 different keratins among the top ten most abundant proteins in all samples. On the other hand, the peptides of thymocyte-specific markers CD4 and CD8 were undetectable in all samples whereas CD3 was detected at very low levels (four orders of magnitude lower than the top keratins) in two out of nine samples. Thus, although a minor contribution from hematopoietic cells can't be excluded, the detected proteome mostly reflects the changes occurring in the thymic stromal compartment.

Among the unique proteins detected, the keratins, serpins, and S100A family were represented by 42, 14 and 9 members, respectively (**Figure S2**). There was a significant upregulation of 37 and down regulation of 38 proteins during the final stages of mTEC maturation (**Figure 1**; **Table S1**). As expected, the loss of nuclei and compaction of epithelial tissue was reflected by significant downregulation of several proteins with nuclear expression (23 out of 38, **Table S1**) and by downregulation of several proteins known to be highly expressed in the extracellular matrix (**Figures 1, 2**; **Table S1**).

Remarkably, despite the progressive loss of nuclei, the late stages of mTECs were characterized by upregulation of several proteins related to protein translation, folding and intracellular transport (**Figures 1, 2**; **Table S1**). These characteristics also showed up to be significantly altered in the pathway analysis under the cytoplasmic ribosomal complex pathway (**Figure 1**). In addition, there was significant enrichment of proteins involved in exocytosis and extracellular exosome generation (**Figure 1**). As the mTEC-derived exosomes have been shown to carry the keratinocyte-specific autoantigens DSG-1, DSG-3 as well KRT5 and KRT14 (54), the data is compatible with the view suggesting that even after losing their MHCII expression, the post-Aire mTECs and HCs may actively contribute to the induction of central tolerance by synthesis and exocytosis of self-proteins for cross-presentation.

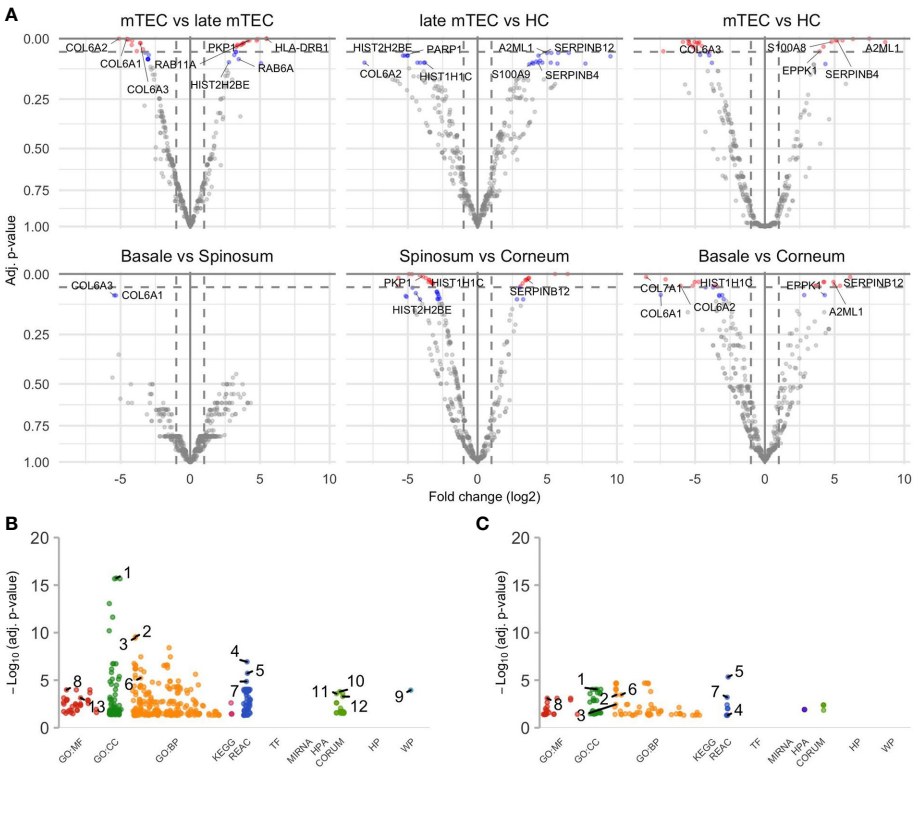
During the final stages of keratinocyte differentiation, we were able to detect 1015 unique proteins in stratum basale, 1092 in stratum spinosum and 718 in stratum granulosum + stratum corneum. Regarding the comparison between the final stages of mTEC and keratinocyte differentiation, we saw several protein groups and pathways being affected similarly in those two different cell types (**Figures 1, 2**; **Table S1**, **S2**). In addition to the expected loss of nuclei and epithelial compaction, the late stages of differentiation were similarly characterized by preferential upregulation of proteins involved in extracellular exosome generation, leukocyte mediated immunity, exocytosis and endopeptidase activity (**Figures 1, 2**; **Table S1**, **S2**). Also, in the thymus and skin, the late stages of differentiation were characterized by increased expression of two auto-antigens, EPPK1 and A2ML1 (**Figures 1, 2**), previously associated with autoimmune skin blistering (55, 56). Altogether, our data confirmed the previously known parallels in mTEC vs keratinocyte differentiation and suggested that at the late stages of differentiation the mTECs can indeed express keratinocyte-specific antigens to be cross-presented by myeloid APCs to the developing thymocytes.

Most importantly, the late-stage mTECs and HCs displayed a clear upregulation of several proteins usually connected to inflammatory processes (**Figures 1, 2**; **Table S1**). This increase in inflammatory proteins was reflected in the pathway analysis showing enrichment for proteins related to leukocyte mediated immunity, immune effector processes and innate immune system (**Figure 1**). Strikingly, among the induced inflammatory proteins were S100A8 and S100A9 and, accordingly, the iNOS-S100A8/A9 complex pathway (**Figure 1**). These S100A proteins, known mainly for their Ca++ binding properties (57), have recently been shown to form a heterodimeric complex, that in turn, through binding and activating TLR4 (57, 58), can induce the expression of several proinflammatory cytokines and mediators. As we also saw an upregulation of the TLR4 binding related processes at the late stages of mTEC differentiation (**Figure 1**), our data strongly suggest that post-Aire mTECs and HCs may play a role in the induction of tonic inflammatory signals by constitutive expression of the endogenous TLR4 agonist, S100A8/A9.

DISCUSSION

The escape of imperfectly selected T cells from the thymus to the periphery has long been considered as a potential mechanism in the development of autoimmune diseases. The thymocyte selection processes are highly dependent on their cross-talk between different thymic cell populations capable of expressing and presenting ectopic proteins to the developing thymocytes (1, 3). Consequently, the signals behind cellular migration, activation, differentiation and communication in the thymus are of critical importance in the development of T cell repertoire in the periphery.

Therefore, the low-grade constitutive expression of type 1 IFNs in the thymus is highly relevant as both the survival of thymocytes (59) as well as induction of thymic Tregs (60) have been shown to depend on signaling through IFNAR, the receptor for type 1 IFNs. Likewise, the expression of MHC and co-stimulatory molecules on APCs is dependent on inflammatory signals as is their activation and migration to the site of inflammation (61–63). Since TLR4 signaling through IRF3 activation on the other hand is a well-characterized inducer of type 1 IFNs (64), the expression of the endogenous TLR4 agonist, S100A8/A9 by post-Aire mTECs and HCs bears the potential to act as an upstream initiator of a constitutive inflammatory cascade capable of modifying all critical counterparts of the thymic cross-talk.



D

ID	Source	Term ID	Term name	adj. p-value (thymus)	adj. p-value (skin)
1	GO:CC	GO:0070062	extracellular exosome	1.83e-16	9.18e-05
2	GO:BP	GO:0002443	leukocyte mediated immunity	2.64e-10	3.43e-04
3	GO:BP	GO:0002252	immune effector process	3.77e-10	3.48e-03
4	REAC	REAC:R-HSA-6799990	Metal sequestration by antimicrobial proteins	1.17e-07	4.85e-02
5	REAC	REAC:R-HSA-6798695	Neutrophil degranulation	1.89e-06	4.59e-06
6	GO:BP	GO:0006887	exocytosis	5.85e-06	3.51e-04
7	REAC	REAC:R-HSA-168249	Innate Immune System	1.37e-05	6.31e-04
8	GO:MF	GO:0004866	endopeptidase inhibitor activity	1.03e-04	7.97e-04
9	WP	WP:WP477	Cytoplasmic Ribosomal Proteins	1.13e-04	ns
10	CORUM	CORUM:3055	Nop56p-associated pre-rRNA complex	1.57e-04	ns
11	CORUM	CORUM:306	Ribosome, cytoplasmic	2.63e-04	ns
12	CORUM	CORUM:6827	iNOS-S100A8/A9 complex	5.27e-04	ns
13	GO:MF	GO:0035662	Toll-like receptor 4 binding	7.34e-04	ns

FIGURE 1 | Results of the differential analysis. **(A)** Volcano plots of differential analysis. The x- and y-axis of the volcano plot show $\log_2$ of fold change (FC) and negative $\log_{10}$ of p-values respectively. There are altogether 6 volcano plots for each groupwise comparison shown in the title of the plot. The first source material name in the title always corresponds to the reference group and thus positive $\log_2$ FC indicates an increase in the later differentiation stage compared to the earlier. Proteins with adj. p-value ≤ 0.05 are colored red, proteins with adj. p-value >0.5 and ≤ 0.1 are colored blue and a selection of them are named by their underlying genes. Only some top genes are shown by names, for full list of genes please see **Table S1**, **S2**. The results of functional enrichment analysis of genes with adj. p-value ≤ 0.1 are visualized by Manhattan plots **(B, C)** that correspond to significant gene sets in the thymus and epidermis respectively. More specifically, these plots convey information about Gene Ontology (GO) with "MF" describing the molecular functions of the gene products, "BP" the biological processes in which they are involved in and "CC" the cellular component where the gene products are located. In addition, there are molecular pathways in which gene sets are enriched in (KEGG, REAC, WP), putative transcription factor binding sites (TF), information about targeted miRNAs (MIRNA), protein complexes (CORUM, HPA) and associated human diseases (HP). For further information please see g:Profiler (https://biit.cs.ut.ee/gprofiler). **(D)** shows further information about the selected GO terms in **(B, C)**. Some highly significant functional terms are not included in **(D)** due to virtual overlap with a functional pathway with even more significant p-value.

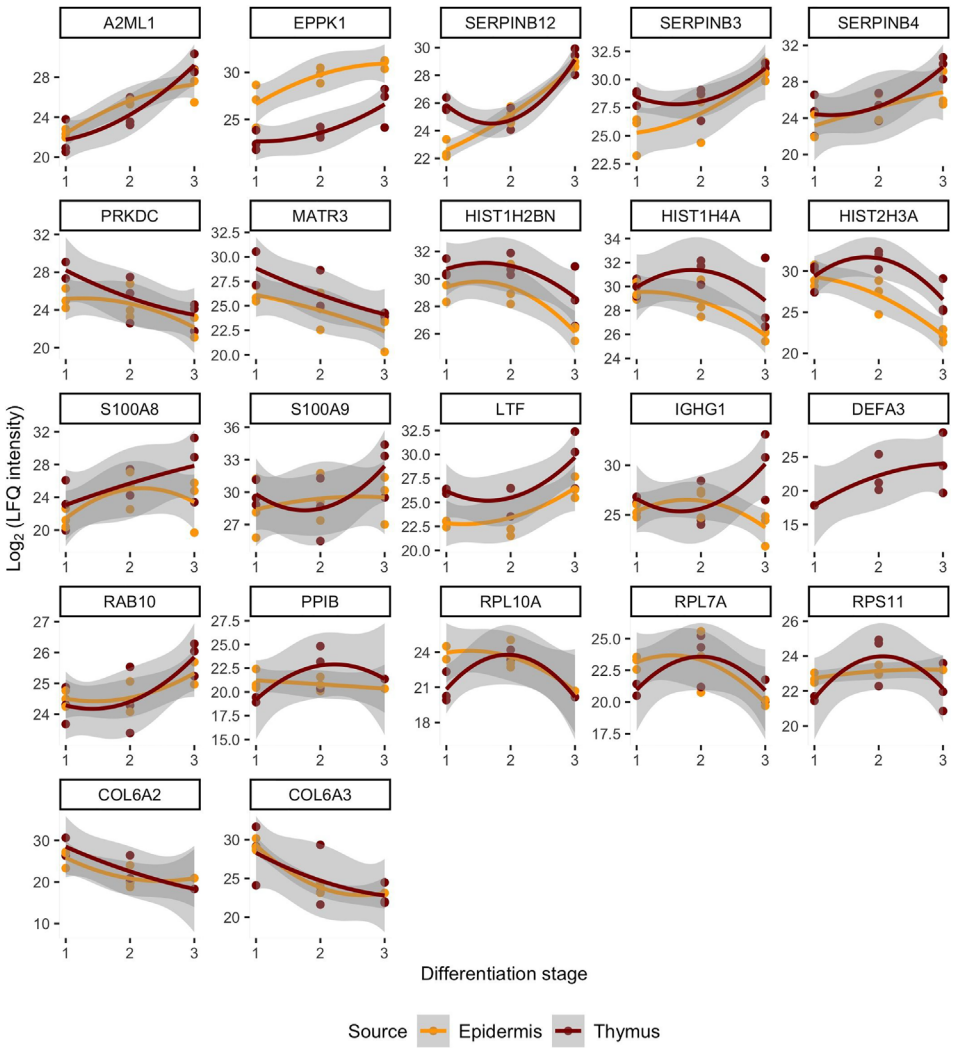


FIGURE 2 | Similarities of protein levels between epidermis and thymus with respect to the differentiation stages and organized based on the localization or function of proteins. The log_2 intensity levels (LFQ) of 22 proteins are shown in all samples. The x-axis corresponds to the source material that is denoted as differentiation stage. The differentiation stages 1, 2, 3 correspond in case of thymus to the mTEC, late mTEC, HC and in epidermis stratum basale, stratum spinosum and stratum granulosum + stratum corneum. The loess regression lines connect the values in those stages representing the change in protein levels during differentiation. Figure's first line shows proteins that are more specific to the epidermis, the second line corresponds to the nuclear proteins, the third one to inflammation related proteins, the fourth line to the translation associated proteins and final fifth one to the collagens.

The proposed role of post-Aire cells/HCs in creating the pro-inflammatory microenvironment by the expression of S100A family members is also supported by previous studies. Thus, in mice the post-Aire cells isolated by using a specific reporter are characterized by high expression of a variety of inflammatory genes, including S100A9 (7), whereas the single cell transcriptomics analysis in humans indicates S100A9 in the top 20 most highly expressed genes in the post-Aire (TEC III) population with several other (DEFB1, ANXA1, CXCL17) inflammation-related genes also belonging to the top 20 list (27).

Alternatively to its role as a TLR4 ligand, S100A8/S100A9 may have intracellular functions in mTECs as they form an LPS-inducible, heterotrimeric complex with iNOS, which elicits S-nitrosylation of GAPDH and a family of other proteins (65) A subsequent relocation of S-nitrosylated GAPDH to the nucleus triggers the cell stress response and apoptosis (66). mTECs constitutively express iNOS, which is upregulated after the contact with self-antigens or with thymocytes activated by TCR stimulation (67), and the expression of S100A8 and S100A9 genes is induced by AIRE (68, 69). We have earlier reported the blockage of AIRE-induced cell death by inhibiting the S-nitrosylation and nuclear translocation of GAPDH (70), suggesting that Aire may mediate the nuclear translocation of GAPDH by so far unknown mechanisms, and induce NO-induced cellular stress and apoptosis in post-Aire mTECs.

Another intriguing topic related to these findings is the previously shown lack of tonic inflammatory signaling in the Aire KO mouse (7) together with the defect in the differentiation of post-Aire populations and the development of autoimmune phenotype (10). Although the role of Aire in regulating mTEC maturation has been established years ago (18, 35), the block in differentiation in Aire KO mouse has mostly been connected to impaired expression of ectopic proteins as a potential mechanism behind the development of autoimmunity. However, as summarized above, there is now an increasing amount of evidence that the developmental block also results in decreased inflammatory signaling in the thymus that seems to be related to the lack of post-Aire mTECs and HCs. As the post-Aire cells and HCs appear immediately after Aire expression during mouse fetal development (34) and are present in high numbers during fetal and perinatal development in humans (42), the local environment modified by these post-Aire populations has the potential to refine the Aire-induced tolerance within the time window when Aire`s effect is the strongest, i.e. during the perinatal stage of development (11, 71). By contrast, another interesting phenomenon, the age-induced inflammatory signaling (26, 32, 72), appears much later when Aire's expression is severely down-regulated (73) and is, accordingly, less likely to have an effect in Aire-related changes. It remains to be determined whether alteration of the final steps in mTEC differentiation or fine-tuning of the inflammatory thymic microenvironment proves to be a useful target for the treatment of autoimmunity caused by Aire-deficiency or possibly for other defects in central tolerance induction.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Review Committee (ERC) on Human Research of the University of Tartu 170/T-i 28.04.2008. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

ML contributed to study design, analysis, presentation, and wrote the paper. AS contributed to data analysis and presentation. AK, KR, and RB contributed to collection of the samples. HP contributed to data analysis and presentation. PP contributed to study design and presentation. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.635569/full#supplementary-material

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REVIEW
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Age-Related Changes in Thymic Central Tolerance

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Thymic epithelial cells (TECs) and hematopoietic antigen presenting cells (HAPCs) in the thymus microenvironment provide essential signals to self-reactive thymocytes that induce either negative selection or generation of regulatory T cells (Treg), both of which are required to establish and maintain central tolerance throughout life. HAPCs and TECs are comprised of multiple subsets that play distinct and overlapping roles in central tolerance. Changes that occur in the composition and function of TEC and HAPC subsets across the lifespan have potential consequences for central tolerance. In keeping with this possibility, there are age-associated changes in the cellular composition and function of T cells and Treg. This review summarizes changes in T cell and Treg function during the perinatal to adult transition and in the course of normal aging, and relates these changes to age-associated alterations in thymic HAPC and TEC subsets.

Keywords: central tolerance, life span, thymus, thymic epithelial cells, dendritic cells

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INTRODUCTION

Throughout life, the immune system must balance the opposing goals of mounting protective responses against diverse pathogens, while preventing a breakdown in self-tolerance. Maintaining this tenuous balance is complicated by age-related changes in the number and composition of cells that comprise the innate and adaptive immune systems, as well as by changes in hematopoiesis, lymphoid and non-lymphoid tissue microenvironments, and an individual's history of pathogen exposure. Neonates encounter a barrage of new pathogens, requiring broad and rapid immune protection, at a time when their immune system is skewed towards mounting tolerogenic responses essential for tissue homeostasis (1). In contrast, following a lifetime of pathogen encounters, the T-cell compartment in older individuals contains a higher frequency of memory T cells, which can combat previously encountered pathogens, but often mounts poor responses to newly encountered pathogens and vaccines (2). (**Figure 1**). Immune responses to self-antigens also exhibit age-associated trends with the onset of many autoimmune disorders peaking in middle age (3) (**Figure 1**). Notably, there are some similarities between manifestations of immune dysregulation at both ends of the age spectrum, as neonates and elderly individuals have elevated susceptibility to various pathogens relative to adults, but less susceptibility to new-onset autoimmune disorders. For example, neonates are highly susceptible to respiratory syncytial

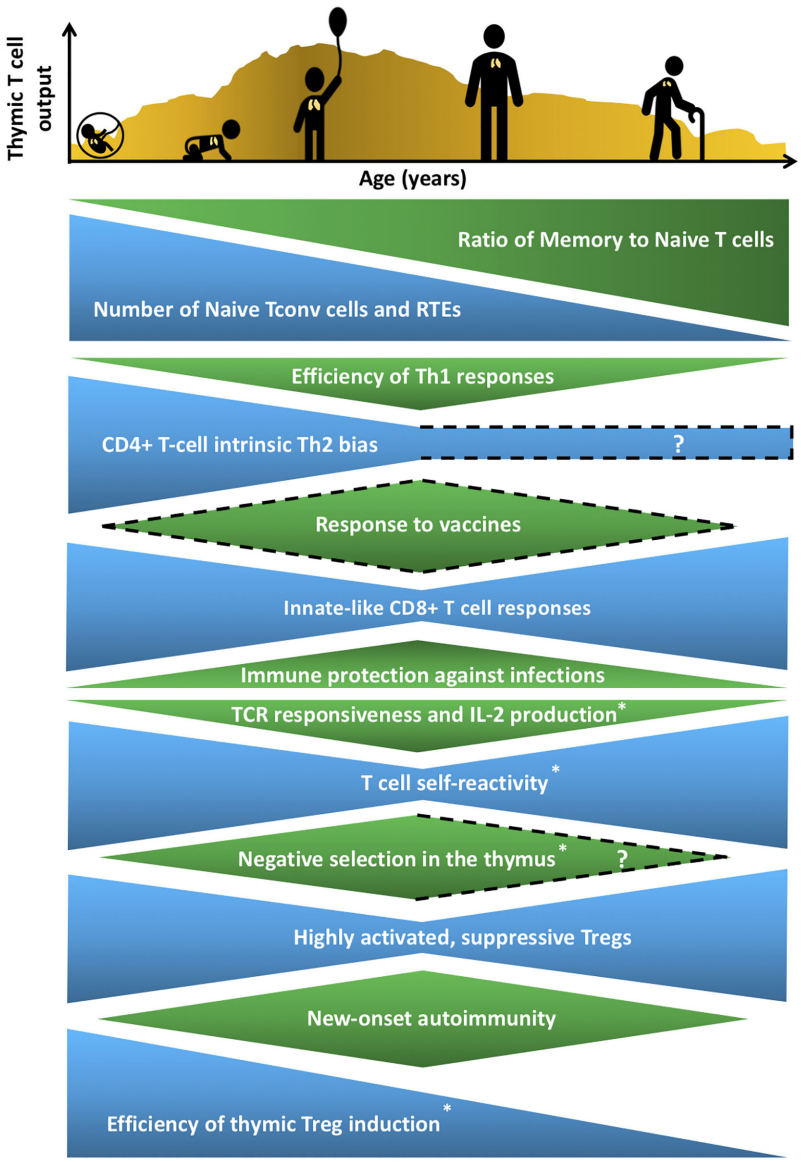

FIGURE 1 | Age-associated changes in T cell generation and function throughout the lifespan. The T cell landscape is in flux throughout life, shaped by age-associated changes in T-cell subset composition and function, which are influenced by cell-intrinsic factors as well as microenvironmental cues that support T cell development and differentiation. While the perinatal T cell pool is dominated by naive conventional T cells (Tconv) and recent thymic emigrants (RTEs), the aged T cell pool contains a higher proportion of memory T cells. Perinatal and aged T cells share several striking similarities in phenotypes and functions. The perinatal and aged CD8+ Tconv cells, including virtual memory T cells (Tvm), are shifted towards short-lived, innate-like, effector responses characterized by increased proliferative potential and rapid cytokine production, at the expense of long-lasting memory generation. Naive CD4+ T cells also display age-associated changes at both ends of the age spectrum, such as reduced T cell receptor (TCR) responsiveness and IL-2 production. In addition, T cells are more self-reactive both early and late in life, which may reflect age-associated changes in thymic selection and/or peripheral maintenance. Regulatory T cells (Treg) generation in the thymus peaks in the perinatal period, but Tregs at both ends of the age spectrum have superior suppressive capacity compared to adult Tregs. These age-associated changes implicate the thymic microenvironment in selecting Tconv cells and Tregs that cater to rapidly changing immune challenges throughout life, while at the same time curbing the risk of triggering autoimmunity. T cell output from the thymus is also lower in both fetal/neonatal periods as well as in the elderly. The uneven pattern of thymic output depicted in the histogram reflects variability throughout life due to numerous extrinsic stressors, such as infections and pregnancy, that alter thymic cellularity and output. In keeping with the above similarities between T cells in the perinatal and elderly stages, immune outcomes, such as overall responsiveness to vaccines and pathogens, as well susceptibility to new onset autoimmunity change in similar directions at both extremes of the lifespan. Phenotypes with question marks are yet to be defined clearly, and dotted lines indicate variable findings in the indicated attributes. All features have been reported in both humans and mice, except those denoted with an asterisk that indicates findings currently reported only in mice in the perinatal to adult and/or adult to aged transitions.

virus (RSV) (4), whereas elderly individuals often mount inadequate immune responses to influenza and West Nile viruses (5, 6). T cells play a central role in modulating the outcome of immune responses by integrating initial signals from the innate immune system with T cell receptor (TCR)-mediated antigen recognition to shift the balance in favor of pathogen-directed protective versus tolerogenic outcomes. Distinct T-cell subset composition, phenotypes, and effector functions have been identified in neonates and in aged individuals compared to younger adults, but the underlying mechanisms responsible for the distinctive age-associated features of T-cell immunity have not been fully established.

T cells develop in the unique tissue microenvironment of the thymus (**Figure 2**), in which thymic epithelial cells (TECs) and hematopoietic antigen presenting cells (HAPCs) provide indispensable signals for T-cell maturation and/or the establishment of self-tolerance. Bone-marrow derived hematopoietic progenitors are recruited from circulation into the postnatal thymus. These $CD4^-CD8^-$ "double negative" (DN) precursors then undergo T-cell lineage specification and differentiation in the thymic cortex. Following productive rearrangement of TCRβ gene segments, DN thymocytes initiate expression of the TCR co-receptors CD4 and CD8 and are referred to as "double positive" (DP) cells. DPs undergo TCRα gene rearrangements resulting in expression of functional αβTCR heterodimers that scan self-peptide/MHC complexes (pMHC) presented by cortical thymic epithelial cells (cTECs). Only thymocytes that express a TCR of sufficient affinity for either MHCI- or MHCII-peptide complexes are signaled to survive and further differentiate to $CD8^+$ or $CD4^+$ single positive (SP) lineages, respectively, through the process of positive selection (7, 8). A range of TCR affinities is compatible with positive selection, and the level of thymocyte self-reactivity has been shown to affect the subsequent threshold of peripheral T cell activation. Positively selected thymocytes migrate into the medulla, a region specialized for the induction of central tolerance. Within the medulla, TECs and HAPCs display a diverse array of self-peptides. Thymocytes expressing TCRs of relatively high affinity for self-pMHC are either triggered to undergo apoptosis, through the process of negative selection, or are diverted to a regulatory T cell (Treg) lineage to establish central tolerance (9). The combined outcomes of positive selection and central tolerance shape the specificity, diversity, and self-reactivity of the TCR repertoire in the peripheral T cell compartment.

Changes in thymus size and thymocyte cellularity are the most apparent age-related changes in the thymus. In both humans and mice, thymus size continues to increase in the neonatal period, then transitions to a homeostatic phase during early life, prior to the initiation of progressive age-associated involution. While the age-associated decline in size and output of T cells is conserved between mice and humans, one notable difference is that only human thymuses accumulate high levels of lipid laden adipocytes, which are interspersed with relatively small functional regions of thymic tissue (**Figure 3**). Accumulating evidence discussed below indicates that the cellularity and composition of TECs and thymic HAPCs change with age. As TECs and thymic HAPCs play critical roles in establishing central tolerance, age-related changes in the thymic microenvironment likely impact thymocyte selection, tolerance, and thus peripheral T cell responses throughout the lifespan. In this review, we focus on age-associated changes in the thymic microenvironment that can affect the diversity and self-reactivity of T cells that emigrate into the periphery to participate in immune responses. We first review the establishment of central tolerance and the roles of TECs and HAPCs in this process. We then discuss age-associated characteristics of conventional and regulatory T cell responses and how they may be linked to changes in thymic selection. Finally, we explore age-associated changes in the composition of HAPC and TEC subsets that may contribute to altered central tolerance and T cell activity throughout life.

THE PLAYERS IN THYMIC CENTRAL TOLERANCE

TCR gene rearrangements can generate $>10^{15}$ distinct TCRs, enabling recognition of an extensive array of diverse antigens (10, 11). Given the random nature of the TCR gene rearrangement process, it is inevitable that some TCRs will recognize self-antigens. To achieve self-tolerance, thymocytes must be screened for autoreactivity and either purged or directed into the Treg lineage to prevent autoimmunity. Multiple factors influence whether a self-reactive thymocyte will undergo negative selection or Treg lineage diversion. One critical determinant is the avidity of TCR binding to pMHC complexes presented by thymic APCs, which is a combined function of both individual TCR-pMHC binding affinities and the abundance of pMHC on APC surfaces. High-avidity binding results in thymocyte negative selection, eliminating autoreactive clones from the TCR repertoire (7). Selection into the Treg lineage is generally induced by somewhat lower avidity TCR-pMHC interactions (9). However, the broad and partially overlapping TCR repertoires of conventional T cells (Tconv) and Tregs (12) demonstrate that this cell fate decision is not dictated solely by TCR avidity. Another factor influencing fate choice is intraclonal competition for limited Treg niches. Thymocytes expressing a Treg-derived TCR transgene efficiently divert to the Treg lineage only when present at low clonal frequencies (9, 13, 14). Thus, the fate of a self-reactive thymocyte is determined by cell-intrinsic and -extrinsic factors. Cell-extrinsic factors include the abundance and local availability of self-pMHC (15–17), CD80 and CD86 co-stimulatory molecules (18, 19), and IL-2, with some contribution from IL-15 and IL-7 (20, 21). Altogether, multiple factors in the thymic environment shape the self-reactivity and diversity of emerging T cells, regulating their responsiveness to self- and foreign antigens.

A variety of thymic APC types present self-peptides to induce central tolerance. The importance of the thymic medulla in negative selection is well-established. Nevertheless, two studies

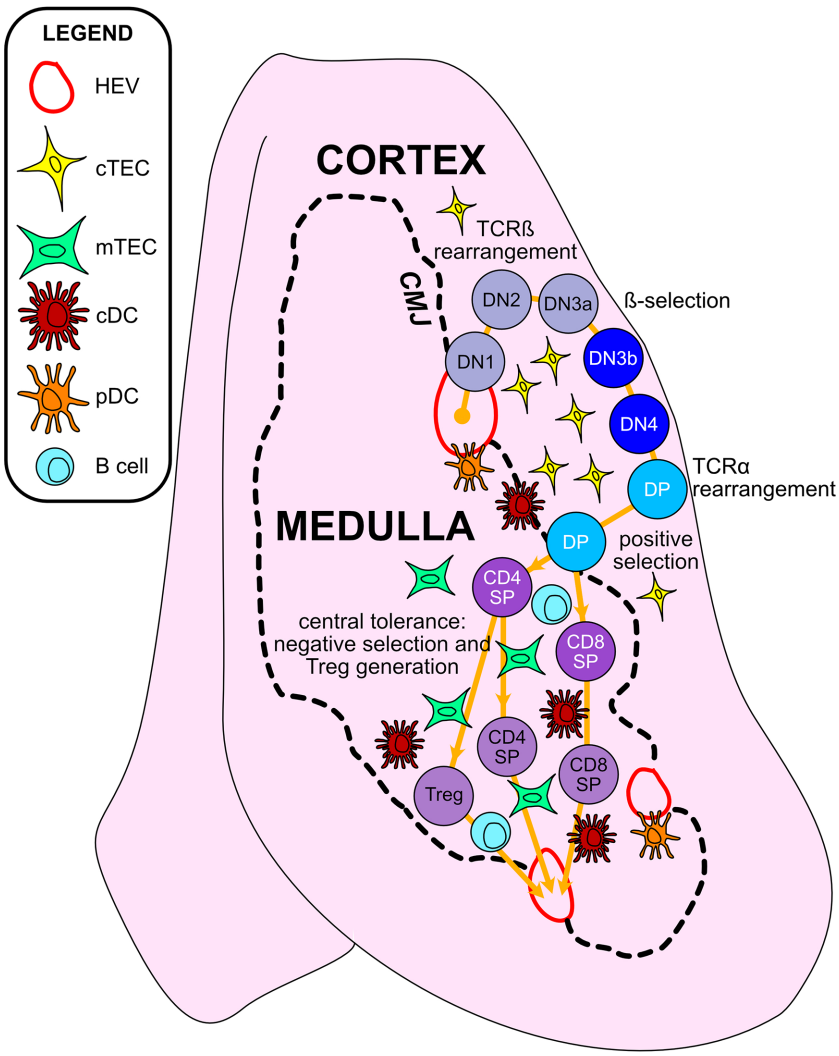

FIGURE 2 | Thymic epithelial cells and hematopoietic antigen presenting cells provide essential signals to guide αβT cell maturation and the induction of central tolerance in the thymus. Cross-sectional view of a thymus lobe reveals cortical and medullary regions, through which thymocytes must travel in an orchestrated manner to encounter heterogeneous stromal cell subsets. Progenitor cells from the bone marrow migrate through the vasculature to seed the thymus at the cortico-medullary junction (CMJ). DN1-DN4 thymocytes require signals from cortical thymic epithelial cells (cTECs) to support their survival, proliferation, and T-lineage commitment. During the DN2-DN3 stages, TCRβ gene segments are recombined, and thymocytes that successfully express TCRβ and signal through the pre-TCR undergo proliferation and further differentiation through the process of β-selection. Subsequently, thymocytes upregulate CD4 and CD8 to become double-positive cells (DPs), which initiate TCRα gene rearrangements. DPs that successfully express a TCRαβ heterodimer are tested for reactivity with self-peptide MHC complexes presented by cTECs. Only those DPs that receive a TCR signal pass positive selection, enabling them to survive and further differentiate. Positively selected DPs transit from the cortex into the medulla. Along the way, some clones may be deleted in an early wave of negative selection in the cortex, driven by strong TCR reactivity to self-peptide MHC complexes displayed by dendritic cells (DCs). In the medulla, DPs downregulate either CD4 or CD8 to become single-positive thymocytes (CD8SP or CD4SP) and interact with medullary APCs to establish central tolerance to a broad array of self-antigens. Strong TCR signals, induced by self-antigens displayed by medullary thymic epithelial cells (mTECs), conventional DCs (cDCs), plasmacytoid DCs (pDCs), or B cells result in either negative selection (apoptosis) or Treg diversion of the autoreactive T cell clones, enforcing central tolerance. SPs that survive these collective thymic selection processes emigrate from the thymus to join the peripheral T cell pool.

reported that most antigen-induced clonal deletion occurs in DP thymocytes, suggesting that cortical APCs can induce negative selection (22, 23). Although antigen presentation by cTECs is required for positive selection, cTECs have not been associated with negative selection (7). Instead, several studies indicate that HAPCs, such as DCs, in the cortex and near the cortico-medullary junction (CMJ) present ubiquitous self-antigens to induce cortical negative selection (24–26). Thymocytes clearly undergo negative selection at the DP stage, but further studies are needed to determine if DPs undergo negative selection in the cortex or medulla. Prior research predominantly relied on CCR7 expression as a proxy for cortical versus medullary localization of thymocytes undergoing negative section. However, live imaging studies indicate that positively-selected DPs can enter the

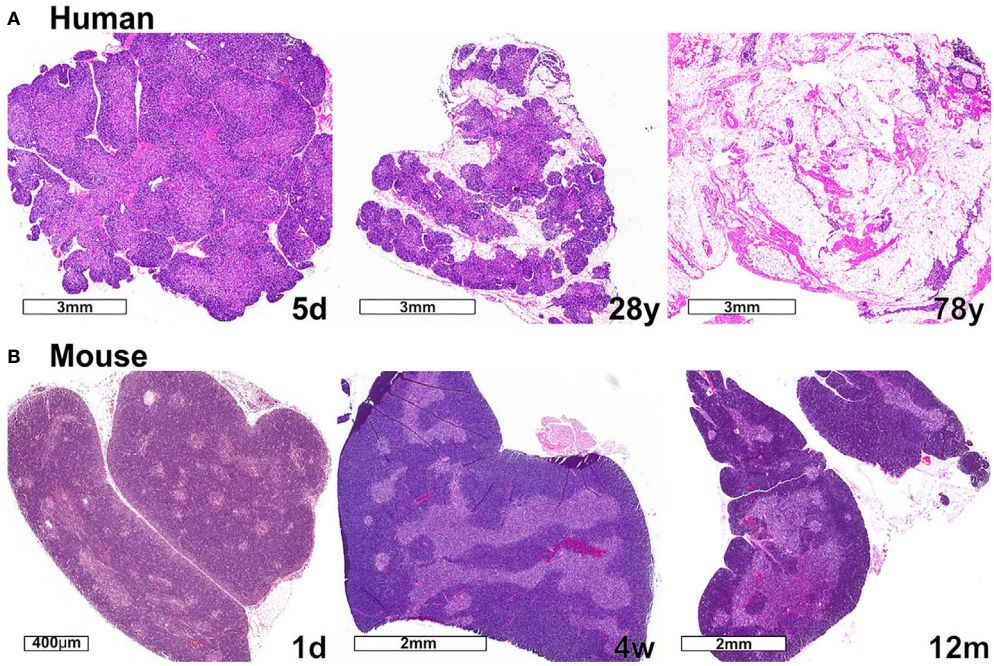

FIGURE 3 | Changes in thymic size, organization, and/or lipid content accompany age-associated thymic involution in humans and mice. **(A)** In humans, the percentage of the thymus comprised of functional thymic tissue progressively declines with age, and is replaced by adipose tissue, as shown in these hematoxylin and eosin-stained images. The percent of thymus area containing thymic epithelium, representing functional thymic tissue, was calculated *via* morphometric analysis of cytokeratin immunohistochemical slides. The results for the subjects shown are 91% at 5 days (5d), 55% at 28 years (28y), and 0.5% at 78 years (78y). **(B)** The mouse thymus grows substantially between postnatal day 1 (1d) (scale bar = 400 µm) and 4 weeks of age (4w) (scale bar = 2 mm), and then declines steadily and is highly involuted by 12 months of age (12m). The small islands of medullary tissue seen at 1d expand and coalesce to form the larger, more organized medullary regions characteristic of adult thymus (4w). Age-associated replacement by adipose tissue is not a prominent characteristic of involution in mice. The corresponding weights (mean ± SD) of murine thymus at the ages shown are 5 ± 0.5 mg at 1d (n = 3), 57 ± 8 mg at 4w (n = 8), and 38 ± 2 mg at 12 m (n = 3).

medulla before upregulating CCR7 (27, 28), raising the possibility that medullary APCs may also contribute to this early wave of DP negative selection.

Thymocytes are screened for reactivity against non-ubiquitous self-antigens primarily in the medulla. The requirement for medullary localization was demonstrated in mice deficient for the chemokine receptor CCR7 for its ligand CCL21, which together promote the medullary accumulation of post-positive selection SP thymocytes (29–33). In the absence of CCR7 signaling, thymocyte migration into the medulla is compromised, resulting in diminished central tolerance and subsequent autoimmune exocrinopathy (34). mTECs play a key role in negative selection due to their unique ability to collectively express >85% of the proteome, allowing them to induce central tolerance against a wide array of self-antigens (35–37). Importantly, mTECs express tissue-restricted antigens (TRAs), encoded by 2,000-3,000 genes that are otherwise expressed only in a small number of terminally-differentiated tissues (38–40). TRA expression is largely under control of the transcriptional regulator AIRE (35–37), which is expressed predominantly by MHCII^{hi} CD80^{hi} mature mTECs (41, 42). AIRE deficiency impairs mTEC maturation and prevents expression of Aire-dependent TRAs, resulting in failed central tolerance and export of autoreactive T cells that induce multi-organ autoimmunity (43, 44). Analogous to *Aire*-deficient mice, autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED) patients have mutations in the *AIRE* gene, resulting in autoimmunity affecting multiple endocrine glands (45, 46). TCR repertoire analysis of Treg and Tconv cells from *Aire*-deficient and -sufficient mice demonstrated that AIRE is also required to select autoreactive clones into the Treg lineage (47, 48).

In addition to expressing diverse self-antigens, mTECs play a critical role in central tolerance by directly presenting self-antigens to thymocytes. MHCII^{hi} mTECs express the costimulatory molecules CD80 and CD86, which are required for negative selection and Treg induction (49, 50). Reducing MHCII expression selectively in mTECs revealed that efficient negative selection requires antigen presentation by mTECs (51). Moreover, tolerance to the *Aire*-dependent RIP-mOVA neoantigen remained intact when MHC was expressed only by TECs, and not by thymic HAPCs (52, 53). Furthermore, our imaging studies using thymic slices from RIP-mOVA transgenic mice, in which MHC is physiologically expressed by both mTECs and HAPCs, revealed that AIRE+ mTECs contribute to roughly half of the negatively-selecting interactions with both MHCI and MHCII-restricted thymocytes (54). mTECs also have the capacity to induce Tregs independently of HAPCs (55).

Interestingly, while AIRE^{+}MHCII^{hi} mTECs clearly express and present diverse TRAs that are essential for central tolerance, recent findings demonstrate that MHCII^{lo} mTECs also contribute to selection of the T cell repertoire (56). Thus, mTECs play a pivotal role in central tolerance, not only as sources of TRAs, but also as APCs that directly present self-antigens to thymocytes to induce central tolerance.

The importance of HAPCs, in particular thymic dendritic cells (DCs), in negative selection is well-established, as genetic ablation of DCs leads to defective central tolerance and autoimmunity (57). DCs efficiently mediate negative selection and Treg induction in reaggregate thymic organ cultures (RTOCs) (58). *In vivo*, DCs can acquire antigens in peripheral tissues and traffic them into the thymus to induce tolerance (59), and those positioned near thymus vasculature can acquire and present blood-borne antigens (60, 61). Thymic DCs also acquire and present mTEC-derived TRAs (53, 62, 63). Thymic DCs with an activated signature, including elevated expression of MHCII and CD86, function as highly efficient APCs (64). Thymocyte-DC crosstalk is important for DC maturation and function. CD40L on SP thymocytes induces CD40 signaling in DCs, which is required for DCs to induce Tregs *in vitro* (65). Interestingly, TCR repertoire sequencing demonstrated that mTECs and DCs select distinct clones into the Tconv and Treg repertoires (48, 66). Our imaging studies also indicated that DCs present mTEC-derived TRAs to MHCI- and MHCII-restricted thymocytes (54). For both monoclonal TCR transgenic and polyclonal thymocytes, DCs were engaged in slightly more than half of the interactions between thymocytes and APCs that induced TCR signaling (54), further supporting the fundamental contribution of DCs to central tolerance. Collectively, these studies demonstrate that both mTECs and DCs are required to establish self-tolerance.

DCs are a heterogenous group of HAPCs that include conventional DCs (cDC) and plasmacytoid DCs (pDCs) (67). The mouse cDC1 subset expresses CD8α and XCR1, and the cDC2 subset expresses CD11b and Sirpα/CD172a (68). While both cDC subsets contribute to tolerance induction, thymic cDC2s have greater CD4+ T cell stimulatory capacity (69) and are especially proficient at Treg induction (70, 71). The cDC1 subset plays a role in clonal deletion, but was reported to be dispensable for Treg induction and to have a negligible impact on the Treg TCR repertoire (72, 73). In contrast, other studies concluded that cDC1s are essential for inducing Tregs in response to mTEC-derived antigens (48, 66). Thus, further studies are needed to resolve the contributions of distinct DC subsets to central tolerance. When compared to cDCs, pDCs in the thymus have a reduced capacity to stimulate T cells and to acquire antigens from TECs (74). However, CCR9+ pDCs can transport peripheral antigens to the thymus to induce negative selection (75). In humans, comparable cDC1 and cDC2 subsets have been identified and defined by expression of CD141 and CD1c, respectively (68). It has been technically challenging to dissect the roles of human thymic APC subsets in establishing central tolerance, although *in vitro* studies have confirmed that DCs have tolerogenic capacity (76), including the ability to induce Tregs (77). Human thymic cDCs are activated by thymic stromal lymphopoietin (TSLP), which is expressed by Hassall's corpuscles that consist of terminally differentiated mTECs, and the activated CD80^{hi} CD86^{hi} cDCs can induce Tregs *in vitro* (78). Like cDCs, human thymic CD123+ pDCs support Treg induction *in vitro* (79, 80). Thus, multiple thymic DC subsets have been shown to promote central tolerance, though the distinct contributions of DC subsets are not entirely resolved.

B cells have also been shown to contribute to central tolerance. Thymic B cells are localized in the medulla and express high levels of MHCII, CD80, and CD86, distinguishing them from splenic B cells (81, 82). Thymic B cells with specificity for self-antigens can present self-peptides to CD4SPs, driving activation-induced cytidine deaminase (AID)-dependent B cell class switching. Class-switched thymic B cells promote negative selection (81, 82). CD40 activation in thymic B cells, driven by CD40L on SP thymocytes, is required to support B cell proliferation, differentiation, and class switching, as well as upregulation of *Aire*, and these licensed B cells present self-antigens to induce negative selection (83, 84). Thus, B cells may play a significant role in central tolerance, but whether licensed B cells are autoreactive and the nature of the self-antigens they present remain to be resolved. Collectively, these studies demonstrate the cooperative roles of multiple TEC and HAPC subsets in enforcing central tolerance against a broad range of self-antigens.

CHANGES IN Tconv FUNCTION AND THYMIC SELECTION THROUGHOUT THE LIFESPAN

Function of Tconv Cells in the Perinatal Period

Tconv cells generated during the perinatal period face the daunting task of mounting a rapid, protective immune response against a sudden surge of pathogen encounters, while at the same time ensuring they do not trigger autoimmunity. Neonatal T cells differ substantially from adult T cells in composition and function, helping them to achieve this balance (85). Generally, T cell responses in neonates are diminished relative to those of adults. This may partly be attributed to a shift in the ratio of naive to memory subsets, as naive T cells are predominant in perinatal tissues, whereas memory T cells become more abundant in adults (86). In keeping with this concept, T cells from pediatric lymph nodes (LNs) produce relatively lower levels of cytokines, including IFN-γ, IL-2, and IL-4 relative to adult T cells (86). Moreover, in the context of infections such as malaria (87) and congenital Cytomegalovirus (CMV) (88) human neonatal T cells express lower levels of Th1 and Th2-associated cytokines compared to adult T cells. Cell-intrinsic properties of perinatal T cells such as high PD-1 expression (88), low NFAT expression (89) and diminished Ca^{2+} influx after TCR stimulation (90) may

contribute to the diminished responsiveness of neonatal T cells. Overall, the relative paucity of memory T cells and reduced functionality of T cells in the perinate are consistent with increased susceptibility to infection in early life.

Multiple studies in mice and humans have demonstrated that neonatal T cell responses are strongly skewed towards Th2 versus Th1 differentiation (91–95). Mouse neonatal T cells and human cord blood T cells readily produce the Th2 cytokines IL-4 and IL-13 following *in vitro* stimulation (92, 94–96). Also, CD4+ T cells isolated from neonatal mice immunized with bacille Calmette-Guerin (BCG), Tetanus toxoid and other vaccines expressed higher levels of IL-5 and lower IFN-γ upon antigen re-stimulation *in vitro*, compared to adults (97). The neonatal Th2 bias is due at least in part to permissive epigenetic regulation of Th2-associated cytokine genes (95, 98). Also, the IFN-γ promoter region is hypermethylated in cord blood CD4+ T cells consistent with their deficient production of IFN-γ after *in vitro* stimulation (99). Moreover, stimulated CD4+ cord blood T cells express higher levels of GATA3, a key transcriptional regulator of Th2 fate (94, 100). The strong Th2 bias could be beneficial both in suppressing development of damaging inflammatory Th1 responses, as well as in promoting tolerance towards allogeneic maternal antigens *in utero*. Consistent with the latter idea, cord blood from preterm infants contains higher levels of proinflammatory cytokines and alloreactive Th1-like central memory CD4+ T cells, which were absent in term infants, suggesting their potential role in promoting premature uterine contractions (101). However, Th2-skewing could leave the newborn vulnerable to infections and unable to respond to some vaccines, which require Th1 responses (102–104).

Interestingly, studies have demonstrated that with appropriate stimuli, such as exogenous IFN-γ and IL-12 (105, 106), exposure to helminth and mycobacterial antigens (107), low viral doses (108), various adjuvants (93), or DNA vaccines (109), neonates can mount Th1-like responses in addition to Th2 responses (110–113). In contrast to findings in mice (97), BCG vaccination of infants induces a strong Th1 response, comparable to adults, supported by high IFN-γ and low IL-4/IL-5 expression after antigen re-stimulation *in vitro* (112, 113). Moreover, Th1 responses are elicited by CMV in the fetus and *B. pertussis* in infants (88, 114). The capacity of neonatal T cells to mount a Th1 response under some conditions may reflect the extent of DC maturation, as mycobacterial and pertussis toxin antigens are particularly effective at activating DCs (115, 116). Nonetheless, studies with neonatally immunized mice suggest that while Th1 responses can be induced in adults following antigen re-challenge, Th2 memory responses still predominated (93, 117). In addition, while a balanced Th1 and Th2 primary response could be induced in neonates early after exposure to a foreign antigen, a Th2 secondary response was dominant in mice re-challenged as adults (111).

Cell-intrinsic properties of neonatal T cells, as well as extrinsic microenvironmental cues have been implicated in driving the reduced responsiveness and Th2 bias of neonatal T cell responses. Adoptive transfer experiments in mice revealed that Th2 skewing was observed only when fetal, but not adult CD4+ T cells were primed regardless of whether the host microenvironment was fetal or adult (110, 118, 119). These results suggest a cell-intrinsic difference in the fate potential of neonatal versus adult CD4+ T cells. Interestingly, when both Th1 and Th2 responses were elicited by primary antigen challenge in neonates, Th1 cells upregulated IL-13Rα1 which associated with IL-4Rα (119). Upon antigen re-challenge, the activated Th2 cells secreted IL-4 which bound the IL-4Rα/IL-13Rα1 heterodimer, triggering Th1 apoptosis, tipping the balance towards Th2-mediated immunity. Moreover, upregulation of IL-13Rα expression during initial activation of Th1 cells is developmentally regulated; antigen exposure after postnatal day 6 does not induce IL-13Rα expression. These results are due to the delayed maturation of a subset of splenic CD8α+ cDC1s, which secrete IL-12 that inhibits IL-13Rα expression on Th1 cells (120). These findings demonstrate that cell extrinsic factors can regulate the Th2 bias in neonates.

Neonatal CD8+ T cell responses also differ from their adult counterparts (reviewed in (85)). Co-transfer of neonatal and adult CD8+ T cells into adult recipients revealed a cell-intrinsic bias of neonatal cells towards a short-lived effector fate, whereas adult T cells differentiated into both effector and memory subsets (121). Thus, upon pathogen re-challenge, the immune response was dominated by adult CD8+ T cells. Further studies demonstrated that neonatal and adult CD8+ T cells are derived from distinct hematopoietic progenitors (122). Notably, neonatally-derived CD8+ T cells persist into adulthood, where they continue to play an important role in responding to pathogens due to their preferential differentiation into effectors that proliferate rapidly and produce cytokines (123, 124). In contrast, adult-derived CD8+ T cells in the same environment have a greater propensity to generate memory T cells (124). In uninfected mice, CD8+ T cells generated during the neonatal period tend to differentiate into "virtual memory" T cells (Tvm), expressing high levels of CD44, Eomes, and CD122, and they proliferate more rapidly and differentiate into short-lived effector cells following pathogen challenge, mirroring the neonatal CD8+ T cell pool (122, 124, 125). Consistent with findings in mice, human cord blood CD8+ T cells are also highly proliferative upon TCR stimulation (123), and undergo bystander activation, producing IFN-γ, TNFα, or IL-4, depending on the cytokine receptor (126, 127). Collectively, these findings suggest that the functional potential of neonatal naive CD8+ T cells is biased towards an innate-like effector phenotype.

Thus, perinatal CD4+ and CD8+ Tconv cells have distinct functional properties compared to their adult counterparts. Both cell-intrinsic changes in differentiation potential and priming by different microenvironmental cues result in CD4+ T cell responses biased towards a Th2 or Treg (see below) fate, which may protect the neonate from damaging inflammatory Th1 responses at a time when tissue homeostasis, including responses to commensal colonization, is being established. During this period, CD8+ T cells are biased to differentiate into short-lived effector cells, which can rapidly combat pathogenic threats at the expense of generating memory responses.

Selection of Tconv Cells in the Perinatal Period

Previous studies suggest that negative selection is impaired in the perinatal compared to the adult thymus, resulting in decreased deletion of self-reactive thymocytes (69, 128, 129). In mice, susceptibility to experimental autoimmune encephalomyelitis (EAE) decreases between the perinatal and adult period, which correlates with increasing age-dependent negative selection of MBP (myelin basic protein) specific T cells (129). Also, in mice and humans, Tconv cells that mature in the perinatal thymus express higher levels of CD5 and Nur77 compared to those generated in adults (130, 131). CD5 and Nur77 levels correlate with TCR affinity for peptide-MHC (132, 133), suggesting that perinatal Tconv cells are more self-reactive compared to those generated in the adult thymus. While heightened self-reactivity could reflect impaired central tolerance, as discussed above, it is also possible that the threshold for positive selection is higher in the perinatal thymus, such that thymocytes with low-affinity TCRs are not efficiently positively selected, resulting in elevated CD5 levels on perinatal T cells (130). Regardless, higher TCR self-reactivity could enable T cells to respond quickly and effectively against multiple foreign antigens, despite the limited perinatal TCR repertoire (131, 134). Studies in mice have suggested another potential advantage of increased TCR self-reactivity: neonatal recent thymic emigrants enter a lymphopenic periphery, where $CD5^{hi}$ T cells outcompete their $CD5^{lo}$ counterparts for CD28 co-stimulation due to their increased affinity for self-pMHC. Their resultant increased sensitivity to the homeostatic cytokines IL-7 and IL-15 promote lymphopenia-induced proliferation to fill empty niches (135–139). Moreover, $CD5^{hi}$ T cells have skewed effector potential in the periphery. $CD5^{hi}$ CD4+ T cells are more prone to differentiate into Tregs (140), while $CD5^{hi}$ CD8+ T cells express effector molecules such as Eomes, T-bet and Helios that promote T cell differentiation to an effector or virtual memory fate (134, 139). Thus, the increased self-reactivity of T cells selected in a neonatal thymus likely contributes to the characteristic rapid proliferation of neonatal CD4+ and CD8+ T cells in response to cytokine or antigen stimulation, as well as the altered differentiation potential biasing neonatal CD8+ T cells to become short-lived effector cells or Tvm, and CD4+ T cells to adopt a Treg fate. These studies emphasize that the strength of TCR signaling during thymic selection not only determines lineage fate decisions in the thymus but also influences peripheral effector T cell function.

Declining Function of Tconv Cells During Aging

It is well established that T cell function declines with age, correlating with increased morbidity and mortality to infectious diseases and reduced responses to vaccination (2, 141, 142). While following a general pattern of age-associated decline, there is increased variability in immune responses between individuals with age, due in part to their lifetime histories of acute and persistent pathogen encounters (143, 144). As in the perinatal period, both cell-intrinsic and microenvironmental changes contribute to the age-associated decline in T cell function; however, the complex mechanisms that drive waning T cell immunity are not yet fully resolved (2).

CD4 T cells exhibit multiple functional changes with age. Reduced expression and production of IL-2 has been demonstrated following stimulation of mouse CD4+ T cells (145, 146). There is evidence for reduced IL-2 production in CD4+ T cells from elderly humans, but this decline has not been universally observed (2). CD4+ T cells from old mice were found to be functionally deficient in B cell activation, indicating reduced T follicular helper cell (Tfh) activity with age (147). Consistent with this notion, an age-associated decline in Tfh responses, along with diminished class-switched antibody levels were reported following viral infections in mice, non-human primates, and humans (143, 148). In addition, aging is associated with reduced IFN-γ^{+} CD4 T cell responses to viral pathogens (143, 149). Age associated defects in CD4 activity could result from impaired T cell priming as aged CD4+ T cells exhibit cytoskeletal defects and poor immunologic synapse formation (150), reduced calcium flux upon TCR cross-linking (151), and defective metabolic reprogramming upon activation (152). These findings suggest cell-intrinsic defects impair CD4+ T cell responses in aged individuals. Cell-extrinsic defects also contribute to the decline in CD4 T cell function with age. For example, aged CD4+ T cells showed reduced homing to LNs following viral infections, despite the finding that expression levels of LN homing molecules (CCR7, CXCR4, PSGL1, and LFA1) were not decreased (148). However, levels of CCL21, which recruits naive T cells to LNs, were lower in draining LNs from old mice early after infection (148). Further support for cell-extrinsic defects was demonstrated by studies showing that the LN microenvironment deteriorates with age due, in part, to reduced IL-7 presentation and increased fibrosis (2, 153, 154),

Because T cells consist of multiple functionally distinct subsets, the defects in T cell function with age described above could reflect a change in subset composition and/or alterations in activity on a per-cell basis. Indeed, phenotypic analyses revealed an age-associated reduction in the proportion and numbers of naive T cells in humans, non-human primates, and mice (154–158). Recent comprehensive single-cell transcriptional profiling studies confirm the shift towards a higher frequency of effector-memory T cell subsets with age (159, 160). Notably, in mice, aging was associated with a stark increase in representation of cytotoxic CD4+ T cells, exhausted CD4+ T cells, and activated Treg (159). The shift to an increased frequency of these CD4+ T cell subsets correlated with elevated levels of cytokines associated with inflammaging, such as IL-27, IFN-β, and IL-6. Thus, the altered distribution of CD4+ T cell subsets likely has a profound impact on immune responses with age. However, such alterations do not fully account for age-related changes in T cell function. For example, antigen-inexperienced CD4+ recent thymic emigrants (RTEs) from old mice produce less IL-2 and proliferate poorly after *in vitro* stimulation compared to young RTEs (161). In addition, naive CD4+ T cells from older mice have a longer lifespan, reflecting increased *Bim* expression, but proliferate poorly after *in vitro* and *in vivo* stimulation (162, 163). Although profound functional deficiencies in naive human CD4+ T cells have not been reported, naïve CD4+ T cells from elderly humans exhibit reduced TCR signaling and expansion following *in*

vitro stimulation due, at least in part, to the age-associated decline in miR-181a expression (164–166). Interestingly, naive polyclonal CD4+ T cells in aged mice are more self-reactive, as indicated by increased CD5 expression, display higher TCR affinity for foreign antigens, and are more promiscuous in antigen recognition. The increase in self-reactivity and promiscuity of the aged CD4+ T cell compartment implicate altered thresholds of CD4+ T cell selection in the thymus with age (155).

Defects in CD8+ T cell responses with age are well established. Early studies reported defective CD8+ T cell responses following primary and secondary influenza challenges (167). Additionally, CD8+ T cells have an age-associated decrease in their capacity to proliferate and produce effector molecules, such as IFN-γ, following *in vitro* stimulation or infection with viral or bacterial pathogens (168–175). In humans, the frequency of activated CD8+ T cells induced by yellow fever vaccination was significantly diminished with age (176), underscoring the potential impact of a declining CD8+ T cell compartment on vaccine-induced as well as on natural protection against pathogens (143).

Similar to CD4+ T cells, the overall decline in CD8+ T cell function with age could reflect changes in the proportions of functionally distinct CD8+ T cell subsets. Indeed, one of the most notable hallmarks of the aged immune system in humans and mice is a substantial decline in both the number and frequency of naive CD8+ T cells (143, 173, 177). At the same time, the CD8+ T cell pool becomes progressively enriched in clonally expanded, antigen-inexperienced CD8+ Tvm cells in mice and in humans (139, 174, 177–180). The homeostatic cytokine IL-15 is required for differentiation and function of Tvm cells (139), which in turn respond to IL-12 and IL-18 stimulation in a TCR-independent manner, resulting in secretion of IFN-γ (139, 174, 177–179). Notably, Tvm can provide antigen-independent bystander protection in bacterial infections, proliferating more rapidly than naive T cells, but differentiating preferentially into short-lived effector cells (181), strikingly reminiscent of perinatal Tconv cells. While Tvm cells can provide effective protection against pathogens in a bystander or TCR-dependent manner (139, 181), and increase in frequency with age, there is a seemingly incongruous age-associated decline in the overall response of CD8+ T cells to pathogen challenge. Previous studies partially resolved this conundrum by showing that aged Tvm in mice and humans have a reduced capacity to proliferate in response to TCR stimulation relative to young Tvm. The mouse Tvm response to homeostatic cytokines is sustained with age, but whether human Tvm have a similarly sustained response has not been tested (173, 174). Regardless of age, Tvm mount a monofunctional cytokine response to TCR stimulation, while naive CD8+ T cells produce multiple cytokines in response to mouse influenza infection (173, 174). These studies indicate that with age Tvm cells accumulate in the CD8+ compartment, respond poorly to TCR stimulation, and produce a less diverse cytokine response. In contrast, while naive CD8+ T cells retain a robust capacity to proliferate to TCR stimulation with age, they do not survive or proliferate well in response to the homeostatic cytokines IL-2 and IL-15, explaining the decreased proportion of naive CD8+ T cells relative to Tvm with age (173, 174). Transcriptional profiling revealed that Tvm cells that accumulate with age have a senescent signature, consistent with the lower frequency of cells that respond to TCR stimulation as well as the reduced burst size of individual responding cells (173). Together, these results partially explain why the composition and function of the CD8+ T cell compartment changes with age. Additional insights into the declining function of aged CD8+ T cells were revealed in a recent single cell transcriptional profiling study that identified a subset of CD8+ T cells that expresses and secretes granzyme K (GZMK) and accumulates with age in mice and humans (160). In contrast to Tvm, age-associated GZMK+ CD8+ T cells have a transcriptional profile and surface marker phenotype (PD-1+ Tox+) consistent with a state of terminal exhaustion (160). Strikingly, upon TCR stimulation, these cells secrete GZMK, which alone or in combination with IFN-γ, induces fibroblasts to secrete pro-inflammatory factors, such as IL-6 and CCL5. Thus, GZMK+ CD8 T cells may contribute to inflammaging. GZMK+ CD8+ T cells also express the integrin CD49d, reminiscent of a previously described clonally expanded CD49d+ CD8+ T cell subset in aged mice (182). These cells home to multiple tissues and fail to secrete granzyme B (GZMB) or IFN-γ upon TCR stimulation, further distinguishing them from Tvm (160). Notably, single-cell TCR repertoire analysis of human PBMCs revealed that the well-documented clonal restriction of the CD8+ T cell pool with age (177, 183–185) was due in part to clonal expansion of this novel GZMK+ CD8+ T cell subset, which was distinct from the clonally expanded GZMB-producing cells that are enriched for recognition of CMV or Epstein-Barr virus (EBV) (158, 186). Clonal expansion of Tvm with age has also been reported (174, 177, 185). Collectively, these studies reveal that aging is associated with a profound shift in the composition of CD8+ T cell subsets, resulting in reduced responses to newly encountered antigens and a shift towards a pro-inflammatory phenotype.

Age-associated changes in the composition of the CD8+ T cell compartment could reflect cell-intrinsic and/or extrinsic influences. Several lines of evidence indicate that the aged environment is a causative factor in the decline in CD8+ T cell functionality. When young naive CD8+ T or Tvm cells are transferred into an aged host, their proliferative potential declines (173). Similarly, an aged host environment induces young CD8+ T cells to adopt an exhausted phenotype, including upregulation of GZMK (160). Additionally, in heterochronic parabiosis experiments fewer young CD8+ T cells were recovered in old compared to young partners (187). Conversely, in each of these studies, the young environment did not restore function, cellularity or phenotype to old CD8+ T cells. Strikingly, the number of T cells declined in the lymph node of a young mouse when parabiosed to an old partner (187). Together, these data indicate that the old environment contains soluble factors that negatively impact CD8+ T cell cellularity and function. Additional cell-extrinsic influences that can diminish CD8+ T cell responses with age include ineffective antigen presentation by aged DCs (188, 189) and disrupted architecture of secondary lymphoid organs that could impair recruitment, maintenance or priming of CD8+ T cells (153, 154, 190, 191). Despite clear evidence that cell-extrinsic factors in the aged environment modulate CD8+ T cell responses, there is evidence that age-associated cell-intrinsic changes also contribute to diminished T cell responses with age.

In addition to the declining responsiveness of aged Tvm to TCR stimulation (173, 174), another characteristic of Tvm cells that accumulate with age is their increased self-reactivity, as reflected by elevated expression of CD5 (139, 185). Furthermore, there is an apparent enrichment in naive T cells with higher CD5 levels in the CD8+ T cell repertoire with age (177, 192), and naive CD8+ T cells expressing higher levels of CD5 have an increased propensity to differentiate into Tvm cells. Together, these data indicate that the naive CD8+ T cell pool is more self-reactive with age. Further studies are needed to determine whether the increased self-reactivity of naive T cells is driven by age-associated cell-extrinsic changes in the thymic microenvironment that affect selection thresholds, peripheral maintenance of self-reactive T cells, and/or intrinsic transcriptional profiles of T cells that alter their capacity to respond to TCR signals.

Changes in Negative Selection of Tconv Cells During Aging

Aging induces profound changes in the thymic microenvironment (see section *Changes In Thymic Apcs And Implications For Selection Throughout The Lifespan*), which could negatively affect central tolerance. For example, TRA expression decreases with age (193, 194), reflecting both a decline in the frequency of *Aire*+ mTECs and reduced *Aire* expression per mTEC (195, 196). Thus, thymocytes may not encounter the full spectrum of self-antigens responsible for central tolerance in an aged thymus, potentially contributing to the increased incidence of autoimmunity with age. Consistent with this possibility, *Aire* haploinsufficiency results in decreased negative selection and an increased incidence of diabetes (197). Also, in an inducible *Foxn1*-deletion model of accelerated thymic atrophy, TRA expression was reported to decline, and negative selection was impaired (198). In addition to age-associated changes in TECs, changes in thymic B cells could impact central tolerance during aging. The number and frequency of thymic B cells increase in old mice; however, their expression of *Aire* and TRAs diminishes with age (199–201). A decline in AIRE-dependent TRA expression is also observed in human thymic B cells (199). Despite the clear association between aging and thymic involution, and recognition that the thymic microenvironment is critical for establishing self-tolerance, surprisingly little is known about the impact of aging on central tolerance. Further investigations are needed to determine if central tolerance is altered during aging, to elucidate the underlying mechanisms, and to determine the impact on autoimmunity.

CHANGES IN Treg FUNCTION AND THYMIC SELECTION THROUGHOUT THE LIFESPAN

Function of Tregs in the Perinatal Period

The critical role of Tregs in suppressing damaging inflammatory immune responses in a broad range of tissues has been well documented [reviewed in (202)]. Immunodysregulation polyendocrinopathy enteropathy X-linked (IPEX) patients, in whom Treg lineage differentiation is impaired, develop severe gastrointestinal pathology, type-1 diabetes mellitus and severe skin inflammation, in addition to other autoimmune manifestations within the first few weeks to months after birth (203–206). Studies in mice have demonstrated that organ-specific Tregs play a crucial role in promoting peripheral tolerance in both lymphoid and non-lymphoid organs (207–209). Tregs control inflammatory T cell responses towards food antigens (210) and commensal microbiota in the gut (211), and intestinal Tregs have been shown to expand in response to microbial cues (211–214). Tregs also migrate to the hair follicles in the skin, where they are critical for tolerance to skin commensals (215, 216). Retinal antigen-specific Tregs in the eye control inflammation in experimental autoimmune uveitis and help resolve disease pathology (217, 218). Other experimental models of organ-specific diseases such as diabetes (219) and EAE (220) have reinforced the crucial role played by Tregs in suppressing autoimmune pathology.

Tregs control T cell responses through multiple mechanisms (221, 222). For example, expression of CTLA-4 on Tregs reduces the ability of DCs to stimulate T cell responses by masking the costimulatory molecules CD80 and CD86 (223). Tregs also express CD39 and CD73, which catalyze the release of adenosine into the extracellular milieu, thus inhibiting effector T cell proliferation (224). In addition, Tregs outcompete effector T cells for IL-2, inhibiting their proliferation (225), and Tregs produce suppressive cytokines like IL-10 and TGFβ (226, 227). Tregs can also suppress effector T-cell differentiation and induce apoptosis of Tconv cells (228, 229).

The importance of intrathymic Treg generation in the neonatal period is illustrated by an experiment performed nearly 50 years ago in which neonatal thymectomy in mice was shown to cause autoimmune pathology in the ovaries (230). Later studies showed that transfer of adult T cells, in particular CD25+ Tregs, prevented autoimmune destruction of ovaries in these mice, implying that a defect in neonatal thymic Treg generation failed to curb activation of autoreactive T cells (231, 232). Differentiation of Foxp3+ CD25+ Tregs in the mouse thymus lags behind that of Tconv cell development (233). In newborn mice, Tregs comprise only 0.09% of CD4SP thymocytes and do not reach adult levels (~4% of CD4SP thymocytes) until 21 days after birth (233). In contrast, CD25+ Tregs constitute ~6-8% of CD4SP thymocytes in humans by 14-17 gestational weeks (GW), and this frequency remains relatively constant after birth (234, 235). Perinatal expansion of the human Treg compartment is observed in the periphery, with a striking surge in the frequency and number of peripheral blood CD25+ Foxp3+ Tregs during the early neonatal period (7-8 days post birth) compared to those in cord blood or present at a later neonatal period (2-4 weeks after birth) (236). Additionally, compared to adult tissues, a higher frequency of Foxp3+ CD25+ Tregs is observed in human fetal as well as in several pediatric lymphoid and mucosal tissues, indicating their importance in early life (86, 237). Tregs in neonatal circulation display an activated phenotype, with a predominantly Foxp3^{hi} CTLA-4^{hi} CCR7^{lo} CD25+ phenotype (236). Similarly, an activated (CD69^{hi} GITR^{hi} CCR7^{lo} CTLA-4^{hi}), memory (CD62L^{lo} CD45RO+)

Treg phenotype was documented in fetal LN and cord blood (235, 237).

The high frequency of Tregs in the fetal and perinatal periods may be due to a higher propensity of fetal hematopoietic progenitors to differentiate into the Treg lineage. HSCs transplanted from human fetal liver or bone marrow into humanized mice give rise to a higher frequency of CD25+ Foxp3+ Tregs compared to HSCs from adult bone marrow (238). Additionally, studies in mice have demonstrated that perinatal CD4SP thymocytes are more prone to differentiate into Tregs upon TCR stimulation when compared to adult CD4SP thymocytes, both *in vitro* and *in vivo* (239, 240). Gene expression profiles of adult Tregs are more similar to fetal naive CD4+ T cells than to adult naive CD4+ T cells, indicating that fetal T-cells may be transcriptionally primed to be suppressive. Consistent with this finding, naive CD4+ T cells from human fetuses give rise to more Tregs than adult CD4+ T cells *in vitro* (236, 238). The increased Treg induction efficiency of perinatal progenitors could be a protective mechanism required to establish initial immune tolerance in multiple peripheral tissues, particularly in light of elevated Tconv self-reactivity in the perinatal period (see above). Supporting this theory, *Aire* expression in the perinatal period is necessary and sufficient to prevent autoimmunity in mice (241), and Treg ablation in perinates induces profound multiorgan autoimmunity characteristic of Aire deficiency (242). Together, these findings suggest that *Aire* expression in the perinatal thymus is essential for selecting perinatal Tregs that suppress multiorgan autoimmunity. Tregs are required for self-tolerance throughout life, as demonstrated by the autoimmunity that ensues following *Foxp3* elimination in adult mice (207). Notably perinatally-derived Tregs persist into adulthood, and relative to adult-derived Tregs, are uniquely capable of protecting against autoimmunity when transplanted into Aire-deficient mice. Perinatally-derived Tregs also express an activated gene signature and have an increased capacity to suppress Tconv cell proliferation *in vitro* relative to adult-derived Tregs (242). These mouse studies are consistent with human studies showing distinct gene expression patterns in fetal versus adult Tregs (238), as well as increased protein expression and suppressive activity of pediatric compared to adult Tregs (86). Taken together, these findings suggest that perinatally-derived Tregs persist into adulthood, where they suppress damaging autoreactive T-cell responses in multiple organs.

Many studies of Treg-mediated protection in tissues have been performed in adults, raising questions of whether neonatally derived Tregs play a critical role in these processes, and if so, what mechanisms underlie their suppressive activity. Some progress has been made towards answering these questions. Tregs generated in the neonatal thymus migrate to the skin in a CCR6-CCL20 dependent manner, where they are essential for establishing tolerance to newly colonizing commensal bacteria (216). Recent studies have also reported that a wave of neonatal thymus-derived Tregs migrates to the liver (243, 244). Interestingly, perinatal liver-resident Tregs are more suppressive than their splenic counterparts, and they are activated in a TCR-dependent manner in the liver microenvironment (243). Ablating these perinatal Tregs resulted in Th1-type inflammation and breakdown of lipid metabolism, highlighting their role in establishing liver homeostasis (243). Another study demonstrated that perinatal Tregs promote and maintain anergy of self-reactive PD-1+ CD44+ Tconv cells in the liver; notably development of these perinatal Tregs was Aire independent (244). These results contrast with the Aire-dependence of perinatal Tregs that confer protection against autoimmune infiltrates in *Aire*-deficient mice (242). Thus, the contribution of Aire to selection of perinatal thymic Tregs that suppress tissue-specific autoreactivity requires further investigation. Tolerance to commensals at mucosal barriers is established in the neonatal period and is mediated by peripherally-induced Tregs. In neonatal mice, the lung microbiota induce differentiation of a Helios negative Treg subset that suppresses Th2-like hyper-responsiveness to aeroallergens (245). Additionally, neonatal T cells encounter a wide variety of antigens derived from gut microbiota which induce Treg differentiation required for tolerance to gut commensals throughout life (246). Thus, thymus-derived and peripherally-induced Tregs are generated early in life and are critical for tissue-specific immune homeostasis in multiple organs.

Selection of Tregs in the Perinatal Period

In mice and humans, Foxp3, the master transcriptional regulator of Treg lineage commitment and maintenance, is predominantly induced in self-reactive CD4SP thymocytes, although it can be detected as early as the DP stage (233–235). Perinatal Tregs express higher CD5 levels compared to adult Tregs, suggesting increased self-reactivity (130).Two distinct Treg populations that differ in their affinity towards self-antigens have been identified in adults (247). Triple^{hi} (PD-1^{hi} GITR^{hi} CD25^{hi}) Tregs are more self-reactive, as indicated by higher Nur77 and CD5 levels, and are efficient at suppressing Tconv cell proliferation in lymphoid organs. In contrast, Triple^{lo} (PD-1^{lo} GITR^{lo} CD25^{lo}) Tregs express less Nur77 and CD5, indicative of lower self-reactivity, and more effectively limit the induction of colitis by inducing peripheral Tregs in the gut (247). However, both Triple^{hi} and Triple^{lo} Tregs in the perinatal thymus express elevated CD5 levels relative to their adult counterparts (130). Taken together with the evidence that Tregs selected in the perinatal thymus are critical for suppressing autoimmunity at multiple tissue sites, higher CD5 expression by thymic perinatal Tregs suggests that the perinatal thymic environment may be specialized for selecting tissue-protective Tregs.

Recent studies support the possibility that self-antigen presentation differs in the perinatal versus adult thymus microenvironment, resulting in efficient Treg selection. A self-peptide derived from peptidyl arginine deaminase type IV (Padi4) was found to efficiently induce selection Treg only in the perinatal thymus (248). Interestingly, in adults, Padi4-specific thymocytes were subject to negative selection as early as the post-positive selection DP stage, whereas in perinates, negative selection was delayed until the CD4SP stage. Thus, in the adult thymus, Padi4-specific DP precursors were deleted before they could give rise to CD4SP cells or Tregs, likely underlying the switch from perinatal Treg induction to adult

negative selection (248). The age-associated shift towards clonal deletion could reflect cell-intrinsic changes in signaling downstream of TCR stimulation in perinatal versus adult DP thymocytes and/or changes in the perinatal versus adult thymic microenvironment. In this regard, bone marrow chimera experiments revealed that expression of Padi4 by HAPCs induced negative selection in the adult thymus, but when Padi4 expression was restricted to radioresistant thymic stromal cells, Treg induction was restored in adults. These findings suggest that antigen presentation by adult HAPCs preferentially drives negative selection, as opposed to Treg induction. Conversely, unique properties of the thymic APC compartment in neonates may selectively promote thymic Treg induction over negative selection. The concept that age-associated changes in the thymic microenvironment play a role in the outcome of self-antigen recognition is supported by the lower expression of H2-DO relative to H2-DM in perinatal versus adult mTECs, which would increase the diversity of peptides presented in the perinatal thymus (242), thus altering the TCR repertoire during thymic selection. Collectively, these studies demonstrate that both negative selection and Treg induction differ in the perinatal versus adult thymus, yielding more autoreactive Treg and Tconv cells in the perinatal period. However, the mechanisms driving these age-dependent changes in selection thresholds and TCR specificities, including whether these differences are due to cell-intrinsic changes in T cell progenitors and/or cell-extrinsic factors in the thymic microenvironment, remain to be resolved.

Changes in Treg Function During Aging

The prevalence of Tregs in the blood of adult mice and humans ranges from 5-10% of the CD4+ T cell compartment (249). The frequency of Tregs does not increase in mouse blood with age (250). In contrast, elevated frequencies of circulating Tregs have been reported in aging humans (251, 252). Furthermore, aging is associated with an increase in both the frequency and number of Tregs in mouse spleen and lymph nodes, but not in the lung, liver or peritoneum (160, 250, 251, 253, 254) In fact, a recent single-cell transcriptional profiling study confirmed that the frequency of Tregs increases in aging mouse spleens, but revealed that this increase was driven almost entirely by an emerging subset of activated Tregs (159). Taken together, these studies indicate that the abundance, distribution, and function of Tregs shift with age towards increasing immunosuppression.

Two single-cell transcriptomics reports show that with age, Tregs express elevated levels of genes associated with Treg activation and suppressive activity, including *Foxp3*, *S100a11*, *IL1r2*, *Pdcd1*, *Tigit*, *Lag3*, and *Batf* (159, 160). Moreover, expression of proteins that promote Treg suppressive activity, such as FOXP3, CD25, CTLA-4, and GITR, is maintained, and in some cases increased in aged Tregs (251, 252, 254, 255). A recent study reported that old activated Tregs are more suppressive than young Tregs (159), consistent with previous findings showing increased functional activity of Tregs with age (251). In contrast, other studies report that the *in vitro* suppressive capacity of Tregs does not differ between young and aged mice (256, 257) or humans (255). Nevertheless, whether due to increased frequency or increased suppressive capacity, it is likely that aged Tregs may impair T-cell mediated control of infection with age, thus contributing to pathology. In keeping with this concept, young mice are able to resolve primary *Leishmania major* infection, whereas aged mice experience increasing reactivation of lesions. However, Treg depletion in the aged mice increased cytokine production by effector T cells and decreased disease severity (251). In addition, $CD4^+$ $CD25^{hi}$ Tregs recovered from Alzheimer's disease and Parkinson's disease patients displayed increased suppressive activity *in vitro* when compared to young and control elderly donors, suggesting that Treg suppressive capacity is also associated with age-related neurodegeneration (252). Increasing Treg activity may also contribute to diminished anti-tumor responses with age. Whereas young mice were able to reject transplanted BM-185 tumor cells, aged mice succumbed, and their ability to reject tumors was restored by Treg depletion (254). Because there are multiple subsets of functionally distinct Tregs (258), some of the discrepancies above regarding alterations of Treg functionality with age may reflect changes in the composition of Treg subsets, which could be impacted by organ sites and the assays chosen to measure Treg suppressive activity. Consistent with this possibility, Tregs were found to be more abundant in the oral mucosa of aged mice and humans, although, counterintuitively, inflammation associated with *Candida albicans* infection was exacerbated despite pathogen control (259). Notably, an age-related shift in favor of IFN-γ-producing relative to IL-17-producing Tregs and Tconv cells was associated with decreased IL-1β and increased IL-6 levels in the mucosa. IL-1R1 deficiency decreased induction of IL-17-producing Tregs after *Candida albicans* infection, whereas there was a relative increase in IFN-γ-producing Tregs, which required IL-6 for their expansion (259). In a mouse model of autoimmune colitis, aged Tregs could suppress IFN-γ+ Th1 cells, but not IL-17+ Th17 cells (260). Restraint of Th17 cells requires STAT3 activation in Tregs (261), and aged Tregs do not activate STAT3 in response to inflammatory IL-6 to the same extent as young Tregs (260). Collectively, these studies demonstrate that age-associated changes in the relative abundance of different cytokines, as well as the responsiveness of aged Tregs to cytokine stimulation, can alter Treg subset differentiation and thus, the ability to suppress inflammatory T cell responses to self-antigens, pathogens, and commensals in a tissue-specific manner. While changes in cytokine levels would contribute to extrinsic alterations in Treg differentiation and function with age, changes in the ability of aged Treg to respond to cytokines suggest that age-associated cell-intrinsic changes affect Treg function.

Selection of Treg With Age

The absolute number of Tregs in the thymus decreases with age, reflecting the reduction in cellularity that accompanies age-associated thymic involution (250, 253). Although the frequency of FOXP3+ cells does not change with age (250, 253), initial studies did not distinguish between thymic Tregs generated in the aged thymus versus those that had recirculated into the thymus from the periphery. Subsequent studies using

RAG2p-GFP mice revealed that the frequency of newly generated Tregs declines rapidly with age, while the proportion of recirculating Tregs increases (262, 263). Moreover, mature Tregs inhibit *de novo* generation of Tregs in fetal thymic organ cultures, suggesting that recirculating Tregs reduce selection of new Tregs in the aged thymus, perhaps by sequestering IL-2, a limiting cytokine required for Treg induction (264). In this regard, Treg generation was increased in the presence of exogenously administered IL-2 (262). These studies suggest that thymic Treg induction is reduced with age. In contrast, Treg selection was favored over clonal deletion in an inducible *Foxn1*-deletion model of accelerated thymic involution, in which TECs are precipitously depleted (198). Thus, it remains to be resolved whether Treg generation is generally reduced in an aged thymus, or is actually increased under some conditions, such as limited self-antigen availability.

Given that the number of Tregs in the periphery does not decline with age, and in fact increases in some organs (see above), the decline in thymic output of newly generated Tregs during age-associated thymic involution must be compensated for in the periphery either by increased proliferation/survival of extant Tregs or increased Treg induction. Naïve $CD4^+$ T cells from old mice have a diminished ability to differentiate into Tregs *in vitro* and *in vivo* (188, 265). However, aged Tregs have a survival advantage relative to young Tregs due to lower expression of the pro-apoptotic factor *Bim* (253, 266). It is important to note that there are multiple subsets of peripheral Tregs (267–271), such that age-associated increased Treg survival could reflect an increased proportion of a long-lived subset. In keeping with this possibility, $CD25^{lo}$ Tregs accumulate with age in the periphery (256, 266). $CD25^{lo}$ Tregs express lower levels of *Bim* than $CD25^{hi}$ Tregs, even though *Bim* levels decline in $CD25^{hi}$ Treg with age (266). Notably, IL-2 is critical for homeostasis of $CD25^{hi}$ Tregs and IL-2 levels decline with age, whereas the $CD25^{lo}$ subset requires IL-15 for survival (266). Thus, altered access to homeostatic cytokines could impact the relative proportions of different Treg subsets with age, which would be in keeping with both the observed decline in circulating IL-2 and the age-associated deterioration of a supportive T-cell microenvironment in secondary lymphoid organs (2), especially given that autoreactive CD4+ T cells in secondary lymphoid organs are an important source of IL-2 for Tregs (272). Thus, there are age-related consequences for Treg selection, induction, and maintenance in the thymus and in the periphery.

CHANGES IN THYMIC APCs AND IMPLICATIONS FOR SELECTION THROUGHOUT THE LIFESPAN

Changes in TECs Across the Lifespan

The composition and function of TEC subsets undergo major changes throughout the lifespan, and there is mounting evidence that the dynamic nature of the TEC compartment is a critical determinant of age-associated alterations in the immune response. As previously discussed, mTECs play a critical role in establishing and maintaining central tolerance. Not only are mTECs uniquely capable of expressing and presenting Aire-dependent and Aire-independent TRAs (38, 43), but they also transfer TRAs to DCs for subsequent cross-presentation to thymocytes (53, 62, 63). In addition, mTECs produce chemokines such as XCL1, CCL19, and CCL21 that promote DC medullary recruitment and localization (70, 273, 274). Moreover, in response to Toll-like receptor (TLR) signaling, mTECs secrete chemokines that recruit CD14+ monocyte-derived DCs into the medulla to promote Treg generation (275). Thus, mTECs play multifunctional and essential roles in negative selection and Treg generation.

The mTEC compartment in both humans and mice is phenotypically and functionally heterogeneous. Initially, mTECs were classified into two major subsets, namely an immature $MHCII^{lo}$ $CD80^{lo}$ $AIRE^-$ subset ($mTEC^{lo}$) and a functionally mature $MHCII^{hi}$ $CD80^{hi}$ $AIRE^+$ subset ($mTEC^{hi}$). There is long-standing evidence that the $mTEC^{lo}$ compartment contains progenitor cells that generate $mTEC^{hi}$ progeny (276–278). However, it is now evident that the $mTEC^{lo}$ population is highly diverse and contains multiple functionally and developmentally distinct subsets that have been identified in investigations using flow cytometric as well as lineage tracing and transcriptomic analyses of mouse and human mTECs. For example, a subset of $mTEC^{lo}$ cells expresses CCL21, indicating their functional importance in recruiting positively selected thymocytes into the medulla (279–283). Interestingly, despite the initial association of an $mTEC^{lo}$ phenotype with an immature stage of differentiation, the $mTEC^{lo}$ subset also contains mature cells that have downregulated Aire and MHCII expression (44, 279, 280, 282). Studies employing single-cell RNA sequencing (scRNAseq) analyses have shown that post-Aire mTECs include a unique population of thymic tuft cells, which are sensory epithelial cells similar to those present in the intestine and other mucosal sites (279, 280, 282). It has been suggested that tuft cells play a role in central tolerance, as the abundance of $Foxp3^{lo}$ Treg precursors decreases in tuft cell-deficient mice (284). Hassall's corpuscles (HCs) are another cell type in the heterogeneous post-Aire $mTEC^{lo}$ subset. HCs form distinctive concentric structures of flattened epithelial cells that are prominent in the human thymus medulla, and small clusters of TECs that may be analogous are found in mouse medullary regions. Transcriptional profiling studies have identified genes that are highly expressed by both HCs and terminally differentiated keratinocytes (279, 282). Moreover, HCs resemble keratinocytes in that both cell types produce proteins found in terminally differentiated epithelial cells such as keratin 10, involucrin, filaggrin and TSLP (44, 285–287). It has been suggested that HCs play a role in regulating central tolerance as TSLP produced by human HCs activates DCs to express co-stimulatory molecules that enhance Treg induction (78). A recent study in which scRNAseq analysis was performed on index-sorted TECs identified a novel TEC subtype, referred to as intertypical, which has both mTEC and cTEC characteristics (193). Thus, studies to date have shown that the mTEC

compartment is highly diverse, consisting of multiple subsets whose phenotypic and functional characteristics, as well as lineage relationships have not yet been fully deciphered. Nevertheless, there is mounting evidence that various mTEC subsets significantly impact the establishment and/or maintenance of central tolerance.

The TEC compartment is highly dynamic during the perinatal to adult transition. TEC numbers expand exponentially during mouse fetal thymus development, and TEC cellularity continues to increase in the perinatal period prior to temporarily leveling off in young adults (288, 289). In parallel, there is a higher frequency of proliferating TECs in the perinatal compared to adult thymus (279, 288, 290). Remodeling of the TEC compartment during the perinatal period in mice is reflected by an increase in the percentage of mTECs and a corresponding decline in the percentage of cTECs (289, 291). Interestingly, functional blockade of vascular endothelial growth factor (VEGF) receptors in neonatal mice inhibits perinatal thymus expansion and accelerates the shifted mTEC to cTEC ratio despite the lack of VEGF receptors on thymocytes and TECs (292). These effects were independent of changes in the vasculature; however, VEGF inhibition altered expression of genes regulating cellular adhesion, migration, adipogenesis and inflammation in CD140a+ mesenchymal cells suggesting that VEGF-mediated effects on mesenchymal stromal cells influences changes in the TEC compartment during the perinatal period (293). The relative increase in mTECs during the perinatal to adult transition was found not only by flow cytometric analysis, but also by microscopic analysis of histological sections (289) showing that this change is not merely an artifact of the enzymatic digestion procedure required to obtain single thymus cell suspensions. This is a matter of concern because enzymatic disaggregation results in suboptimal recovery of cTECs, particularly those present in cage-like structures, referred to as thymic nurse cells, which encompass DP thymocytes (288, 289, 294). An increase in the frequency of mTECs relative to cTECs was also demonstrated by single cell transcriptional profiling of neonatal versus adult human thymuses (295). Furthermore, a recent scRNAseq analysis revealed the presence of a unique cTEC subset in the perinatal mouse thymus that rapidly declined and was replaced by mature cTECs in the adult thymus (193). The composition of the mTEC compartment also changes as perinates transition into adulthood. For example, few tuft cells are present in the neonatal mouse thymus, but their numbers increase substantially in adults (279, 281). Similarly, HCs become more abundant after the perinatal to adult transition (296). Taken together, these studies show that the network of TEC subsets undergoes extensive remodeling during the perinatal to adult transition.

TECs also undergo dynamic changes at the opposite end of the age spectrum as the thymus undergoes involution, a general feature of vertebrate aging. Thymus involution is characterized by progressive organ atrophy, reduced T cell output, disruption of thymus architecture and collapse of the TEC compartment (193, 297–300). Although both thymocyte and TEC cellularity decline as the thymus undergoes involution (161, 194, 288, 289, 291, 297), TEC depletion is a primary factor driving thymus involution. FOXN1, a transcription factor required for TEC development and maintenance, declines with age in mice and humans (291, 301–303). Genetic models in which *Foxn1* expression is upregulated in TECs prior to or after thymus involution can attenuate or reverse this process (195, 304), whereas downregulation of *Foxn1* results in early degeneration of the TEC compartment and premature involution (291). Furthermore, thymus involution can be prevented by expressing either a *Cyclin D1* or *c-myc* transgene in TECs, or by deleting *Retinoblastoma* family genes, all of which result in a continuous thymus growth phenotype despite the fact that thymocytes are not genetically manipulated (290, 305, 306). Furthermore, heterochronic parabiosis experiments showed that migration of thymus-seeding hematopoietic cells from a young partner into the thymus of an aged partner failed to restore cellularity of the old, involuted thymus (307). Collectively, these investigations indicate that degradation of the TEC compartment is a major factor contributing to thymus involution, a finding that is not surprising given that TEC-derived signals are indispensable for T cell differentiation and selection.

Although thymus involution is generally thought to result in a progressive decline in the number of both cTEC and mTEC compartments, this view was challenged by a recent investigation showing that the extensive cytoplasmic projections characteristic of cTECs contract during involution (308). Based on these findings, it was suggested that changes in cTEC morphology, rather than cellular depletion, are responsible for the apparent reduction in cTEC cellularity and associated cortical thinning (308). In contrast, morphological changes in mTECs were not observed during thymus involution consistent with an age-associated decline in the number of mTECs. In addition, changes in mTEC gene expression patterns, including increased expression of inflammatory pathway genes (193, 309) occur during thymus involution. The mechanisms responsible for transcriptional changes may reflect altered mTEC subset composition and/or intrinsic alterations in transcriptional regulation (193, 195, 196, 309). With regard to the former possibility, a recent study combining scRNAseq and lineage tracing approaches demonstrated marked changes in TEC subset composition with age (193). Taken together, these studies show that remodeling of the TEC compartment is a characteristic and progressive feature of age-related thymus involution.

Depletion of the mTEC compartment during aging, particularly the decline in mature mTECs that express Aire-dependent TRAs (195, 196, 288, 291), is likely to compromise central tolerance and result in increased export of self-reactive T cells. Indeed, a decline in expression of Aire-regulated as well as Aire-independent TRAs has been associated with age-related thymus involution (193, 194). Interestingly, however, neither the expression of *Aire* nor *Fezf2* (required for expression of *Aire*-independent TRAs) was altered in TECs obtained from aged, involuted thymuses suggesting that TRA expression depends on additional, as yet undefined, factors (193, 194). Collectively, these

data suggest that the decline in mTEC cellularity, changes in mTEC subset composition and altered transcriptional signatures of mTEC subsets are features of thymus involution that may affect central tolerance and contribute to the age-associated increase in autoimmunity (3, 310).

Changes in HAPCs Across the Lifespan

While mTECs present self-antigens directly to thymocytes to mediate central tolerance, DCs also cross-present mTEC-derived TRAs to induce negative selection and Treg generation (48, 53, 54, 62, 66, 71, 73, 74). Changes have been documented in the composition of thymic DCs during the perinatal to adult transition. Some studies indicate that CD8α$^+$ cDC1s increase in the thymus of adult relative to fetal and neonatal mice (242, 311). However, another study showed that CD8α$^+$ cDC1s decrease during the transition from neonate to adult, whereas Sirpα+ cDC2s and pDCs increase with age in the thymus (69). Adult cDC2s express higher levels of genes associated with antigen processing and presentation and are more efficient at MHCII-dependent antigen processing and presentation to T cells compared to newborn cDC2s. Interestingly, this study indicates that the efficiency of negative selection is diminished in perinatal mice, compared to adults, correlating with a lower frequency of cDC2 cells (69). In the human thymus, XCR1+ cDC1s increase in the second trimester of pregnancy and decline postnatally with increasing age (295), consistent with the trend reported in mice (69). Thus, while multiple studies have established that the DC compartment changes with age, the cellular composition and molecular alterations remain to be elucidated.

Since mTECs influence thymic DC localization and composition, mTEC-DC crosstalk likely plays an important role in mediating central tolerance. For instance, in the adult thymus, mTECs express CCL2, XCL1, JAG1, CCL19, and CCL21, which could promote recruitment, localization and/or maturation of thymic DCs (30, 33, 34, 61, 274, 295, 312–314). CCR7 is expressed not only by thymocytes, but also by some thymic DCs, indicating that expression of CCL19 and CCL21 by mTECs could recruit not only post-positive selection thymocytes to the medulla, but also CCR7$^+$ DCs. CCR7 is upregulated on thymic DCs by interactions with autoreactive thymocytes in a CD40-CD40L dependent manner (65). CCR7+ DCs in human and mouse fetal and postnatal thymuses express high levels of MHCII and costimulatory molecules, suggesting they may have an increased capacity to present self-antigens to medullary thymocytes (64, 65, 295). Indeed, compared to CCR7- cDC1s, CCR7+ cDC1s are more efficient at acquiring and presenting *Aire*-dependent TRAs to CD8SP cells (64) and CCR7+ cDC1s have also been implicated in playing a central role in presenting mTEC-derived antigens to promote Treg selection (48, 66). We found that CCR7 deficiency results in increased apoptosis of MHCII^{hi} cDC1s and reduced antigen transfer from mTECs, with a concomitant increase in cDC2 and, surprisingly, Treg induction (70). Thus, production of CCR7 ligands by mTECs alters the composition of the thymic DC compartment, with downstream consequences for central tolerance. Taken together with the age-associated changes in DCs and mTECs, these findings suggest that altered mTEC-DC crosstalk in the perinatal period likely impacts central tolerance induction.

While the perinatal thymic HAPC compartment has slowly garnered interest over the last two decades, there is relatively less information regarding the impact of age-associated thymic involution on thymic HAPCs. The numbers and proportions of CD8α+ cDC1s and pDCs decrease gradually with age in the mouse thymus, while migratory Sirpα$^+$ cDC2s remain constant in number, thus comprising an increased proportion of the thymic DC compartment (315). Though reduced in number, DCs in aged mice express similar levels of the activation markers CD40, CD80, CD86, and MHCII compared to young DCs, suggesting they are functionally intact (315). In humans, the number of total thymic DCs declines in proportion to the overall decrease in cellularity of the thymus with age (316). Similar to the mouse thymus, the proportion of DCs expressing MHCII, CD80, and CD86 was not altered with age, though expression of CD40 was diminished with age (316). Our transcriptional profiling analysis showed that murine thymic cDC1s and cDC2s express an increasingly proinflammatory gene signature with age, including expression of *Il1a, Il6, Tnf* and *Il18* (309). These aging DCs could potentially contribute to the age-associated inflammation observed in the thymus (317) and alter central tolerance. As previously mentioned, B cells mediate negative selection (81–83) and Treg induction in the thymus (318), underscoring their crucial contribution to central tolerance. Although the frequency of thymic B cells increases with age, there is a dramatic age-associated decline in Aire and Aire-dependent TRA expression in mouse and human thymic B cells (199), which could impair negative selection and Treg induction. These studies suggest that age-associated changes in HAPC subset composition and/or gene expression may impact central tolerance, highlighting the need for more comprehensive studies to determine how age-associated changes in thymic HAPCs influence negative selection, Treg induction, and the incidence of autoimmunity.

CONCLUSIONS

The cellular composition and transcriptional profiles of TECs and HAPCs undergo profound changes throughout life, suggesting that a concomitant change in central tolerance likely occurs. Indeed, multiple lines of evidence presented above suggest that the perinatal thymic microenvironment is inefficient at inducing negative selection of Tconv cells, but is biased towards selection of auto reactive Tregs that are critical for suppressing autoimmunity in various tissues throughout life. On the other end of the aging spectrum, evidence suggests that the involuting thymus becomes inefficient at supporting both negative selection and Treg induction. The impact of age on the ability of the thymus to support both arms of central tolerance warrants further investigation. Studies to date raise several important issues. For example, while negative selection may be inefficient in both the perinatal and aged thymus, only the aged thymus appears to be impaired in supporting Treg generation, invoking a possible link with the age-associated

increase in incidence of autoimmune disorders. Moreover, given that age-related changes in peripheral T cell differentiation and maintenance can impact the outcome of T cell responses to self and foreign antigens, it will be important to distinguish thymic from peripheral contributions with regard to changes in Tconv and Treg cells as a function of age.

Given that expression levels of CD5 on T cells are set during positive selection in the thymus, and that the level of CD5 on Tconv and Treg cells correlates with altered activity in the periphery, elevated CD5 levels on Tconv and/or Treg cells in the neonatal and aged periods indicate that age-associated changes in thymic selection impact peripheral T cell responses. However, changes in thymic selection could reflect either an altered capacity of thymic APCs to induce selection or cell-intrinsic changes in the differentiation potential of hematopoietic progenitors that seed and differentiate within the thymus. For example, the bias of neonatal CD8 T cells towards short-lived effectors reflects altered functional potential of neonatal versus adult hematopoietic cells (122). Whether preferential differentiation of CD4SP cells into Th2 effectors and Tregs in perinates reflects changes in T cell differentiation in the periphery and/or altered thymic selection, due to either an altered microenvironment or changes in hematopoeitic progenitors, requires further investigation. Furthermore, the mechanisms underlying thymic selection of T cells with altered self-reactivity have not been firmly established. Elucidating altered functions of Tconv and Treg cells with age will enhance understanding of how the immune system responds to pathogens throughout life without invoking autoimmunity. Furthermore, determining the mechanisms underlying altered thymic selection and peripheral maintenance of functionally distinct T cell subests will inform future strategies for enhancing T cell mediated immune protection and suppressing autoimmunity.

While studies to date have identified unique aspects of immune responses at both extremes of the age spectrum, and suggested that central tolerance is subject to age-related restrictions, a number of questions remain unanswered. Some of these unresolved questions include:

1. What are the precise roles of diverse mTEC and HAPC subsets in selection across the lifespan?
2. Is the bias of perinatal Tconv towards Th2 and short-lived effector cells due to altered thymic selection? Although this was found not to be the case for short lived CD8 effector cells (85), this question remains open for $CD4^+$ T cells.
3. Do Aire-dependent versus Aire-independent Treg subsets induce anergy in Tconv cells in a tissue-specific manner?
4. What cellular subsets and molecular mechanisms account for the propensity of the perinatal thymic microenvironment to select $CD5^{hi}$ Tregs?
5. Are the activated Tregs that accumulate with age and have heightened suppressive ability (159) essential for maintaining tolerance with age? A related issue is whether Tregs generated in adulthood contribute to tolerance.

AUTHOR CONTRIBUTIONS

JS and JL reviewed the literature. JS, JL, NS and LH prepared figures and legends, and commented on manuscript drafts. LE and ER revised the manuscript. All authors contributed to the article and approved the submitted version.

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Premature Senescence and Increased Oxidative Stress in the Thymus of Down Syndrome Patients

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Down syndrome (DS) patients prematurely show clinical manifestations usually associated with aging. Their immune system declines earlier than healthy individuals, leading to increased susceptibility to infections and higher incidence of autoimmune phenomena. Clinical features of accelerated aging indicate that trisomy 21 increases the biological age of tissues. Based on previous studies suggesting immune senescence in DS, we hypothesized that induction of cellular senescence may contribute to early thymic involution and immune dysregulation. Immunohistochemical analysis of thymic tissue showed signs of accelerated thymic aging in DS patients, normally seen in older healthy subjects. Moreover, our whole transcriptomic analysis on human Epcam-enriched thymic epithelial cells (hTEC), isolated from three DS children, which revealed disease-specific transcriptomic alterations. Gene set enrichment analysis (GSEA) of DS TEC revealed an enrichment in genes involved in cellular response to stress, epigenetic histone DNA modifications and senescence. Analysis of senescent markers and oxidative stress in hTEC and thymocytes confirmed these findings. We detected senescence features in DS TEC, thymocytes and peripheral T cells, such as increased β-galactosidase activity, increased levels of the cell cycle inhibitor p16, telomere length and integrity markers and increased levels of reactive oxygen species (ROS), all factors contributing to cellular damage. In conclusion, our findings support the key role of cellular senescence in the

pathogenesis of immune defect in DS while adding new players, such as epigenetic regulation and increased oxidative stress, to the pathogenesis of immune dysregulation.

Keywords: Down syndrome, thymus, thymic epithelial cells, senescence, oxidative stress

INTRODUCTION

Down syndrome (DS) is the most common chromosomal anomaly among live-born infants, typically characterized by complete or partial trisomy of chromosome 21 (Chr21) [OMIM#190685] (1). Its incidence ranges between 1:100 and 1:1000 live births in general population and is influenced by maternal age (2, 3). DS is one of the most common genetic cause of intellectual disability (4) and its complex phenotype results from a dosage imbalance of genes located on human Chr21. In addition to learning disabilities, there are various common features occurring in all DS patients, such as craniofacial abnormalities and hypotonia in early infancy (5). Congenital heart disease (CHD) is regarded as one of the most important clinical phenomena in children with DS, due to its significant impact on morbidity and mortality (6). DS is also associated with a group of clinical manifestations of accelerated aging (7).

Previous studies in *Ts65Dn* mice, a well-characterized mouse model of DS, have evidenced defects in hematopoietic progenitor cell development and function at the level of both hematopoietic stem cells (HSC) and lymphoid progenitors, which have thymus-seeding potential, with increased oxidative stress and decreased IL7Rα expression as indicated causes of these alterations (8). Further studies in *Ts65Dn* mice have also shown a decrease in the number and proportion of immature, double negative (DN) thymocytes, double positive (DP) and single positive (SP) CD4 thymocytes (9). Similarly to lymphoid progenitors, a reduced expression of IL7Rα was also detected in immature thymocyte subsets, likely mediated by higher oxidative stress and Notch pathway inhibition (9). A senescent phenotype was suggested by reduced naïve T cells in the spleen and reduced proliferation to polyclonal stimulation of peripheral T cells (8).

Several studies indicated alterations in the thymic stroma and in the thymocytes of DS patients, with defects in both immature progenitor cells and mature peripheral lymphocytes (10, 11). Early reports showed that the thymus is smaller in DS subjects, with an abnormal structure showing signs of premature thymic involution, with loss of cortico-medullary demarcation and markedly enlarged Hassall's corpuscles (12). Our previous study confirmed the significant weight reduction of DS thymi as compared to age-matched HDs (11). Additionally, the thymic tissue from the DS patients that we analyzed showed an accelerated maturation of the thymic epithelial compartment, with signs of premature involution (11). Increased frequency of peripheral γδ-T cells and lower frequency of naïve T cells have also been reported (13). Decreased numbers of recent thymic emigrant (RTE) cells (13) and significantly lower TREC levels have been observed, suggesting decreased thymopoiesis (11–14). Expression of thymic-specific proteasome subunit β5t, but not of cathepsin, has also been reported as markedly reduced in DS thymi (15). These findings suggest that abnormal thymic architecture and decreased expression of functionally important molecules in thymic stroma may contribute to altered thymic function and constitute a causative factor for immunological abnormalities in DS patients. Impairment of thymic function in DS patients could indeed explain their higher risk to develop autoimmune phenomena, as compared to age-matched individuals. Impaired function of natural T regulatory cells (nTreg), generated in the thymus, has been shown in several reports on DS patients (16). Of note, we showed that, although Treg cells were higher in number both in the periphery and in thymus, they were impaired in their suppressive ability (11), suggesting that the profound anatomical and architectural abnormalities of DS thymus may affect nTreg cell functionality.

To the best of our knowledge, no studies focused on TEC of DS patients have been reported to date. Indeed, published gene expression analyses have been performed on the whole thymus without distinguishing the epithelial component and thymocytes (16, 17). Two studies reported that the expression of *AIRE* gene, located on 21q22.3, is reduced in DS thymus, as compared to age-matched controls, leading to global thymic hypofunction and central tolerance failure (17, 18). Remarkably, we detected a statistically significant increased expression of AIRE and Ins2, a tissue-restricted antigen (TRA) induced by AIRE, in DS thymic tissue of children under one-year of age, when compared to age-matched controls (11). These results are in contrast with what we noticed in DS children aged between 2 and 5 years, in which we detected a remarkable decrease in AIRE expression, which correlates well with the presence of large cystic involutions, a sign of premature aging, and decreased thymopoietic activity (11).

Transcriptome analysis of the whole thymus revealed contrasting data, showing down regulation of genes involved in antigen processing and presentation and in thymic T-cell differentiation/selection, as well as downregulation of TRAs (18), while another study performed on thymocyte-depleted thymic specimens showed elevated expression of AIRE mRNA and a trend toward increased expression of some AIRE-dependent TRA genes in DS patients (19). Increased frequency of $AIRE^+$ mTEC and $CD11c^+$ DC and enlarged Hassall's corpuscles were also showed, as part of altered cell composition and architecture of thymic medulla in these patients (19). A recent study evaluated the impact of trisomy 21 on thymic gene interaction networks, through gene co-expression network and miRNA-target analyses and showed that epigenetic mechanisms acting at chromatin level and through the miRNA control of transcriptional programs involving the networks high-hierarchy genes contribute to thymic tissue adaptation occurring in trisomy 21 genomic

dysregulation (20). We also detected alterations in the kinetics of DS thymocytes differentiation with a skewing towards increased thymocyte maturation (11). Altogether these results support central tolerance perturbation in DS patients contributing to increased susceptibility to develop autoimmune signs.

To further dissect the contribution of the epithelial and the lymphoid component to the increased susceptibility to develop immunodeficiency and immune dysregulation, we set out to study thymic tissue removed from pediatric DS patients undergoing cardiac surgery. We performed immunohistochemistry studies on the thymic tissue and transcriptomic analysis on EpCam-enriched cells. We then validated transcriptome-specific alterations found in DS patients in TEC, thymocytes and peripheral T cells.

MATERIALS AND METHODS

Human Thymic Specimen Collection

In order to collect human post-natal thymic specimens, collaborations with the Pediatric heart surgery departments of "Bambino Gesù" Children's Hospital (Rome), Policlinico San Donato (Milan) and Regina Margherita Children's Hospital (Turin) were established. The required protocols for biological sample collection for research purpose and relative informed consents were prepared and approved by local Ethical Committees (DGS_Project_OPBG_2015 and TIGET07 protocol). Parents or legal guardians of enrolled subjects signed the informed consent forms. Enrolled patients were either pediatric patients affected by DS, or age-matched children with congenital heart disease (CHD) without known immune defects (control group), undergoing heart surgery for the first time with median sternotomy. Patients affected by a known infectious or immune system disease and characterized by a previous history of chemo/radiotherapy were excluded from the study. Thymic samples were collected during heart surgery, during which thymic tissue is usually removed, entirely or partially, to access the operating field. After surgery, thymic specimens were kept in normal saline solution at +4°C and processed within 24-48 hours.

Human PBMC Collection

Peripheral blood of HD and DS patients were obtained according to The Code of Ethics of the World Medical Association (Declaration of Helsinki) with the approval of the local Ethical Committees of the Policlinico San Donato and San Raffaele Scientific Institute (TIGET07).

In our study we enrolled 13 Healthy Donors (HD) and 12 DS patients both undergoing cardiac surgery. To increase the number of DS patients included in the study, we collected peripheral blood samples from the Pediatric Department of San Raffaele Hospital. Samples were obtained according to the Helsinki Declaration with the approval of the local Medical Ethical Committees of the San Raffaele Scientific Institute Internal Review Board (TIGET06). Written informed consent was obtained from parents and/or legal guardians for sample collection. A description of all HDs and patients is reported in **Supplemental Tables 1–3**, respectively. In our study patients have been divided in different age groups, known to differentiate as regards thymic maturational processes, which were determined based on previous reports (21) (**Supplemental Table 4**).

Thymic Tissue Processing: Thymocyte Recovery and htec Isolation and Enrichment

After arrival at SR-Tiget, thymic tissue was cleaned from blood vessels, clots and surrounding fat and connective tissue, weighted and cut in small pieces. About 1 gram of tissue was then collected and fixed in formalin for histological studies. Thymocytes were recovered by mashing thymic fragments with a sterile syringe plunger. Supernatant containing released thymocytes was removed and replaced with fresh PBS. This was repeated until the supernatant became relatively transparent. All these steps were performed on ice. Thymocytes were kept on ice in PBS (CORNING, Corning, NY, USA) containing 1% of penicillin/streptomycin (P/S) (ThermoFisher scientific, Waltham, Massachusetts, USA) and 10% FBS (Sigma-Aldrich, Saint Louis, Missouri, USA) to preserve their viability.

hTEC isolation was performed using a published protocol (22), further optimized by our group. Thymic samples were digested at 37°C with a solution containing Liberase TL (Roche, Basel, Switzerland) and DNase I (Sigma-Aldrich, Saint Louis, Missouri, USA) in 3 steps of 40, 40 and 30 minutes of length, respectively. After each digestion step, supernatant was collected and kept at 37°C, upon addition of an equal volume of RPMI (CORNING, Corning, NY) containing 10% FBS and 1% P/S. At the end of the whole digestion process, all 3 fractions were pooled and centrifuged at 1,500 rpm for 5 min. The thymic single cell suspension was then incubated for 15 minutes at +4°C with anti-human CD45 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) and then processed with the autoMACS Pro Separator (Miltenyi Biotec). The CD45-negative fraction, enriched in thymic stromal cells and depleted from hematopoietic cells, was retrieved and then tested by multicolor FACS analyses for the expression of TEC markers or processed for further sorting.

Sorting of Human TEC

Human TEC (hTEC) sorting was performed on freshly digested thymic stromal cells or after thawing human thymic samples, previously frozen soon after digestion, and after CD45+ cell-depletion with AutoMACS Pro Separator. Briefly, cells were stained with the following antibodies: anti-CD45 VioBlue (clone 5B1), anti-Epcam PeVio770 (clone HEA-125), anti-CD31 APCVio770 (AC128), anti-HLA-DR, DP, DQ APC (REA332) (all from Miltenyi Biotec), anti-Ulex-1 FITC (FL 1061, Vector), and anti-CD205 PE (HD83, Biolegend) antibodies and sorted with a FACSAria Fusion (Becton Dickinson) cell sorter (FRACTAL facility of San Raffaele Hospital, Milan, Italy), with a 85 micron nozzle. Non-viable cells were excluded from analyses using 7-AAD (BD Pharmingen). Cells were sorted directly in lysis buffer (LB) for next RNA extraction. Lysis buffer from "ReliaPrepTM RNA Cell

Miniprep system" kit was used. 1-thioglicerol (TG) was added to LB at a 1:100 dilution, according to manufacturer's instructions. Sorted cells in LB+TG were then stored at -80° until use.

RNA-Seq

RNA was extracted from Epcam-enriched hTEC subsets by using ReliaPrepTM RNA MiniPrep System (Promega) according to manufacturer's recommendations. RNA was then stored at -80°C until use. Full-length RNA-seq libraries were prepared using the SMART-Seq2 protocol (23), with minor modification. Briefly, RNA (1–5 ng) was reverse transcribed using custom oligodT and template-switching LNA oligos (sequences), followed by PCR amplification and clean-up (Ampure XP beads, Beckman Coulter). The resulting cDNA (0.5–1 ng) was tagmented at 55°C for 30 min and final RNA-Seq libraries generated using reagents from the Nextera XT DNA Library Prep Kit (Illumina). Sequencing was performed on a NextSeq 500 machine (Illumina, San Diego, CA) using the NextSeq 500/550 High Output v2 kit (75 cycles).

Nextseq 500 high output v2 kit. All RNA-seq data represent pooled data from at least two distinct biological replicates. Sequencing was performed by the Center for Translational Genomics and Bioinformatics of San Raffaele Scientific Institute, Milan.

Quality of the input reads was assessed using FastQC and read trimming was performed with Trimmomatic to remove adapters and low-quality sequences. Then, trimmed reads were given as input to the STAR aligner software to align them against the human reference genome (GRCh38/hg38) with standard parameters. After that, gene counts were obtained using 'Subread feature Counts' with Genocode (v29) gene annotation. Gene expression counts were processed with R using the R/Bioconductor package edgeR, normalizing for library size using trimmed mean of M-values, and correcting p-values using FDR. Lists of differentially expressed genes (FDR < 0.05) were analyzed with clusterProfiler for functional enrichment analysis. Enrichment p-values were corrected for multiple testing using FDR. Gene Set Enrichment Analysis (GSEA) was performed on Gene Ontology and Pathway databases by pre-ranking genes according to log2 (FoldChange) values.

Flow Cytometric Analysis

Flow cytometric analysis of human TEC was performed by multi-color staining and using the following mAbs: anti-CD326 (EpCam) PE-Vio770 (clone HEA-125), anti-CD45 APC (5B1), anti-HLA-DR, DQ, DP PE (all from Miltenyi Biotec), anti-Ulex-1 FITC (FL 1061, Vector Laboratories, Burlingame, California, USA).

Thymocytes were characterized using a multi-color staining. To discriminate DN, DP, SP4 and SP8 thymocyte subsets, we performed a three-color staining using the following mAbs: CD8 Vio-Blue (clone BW135/80), CD45 APC (5B1) and CD4 PerCP (VIT4) (all mAbs are from Miltenyi Biotec).

Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation using Lymphoprep (density: 1.077 g/ml; STEM CELL Technologies, Vancouver, Canada). To isolate total T cells we used the anti-human Pan T cells isolation kit (Miltenyi Biotec). To discriminate $CD4^+$ and $CD8^+$ T cell subsets we performed a four-color staining using the following mAbs: CD45 APC (clone 5B1), CD3 VioGreen (REA613), CD4 PE-Vio770 (M-T321) and CD8 PE (BW135/80) (all from Miltenyi Biotec).

Surface stainings were performed in PBS with 2% FBS and 0,1% sodium azide for 20 min at 4°C. Cells were acquired using a FACS CantoII (BD Biosciences, San Jose, CA, USA) and analyzed with Flow Jo Software (FLOWJO, LLC, Ashland, OR, USA).

Senescence Associated - Beta Galactosidase (SA-β-Gal) Analysis

SA-β-gal activity was assessed in TECs, thymocytes and PBMCs. We used a fluorescence-based protocol based on the alkalinization of lysosomes. Cells were plated at a concentration of 2 million cells per well in 12-well plates (CORNING) in pre-warmed fresh RPMI culture medium (CORNING). Cells were treated with a final concentration of 100nM of lysosomal inhibitory drugs bafilomycin A1 (INVIVOGEN, San Diego, California, USA). Bafilomycin A1 is used to neutralize the acidic pH of lysosomes and to allow the detection of SA-β-gal. Cells were incubated for 1 hour at 37°C and 5% CO_2. Cells were then resuspended in cold PBS (CORNING) containing 5-dodecanoylaminofluorescein di-ß-D-galactopyranoside (C_{12}FDG) (ThermoFisher Scientific, Walthman, Massachusetts, USA) a fluorogenic substrate for β-galactosidase. This compound was added at a final concentration of 33uM and cells were incubated for 2 hours at 37°C and 5% CO_2. C_{12}FDG is a substrate which, when hydrolyzed by SA-β-gal, becomes fluorescent and membrane impermeable. Cells were then washed, resuspended in 200 µl PBS and analyzed immediately using a FAS Canto II (BD Biosciences, San Jose, CA, USA) or an EVOS fluorescence microscope (ThermoFisher).

Cells acquired by FACS Canto II (BD Biosciences) were analyzed using the Flow Jo Software (FLOWJO, LLC, Ashland, OR, USA) and the analysis was based on two parameters: forward scatter (FSC) versus side scatter (SSC) region to exclude dead cells and subcellular debris. SA-β-gal activity was expressed as median fluorescence intensity (MFI) on one-parameter histogram displaying FL1, 488, FITC in different cell subpopulations: TECs, thymocytes and PBMCs.

TEC analyzed by the EVOS fluorescence microscope (Leica Microsystems, Rijswijk, The Netherlands) were seeded in Permanox 4-chamber slides (ThermoFisher) at 4 × 10 (3) cells/chamber and allowed to attach for 4 h. After treatment, TECs were fixed with 4% paraformaldehyde (Sigma-Aldrich) in PBS (CORNING) for 4 min. Slides were mounted with Vectashield Fluorescent Mounting Medium (Vector Laboratories, Burlingame, CA) and photographed with the EVOS fluorescence microscope (Leica Microsystems). One-hundred randomly chosen cells per sample were assessed for SA-β-gal positivity density (% of area analyzed).

RNA Extraction and Gene Expression Analysis

RNA was extracted from TECs after digestion and CD45 cell-depletion, from thymocytes and PBMCS using the RNeasy Micro

kit (QIAGEN, Hilden, Germany). Reverse transcription of mRNA was performed with the High Capacity Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). Real-time PCR was performed using TaqMan Gene expression Assays (Applied Biosystems) and the EagleTaq Universal Master Mix (Roche, Basel, Switzerland). PCR reactions were performed in MicroAmp®Optical 96-well reaction plates (Applied Biosystems) in a final volume of 25 µl and run on the Viia-7 Real-Time PCR machine (Applied Biosystems). Relative quantification of genes was performed with the 2–ΔΔCt method and expressed as fold change relative to the expression of the endogenous control, RPLP0.

Histology and Morphometric Analysis

Human tissue samples were formalin-fixed and paraffin-embedded. Sections (1.5 µm) were used for routine hematoxylin and eosin (H&E) staining. The following primary antibodies were used: rabbit anti-human Involucrin (Abcam, Cambridge, UK; 1:100; art: microwaves in EDTA buffer pH 8.0; inc: 1 hr at RT), mouse anti-human AIRE (kindly provided by Prof P. Peterson, University of Tartu, Tartu, Estonia; 1:3.000; art: thermostatic bath in EDTA buffer pH 8.0; inc: 1 h at RT), mouse anti-human p16 (BioGenex, Fremont, California, USA; 1:1; art: microwaves in EDTA buffer pH 8.0; inc: 1h at RT). Primary antibodies were incubated with MACH 1™ Universal HRP Polymer Kit (Biocare Medical), and reactions were developed in Biocare's Betazoid DAB and nuclei counterstained with hematoxylin.

Digital images were acquired by an Olympus XC50 camera mounted on a BX51 microscope (Olympus, Tokyo, Japan) with CellF Imaging software (Soft Imaging System GmbH, Münster, Germany). Morphometric analysis was performed using Olympus Slide Scanner VS120-L100 (Olympus, Tokyo, Japan) to acquire digital images and Image-pro software (Olympus) to analyze them.

Oxidative Stress Detection

PBMC from DS patients and age matched HDs were also analyzed to assess the level of oxidative stress. To this end, we used two different methods based on the mitochondrial evaluation: MitoTracker Green kit (ThermoFisher) and Tetramethylrhodamine ethyl ester (TMRE) assay.

For mitochondrial staining evaluated by MitoTracker Green kit, used at a final concentration of 300 nM in PBS (CORNING), cells were fully immersed in 1 milliliter of staining solution and incubated on ice for 25 minutes with general agitation. Cells were then washed in PBS for 5 minutes and then to block non-specific binding of the antibodies, cells were submerged in 5% bovine serum albumin (Sigma-Aldrich) for 1 hour at room temperature. Cells were then washed and resuspended in fresh pre-warmed (37°C) PBS (CORNING), acquired using a FACS Canto II (BD Biosciences, San Jose, CA, USA) and analyzed with Flow Jo Software (FLOWJO, LLC). In parallel, TMRE (tetramethylrhodamine, ethyl ester) assay evaluates the mitochondrial membrane potential, a parameter also directly linked to cellular oxidative stress. TMRE (Thermofisher) was dissolved in methanol and used directly adding this compound to PBS at a final concentration of 20nM. Cells were incubated for 30 minutes in the incubator (37°C and 5% CO_2), then washed in fresh pre-warmed PBS (CORNING) and directly acquired using a FACS Canto II (BD Biosciences, San Jose, CA, USA). Flow cytometric analysis was performed with Flow Jo Software (FLOWJO, LLC).

Oxidative stress was detected also in plasma samples using the OxiSelect *in vitro* ROS Assay Kit (CELL BIOLABS, San Diego, California, USA), following the protocol recommended by the manufacturer.

Statistical Analyses

Statistical analyses were performed with GraphPad Prism 5.0 (GraphPad Software, San Diego, California, USA). All results are expressed as the mean ± SEM if not stated otherwise. Comparisons between proportions were calculated by using the chi-square test (χ^2 test) (with continuity correction) as stated in the Figure legends. To assess significance, we used one-way ANOVA with Bonferroni post-correction or two-way ANOVA analysis of variance when specified. We also used two-tailed Mann-Whitney test where specified. p-values <0.05 were considered significant.

RESULTS

Premature Aging in Thymi of DS Patients

In our previous work we showed tissue abnormalities in DS thymi, which are smaller in size and display accelerated kinetics of maturation, as compared to age-matched controls, with signs of premature involution (11). We found an expansion of the medullary area caused by the presence of large cystic involutions positive for involucrin, a marker expressed by terminally differentiated medullary TEC (11). Based on these observations and data reported in literature suggesting immunosenescence in DS patients (10, 12), we evaluated thymic epithelium maturation by immunohistochemical analysis on thymic tissues of DS patients and compared them to age-matched healthy donors. We considered three age groups: 2-5 months, 5-9 months, 2-5 years. As a marker of cellular senescence we analyzed p16, a cyclin-dependent kinase inhibitor that tends to accumulate in cell cycle arrested cells (24). Immunohistochemistry revealed an increased p16 expression associated with large size of Hassall's bodies in DS patients, as compared to age-matched HDs, which becomes more evident in the oldest patient-group (**Figure 1A**). Moreover, we analyzed the thymic tissue of a 4 year-old DS patient, and the thymic tissue from a 3 year-old healthy donor, evaluating their morphology by H&E staining and by immunohistochemistry the expression of Involucrin which detects terminally differentiated mTECs. In the 4 year-old DS thymus, H&E staining showed a similar cortico-medullary ratio as compared to the healthy control (**Figure 1B**). However, we observed in DS thymic samples an increased size of Hassall's bodies, identified by the staining with Involucrin, and the amount of medullary area occupied by the Hassall's bodies in the medulla (**Figure 1C**). Additionally, the 4 year-old DS sample showed a reduced number of $AIRE^+$ cells [**Figure 1C** and our previous published data (11)]. No relevant differences were

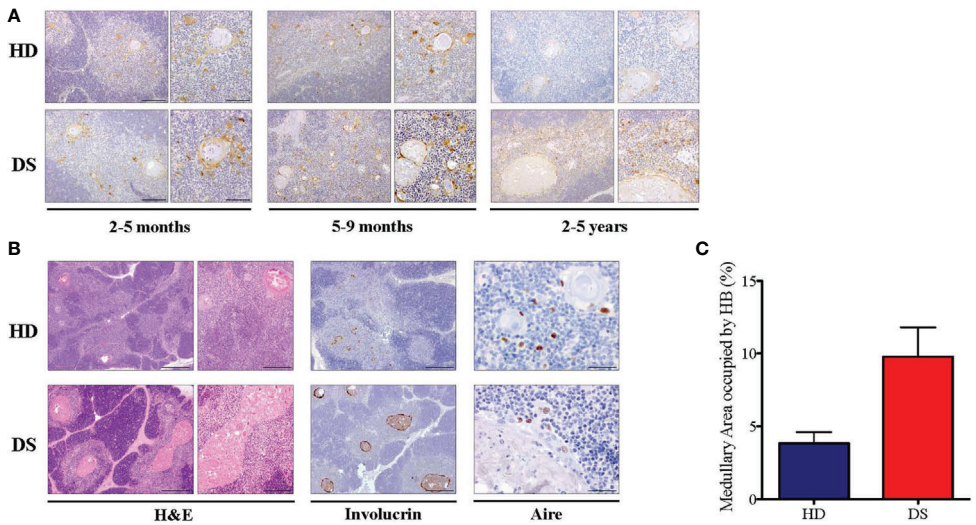

FIGURE 1 | Histological analysis of DS patients thyme. **(A)** Representative histological images of p16 immunohistochemical staining in three different groups of age-matched HD and DS thymic tissue sections (original magnification: 10x = 200 μm left panel – 20x = 100 μm right panel). For each age group, a minimum of 1-3 thymi were analyzed, according to sample availability. **(B)** H&E staining and Involucrin and AIRE immunohistochemical staining of HD and DS thymic samples from a 4 year-old DS patient and a 3 year-old healthy donor (original magnification: H&E 4x = 500 μm left panel -10x = 200 μm right panel; Involucrin 4x = 500 μm: AIRE 40x = 50 μm) (HD sample 12 in **Supplemental Table 2**; DS sample 1 in **Supplemental Table 3**) **(C)** The graph shows the proportion of medullary area occupied by the Hassall's bodies in thymic samples (HD samples: 2, 7 and 12 in **Supplemental Table 2**; DS samples: 1 and 6 in **Supplemental Table 3**). Median with interquartile range is represented.

detected by immunohistochemistry in the thymocyte compartment, which showed no alterations in T cell-maturation and expression of developmental markers (TdT, CD3, CD4, CD8).

Transcriptomic Profile of Human TEC in DS Patients

In order to investigate the mechanisms underlying thymic dysfunction in DS patients, we performed a whole transcriptomic analysis *via* RNA-Seq analysis on sorted CD45$^-$ Epcam-enriched hTEC from DS patients and HD. Frozen samples of 3 normal HD and 3 DS patients were analyzed (**Supplemental Table 1**). Differential gene expression (DE) and gene set enrichment analysis (GSEA) provided interesting insights into Epcam-enriched hTEC transcriptome in samples from both HD and DS patients, highlighting distinct transcriptomic profiles (**Supplemental Figure 1A** and **Supplemental Table 5**). First of all, DE analysis confirmed that we were able to substantially enrich TEC in Epcam-positive (Epcam$^+$) sorted thymic samples, as genes typically expressed in cTEC and mTEC were significantly more expressed in Epcam$^+$ subsets, as compared to Epcam-negative (Epcam$^-$) fraction (**Table 1**, **Supplemental Figure 1B**). When we compared Epcam$^+$ versus Epcam$^-$ samples from both HD and DS patients, enrichment analysis using the REACTOME Pathway and GO (Biological Processes) databases on differentially expressed genes (DEGs), revealed a statistical increased expression of genes involved in extracellular matrix organization, collagen formation and epithelial development (**Supplemental Figure 1C**).

Interestingly, majority of gene expression differences was observed within the Epcam$^+$ positive subsets, while Epcam$^-$ fraction showed more similarities between patients and healthy subjects, suggesting that main differences between DS patients and normal donors reside in TEC (**Table 2**, **Figure 2A**). Among the most significantly upregulated genes in DS TEC-enriched samples, many transcripts encoding for histones emerged (**Figure 2B**). Furthermore, GSEA revealed several gene sets particularly enriched in DS samples, as compared to HD, suggesting the presence of a disease-specific transcriptomic signature at TEC level. Noteworthy, GSEA results obtained from the comparison between DS and HD Epcam$^+$ cell subsets showed a statistically significant enrichment of categories related to cellular senescence, cellular response to stress and epigenetic histone and DNA modifications in DS (**Figure 2C**). In particular, the positive enrichment of cellular senescence, cellular response to stress, and cellular responses to external stimuli terms, strongly suggesting that cells in the Epcam$^+$ subset in DS patients present an over-expression of genes involved in the senescence process when compared to normal subjects (**Figure 2D**).

Thymic Epithelial Cells in DS Show Signs of Senescence

Senescence associated β-galactosidase (SA-β-gal) activity is one the most commonly used biomarkers for senescent cells detection (24). To further characterize TEC senescence in DS patients, SA-β-gal levels were quantified by fluorescent microscopy (**Figures 3A, B**) and flow cytometry (**Figure 3C**) and we reported a significant increase in DS patients as compared to age-matched HDs.

To analyze more in depth the senescence profile of TEC in DS patients we evaluated the expression of four genes normally

TABLE 1 | Differential expression of cTEC-specific genes.

	HD		DS	
	logFC	**FDR**	**logFC**	**FDR**
Ccl25	4,5547	2,76E-06	3,5524	0,0002
Cd74	1,2122	0,1379	2,4771	0,0004
Ctsl	-2,278	7,96E-06	-2,4404	1,68E-06
Foxn1	6,7714	5,79E-08	5,6012	3,24E-06
Ly75	3,3015	1,87E-05	3,1349	5,44E-05
Pax1	5,5748	1,93E-07	3,8492	0,0002
Prss16	3,6074	2,3E-05	3,7218	1,26E-05
Psmb11	3,5666	0,0537	3,3808	0,0563

Log2-fold change and FDR adjusted p-values (padj) are reported, obtained from the comparison of Epcam$^+$ and Epcam$^-$ cell subsets within each subject's group. Positive log2-fold change values indicate upregulation of that specific gene in Epcam$^+$ cells.

TABLE 2 | Differentially expressed genes at FDR < 0.05.

	DS Epcam$^+$/DS Epcam$^-$	HD Epcam$^+$/HD Epcam$^-$	DS Epcam$^+$/HD Epcam$^+$	DS Epcam$^-$/HD Epcam$^-$
Upregulated	2419	1903	125	10
Downregulated	2814	2082	424	4
Total DE genes	5233	3985	549	14
(FDR < 0.05)				

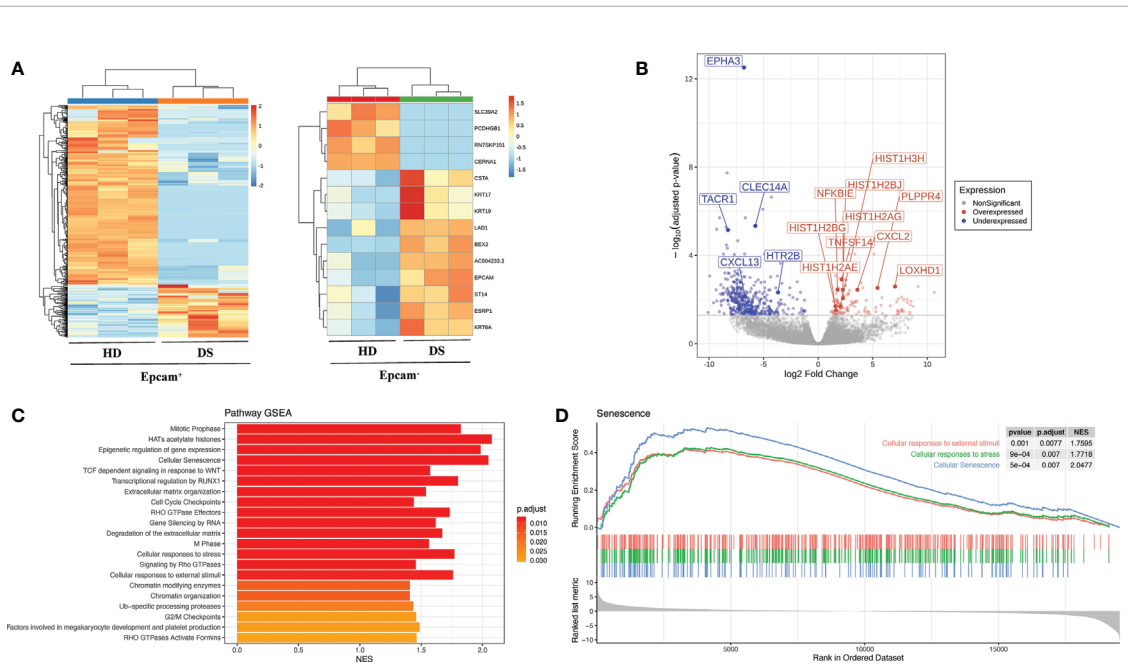

FIGURE 2 | Transcriptomic profile of human TEC in HD and DS patients. **(A)** Heatmaps showing the expression of differentially expressed genes (DEGs) between normal subjects (HD) and DS patients in Epcam+ (left) and Epcam- (right) cell subsets. **(B)** Differentially expressed genes (DEGs) between DS and HD Epcam$^+$ cell subsets. Log2 fold change and FDR-adjusted p-values are reported for each gene. Volcano plot showing significant (FDR < 0.05) down- (blue) and up- (red) regulated genes between DS and normal patients in Epcam+ cell subset. Differentially expressed genes of interest are highlighted. **(C)** Significantly enriched terms resulting from the GSEA against the REACTOME Pathway database of genes ranked according to logFC values, resulting from the comparison between DS and normal subjects (HD) in Epcam+ samples. **(D)** Enrichment plot showing the GSEA results of three senescence-related terms on gene pre-ranked based on logFC values resulting from the comparison between DS and normal patients in Epcam$^+$ cell subset.

associated to cellular senescence: H2AX, p16, TERF1 and TERF2 (**Figure 3D**). H2A histone family member X (usually abbreviated as H2AX) is a type of histone protein from the H2A family involved in all senescence mechanisms (25). TERF1 is a negative regulator of telomere length, while TERF2 protects telomers from degradation and fusion and both of them are involved in cellular senescence and in aging processes (26). Real time PCR analysis detected higher expression of all these genes in digested

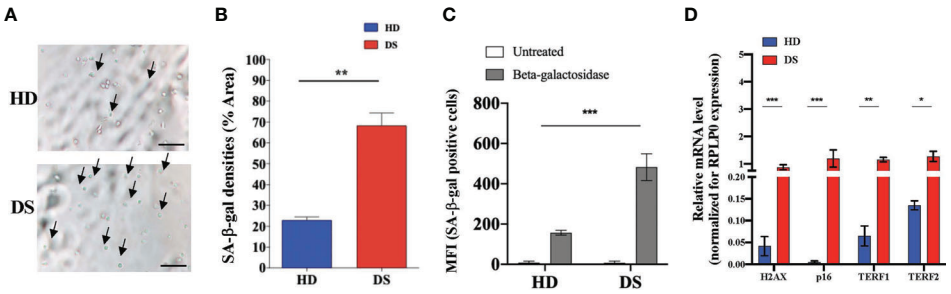

FIGURE 3 | Senescence detection in thymic epithelial cells. **(A–C)** SA-β-gal staining in TEC isolated from DS patients and age-matched HDs. Analyses performed by microscopy **(A, B)** (SA-β-gal$^+$ cell counts were normalized per % of Area; scale bar = 100 μm) and flow cytometry **(C)** (HD, n = 9; DS, n = 9; HD 2-5 months: 1,35; 5-9 months: 4,6,9 and 2-5 years 2,7,10 referring to **Supplemental Table 2**; while DS 2-5 months: 2,3,5; 5-9 months: 4,8,9 and 2-5 years 1,6, 10 referring to **Supplemental Table 3**). **(D)** Comparison of mRNA expression of senescence-associated genes (H2AX, p16, TERF1 and TERF2 in thymic tissue from DS patients and age-matched HDs, normalized for the expression of the housekeeping gene RPLP0 (HD, n = 12, sample 13 in **Supplemental Table 2** was excluded from the analyses; DS, n = 9, from sample 1 to sample 9 in **Supplemental Table 2**). Mean ± SEM are represented (Mann-Whitney test; *p-value < 0.05; **p-value < 0.002; ***p-value < 0.0001).

thymic tissue from DS patients after depletion of CD45+ cells, further confirming the establishment of a senescence program in thymic stroma of these patients.

T-Cell Compartment Is Compromised in DS Patients

Next, we moved to the lymphoid compartment in which we evaluated SA-β-gal expression in thymocytes isolated from DS patients and HDs. To obtain a better characterization, we divided our samples, both HDs and DS, in three age-groups that represent different stages of thymic maturation and development: 2-5 months, 5-9 months and 2-5 years (27). Flow cytometric analysis was performed in 4 different thymocyte subsets: DN, DP, SP and SP8. In all thymocyte subsets and age-groups analyzed the expression of SA-β-gal was statistically increased in DS patients as compared to age matched HDs. Interestingly, SA-β-gal expression increased more dramatically from 2 months to 5 years in DS patients, as compared to HDs, suggesting an accelerated aging (**Figure 4A**). Similar to TECs, H2AX, TERF1 and TERF2 expression was significantly increased in DS patients as compared to HDs (**Figure 4B**). p16 gene expression in thymocytes from DS patients was increased although it did not reach statistical significance (**Figure 4B**). These results indicate that also the thymocyte compartment in DS patients has increased senescence. To evaluate whether peripheral T cells were also affected, we analyzed β-gal expression in peripheral blood CD4$^+$ and CD8$^+$ T cells of DS patients and age-matched controls. We noticed an increased SA-β-gal expression in terms of MFI in both CD4$^+$ and CD8$^+$ T cells in all DS patients as compared to age-matched HDs (**Figure 4C**). Concomitantly, we also found a consistent increased of H2AX, TERF1 and TERF2 expression in total PBMCs from DS patients with data observed in the thymic compartment (**Figure 4D**).

Overall, these data indicate an accelerated senescence establishment affecting thymic epithelial cell compartment as well as thymocytes and leading to the egress of exhausted and senescent lymphocytes to the periphery.

High Level of Cellular Oxidative Stress in Thymic Compartment and in Peripheral Blood in DS Patients

Growing evidence supports the close link between cellular senescence and oxidative stress (28–30), due to the excessive production of reactive oxygen species (ROS), which leads to the accumulation of oxidative damage and induces changes in molecules, cells and tissues (31). ROS are produced by several endogenous and exogenous processes and their negative effects are neutralized by antioxidant defenses. Oxidative stress occurs from the imbalance between ROS production and antioxidant defenses (30). Based on these observations and on our previous results, we investigated the correlation among senescence, oxidative stress and mitochondrial dysfunction in DS (**Figure 5**). Indeed, mitochondria are deeply involved in the production of reactive oxygen species and are also very susceptible to oxidative stress. Oxidative stress can induce apoptotic death, and mitochondria have a central role in this and other types of apoptosis (32). To investigate whether the oxidative stress might have a role in accelerated senescence in DS patients, we analyzed the expression of oxidative stress promoter Superoxide Dismutase (SOD1), Amyloid Beta Precursor Protein-(APP), which is induced by oxidative stress (32), and BACH1, an antioxidant gene. The analysis was performed on TEC (**Figure 5A**), total thymocytes (**Figure 5B**) and PBMCs (**Figure 5C**) isolated from DS patients and HDs. Our results indicate an increased expression of SOD1 and APP genes in DS patients as compared to age-matched HDs in all three different cell types (**Figures 5A–C**); conversely the antioxidant gene BACH1 resulted significantly decreased in DS patients as compared to age-matched HDs in TEC, thymocytes and PBMCs (**Figures 5A–C**, respectively).

We then evaluated mitochondria functionality, by quantifying their number and their surface membrane potential. The analysis was performed on total T lymphocytes isolated from DS patients and age-matched HDs. In both flow cytometric analyses performed using two different methods, Mitotracker (**Figure 5D**) and TMRE analysis (**Figure 5E**) to evaluate mitochondria and label active mitochondria respectively, we noticed an alteration of cellular

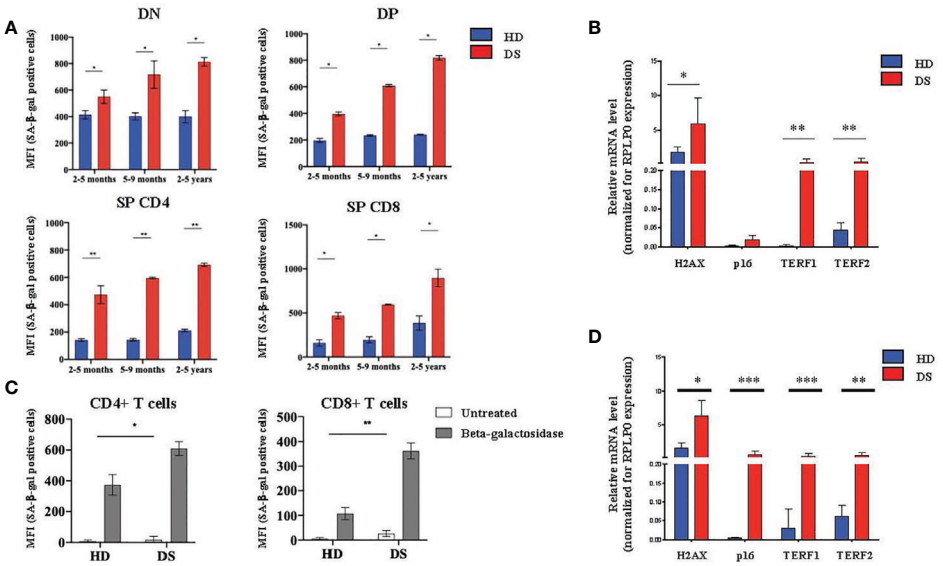

FIGURE 4 | Senescence detection in thymocytes and PBMCs. **(A)** MFI of SA-β-gal positive cells calculated on the gate of DN ($CD45^+CD4^-$ $CD8^-$), DP ($CD45^+$ $CD4^+$ $CD8^+$), SP4 ($CD45^+$ $CD4^+$ $CD8^-$) and SP8 ($CD45^+$ $CD4^-$ $CD8^+$) thymocytes. HD and DS samples were divided in three age-groups: 2-5 months, 5-9 months and 2-5 years (2-5 months: HD, n = 3, samples 1, 3, 5 in **Supplemental Table 2**; DS, n = 3, samples 2, 3, 5 in **Supplemental Table 3**. 5-9 months: HD, n = 3, samples 4, 6, 9 in **Supplemental Table 2**; DS, n = 3, samples 4, 8, 9 in **Supplemental Table 3**. 2-5 years: HD, n = 3, samples 2, 7, 10 in **Supplemental Table 2**; DS, n = 3, sample 1, 6, 10 in **Supplemental Table 3**). **(B)** Comparison of mRNA expression of senescence associated genes (H2AX, p16, TER1 and TERF2) in thymocytes isolated from DS patients and age matched HDs, normalized for the expression of the housekeeping gene RPLP0 (HD, n = 12, sample 13 in **Supplemental Table 2** was in excluded from the analyses; DS, n = 9, from sample 1 to sample 9 in **Supplemental Table 3**). **(C)** MFI of SA-β-gal positive $CD4^+$ T cells (*left graph*) and $CD8^+$ T cells (*right graph*) calculated on the gate of $CD45^+$, $CD3^+$ T lymphocytes (HD, n = 12, sample 13 in **Supplemental Table 2** was excluded from the analyses; DS, n = 12, from sample 1 to sample 9 in **Supplemental Table 3**). **(D)** Comparison of mRNA expression of senescence-associated genes (H2AX, p16, TER1 and TERF2), in peripheral T cells isolated from DS patients and age-matched HDs, normalized for the expression of the housekeeping gene RPLP0 (HD, n = 12, sample 13 in **Supplemental Table 2** was in excluded from the analyses; DS, n = 9, from sample 1 to sample 9 in **Supplemental Table 3**). Mean ± SEM are represented (Mann-Whitney test; **p*-value < 0.05; ***p*-value < 0.002; ****p*-value < 0.0001).

organelles in DS patients' cells. The frequency of mitochondria was significantly increased as compared to age-matched HDs, while analysis of mitochondria membrane potential showed an increased frequency of active mitochondria in DS patients as compared to age-matched HDs.

Altogether, our results indicate an increased level of oxidative stress in TEC, thymocytes and PBMCs isolated from DS patients in terms of increased ROS production associated to a decreased antioxidant function and increased mitochondria numbers and membrane potential.

DISCUSSION

DS patients present with specific conditions associated with earlier aging as compared to general population, including premature skin wrinkling, greying of hair, hypogonadism, early menopause, hypothyroidism, Alzheimer's disease and declining immune function (12). Premature aging in DS is atypical and segmental, involving some but not all organs and tissues, particularly the brain and the immune system (12). Several studies have reported alterations of adaptive immune system including defects affecting thymocytes and mature peripheral lymphocytes (9, 12). These defects result in a high predisposition to develop recurrent infections, especially of the respiratory tract, autoimmune diseases and leukemia's or other lymphoreticular malignancies (8, 9, 13). The recent description of DS individuals showing increased risk of dying from COVID-19 infection has further highlighted their defective and senescent immune system (33). Several are the mechanisms underlying immune perturbation. Various observations, including the fact that chronic diseases associate with aging, point to an accelerated senescence process as the major culprit in DS (12, 13). Consistently, DS patients' immunological profile is suggestive of precocious immunosenescence, characterized by earlier thymic involution with low thymic output and decreased number of naïve $CD4^+$ and $CD8^+$ T lymphocyte (10–13). The lack of data on TEC and thymocytes vs PB obtained from the same patient and how these immune parameters change over time, prompted us to further evaluate the contribution of various cellular players (epithelial vs thymocytes and lymphocytes) on DS pathogenesis focusing on senescence process. We speculated that accelerated senescence both in TEC and thymocytes might act as disruptive element of central tolerance homeostasis (12–14). Data from the *TS65Dn* mouse model show increased oxidative stress and reduced cytokine signaling thus pointing to the major role of premature senescence in the pathogenesis of the disease (8, 9). More recently, the demonstration of a perturbed Nrf2 signaling, a pathway required to prevent oxidative damage in DS human fibroblasts and *Dp16*

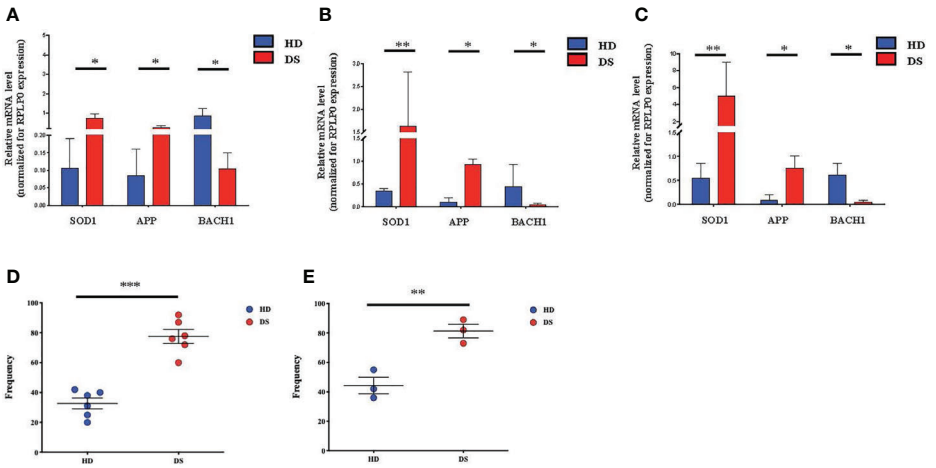

FIGURE 5 | Oxidative stress detection in TEC, thymocytes and PBMCs. **(A–C)** Comparison of mRNA expression of oxidative stress-associated genes (SOD1, APP, BACH1), in TEC **(A)**, thymocytes **(B)** and PBMCs **(C)** isolated from DS patients and age-matched HDs, normalized for the expression of the housekeeping gene RPLP0 (TEC and thymocytes: HD, n = 12, sample 13 in **Supplemental Table 2** was excluded from the analyses; DS, n = 9, from sample 1 to sample 9 in **Supplemental Table 3**. PBMCs: HD, n = 12, sample 13 in **Supplemental Table 2** was excluded from the analyses; DS, n = 9, from sample 1 to sample 9 in **Supplemental Table 3**). **(D)** Frequency of mitochondria function and number in PBMCs evaluated on the gate of total $CD45^+$ $CD3^+$ T lymphocytes isolated from DS patients and age-matched HDs (HD, n = 6, samples 2, 7, 9, 10, 11,12 in **Supplemental Table 2**; DS, n = 6, samples 1, 6, 9, 10, 11, 12 in **Supplemental Table 3**). **(E)** Analysis of mitochondrial membrane potential, expressed as frequency and calculated on the gate of total $CD45^+$ $CD3^+$ T lymphocytes isolated from DS patients and age-matched HDs (HD, n = 3, samples 2, 7, 11 in **Supplemental Table 2**; DS, n = 3, samples 10, 11, 12 in **Supplemental Table 3**). Mean ± SEM are represented (Mann-Whitney test; **p*-value < 0.05; ***p*-value < 0.002; ****p*-value < 0.0001).

mouse embryonic fibroblasts (34) has provided an additional layer of complexity to DS pathogenesis and focused the attention to the effect of trisomy on the mitochondrial activity. Given these data, we analyzed DS thymic specimens obtained from three different cohort of pediatric patients, confirming the already known signs of thymic involution characterized by a general reduction of thymic size and premature increase of medulla mainly due to enlargement of Hassall's bodies including highly enriched involucrin positive cells and cystic involutions (10, 11, 18). First, we tested the expression of p16, a marker accumulating in senescent cells and indicating the accelerated maturation process leading to a premature thymic involution. Immunohistochemistry revealed the dramatic increase of p16 in all thymi analyzed, irrespective of the age. Transcriptomic profile was performed on the total epithelial component obtained after depletion of $CD45^+$ cells and enriched in TEC cells based on the Epcam surface expression. Noteworthy, for technical reasons, we could not isolate mTEC versus cTEC subpopulations and the different ratio between these cell types might contribute to gene expression changes. However differential gene expression analysis based on TEC subset specific genes did not reveal significant differences between HD and DS Encamp+ cells (data not shown). Overall, the comparison between $Epcam^+$ and $Epcam^-$ cell fraction transcriptomic profiles highlighted the effect of trisomy on $Epcam^+$ subset transcriptomic modulation. Consistently, within this cellular subset in DS we found the majority of differences in gene expression, further supporting the hypothesis of immune dysregulation as an effect of perturbed central tolerance mechanism (18). Conversely to the assumption that perturbation of AIRE and promiscuous gene expression is the main driver of immune dysregulation in DS sustained by some authors (18), here we evaluated whether other players may cause the altered thymic function. Among the genes overexpressed in DS $Epcam^+$-enriched subset, we found an increased expression of gene sets involved in cellular response to stress and in senescence process. Many transcripts encoding for histones emerged among the most significantly overexpressed genes in DS TEC-enriched sample corroborating the hypothesis that a leverage of epigenetic mechanisms may counteract immune dysregulation induced by trisomy 21 as originally speculated by Moreira-Filho et al. (20).

These data are in line with the peculiar feature of senescent cells showing distension of pericentromeric satellite sequences and profound changes in epigenome organization (35). To further assess the establishment of the senescent process in TEC, epithelial cells were stained with SA-β-galactosidase showing an increased frequency of positive cells in the area analyzed together with augmented MFI expression detected by flow cytometry. These cells express higher level of H2AX, an indicator of DNA damage response activation, and increased expression of senescent markers p16 and TERF1/TERF2, two proteins of the sheltering complex involved in the maintenance of capping function and telomere length (36). Increased expression of SA-β-gal, p16 and telomerase markers were also found in thymocytes at various differentiation stages irrespectively to the age, confirming that both component epithelial and thymocytes undergo accelerated senescent process.

Extensive studies have shown the interplay between oxidative stress, aging and senescence (35). Reactive oxygen species (ROS) act as signaling molecules that may be detrimental for the cells if not counteracted by antioxidant molecules such as superoxide dismutase (SOD1), glutathione peroxidase and other molecules. In thymocytes and PBMC of DS patients, analysis of SOD1

showed increased levels of expression together with Amyloid Beta Precursor Protein (APP), a molecule mapping on chromosome 21 and induced by oxidative stress (37). BACH1, the transcription regulator protein binding to the promoter of genes containing antioxidant response elements (ARE) to repress cellular antioxidant responses (38), was found decreased in DS patients as compared to healthy donors. All these findings confirmed the increased stress response observed by RNA-seq analysis. Increased oxidative stress has been linked to the accumulation of dysfunctional mitochondria (39), which in turn play a relevant role in triggering senescent process (40). To assess mitochondria function in DS T cells, we measured the frequency of activated mitochondria by measuring membrane potential by TMRE assay in circulating lymphocytes. We found an increased number of mitochondria with increased membrane potential in DS indicating a perturbation of mitochondrial fitness. This perturbed homeostasis further sustains the increased levels of ROS contributing to cellular damage.

Overall, our data provide evidence that immune dysregulation in DS is caused by multiple dysfunctional layers acting in both thymic epithelial cells and thymocytes. Telomere damage, increased senescent process induced by mitochondrial dysfunction and accumulation of ROS all contribute to premature thymic involution with consequent alterations in thymocytes maturation kinetics and egression of exhausted lymphocytes. These data are supported by changes in Epcam-enriched TEC gene expression profile suggesting a disease specific transcriptomic signature. Further dissection of the mechanisms underlying the senescent process in DS, possibly using single cell-based analysis, will be instrumental to envisage novel therapies slowing disease progression in DS patients.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by San Raffaele Ethics Committee. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

GM, FF, and IB performed experiments, analyzed data and wrote the manuscript. MG performed RNA-Seq profile. StB and IM analyzed data of RNA-Seq TEC. EF performed histological analyses on human tissue samples; ACo performed senescence experiments in **Figures 3A–C** and **4A–C** and interpreted data. DM, MC, SoB, VD'O, AG, DA, CC, and ACa provided samples of patients and healthy donors. RM designed and supervised experiments. AV and MB designed research experiments, supervised the study and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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Thymic Epithelial Cell Alterations and Defective Thymopoiesis Lead to Central and Peripheral Tolerance Perturbation in MHCII Deficiency

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Major Histocompatibility Complex (MHC) class II (MHCII) deficiency (MHCII-D), also known as Bare Lymphocyte Syndrome (BLS), is a rare combined immunodeficiency due to mutations in genes regulating expression of MHCII molecules. MHCII deficiency results in impaired cellular and humoral immune responses, leading to severe infections and autoimmunity. Abnormal cross-talk with developing T cells due to the absence of MHCII expression likely leads to defects in thymic epithelial cells (TEC). However, the contribution of TEC alterations to the pathogenesis of this primary immunodeficiency has

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not been well characterized to date, in particular in regard to immune dysregulation. To this aim, we have performed an in-depth cellular and molecular characterization of TEC in this disease. We observed an overall perturbation of thymic structure and function in both MHCII$^{-/-}$ mice and patients. Transcriptomic and proteomic profiling of murine TEC revealed several alterations. In particular, we demonstrated that impairment of lymphostromal cross-talk in the thymus of MHCII$^{-/-}$ mice affects mTEC maturation and promiscuous gene expression and causes defects of central tolerance. Furthermore, we observed peripheral tolerance impairment, likely due to defective Treg cell generation and/or function and B cell tolerance breakdown. Overall, our findings reveal disease-specific TEC defects resulting in perturbation of central tolerance and limiting the potential benefits of hematopoietic stem cell transplantation in MHCII deficiency.

Keywords: thymus, thymic epithelial cells, primary immunodeficiency, MHCII, central tolerance

INTRODUCTION

Major Histocompatibility Complex (MHC) class II (MHCII) deficiency (MHCII-D), also known as Bare Lymphocyte Syndrome (BLS), is a rare autosomal recessive combined immunodeficiency (CID) due to mutations in genes regulating expression of MHCII molecules [OMIM#209920] (*CIITA*, *RFXANK, RFX5*, *RFXAP*) (1–4). MHCII molecules play a crucial role in immune responses, due to their fundamental role in presenting exogenous peptides to the antigen T-cell receptor (TCR) of CD4$^+$ T lymphocytes. In the thymus, complexes formed by self-peptides and MHCII, expressed on thymic epithelial cells (TEC) and dendritic cells, drive the positive and negative selection processes, which are critical for the development of CD4$^+$ T cells and the establishment of a non-self-reactive TCR repertoire (1, 4). In the periphery, MHCII molecules contribute to the induction of antigen-specific immune responses against pathogens and tumors and, through the presentation of self-peptides, to the maintenance of peripheral T-cell tolerance.

Defects of antigen processing and presentation via MHCII molecules in patients with MHCII deficiency lead to impairment of thymic education and peripheral T-cell help (5, 6). Thymopoiesis is defective in this disease, presumably due to impaired thymic selection in the absence of MHCII on TEC (4, 7). Examination of thymic biopsies from two patients with CID and defective expression of MHC I and II molecules revealed normal lobular thymic architecture, with distinct cortex-medullary areas, well-differentiated epithelium, and presence of Hassall's corpuscles. However, MHCII antigen expression was not detectable on epithelial cells in the cortex and absent/reduced in the medulla, in contrast to what was observed in normal controls (8). Study of thymic function in 8 MHCII-deficient patients (7) showed that, despite normal TCR-Vβ repertoire of total CD3$^+$ T cells, clonal abnormalities emerged at flow cytometric evaluation of TCR-Vβ repertoire on CD4$^+$ T cells and at spectratyping evaluation of TCR-Vγ repertoire on total CD3$^+$ lymphocytes (7). These findings suggest a reduced global thymic activity in MHCII deficiency and emphasize the key role of MHCII molecules in the process of normal thymic maturation of T lymphocytes. However, interestingly, TCR excision circles (TREC) were detectable in patients' total lymphocytes and sorted CD4$^+$ cells, reflecting normal early T-cell development (4, 7).

In MHCII-D patients, lack of MHCII molecules expression on the cell surface results in severe CD4$^+$ T-cell lymphopenia and impaired antigen-specific cellular and humoral immune responses. Usually hematopoiesis is not affected, and most patients have normal numbers of circulating B and total T cells. Despite intact response to mitogen stimulation, T-cell mediated responses to foreign antigens are impaired. Hypogammaglobulinemia is a common finding (1, 4) and antibody responses to immunizations and to infections by microbial agents are generally absent or markedly reduced (6). On the other hand, autoantibodies have been demonstrated in several patients (6) and autoimmune manifestations, such as autoimmune cytopenias, have been observed in 6–20% of patients (4, 9, 10). Patients suffer from recurrent and severe infections (viral, bacterial, fungal, and protozoan), primarily involving the respiratory and gastrointestinal tract (4, 11) since early in life. Inflammatory enteropathy is frequently observed and reflects the multifaceted disturbance of intestinal epithelial cell regulation of adaptive mucosal immunity due to the lack of MHCII expression on enterocytes (12). If untreated, MHCII deficiency is often fatal early in life (3, 4). Allogeneic hematopoietic stem cell transplantation (HSCT), preferably from an HLA-identical sibling donor, is the treatment of choice, but overall success rate is limited (10, 13–15). In recent years HSCT survival has improved, but CD4$^+$ T-cell lymphopenia may persist (16).

The Aβ$^{0/0}$ mouse model, lacking MHC class II antigens, shares some phenotypical features with MHCII-D (17, 18). Aβ$^{0/0}$ mice have barely detectable numbers of CD4$^+$ T lymphocytes in secondary lymphoid organs, while in the thymus, immature CD4 single-positive (SP) thymocytes are present, indicating impairment of positive selection process, particularly in its initial stages, when TCR/MHC interactions are required (19). Interestingly, it has been shown that a large proportion of residual CD4$^+$ T cells correspond to

CD1-restricted natural killer T (NKT) cells in MHCII-deficient mice (20, 21). *In vivo* treatment of $A\beta^{0/0}$ mice with anti-TCR antibody has been shown to restore the generation of circulating $CD4^+$ T cells and to normalize the thymic medulla (22). A reduction of the medullary TEC (mTEC) compartment has been described also in another MHCII ko mouse model ($A\alpha^{-/-}$ mice) (23). Reduced number of mature mTECs and decreased expression of Aire and Aire-dependent and -independent tissue restricted antigens (TRA) have been detected in the thymus of $A\alpha^{-/-}$ mice (24). The demonstration of $CD8^+$ T cell infiltrates in multiple organs of $A\alpha^{-/-}$ mice suggested defects of central tolerance and/or of regulatory T (Treg) cells (24). While $CD4^+$ $FoxP3^+$ Treg cells were not found in the thymus of $A\alpha^{-/-}$ mice, they were present in the periphery and seemed functional and efficient in mediating immune suppression (25). Furthermore, in experimentally induced colitis models, regulatory $CD25^+$ double-positive (DP) T cells generated in MHCII ko mice ($A\alpha^{-/-}$ or $A\beta^{0/0}$), probably arising from SP $CD8^+$ T cells, have been demonstrated to control the colitogenic potential of $CD25^-CD4^+$ T cells (26). Indeed, $CD8^+$ T cells constitutively expressing CD25 and bearing characteristics similar to regulatory $CD4^+CD25^+$ T cells have been also detected in the thymus of $MHCII^{-/-}$ mice (27).

In conclusion, it is currently unclear if TEC defects are responsible, at least in part, for the pathogenesis of MHCII deficiency. To better define this issue, here we report on thymic defects in both patients and in the $A\beta^{0/0}$ mouse model of MHCII-D and describe how these alterations lead to peripheral immune dysregulation.

MATERIALS AND METHODS

Human Samples

A thymic biopsy was obtained from a 23-month-old infant with MHCII deficiency upon informed consent in accordance with the Research Ethics Board at The Hospital for Sick Children in Toronto (Canada). Patient's data were compiled prospectively and retrospectively from medical records and entered into the Primary Immunodeficiency Registry and Tissue Bank (REB protocol no. 1000005598). The patient presented at 18 months of life with a history of recurrent respiratory tract infections, chronic diarrhea, and CMV hepatitis. She had a family history of MHCII deficiency and was found to be homozygous for a mutation in the *CIITA* gene. Immunological data are reported in **Table 1**. The patient received a matched related HSCT, but engraftment was poor.

A human thymic sample from a healthy control used for comparison was analyzed retrospectively in compliance with Declaration of Helsinki and policies approved by Ethics Board of Spedali Civili in Brescia. Specifically, for retrospective and exclusively observational study on archival material obtained for diagnostic purposes, patient consent was not needed (Delibera del Garante n. 52 del 24/7/2008 and DL 193/2003).

Blood samples were collected from 11 patients with MHCII deficiency [peripheral mononuclear cells (PBMC), n = 4; serum or plasma, n = 9]. Each patient was attributed a numerical code (MHCII_xx). Questionnaires to gather patient's relevant clinical data were sent to referring clinicians, who were responsible for the collection of informed consent for biological samples' collection and anonymized biological sample/data sharing from their own patients, according to local research protocols, reviewed and approved by local ethics committees or institutional review board (IRB) [for NIH patient's samples, protocols 18-I-0041 and 18-I-0128, approved by the NIH IRB]. Data about clinical history, immunological features and HSCT of this cohort of MHCII-D patients are reported in **Tables 2**–**4** respectively.

TABLE 1 | Immunological work-up of the MHCII-D patient who underwent thymic biopsy.

Test	Results
Proliferative response to mitogens	
–PHA	40–50% of control
–Response to specific Ag at day 6 (PPD, VZV, CMV, HSV, Candida)	No
Serum immunoglobulins	Normal
Immune phenotype	
CD3	64% (3,184/μl)
CD4	7.1% (356/μl)
CD8	44% (2,241/μl)
CD56	3% (151/μl)
CD19	34% (1,713/μl)

Abnormal results according to age-based reference ranges (28) are reported in bold. PHA, phytohemagglutinin; Ag, antigen; PPD, tuberculin-purified protein derivative; VZV, Varicella-Zoster virus; CMV, Cytomegalovirus; HSV, Herpes Simplex virus.

Peripheral blood (PB) from healthy controls (HD) was obtained in accordance with the 1964 Declaration of Helsinki and its subsequent amendments, under biological material collection protocols approved by the Institutional Ethical Committee of San Raffaele Hospital (Tiget07, Tiget09). Informed consent was signed directly by the subject or by parents or legal representatives in case of minors.

Mice

$A\beta^{0/0}$ mice were kindly provided by Dr. Matteo Iannacone (San Raffaele Scientific Institute, Milan, Italy). $Rag1^{-/-}$ mice were purchased from The Jackson Laboratories. C57/black (BL) 6 control wild-type (WT) mice were purchased from Charles River Laboratories Inc. All mice were housed in specific pathogen-free conditions and treated according to protocols approved by the Animal Care and Use Committee of the San Raffaele Scientific Institute (Institutional Animal Care and Use Committee protocol no. 710 and 712).

Histological Analysis

Following sacrifice, thymus and gut tissue isolated from mice were formalin-fixed and paraffin-embedded. Hematoxylin and Eosin (H&E) staining was used to assay basic histopathological changes. Paraffin sections were de-waxed, rehydrated, endogenous peroxidase activity was blocked by 0.1% H_2O_2, and nonspecific background reduced with Rodent Block M (Biocare Medical, Concord, CA, USA) before heat-based antigen-retrieval treatment and incubation with antibodies. Depending on the primary antibodies used, sections were

TABLE 2 | MHCII-D patients' features.

Patient code	Mutated gene	Age at sampling (years)	Pre/Post HSCT	Infections	Immune dysregulation	Chronic diarrhea
MHCII_01	NK	0.5	Pre	Yes	No	Yes
MHCII_02	RFXANK	12.8	Pre	Yes	AIHA	Yes
MHCII_03	RFXANK	0.9	Pre	Yes	No	No
MHCII_04	RFXANK	4.0	Pre	Yes	No	Yes
MHCII_05	RFXAP	16.0	Pre	Yes	Autoimmune enteropathy and polyendocrinopathy[#]	Yes
MHCII_08	RFXANK	6.5	Post (+2 years)	Yes	No	Yes
MHCII_09*	RFXANK	4.7	Pre	Yes	No	Yes
MHCII_10*	RFXANK	4.6	Pre	Yes	No	Yes
MHCII_11	NK	0.4	Pre	Yes	No	NK
MHCII_12	RFXANK	24.1	Pre	Yes	No	Yes
MHCII_13	RFXANK	15.5	Pre	Yes	AIHA, autoimmune thyroiditis, adrenal insufficiency	No

NK, not known; HSCT, hematopoietic stem cell transplantation; AIHA, autoimmune hemolytic anemia; mo., months; Ab, antibody. [#]Insulin-dependent diabetes mellitus (IDDM) type 1, hypothyroidism.

**These patients are described in greater detail in (16).*

TABLE 3 | Immunophenotype and serum Ig level in our cohort of MHCII-D patients.

Patient code	$CD3^+$ cells ($\times10^9$/l)	$CD4^+$ cells ($\times10^9$/l)	$CD8^+$ cells ($\times10^9$/l)	$CD19^+$ cells ($\times10^9$/l)	$CD16^+/CD56^+$ cells ($\times10^9$/l)	$HLA\text{-}DR^+$ cells ($\times10^9$/l)	IgM (g/L)	IgG (g/L)	IgA (g/L)
MHCII_01	1,495	402	1,087	491	30	Absent	0.06	4.94	<0.02
MHCII_02	1,121	240	741	261	20	Absent	0.31	9.93	<0.05
MHCII_03	1,021	484	480	2,178	77	7	0.29	3.57	0.2
MHCII_04	NA	NA	NA	NA	NA	NA	NA	NA	NA
MHCII_05	NA	NA	NA	NA	NA	NA	NA	NA	NA
MHCII_08	1,540	901	526	1,315	714	Present[§]	1.17	9.26	<0.05
MHCII_09*	207	71	117	58	34	Absent	0.27	4.1°	0.0
MHCII_10*	1,247	535	563	243	<16	Absent	0.21	18.2	0.07
MHCII_11	NA	NA	NA	NA	NA	NA	NA	NA	NA
MHCII_12	725	136	444	136	45	11 (tot. ly.), 1 (B cells)	0.004	12.2	0.006
MHCII_13	1,947	331	1,512	62	21	41	<0.13	8.07	<0.07

Data are reported at blood sampling. Abnormal results according to age-based reference ranges (28) are reported in bold.

°Not on Ig replacement therapy at sampling. [§]Patient with 100% donor chimerism after HSCT. ND, not done; NK, not known; NA, not available; ly., lymphocytes.

**These patients are described in greater detail in (16).*

incubated with Rat-on-Mouse HRP-Polymer (Biocare Medical) or MACH 1™ Universal HRP Polymer Kit (Biocare Medical) or 4plus Streptavidin HRP label (Biocare Medical); reactions were developed in Biocare's Betazoid DAB and nuclei counterstained with Hematoxylin. Digital images were acquired with an Olympus XC50 camera mounted on a BX51 microscope (Olympus, Tokyo, Japan), with CellF Imaging software (Soft Imaging System GmbH, Münster, Germany) or with a Nikon Eclipse E600 Microscope (Nikon) with NIS Elements Software (Nikon). The following primary antibodies were used on thymic sections: rabbit anti-cytokeratin 5 (CK5) (1:200; Covance), rat anti-cytokeratin 8 (CK8) (1:200; Developmental Studies Hybridoma Bank); rat anti-mouse AIRE (1:300; Millipore); rat anti-mouse FOXP3 (1:100; eBioscience); biotin-UEA (Biotinylated Ulex Europaeus Agglutinin I) (1:800; Vector Laboratories). Rabbit anti-CD3 primary antibody (1:100; ThermoFisher Scientific) was used on gut sections.

To quantify abnormalities of colon pathology, a histological score was used. The degree of colic alterations was blindly graded using combined scores including: the grade of inflammation (grade 0, no evidence of inflammation; grade 1, low; grade 2, moderate; grade 3, high; grade 4, intense and diffuse level of inflammation), the structural changes of intestinal layers (grade 0, absence; grade 1, focal; grade 2, partial; grade 3, diffuse level of structural changes), and the gland secretion alterations (grade 0, normal; grade 1, slight gland secretion alterations; grade 2, moderate; grade 3, diffuse gland secretion alterations). The cumulative total scores ranged from 0 to 10.

Human thymic tissue sample was formalin-fixed and paraffin-embedded. Sections were used for routine H&E staining and treated as described above. Nonspecific background was reduced with Background Sniper (Biocare Medical, Concord, CA, USA) before heat-based antigen-retrieval treatment and incubated with primary antibodies. The following primary antibodies were used: rat anti-human FOXP3 (1:100; eBioscience), mouse anti-human AIRE (1:3000; kindly provided by Prof P. Peterson, University of Tartu, Tartu, Estonia), biotin-UEA (Biotinylated Ulex Europaeus Agglutinin I) (1:800; Vector Laboratories) and Claudin-4 (used according to local standards of Pathological Anatomy Unit of Spedali Civili,

TABLE 4 | MHCII-D patients' treatment features.

Patient code	HSCT	Age at HSCT (years)	Donor	HSC source	Conditioning regimen	Complications after HSCT	Donor chimerism[§]	HSCT outcome[§]
MHCII_01	Yes°	1.5	MUD	PBSC	Bu/Flu/ ATG	GVHD, respiratory insufficiency	NK	Deceased (+1.8 mo. after HSCT)
MHCII_02	No	–	–	–	–	–	–	–
MHCII_03	Yes°	1.1	MUD	BM	Bu/Cy/ ATG	No	31% T cells, 8% B cells	Alive and well
MHCII_04	Yes°	4.0	MUD	BM	Treo/Flu/TT/ Alemtuzumab	Mucositis, CLS, brain hemorrhage	NK	Deceased (+25 days after HSCT)
MHCII_05	Yes°	16.2	MUD	BM	Bu/Flu/TT/ Alemtuzumab	VOD, GVHD, infections, cytopenias	100%	Deceased (+5 mo. after HSCT)
MHCII_08	Yes	4.5	MRD	BM	Bu/Flu/ ATG	GVHD, TMA	100%	Alive and well (but lung sequelae)
MHCII_09*	Yes°	5.2	MUD	PBSC	Treo/Flu/ Alemtuzumab	Viral infections	100%	Alive and well
MHCII_10*	Yes°	6.1	TCRαβ/CD19-depleted Haplo	PBSC	Treo/Flu/TT/ ATG/Rtx	GVHD, severe infections, BM failure	100% (after T-cell add-back + top up haplo tx)	Deceased (+6.5 mo. after first HSCT)
MHCII_11	No	–	–	–	–	–	–	Deceased
MHCII_12	No	–	–	–	–	–	–	–
MHCII_13	No	–	–	–	–	–	–	–

ATG, anti-thymocyte globulin; BM, bone marrow; Bu, Busulfan; CLS, capillary leak syndrome; Cy, cyclophosphamide; Flu, fludarabine; GVHD, graft-versus-host disease; HSC, hematopoietic stem cell; mo., months; MRD, matched related donor; MUD, matched unrelated donor; NK, not known; PBSC, peripheral blood stem cells; Rtx, rituximab; TMA, thrombotic microangiopathy; Treo, Treosulfan; TT, thiotepa; VOD, veno-occlusive disease.

°performed after sample sampling. §at last available follow up.

**These patients are described in greater detail in (16).*

Brescia, Italy). Sections were processed as described above for the murine experiments. Morphometric analysis was performed using Olympus Slide Scanner VS120-L100 (Olympus, Tokyo, Japan) and Image-pro software (Olympus) was used to analyze them. Digital images were acquired with the same instruments and software as described above.

Murine Cell Isolation

Murine TEC isolation was performed on age-matched WT and $A\beta^{0/0}$ mice. Briefly, mice were sacrificed by decapitation to avoid excessive bleeding during surgery. Murine thymus was cleaned from fat and stromal tissues, and then digested at 37°C with an enzymatic solution containing Liberase TL and DNAse I (Roche). Digested tissues were collected in DMEM (Lonza) supplemented with 10% fetal bovine serum (FBS), 1% glutamine and 1% penicillin and streptomycin. Single thymic cell suspensions were then incubated with anti-CD45 micro-beads (Miltenyi Biotec) and processed with the AutoMACS Pro Separator (Miltenyi Biotec). The CD45 negative fraction was retrieved and then tested by multicolor FACS analyses for the expression of TEC markers.

Thymocytes, splenocytes and lymphocytes were freshly isolated by mechanically disrupting the thymus, the spleen and lymph nodes of age-matched WT and $A\beta^{0/0}$ mice. In addition, splenocytes were lysed with Red Cell Lysis Buffer (RCLB) (150mM M NH4Cl, 10 mM KHCO3, and 0.1 mM EDTA). PB samples underwent red blood cell (RBC) lysis twice with RCLB, before further processing. Recovered cells were re-suspended in D-PBS (EuroClone, Pero, Italy) with 2% FBS and subsequently stained for flow cytometric analysis. $CD4^+$ T cells were isolated by pooled spleens and mesenteric lymph nodes (MLNs) from 2–3 mice/pool of age-matched WT and $A\beta^{0/0}$ mice using CD4-specific magnetic beads via negative selection according to the manufacturer's instructions ($CD4^+$ isolation kit Miltenyi Biotec).

Flow Cytometric Analysis of Murine Cells

The list of monoclonal antibodies used for the staining of murine TEC, thymocytes, splenocytes and lymphocytes derived from lymph nodes are reported in the *Materials and Methods* section in this article's **Supplementary Materials**. Cells were acquired on a FACS CANTO (BD Pharmingen) and analyzed with FlowJo software.

Cell Sorting of Murine TEC

TECs were isolated and enriched with the AutoMACS Pro Separator after digestion of thymi from pool of 5–10 age-matched WT and $A\beta^{0/0}$ mice of 4–6 weeks of age, as previously described. To sort mTEC and cTEC, isolated TECs were stained with anti-CD45 (30-F11), anti-CD326 (EpCam; G8.8), anti-MHCII (M5 114.15.2) (all Biolegend), anti-Ulex-1 (FL 1061, Vector) and anti-Ly51 (6C3, Miltenyi) monoclonal antibodies and sorted with the MoFlo Legacy cell sorter (Becton Dickinson), with 100 µl noozle (FRACTAL facility of San Raffaele Hospital, Milan, Italy). Non-viable cells were excluded from analyses using 7-AAD (BD Pharmingen). In order to preserve RNA quality for further analyses, ProtectRNA™ RNase Inhibitor (Sigma-Aldrich) was added to CD45-negative fractions according to manufacturer's instruction, before sorting procedure.

Moreover, TEC subsets were sorted directly in RNAlater (Sigma-Aldrich), kept at +4°C overnight and then at −20°C until use.

RT-PCR

RNA extraction from sorted TEC from WT and $A\beta^{0/0}$ mice was performed with RNeasy microkit (QIAGEN, Hilden, Germany) according to manufacturer's instructions. RNA was then stored at −80°C until use. Reverse transcription of murine-sorted TEC mRNA, synthesis and amplification of cDNA were performed with the Ovation PicoSL WTA System V2 (Nugen), according to the manufacturer's instructions. Purification of amplified cDNA was achieved using the QIAquick PCR Purification Kit (QIAGEN). Quantitative Real-time (RT) PCR was performed using Fast SYBR green master Mix (Thermo Fisher Scientific) and the ViiA7 Real-Time PCR System (Thermo Fisher Scientific). Each sample was analyzed in duplicate. The relative level of expression was determined by normalization to β-actin (Actb) ribosomal RNA. The primers used are listed in **Supplementary Table 1**.

Bulk RNA-Seq

The cDNA libraries of sorted cTEC and mTEC from WT and $A\beta^{0/0}$ mice were constructed using the Takara SMART-Seq v4 Ultra low input RNA Kit. PCR library products were quantified using the Agilent DNA 1000 assay (Agilent#5067-1504) on an Agilent Technologies 2100 bioanalyzer. Pooled libraries were loaded on a Paired End Flow Cell using the cBot System (Illumina) and the HiSeq 3000 platform. At the end of the run, around 30 M of 50 bp paired-end reads per sample were generated. Transcript abundance was estimated with Kallisto (29) and differentially expressed (DE) genes were identified using DeSeq2 (30) R package and a FDR corrected p-value <0.05. Gene set enrichment analysis (GSEA) was applied in order to identify gene sets and pathways that were significantly perturbed across conditions. Collections of gene sets were downloaded from MiSigDB (*http://www.broadinstitute.org/gsea/msigdb/*). We used the Benjamini–Hochberg method to adjust gene set p-values and set 0.1 as the significant threshold. Splicing entropy was calculated on TRA transcripts as described in (31). RNA-Seq data are available under accession number GSE166463.

Sample Preparation and Proteomic Analyses

Murine TEC for proteomic analyses were obtained from three pools of WT or $A\beta^{0/0}$ mice, three mice per pool (5–6 weeks-old). TEC were isolated, enriched and processed through $CD45^+$ cell-depletion as described above. $CD45^-$ cell-samples were pelleted and stored at −80°C until use. Detailed descriptions of proteomic analysis, data processing, and interaction network reconstructions are described in the *Materials and Methods* section in this article's **Supplementary Materials**.

Autoantigen Array

This analysis was performed at the Genomics & Microarray Core Facility at UT Southwestern Medical Center (USA) on human and murine serum or plasma samples. The Autoantigen array contained 95 autoantigens and eight internal control antigens. Profiling of both IgG and IgM autoantibodies was performed. The autoantibodies binding to the antigens on the array were detected with Cy3 labeled anti-IgG and Cy5 labeled anti-IgM and the arrays were scanned with GenePix® 4400A Microarray Scanner. The images were analyzed using GenePix 7.0 software to generate GPR files. The averaged net fluorescent intensity (NFI) of each autoantigen was normalized to internal controls (IgG or IgM).

Induction of Colitis by Adoptive T-Cell Transfer

$CD4^+$ T cells were isolated from pooled spleens and MLNs of 9–10 wk-old WT or $A\beta^{0/0}$ mice as described above. Enriched $CD4^+$ T-cell samples were subsequently sorted into naive $CD4^+CD25^-CD45RB^{hi}$ and regulatory $CD4^+CD25^{hi}CD45RB^-$ cell populations (>98% purity) using a FACS Aria Fusion (Becton Dickinson) cell sorter (FRACTAL facility of San Raffaele Hospital, Milan, Italy). For colitis induction, 4×10^5 WT $CD4^+CD25^-CD45RB^{hi}$ naive T cells were transferred by intraperitoneal injection (i.p.) into 8–9 week-old *Rag1*$^{-/-}$ mice alone or with 1.5×10^5 $CD4^+CD25^{hi}$ Treg cells from WT or $A\beta^{0/0}$ mice. Recipient mice were weighted twice a week and sacrificed at week 4 or 8 after the transfer, when colitis was diagnosed by severe weight loss and/or wasting diarrhea. Gut tissues were isolated and analyzed as described and the colon length was measured at time of sacrifice.

Flow Cytometric Analysis on Human PBMC

PBMC were isolated from PB of HD and patients by density gradient centrifugation using LymphoprepTM (density: 1.077 g/ml; STEMCELL Technologies, Vancouver, Canada). PBMC were maintained in RPMI medium (CORNING) with 2% FBS, 2% L-glutamine, 1% Penicillin/Streptomycin at 4 °C until use or live frozen in FBS + dimethyl sulfoxide (DMSO) 10%. Multi-color immunophenotype of T-lymphocyte subsets on human PBMC was performed by flow cytometry. Details about the monoclonal antibodies used for each staining are reported in the *Materials and Methods* section in this article's **Supplementary Materials**.

ELISA Assay

Levels of B-cell activating factor (BAFF) were measured in duplicate in serum or plasma samples of MHCII-D patients and age-matched HD using a Quantikine Human BAFF/BLyS/TNFSF13B Immunoassay kit (R&D Systems, Minneapolis, USA). The assay was performed according to manufacturer's instructions and the optical density (OD) was determined using a microplate reader.

Statistical Analyses

All results are expressed as median and interquartile range if not stated otherwise. In the present study a descriptive statistical analysis has been performed. No formal inference was performed due to the small sample size. Statistical significance was assessed using a two-tailed Mann–Whitney test to compare continuous outcomes between groups. T test was used for splicing entropy result analysis. Levels of significance were defined as $p \leq 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), and $p < 0.0001$ (****). Statistical testing

was performed using Prism GraphPad (Version 5.0f, La Jolla, CA). Graphs were created using the same software.

RESULTS

Thymic Structure Perturbation in Absence of MHC Class II Expression in a MHCII-D Patient and in Aβ$^{0/0}$ Mice

Data about human thymic histology in patients with MHCII deficiency are limited because of the difficulties in accessing these tissues for technical and ethical reasons. In this study, we had the unique opportunity to perform histological analysis of a thymic biopsy performed in a 23-month-old MHCII-D female patient before HSCT (see **Table 1** for immunological data). The patient carried a homozygous mutation in the *CIITA* gene [c.3317 + 2dup], resulting in an intronic splice variant.

The thymic architecture was perturbed, with reduced representation of thymic medulla (**Figure 1A**), as compared to a control thymus analyzed in parallel. This finding was also supported by staining for Ulex Europaeus Agglutinin I (UEA1) and FOXP3 (**Figure 1B**). Moreover, the MHCII-D thymus

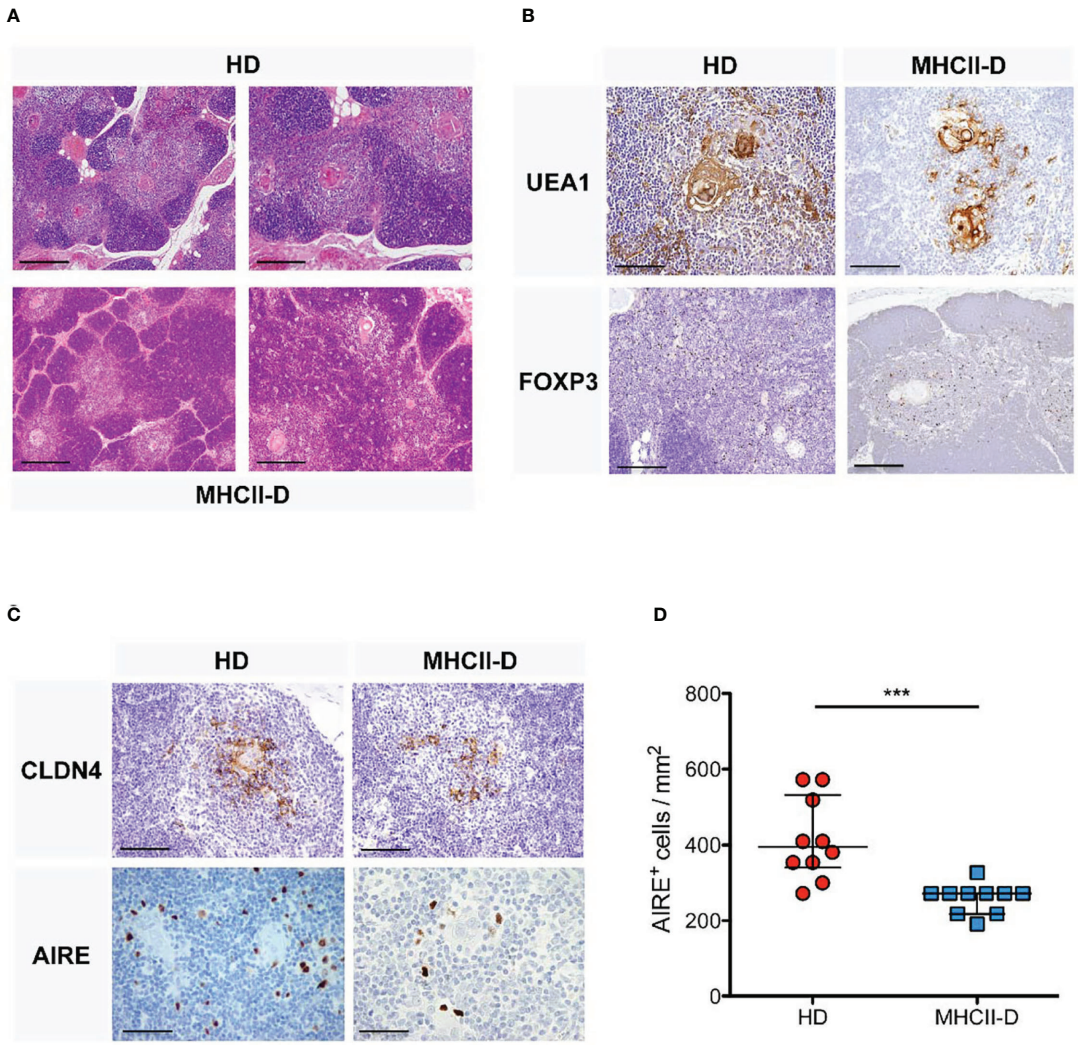


FIGURE 1 | Thymic structure perturbation in a MHCII-D patient's thymus. **(A)** Histological analysis of a MHCII-D patient's thymus. Immunohistochemistry analysis of thymic tissue isolated from a human healthy donor (HD) and a MHCII-D patient. Hematoxylin–eosin (H&E) staining shows a reduced representation of thymic medulla. Original magnification 4×, corresponding to 500 μm. **(B)** UEA1 and FOXP3 staining in MHCII-D thymus. Immunohistochemistry analysis of Ulex Europaeus Agglutinin I (UEA1) and FOXP3 expression in a thymic tissue isolated from a human HD and a MHCII-D patient. Original magnifications: 20× for UEA1 and 10x for FOXP3 staining images, corresponding respectively to 100 and 200 μm. **(C)** AIRE and CLDN4 expression in MHCII-D thymus. Immunohistochemistry analysis of CLDN4 (Claudin 4) and AIRE expression in a thymic tissue isolated from a human HD and a MHCII-D patient. Original magnifications: 20× for CLDN4 and 40× for AIRE staining images, corresponding respectively to 100 and 50 μm. **(D)** AIRE expression is reduced in MHCII-D thymus. Comparison of the concentration of AIRE$^+$ cells per square millimeter (mm^2) in a thymic tissue isolated from a human HD and a MHCII-D patient. Mann–Whitney test, p-value < 0.001. Bars represent median with interquartile range. ***p value < 0.001.

presented a reduced frequency of AIRE$^+$ and CLAUDIN 4 (Cldn4)-expressing TEC, suggesting possible defects of central tolerance (**Figures 1C, D**).

To further corroborate these observations, we analyzed thymic tissue isolated from the MHCII knock out mouse model, Aβ$^{0/0}$ (17, 18). Histological analysis showed similar abnormalities of thymic architecture, with reduced representation of TECs, especially in the medulla, resulting in a significantly increased cortico-medullary (C/M) ratio in Aβ$^{0/0}$ mice, as compared to WT mice (p-value 0.0087) (**Figures 2A, B**). However, CK5, a medulla specific marker, and CK8 staining indicated a correct compartmentalization of cortical and medullary areas in the thymus of Aβ$^{0/0}$ mice (**Supplementary Figure 1A**). Overall reduced total Epcam$^+$ TEC and mTEC frequency and absolute count in Aβ$^{0/0}$ thymi, as compared to WT, were confirmed by flow cytometric analysis (**Figure 2C** and **Supplementary Figure 1B**). Finally, we detected a decreased frequency of AIRE$^+$ TEC and FOXP3$^+$ cells in the medullary area of Aβ$^{0/0}$ mice thymus, as compared to WT mice (**Figures 2D, E** and **Supplementary Figure 1A**).

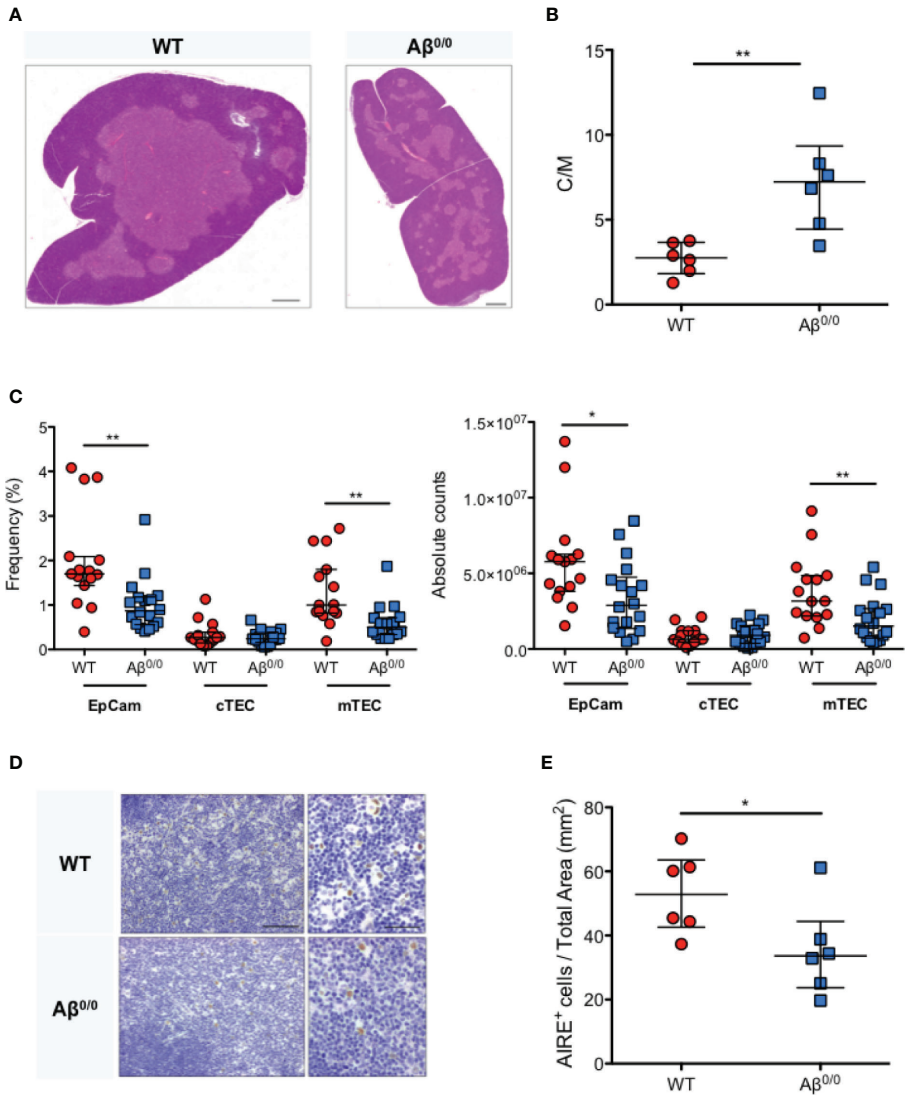


FIGURE 2 | Thymic structure perturbation in Aβ$^{0/0}$ mice thymus. **(A)** Reduced thymic medulla representation in Aβ$^{0/0}$ mice, confirmed by H&E staining as compared to WT mice. Scale bars correspond to 500 μm. **(B)** Increased cortico-medullary (C/M) ratio in Aβ$^{0/0}$ mice. Mann–Whitney test, **p-value <0.01. Median with interquartile range is showed in the graph for each experimental group. **(C)** Reduced frequency (*left panel*) and absolute count (*right panel*) of TEC and mTEC in Aβ$^{0/0}$ mice after enzymatic digestion of thymic tissue and depletion of CD45$^+$ cells. Mann–Whitney test, *p value < 0.05; **p value <0.01. **(D)** Immunohistochemical analysis of AIRE expression in WT and Aβ$^{0/0}$ mice thymi. Original magnification: 20× for left panel and 40× for right panel for each condition, corresponding respectively to 100 and 50 μm. **(E)** Frequency of AIRE-expressing cells (AIRE$^+$) is reduced in Aβ$^{0/0}$ thymus. Comparison of the concentration of AIRE$^+$ cells per mm^2 of total thymic tissue isolated from WT or Aβ$^{0/0}$ mice. Mann–Whitney test, *p-value < 0.05. Median with interquartile range is showed in the graph for each experimental group.

Impaired Generation and Maturation of CD4$^+$ SP Thymocytes in Aβ$^{0/0}$ Mice and MHCII-D Patients

In accordance with previous reports (17, 18, 24), we observed a reduction in the frequency and in the absolute count of CD69^{hi}TCRβ^{hi} thymocytes (**Figures 3A, B** and **Supplementary Figure 2A**), and severe defects of CD4$^+$ SP thymocyte generation and maturation (**Figures 3C, D** and **Supplementary Figure 2B**) in Aβ$^{0/0}$ mice, in line with the known fundamental role of MHCII molecules for the progression from the DP stage to the SP CD4$^+$ T cell stage (18). Moreover, we confirmed that that majority of these residual SP CD4$^+$ thymocytes in Aβ$^{0/0}$ mice corresponds to CD1-restricted NKT cells, as previously described (20, 21) (**Supplementary Figure 2C**). This resulted in peripheral CD4$^+$ T cell lymphopenia, with significant reduction in the frequency and absolute count of CD4$^+$ naïve T cells and

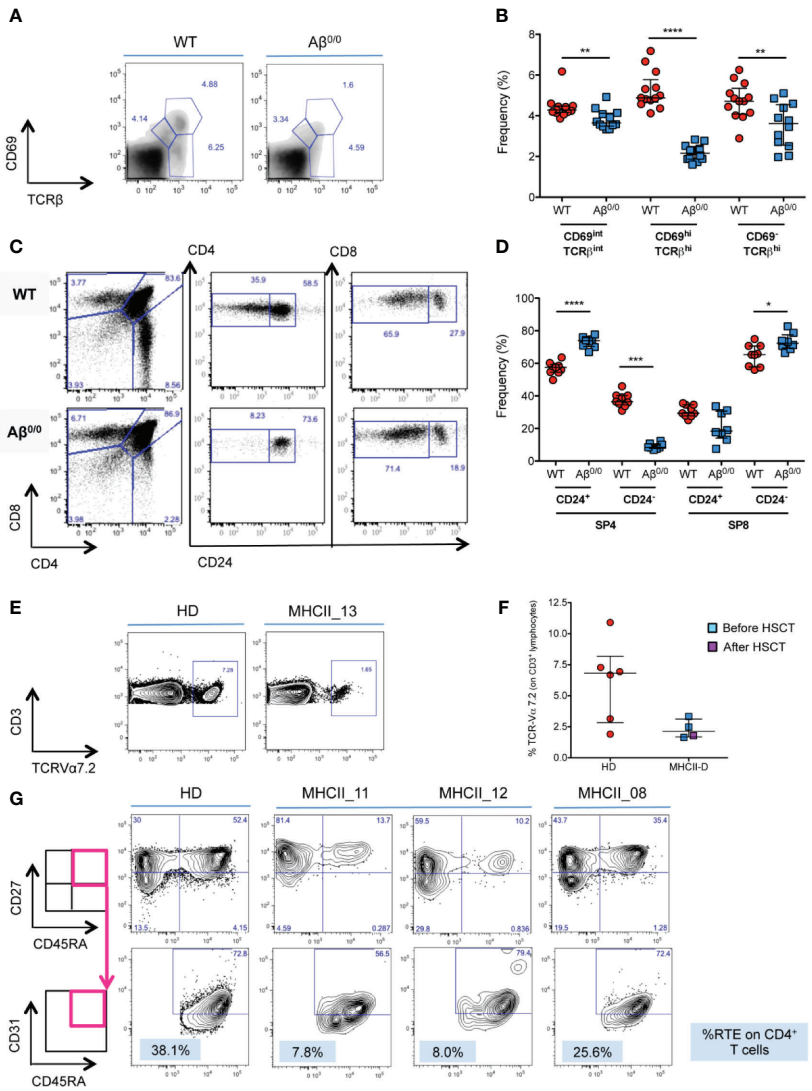


FIGURE 3 | Defective thymopoiesis in MHCII deficiency. **(A)** FACS analysis of thymocytes isolated from WT and Aβ$^{0/0}$ mice, in terms of CD69 and TCRβ expression, shows an impaired positive selection of Aβ$^{0/0}$ thymocytes. Analysis performed on total thymocyte gate. **(B)** Graph shows the summary of all mice analyzed, showing a significant reduction in the frequency of CD69^{hi}TCRβ^{hi} post-positive selection thymocytes in Aβ$^{0/0}$ mice, as compared to WT. **p value < 0.01; ***p value < 0.001. **(C)** FACS analysis of thymocytes isolated from WT and Aβ$^{0/0}$ mice shows a normal development of CD4$^-$CD8$^-$ double negative (DN) subsets. However, Aβ$^{0/0}$ mice CD4$^+$ SP cells (SP4) mostly present an immature phenotype (CD24$^+$), since only few of them are CD24$^-$, as compared to WT SP4 cells. **(D)** Graph shows the summary of all mice analyzed in terms of frequency of CD24$^+$ and CD24$^-$ SP4 and SP8 cells. *p value < 0.05; ***p value < 0.001; ****p value < 0.0001. **(E, F)** TCR Vα7.2 expression on CD3$^+$ T cells is reduced in MHCII-D patients. **(E)** Representative FACS plots on Vα7.2 expression on CD3$^+$ T cells on a healthy control (HD) and a MHCII-D patient (MHCII_13). **(F)** Graph shows the summary of all MHCII-D patients analyzed (n = 4) and the healthy controls (HD) tested in parallel. Median and interquartile range are represented for each group. **(G)** Representative FACS plots of CD4$^+$ naïve T cells and recent thymic emigrants (RTE) of a healthy control (HD) and three MHCII-D patients, two before HSCT (MHCII_11 and _12) and one after HSCT (MHCII_08). CD27$^+$CD45RA$^+$ naïve T cells are gated on CD4$^+$CD3$^+$CD45$^+$ T-cell gate, while CD31$^+$ RTE are gated on CD27$^+$CD45RA$^+$ naïve T cell gate.

increased frequency of activated $CD4^+$ T cells in secondary lymphoid organs (spleen and lymph nodes), as compared to WT $CD4^+$ SP cells (SP4) cells (**Supplementary Figures 2D, E**).

Since we could not get access to thymocytes from MHCII-D patients, we investigated TRAV1 (Vα7.2) expression on patients' PB $CD3^+$ lymphocytes as a surrogate marker of thymocyte maturation and survival (32). Interestingly, we found that all four patients analyzed (three before and one after HSCT) showed a trend towards reduced expression of Vα7.2 on their $CD3^+$ T cells as compared to normal controls tested in parallel (median: MHCII-D 2.1%; HD 6.8%) (**Figures 3E, F**).

Reduced Frequency of Naïve $CD4^+$ T-Cells and RTE in Untreated MHCII-D Patients

In order to evaluate thymic output in MHCII deficiency, we analyzed the proportion of naïve T cells and recent thymic emigrants (RTE) among PBMC from three patients: two untransplanted [of whom one infant (MHCII_11) and one adult (MHCII_12)], and one transplanted patient (MHCII_08). The percentage of naïve $CD45RA^+CD27^+CD4^+$ T cells was reduced in the two untreated patients, as compared to the normal donor tested in parallel. Conversely, naïve $CD4^+$ T cells in the transplanted patient tended to normal levels (**Figure 3G**). No significant differences emerged in the proportion of naïve $CD8^+$ T cells (*data not shown*). We identified RTE as $CD31^+$ cells within naïve $CD27^+CD45RA^+CD4^+$ T cells. In line with data on naïve $CD4^+$ cells, the frequency of RTE in the two untransplanted patients was also reduced (**Figure 3G**). This finding was particularly striking for the infant patient. RTE in MHCII_08, who underwent transplant, were comparable to the adult control tested in parallel (**Figure 3G**).

TEC Transcriptome and Proteome Perturbation in the Absence of MHC Class II

Based on our observations of TEC abnormalities in histologic analysis of thymic samples, we evaluated gene expression profile of $A\beta^{0/0}$ mouse TEC subsets by performing bulk RNA-Seq on sorted WT and $A\beta^{0/0}$ cTEC and mTEC. In order to obtain a sufficient amount of sorted TEC, we pooled 5–10 WT or $A\beta^{0/0}$ mice of 4–6 weeks of age for each biological replicate (n = 3, **Supplementary Table 2**). The gating strategy used to sort cTEC ($Epcam^+CD45^-Ly51^+UEA1^-$ cells) and mTEC ($Epcam^+CD45^-Ly51^-UEA1^+$ cells) is shown in **Supplementary Figure 3A**. Principal component analysis (PCA) showed that samples were properly grouped according to genetic background and tissue of origin (**Supplementary Figure 3B**).

Transcriptome analysis revealed an altered gene expression profile in sorted TEC subsets from $A\beta^{0/0}$ mice, as compared to age-matched WT mice. These differences were especially prominent in mTEC (**Figure 4A** and **Supplementary Table 3**), on which we subsequently focused our analyses. In particular, we identified almost one thousand (n = 929) transcripts that were differentially expressed (DE) in mTEC from WT versus $A\beta^{0/0}$ mice. The majority of these transcripts (n = 679, in red in **Supplementary Figure 4A**) were upregulated in mTEC from WT mice (**Supplementary Table 3**). Among DE genes, transcripts that were enriched in $A\beta^{0/0}$ mTEC (n = 250) were mainly relative to RNA splicing, histone modifications and transcription factors (most of which with repressive function) (**Figure 4B**).

Similarly, even if to a lesser extent, cTEC transcriptome resulted also altered (**Figure 4A** and **Supplementary Table 3**) in $A\beta^{0/0}$ mice. Differentially expressed transcripts upregulated in $A\beta^{0/0}$ cTEC were mainly relative to RNA splicing and translation, carbohydrate metabolism and cytoskeleton. Conversely, in WT cTEC enriched DE transcripts resulted relative to lipid metabolism, peptidases, apoptosis, transmembrane transport, transcriptional regulation, blood vessels/extracellular matrix, cytokine signaling pathway, inflammatory and immune response (**Supplementary Figure 5**).

To further define the cellular features of TEC in $A\beta^{0/0}$ mice, we performed proteomic analysis of bulk CD45-depleted TEC fractions from pools of 4–6-week-old WT and $A\beta^{0/0}$ mice (n = 3, three mice/pool). This analysis could not be performed on sorted TEC subsets due to technical limitations. In these cell subsets, we found 373 DE proteins, 111 of which with high confidence (**Supplementary Tables 4** and **5**). Most of these proteins were upregulated in WT mice (in red in **Supplementary Figure 4B**). Among DE proteins, the only ones that were enriched in $A\beta^{0/0}$ TEC-enriched subsets were relative to chromatin assembly, in particular histones, as indicated by combined cluster-network analysis (**Figure 4C**). We cannot exclude the presence of some fibroblasts and endothelial cells in the analyzed samples.

Next, we performed an integrated network analysis on DE transcripts and proteins in WT and $A\beta^{0/0}$ TEC in order to identify common specific pathways dysregulated in TEC in the absence of MHCII. The only common variation observed in both cTEC and mTEC transcripts and in CD45-depleted TEC fractions was an increased representation of transmembrane transporter activity in WT mice thymi. However, if restricting the analysis to mTEC only, a concordant pattern of variations in both transcripts and proteins within the same cluster between WT and $A\beta^{0/0}$ mice was observed for four clusters (**Figure 4D**). In particular, in WT mice, increased expression was observed for genes and proteins involved in cell metabolism, energy production and cell cycle.

Impaired Promiscuous Gene Expression in the Absence of MHCII Molecules

Next, we interrogated RNA-Seq data for genes known to be involved in the establishment and maintenance of central tolerance. GSEA showed a significant enrichment for both Aire-dependent and Aire-independent transcripts known to have a tissue-restricted pattern of expression, also known as Tissue Restricted Antigen (TRA) genes, in WT mTEC (**Figure 5A**). Reduced expression of TRA genes in $A\beta^{0/0}$ mTEC emerged also at DE analysis and was confirmed by qRT-PCR analysis for a selected number of them (**Figures 5B, C**). Consistent with these observations, *Aire* gene expression was significantly reduced in $A\beta^{0/0}$ mTEC, as compared to WT (Log2 fold change mTEC WT/$A\beta^{0/0}$: 2.12,

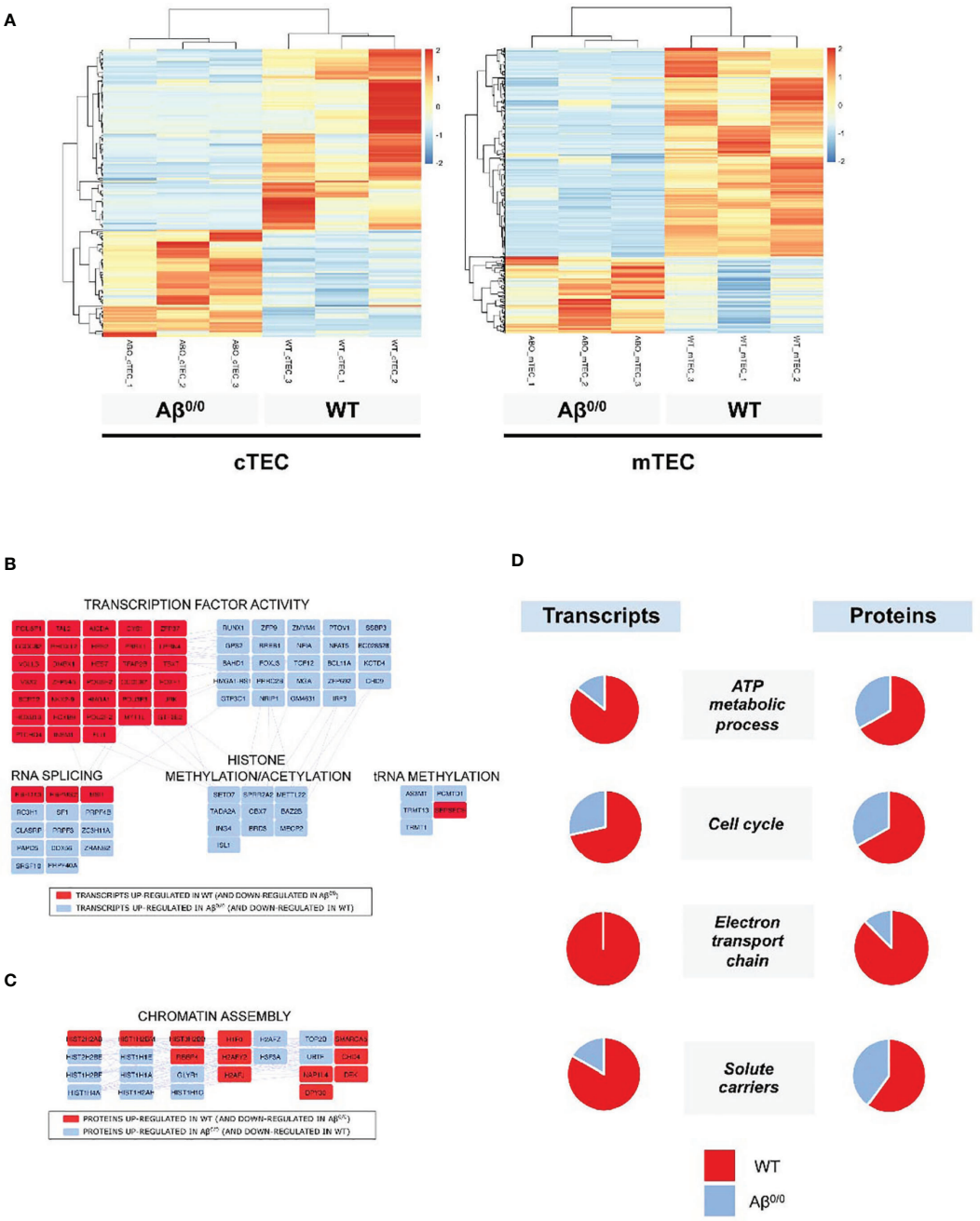


FIGURE 4 | Transcriptome and proteome perturbation in absence of MHCII molecules. **(A)** Heat map of expression values for genes that were differentially expressed between Aβ$^{0/0}$ and WT mice cTEC or mTEC at a p value cut-off (FDR-corrected) of 0.05. Rows (genes) are scaled, i.e., the value (z score) for a gene in a given sample represents its deviation from the mean expression value of the gene across all samples in terms of standard deviations. Up regulation is shown in red, down regulation in blue. Genes and samples are ordered by hierarchical clustering using Pearson's correlation as the distance measure and complete linkage as the clustering method. **(B)** Network analysis of differentially expressed transcripts in mTEC. The figure shows the main results of network/topology analysis based on the combination of the list of DE transcripts in mTEC with *Mus musculus* Protein–Protein Interaction (PPI) network using STRING bioinformatics tool (full figure is reported in **Supplementary Figure 4A**). In red are represented transcripts up-regulated in WT mTEC, in light blue those up-regulated in Aβ$^{0/0}$ mTEC. **(C)** Cluster-Network analysis of differentially expressed proteins in CD45-depleted TEC fractions. The figure shows the main results of network/topology analysis based on the combination of the list of DE proteins in CD45-depleted TEC fractions with *Mus musculus* Protein–Protein Interaction (PPI) network using STRING bioinformatics tool (full figure is reported in **Supplementary Figure 4B**). In red are represented proteins up-regulated in WT mice, in light blue those up-regulated in Aβ$^{0/0}$ mice. **(D)** Transcript and protein expression per functional cluster in WT and Aβ$^{0/0}$ cell subsets. For each cluster, the proportion of enriched transcripts or proteins in WT (red) or Aβ$^{0/0}$ (blue) are represented as percentage in the respective pie charts. Data about transcripts were obtained from sorted mTEC, data about proteins were obtained from CD45-depleted TEC samples.

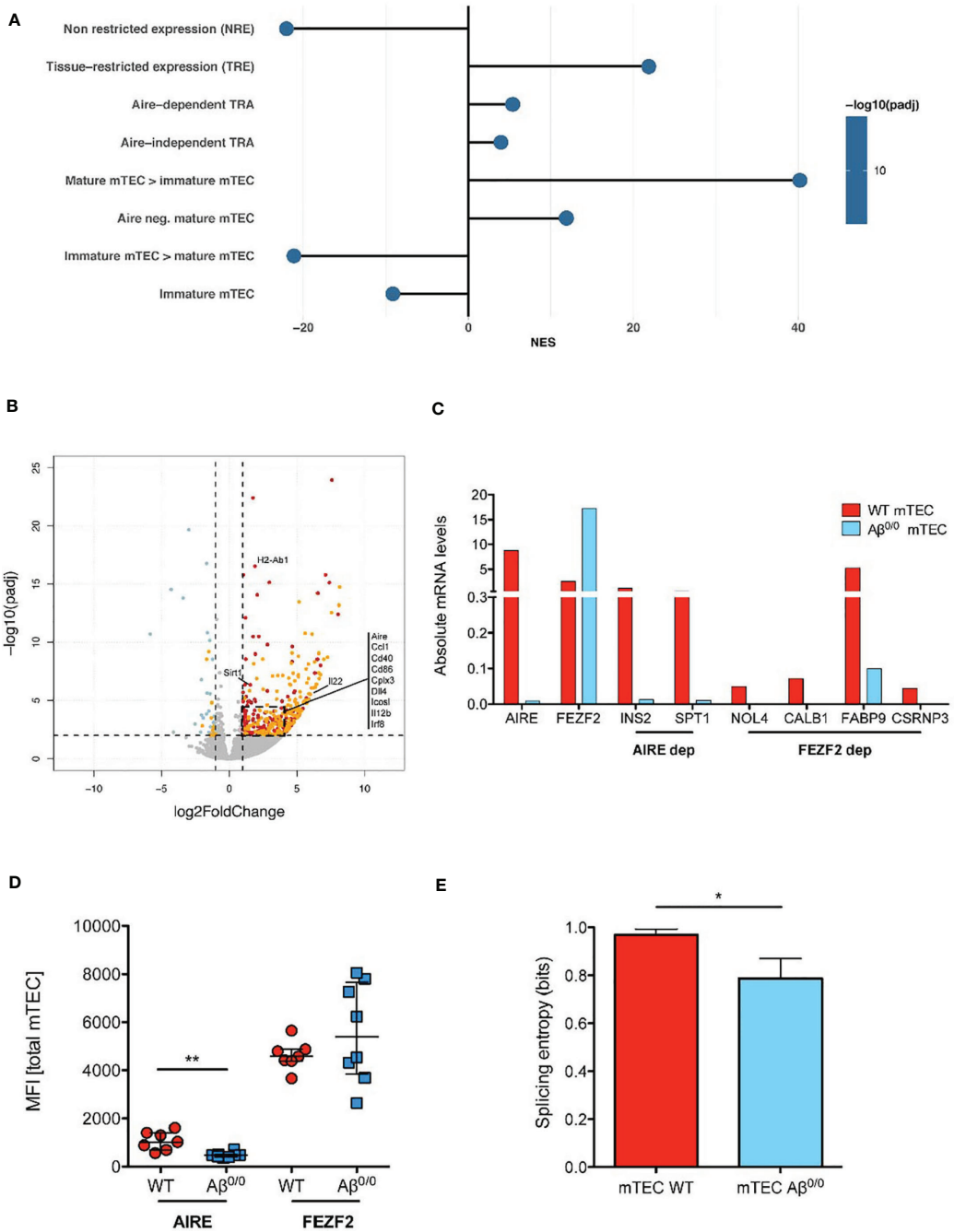


FIGURE 5 | TEC transcriptome perturbation in the absence of MHC class II. **(A)** Enrichment Analysis of Gene Sets involved in promiscuous gene expression and maturation in mTEC. The normalized enrichment score (NES) is reported for each gene set. Positive NES indicate gene sets enriched in WT mTEC, negative NES indicate gene sets enriched in $A\beta^{0/0}$ mTEC. This analysis was performed based on gene lists published in (33). **(B)** Volcano plot representing results of differential gene expression (DE) analysis of genes between WT and $A\beta^{0/0}$ mTEC. In red are represented genes more expressed in WT mTEC, in light blue those more expressed in $A\beta^{0/0}$ mTEC. In orange are evidenced genes with tissue-restricted expression (TRE). **(C)** Absolute mRNA level of Aire, Fefz2, and some TRA (Aire- or Fezf2-dependent), quantified by RT-PCR performed on sorted WT or $A\beta^{0/0}$ mTEC, pooled from 2–3 sorting experiments. Ins2, insulin; Spt1, serine palmitoyltransferase; Nol4, nucleolar protein 4 (testis); Calb1, calbindin (brain, kidney), Fabp9, fatty acid binding protein 9 (testis); Csrnp3, cysteine-serine-rich nuclear protein 3. **(D)** AIRE protein expression is reduced in mTEC lacking MCHII. Graph shows the mean fluorescence intensity (MFI) of AIRE and FEZF2 proteins by flow cytometry in total mTECs after intracellular staining in WT (n = 7) or $A\beta^{0/0}$ (n = 8) mTEC, respectively. **p-value < 0.01. **(E)** Splicing entropy, a measure of the diversity of observed transcripts isoforms in a given sample, resulted decreased in $A\beta^{0/0}$ mTEC. The analysis was restricted to TRA only. The formula used for the calculation of mRNA splicing entropy was derived from (31). Results are expressed as mean and standard deviation. *p value < 0.05.

adjusted p-value: 0.0017) and this result was confirmed by qRT-PCR (**Figures 5B, C**). These results confirm and extend previous observations (24). In addition, we confirmed reduced Aire expression also at the protein level, as shown by intracellular FACS analysis (**Figure 5D**). Analysis of Fezf2 expression did not reveal any relevant differences in $A\beta^{0/0}$ and WT mTEC (**Figures 5C, D**). In addition, DE analysis suggested a disruption of the mechanisms controlling Aire expression in $A\beta^{0/0}$ mTEC, as indicated by a significantly reduced expression of the transcripts encoding for the protein deacetylase Sirtuin-1 (*Sirt1*) and *Irf8* in $A\beta^{0/0}$ mTEC (**Figure 5B**), respectively an essential *Aire* regulator and an *Aire* transcriptional activator. Finally, in order to further investigate mTEC functionality (34), we also calculated TRA splicing entropy (31), a measure of the diversity of transcripts isoforms, which resulted decreased in $A\beta^{0/0}$ mTEC (**Figure 5E**). This disruption of the complex machinery regulating Aire expression could be due to $A\beta^{0/0}$ mTEC impaired maturation. Collectively, these findings suggest that lack of MHCII expression in mTEC is associated with impaired promiscuous gene expression (PGE) and abnormalities of the mechanisms that govern central tolerance.

Impaired mTEC Maturation in $A\beta^{0/0}$ Mice

RNA-Seq results showed a significantly decreased expression of genes involved in mTEC maturation in $A\beta^{0/0}$ sorted mTEC. Accordingly, GSEA showed a significant enrichment in genes known to be expressed in mature mTEC in WT mice, while genes known to be expressed in immature mTEC where significantly enriched in $A\beta^{0/0}$ mTEC (33) (**Figure 5A**). Moreover, DE gene analysis showed a significantly increased expression of co-stimulatory molecules (mainly CD86 and ICOS ligand, which are involved in TEC-thymocyte cross-talk), in WT mTEC, as compared to their $A\beta^{0/0}$ counterpart (**Figure 5B**). A similar pattern was observed for transcripts encoding for molecules involved in NF-κB signaling, and in particular for CD40, which was more abundantly expressed in WT mTEC (**Figure 5B**). These results were confirmed when analyzing numbers of $CD40L^+$ and $RANKL^+$ thymocytes in $A\beta^{0/0}$ mice. Indeed, we detected by flow cytometry a severe reduction of both $CD40L^+$ and $RANKL^+$ thymocyte absolute counts in $A\beta^{0/0}$ mice (**Supplementary Figure 6**), suggesting a low CD40L and RANKL-mediated stimulation of $A\beta^{0/0}$ mTEC. This reduction was particularly evident in mice aged 3 weeks or older (**Supplementary Figure 6**).

Abnormalities of Peripheral Tolerance in $A\beta^{0/0}$ Mice

Based on the alterations detected in the thymus of MHCII deficient patients and mice, we hypothesized that impairment in central tolerance mechanisms could have an impact also on peripheral tolerance. In line with published results for another MHCII mouse model, the $A\alpha^{-/-}$ mice (25), Treg cells were nearly absent in the thymus of $A\beta^{0/0}$ mice, but they appeared relatively enriched in spleen and lymph nodes where they were present at higher frequency than in WT mice (**Figure 6A**, left panel). Nonetheless, Treg absolute count was reduced in all lymphoid organs in $A\beta^{0/0}$ mice (**Figure 6A**, right panel).

We then tested in *vivo* the function of $A\beta^{0/0}$ Treg cells. To this purpose, we made use of a model of colitis induced by adoptive transfer of WT T cells, alone or in combination with WT or $A\beta^{0/0}$ Treg cells, into *Rag1* ko mice (**Supplementary Table 6**), and analyzed the capacity of Treg cells to attenuate colitis. Recipient mice were monitored weekly and sacrificed four or eight weeks after the transfer, when signs of colitis (wasting diarrhea and weight loss) became evident in mice that had received WT T naive cells only or in combination with $A\beta^{0/0}$ Treg cells (**Supplementary Figure 7A**). At sacrifice, the gut tissue was analyzed macroscopically and colon length, an indirect sign of gut inflammation (35), was measured. As shown in **Figure 6B**, colon length in mice receiving co-injection of WT Treg cells together with WT T naïve cells, was similar to that of untreated mice. Conversely, colon length was reduced in mice receiving WT naïve T cells alone and in those receiving co-injection of $A\beta^{0/0}$ Treg cells together with WT naïve T cells. Moreover, in mice that received either WT naïve T cells only or in combination with $A\beta^{0/0}$ Treg cells, the study of intestinal pathology revealed different degrees of spontaneous colitis and wasting diarrhea. Substantial thickening of colonic mucosa, indicative of inflammation, was observed. Histologically, colonic inflammation was characterized by crypt elongation and large inflammatory cell infiltrate, mainly consisting of T lymphocytes, with occasional crypt abscesses (**Figure 6C**). A colitis score was calculated on histological sections of gut tissue based on the sum of the evaluation of architectural alterations, degree of inflammation and muciparous gland activity (see *Materials and Methods* for details). When compared to untreated animals, the colitis score was significantly increased in mice receiving WT naïve T cells alone or in combination with $A\beta^{0/0}$ Treg cells, while it was not statistically different in mice receiving both WT naïve T cells and WT Treg cells (**Figure 6D**). Furthermore, among the four treatment groups, only mice that had received co-transplantation of WT Treg cells showed an increased proportion of Treg cells in the spleen and MLN at sacrifice (**Supplementary Figures 7B, C**). Altogether, these results demonstrate that adoptive transfer of $A\beta^{0/0}$ Treg cells failed to block colitis induction in *Rag1* ko mice, suggesting an impaired function of these cells.

Peripheral Tolerance Impairment in Patients With MHCII Deficiency

To confirm the results obtained in the mouse model of MHCII deficiency, we set out to evaluate peripheral tolerance in MHCII-D patients. To this purpose, we collected PB samples from a cohort of patients with MHCII deficiency, whose main clinical features are summarized in **Table 2**. *RFXANK* gene mutations were identified in eight of them. Median age at sampling was 4.7 years (range: 0.4–24.1 years). All samples were collected before HSCT, except in one patient for whom only post-transplant sample was available. They suffered from severe and recurrent infections and the majority of them also presented with chronic diarrhea. Autoimmune manifestations were described in three

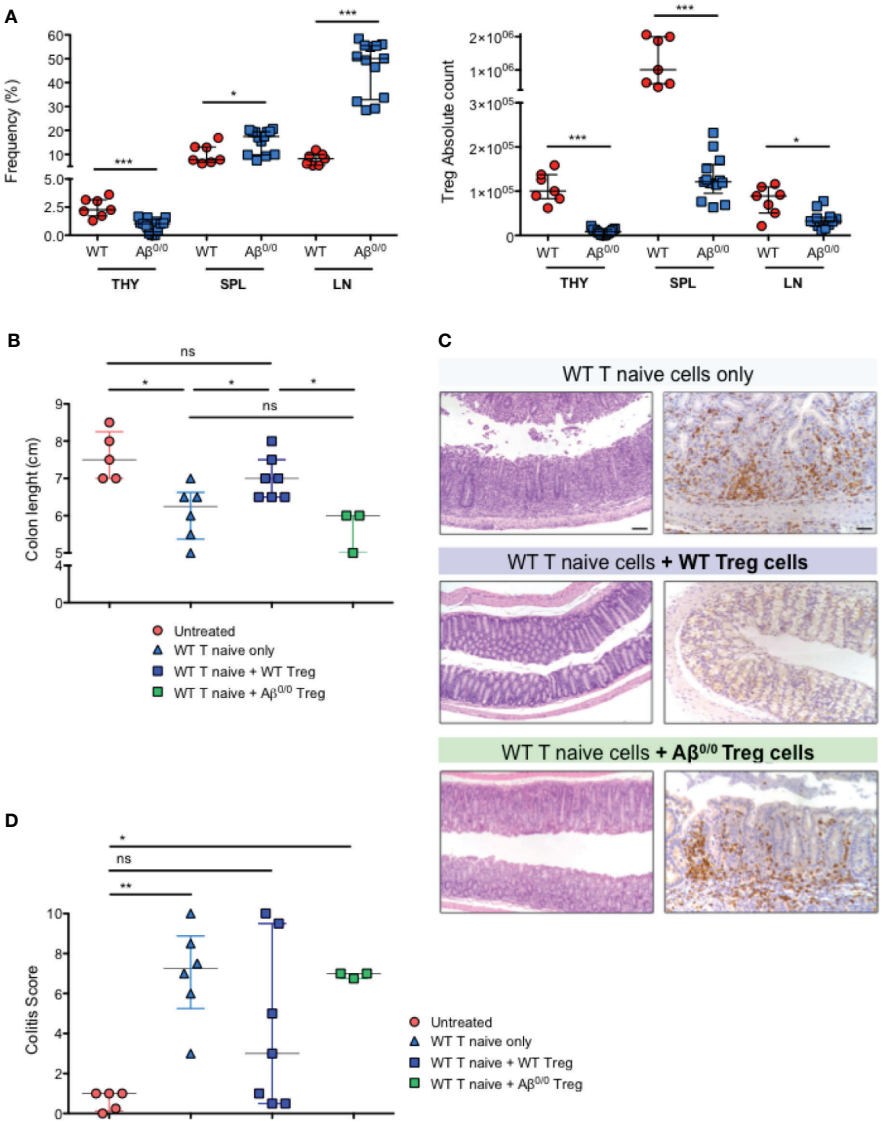


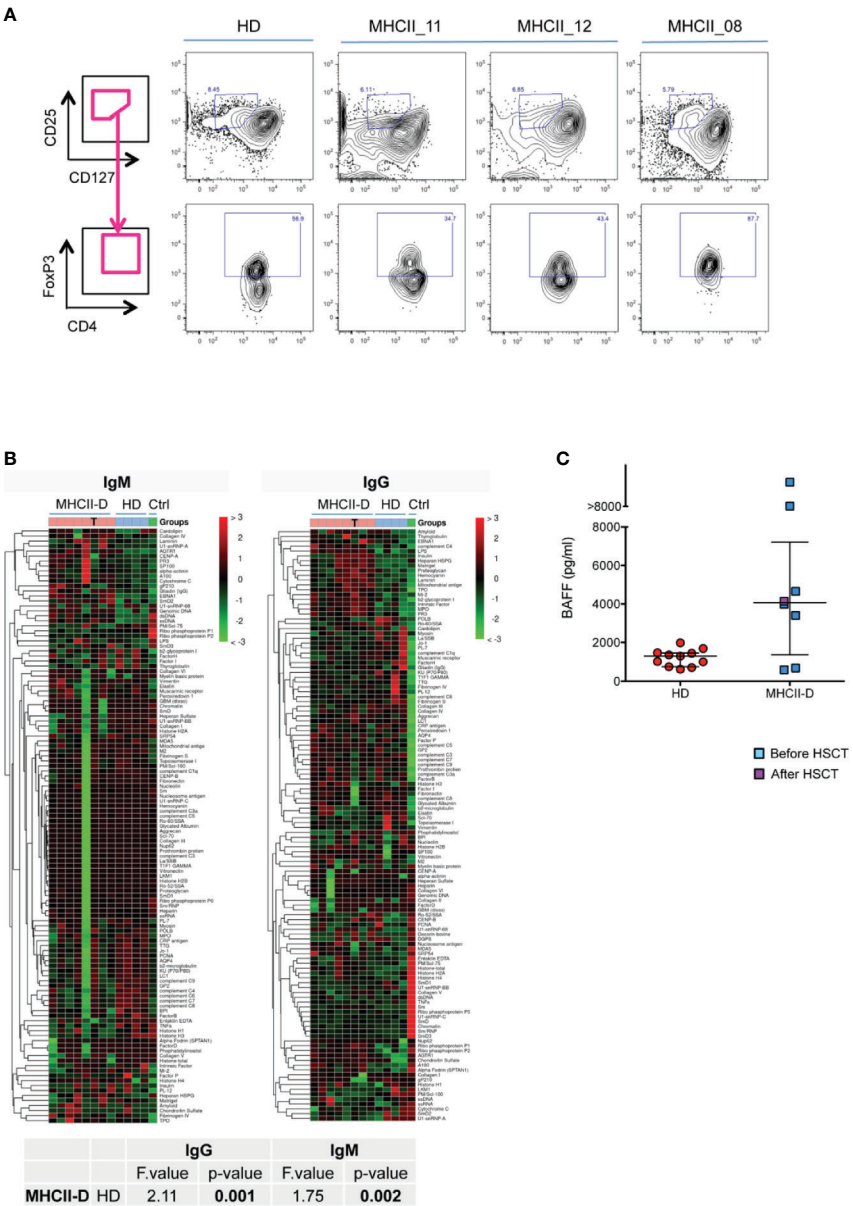
FIGURE 6 | Peripheral tolerance impairment in Aβ$^{0/0}$ mice. **(A)** Frequency and absolute count of Treg cells in WT and Aβ$^{0/0}$ mice. *(Left panel)* Frequency of Treg cells is expressed as the percentage (%) of FoxP3^{+}CD25^{hi} cells gated on CD4^{+} T-cell gate. *(Right panel)* Absolute count of Treg cells. THY, thymocytes; SPL, spleen; LN, lymph nodes. *p-value < 0.05; ***p-value < 0.001. **(B)** Colon length in the different treatment groups of induced colitis experiments. The graph shows the colon length of n = 3–7 mice/group from three experiments. Median score is reported for each treatment group. Error bars represent interquartile range. *p-value < 0.05; ns, not significant. **(C)** Histological analysis of the colon of treated mice. Representative colonic sections from Rag1 ko mice who received administration of WT T cells alone or in combination with WT or Aβ$^{0/0}$ Treg cells, stained with H&E (*left panels*) and CD3 immunostaining (*right panels*). Original magnifications: 10× for H&E, 20× for CD3 staining. **(D)** Colitis score in the different treatment groups of induced colitis experiments. The graph shows the inflammation score in the gut of n = 3–mice/group from three experiments. Median score is reported for each treatment group. Error bars represent interquartile range. *p-value < 0.05; **p-value < 0.01; ns, not significant.

patients. Available data about patients' immune phenotype and serum immunoglobulin levels are reported in **Table 3**. Most patients were treated with immunoglobulin replacement therapy and anti-infective prophylaxis, as standard supportive care. Seven patients were treated with HSCT, at a median age of 4.5 years (range: 1.1–16.2 years). Characteristics and outcome of HSCT are detailed in **Table 4**.

We first investigated the frequency of circulating Treg cells in three MHCII-D patients. Treg cells were defined as CD4^{+}CD25^{hi}CD127^{lo}FOXP3^{+} cells. Moreover, in order to differentiate thymic from peripherally induced Treg cells we also stained cells for the expression of HELIOS. The frequency of Treg cells was slightly reduced in one of the untransplanted patients (MHCII_11), as compared to published reference values (36). However, the other two patients showed a frequency of Treg cells comparable to the normal donor, and within normal range also as compared to published references (**Figure 7A** and **Supplementary Table 7**). Interestingly in all patients, most CD4^{+}CD25^{hi}

$CD127^{lo}FOXP3^{+}$ cells were also positive for HELIOS, suggesting their likely thymic origin (**Supplementary Table 7**).

We then investigated possible tolerance breakdown as a result of impaired T-B cell cross-talk in the absence of MHCII molecules. In order to evaluate the presence of autoantibodies in the serum of MHCII-D patients, we performed a protein microarray (37) to screen for a large panel of IgG and IgM autoantibodies. This assay revealed a significantly increased titer of many different autoantibodies, especially of IgG isotype, in the serum of MHCII-D patients (**Figure 7B**), as compared to healthy



		IgG		IgM	
		F.value	p-value	F.value	p-value
MHCII-D	HD	2.11	**0.001**	1.75	**0.002**

FIGURE 7 | Peripheral tolerance impairment in patients with MHCII deficiency. **(A)** Treg cell frequency in MHCII-D patients. Representative FACS plots of Treg cells in three patients with MHCII-D and a HD. Treg cells are defined as $CD4^{+}CD25^{hi}CD127^{lo}FOXP3^{+}$ cells. In upper panels $CD25^{hi}CD127^{lo}$ cells are represented, gated on $CD4^{+}$ T-cell gate. In lower panels, $FOXP3^{+}$ cells are represented, gated on $CD25^{hi}CD127^{lo}$ cells. FOXP3 gate was set based on fluorescence-minus-one (FMO) control on normal donor cells. **(B)** Autoantibodies are present in the serum of MHCII-D patients. Patients' serum was tested for the presence of a large panel of 95 autoantibodies. Heatmap colors correspond to z-score, i.e. the number of standard deviations from the mean signal intensity of the autoantibody across all samples. Color legend over the panel show: pink box, MHCII-D patients; light blue box, age matched normal controls (HD); green, positive control (serum of a patient with systemic lupus erythematous, kindly provided by Stefano Volpi, Genova, Italy). T symbols identify the sample of a MHCII-D patient after HSCT. Statistical analysis included only not transplanted MHCII-D patients. **(C)** BAFF level in MHCII-D patients' sera. Serum BAFF concentration was determined by ELISA in 11 age-matched healthy controls (HD) and in eight MHCII-D patients (one of whom after HSCT). Each symbol represents an individual, and the median is represented with a horizontal bar. Error bar shows the interquartile range.

controls. Furthermore, BAFF levels, which are known to play a role in B-cell homeostasis and peripheral tolerance (38, 39), were increased in the sera of most of the patients analyzed, as compared to healthy controls (**Figure 7C**). Both findings of multiple serum autoantibodies and increased BAFF levels, even if not linked to overt autoimmune manifestations in most patients, are suggestive for the presence of a defective peripheral B-cell tolerance checkpoint in MHCII-D.

DISCUSSION

Our findings in the MHCII deficient mouse model and in human samples from MHCII-D patients suggest that lack of MHCII molecules leads to altered thymic structure and function, resulting in a tolerance impairment broader than previously recognized, involving both central and peripheral mechanisms. In particular, thanks to the unique opportunity to analyze a human thymic biopsy, we observed perturbation of thymic structure in a MHCII-D patient, with reduction of thymic medulla and decreased frequency of AIRE$^+$ TEC. These findings are consistent with data obtained in murine models showing thymic structure perturbation with reduced total TEC cellularity and mature mTEC representation. The decreased AIRE$^+$ TEC frequency might be caused by medullary area reduction. However, a significantly reduced expression of Aire and Aire-dependent and -independent TRA mRNA by qRT-PCR was previously reported in another MHCII ko mouse model (A$\alpha^{-/-}$) (24), suggesting also a functional impairment. Whether this apparent functional defect can be explained merely by the reduced size of the thymic medulla, due to impaired thymic cross-talk with developing thymocytes, or by intrinsic TEC defects was not clear. To address this point, we performed an in-depth cellular and molecular characterization of TEC in A$\beta^{0/0}$ mice. Transcriptomic and proteomic studies revealed several differences in the total TEC population and particularly in the mTEC subset, indicative of an overall reduced functionality of these cells in A$\beta^{0/0}$ mice. Interestingly, an integrated network analysis combining RNA-Seq and proteomic data, revealed potential common processes dysregulated in MHCII ko TEC, mainly involving cell metabolism, energy production and cell cycle, that were downregulated in A$\beta^{0/0}$ mice. In line with this, previous studies showed reduced mTEC proliferation capacity in MHCII deficient mice (24). Analysis of PGE revealed profound abnormalities in A$\beta^{0/0}$ mTEC. First, we confirmed known reduced expression of Aire at both mRNA and protein level, while no differences in Fezf2 expression emerged between WT and A$\beta^{0/0}$ mice mTECs. Moreover, further to previous published observations, we extended the analysis on RNASeq data to thousands of genes known to have a tissue-restricted pattern of expression, which resulted significantly enriched in WT mice mTEC, as compared to A$\beta^{0/0}$. Following recent evidence highlighting RNA processing as an additional way to expand the diversity of the self-antigen repertoire displayed by mTEC (31, 34), we tested the capacity of WT and A$\beta^{0/0}$ mTEC to express a great variety of alternatively spliced TRA transcripts. Interestingly, TRA splicing entropy was reduced in A$\beta^{0/0}$ mTEC, suggesting also a qualitative defect in PGE. Various transcription factors and regulators, which associate with AIRE for its expression and function (40), have been shown to modulate PGE. Here we have shown for the first time that two such factors, *Sirt1* and *Irf8*, that have been previously shown to participate at the control of central tolerance induction (41, 42), are expressed at low levels in A$\beta^{0/0}$ mTEC. We have further hypothesized that the abnormalities of PGE in A$\beta^{0/0}$ mice may reflect impaired mTEC maturation. Consistent with this hypothesis, GSEA demonstrated that A$\beta^{0/0}$ mTEC express reduced levels of many genes known to be expressed in mature TEC, including co-stimulatory molecules and key components of the NF-κB pathway, and in particular CD40, on which mTEC developmental program primarily depends on (43).

Thymic cross-talk with developing thymocytes is well-known to a have a fundamental role for the maturation of mTEC, in particular, through RANK-RANKL and CD40-CD40L signaling (24, 43, 44). In line with this, we observed a severe reduction of both CD40L$^+$ and RANKL$^+$ thymocyte absolute count in A$\beta^{0/0}$ mice. These results support the hypothesis that the underlying cause of reduced mTEC maturation resides in the severe reduction of CD4$^+$ thymocytes, resulting in a low RANKL-mediated stimulation of A$\beta^{0/0}$ mTEC, in line with previous data reported by Irla et al. (24). This observation would suggest a possible role for exogenous soluble RANKL administration in overcoming mTEC maturation and number defects in this disease, also based on its beneficial effects on thymic cellularity observed in other disease murine models (45, 46).

We also confirmed impairment of generation and maturation of CD4$^+$ SP thymocytes in A$\beta^{0/0}$ mice, which resulted in severe reduction of CD4$^+$ T lymphocytes in the periphery, especially in the naïve subset. Defective thymopoiesis in MHCII-D patients has long been considered a reflection of abnormal CD4$^+$ SP thymocytes thymic selection and maturation resulting from the absence of MHCII expression on TEC (1, 4, 6, 7, 47), but no specific studies on patients thymocytes have ever been reported due to technical difficulties in accessing these cells in patients. To overcome this limitation, we investigated *TRAV1-2* (Vα7.2) expression on patients' PB CD3$^+$ lymphocytes, which has been recently described to be severely reduced in patients with immunodeficiencies caused by V(D)J recombination and DNA repair defects (32). The evaluation of *TRAV1-2* gene usage allows to indirectly assess the presence of a specific bias in TCRα use, reflecting potential alterations in thymocyte lifespan or alterations in a very specific window of their intrathymic maturation during which thymocytes undergo TCRα rearrangement, the DP stage. Indeed, MHCII molecules are known to have a fundamental role for the progression from this stage to the following SP CD4$^+$ stage (18). Interestingly, we found a trend to reduced expression of Vα7.2 on CD3$^+$ T cells from the four MHCII-D patients analyzed, including one patient after HSCT. As compared to published mean values obtained in pediatric and adult controls (32), expression of Vα7.2 on their CD3$^+$ T cells resulted in the lower end of normal range,

suggesting a suboptimal TCRα gene rearrangement in these patients. However, since the expression of Vα7.2 has been shown to be highly heterogeneous (32), both in normal and in general PID population, examining a larger cohort of patients with MHCII-D, both prior and after HSCT, would be needed to confirm this finding. In line with this, previous studies on thymic function in MHCII-D patients reported clonal abnormalities of TCR repertoire and lower TCR gene rearrangement events in patients' T lymphocytes, as compared to healthy controls (7), suggesting an overall reduced thymic activity in MHCII deficiency and emphasizing the key role of MHCII molecules in the thymic T-cell maturation processes. However, TREC were detected in patients with MHCII deficiency, reflecting normal early T-cell development (4, 7) and published data about naïve $CD4^+$ T-cell count in patients with this condition are inconclusive (7). In our study, we observed a reduced frequency of naïve $CD45RA^+CD27^+CD4^+$ T cells and RTE in the two untreated patients, especially marked in the infant one.

Immune dysregulation is still a poorly characterized feature of MHCII deficiency. Autoimmunity is reported in 6–20% patients (6). The presentation of self-peptides by MHCII molecules is critical for the maintenance of peripheral T-cell tolerance. mTEC support intrathymic generation of Ag-specific $FoxP3^+$ Treg cells (48–51), together with negative selection of αβ conventional T cells. Based on the alterations detected at thymic level in the absence of MHCII expression, we hypothesized that impairment in central tolerance mechanisms could impinge also on peripheral tolerance establishment and maintenance, in particular by affecting generation of Treg cells. Previous studies in MHCII ko mice provided evidences in favor of this hypothesis (24). However, it has been previously reported that $CD4^+FoxP3^+$ Treg cells from MHCII ko mice are capable of mediating immune suppression *in vitro* (25). In experimentally induced colitis models, regulatory $CD25^+$ DP T cells generated in MHCII ko mice, probably arising from SP $CD8^+$ T cells, have been demonstrated to control the colitogenic potential of $CD25^-CD4^+$ T cells (26), but data are missing on the *in vivo* $CD4^+$ Treg-specific functionality in MHCII ko models. To address this, we tested $A\beta^{0/0}$ Treg cell capacity to attenuate colitis induced by the injection of WT T naïve cells into *Rag1* ko mice. Our experimental data suggest a functional impairment of $A\beta^{0/0}$ Treg cells *in vivo*, in contrast to previous reports on their *in vitro* functionality. This defect in $A\beta^{0/0}$ mice may be compensated *in vivo* by tolerogenic $CD8^+$ "Treg-*like*" cells which have been described to constitutively express CD25, CTLA4 and FoxP3 and have been demonstrated to be able to produce IL-10 and efficiently inhibit $CD25^-$ T cell responses to anti-CD3 stimulation (27). These cells might also account, at least in part, for reduced signs of overt spontaneous autoimmunity in $A\beta^{0/0}$ mice.

Limited data regarding Treg cells have been reported to date in patients with MHCII-D. The frequency of circulating Treg cells within the $CD4^+$ T-cell population was either normal or slightly reduced in the three patients tested. This is in line with a previously published observation (52) in a pediatric MHCII-D patient, in whom a normal frequency of Treg cells was reported, together with reduced absolute number due to severe reduction in total $CD4^+$ T cells in these patients. Of interest, most Treg cells were $Helios^+$, suggesting their likely thymic origin.

Impaired Treg function may also contribute to the impairment of peripheral B-cell tolerance checkpoint through altered cognate T-B cell interactions. This is in line with a previous report on a MHCII-D patient who displayed a low number of Treg cells and failed to counterselect autoreactive mature naïve B cells. This suggests that peripheral B-cell tolerance also depends on MHCII-TCR interactions and that Treg cells may play an important role in preventing the accumulation of new emigrant/transitional autoreactive B cells in the mature naive compartment of these patients (52). We found a significantly increased level of BAFF in the serum of most of MHCII-D patients in our cohort. Our results confirm the preliminary observation of increased BAFF levels in one MHCII-D patient reported by Hervé et al. (52), which was correlated with defective peripheral B-cell tolerance checkpoint in this disease. Elevated BAFF levels lower the thresholds for the survival of autoreactive B cell clones (38) and inhibit the counterselection of autoreactive new emigrant/transitional B cells that fail to be removed from B-cell population. It would be important to understand the causes underlying elevation in BAFF level in MHCII-D patients. Elevated BAFF serum levels are often present in B cell lymphopenic conditions (53), autoimmune diseases (54, 55) and viral infections (56). Increased BAFF levels in MHCII-D are unlikely due to peripheral B-cell lymphopenia, since most patients have normal B cell levels, but are more suggestive of ongoing autoreactivity/autoinflammation. BAFF is known to be produced by myeloid cells. In particular, neutrophils can contribute to excess serum BAFF levels, through which they have been demonstrated to promote $CD4^+$ T-cell and B-cell responses and enhance $CD4^+$ T-cell proliferation and IFNγ secretion in lupus-prone mice (57). Moreover, BAFF has been reported to promote Th1-mediated inflammation through downstream cellular events (58) and to trigger the production of pro-inflammatory cytokines through the activation of the NF-kB pathway (59). These findings indicate a possible link between the chronic infection-driven inflammatory state in these patients and their B-cell tolerance impairment. However, no clear correlation between BAFF levels and autoimmune manifestations emerged from the study of the patients reported here.

In conclusion, our data highlight the key role of MHCII molecules in both central and peripheral immune tolerance mechanisms, and uncover specific defects in TEC maturation and function, both at transcriptomic and proteomic levels. These results also indicate the need of complementing therapeutic approaches based on HSCT with new therapeutic strategies aimed at correcting the underlying molecular defect also at the TEC level in order to achieve a more effective cure for MHCII-D.

DATA AVAILABILITY STATEMENT

RNA-Seq data are available under accession number GSE166463. Data from proteomic analyses are available in MassIVE

repository, at the following link: ftp://massive.ucsd.edu/MSV000086866/. Other raw data supporting the conclusions of this article will be made available by the authors upon request, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the San Raffaele Ethical Committee. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin. The animal study was reviewed and approved by Institutional Animal Care and Use Committee protocol 710-712.

AUTHOR CONTRIBUTIONS

FF performed experiments, analyzed data, and wrote the manuscript. IB performed experiments and analyzed data. RR, GEM, ED, MCC, and GD performed experiments. EF and PLP performed histological analyses on human and murine tissue samples. PU performed RNA-Seq on murine-sorted TEC and data analysis. FB contributed to sample preparation for proteomic studies. DDS performed proteomic and network analyses. DM, CP, TT, VB, ASc, CS, SG, ASo, ARG, SS, BDS, OMD, LDN, and CMR provided patients' samples and clinical information. LDN, PLP, and PLM contributed intellectual input and data analysis and revised the manuscript. AV and MB designed research experiments, supervised the study, and reviewed the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.669943/full#supplementary-material

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The Early Postnatal Life: A Dynamic Period in Thymic Epithelial Cell Differentiation

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The microenvironments formed by cortical (c) and medullary (m) thymic epithelial cells (TECs) play a non-redundant role in the generation of functionally diverse and self-tolerant T cells. The role of TECs during the first weeks of the murine postnatal life is particularly challenging due to the significant augment in T cell production. Here, we critically review recent studies centered on the timely coordination between the expansion and maturation of TECs during this period and their specialized role in T cell development and selection. We further discuss how aging impacts on the pool of TEC progenitors and maintenance of functionally thymic epithelial microenvironments, and the implications of these chances in the capacity of the thymus to sustain regular thymopoiesis throughout life.

Keywords: thymus, thymic epithelial cells, tolerance, early postnatal life, aging

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INTRODUCTION

The current pandemic caused by the SARS-CoV-2 virus underscores the importance of maintaining a pool of immunologically competent T cells, which are capable of responding to virtually any new foreign threats while tolerant to the host own tissues. The establishment of a diverse T cell receptor (TCR) repertoire arises from the random recombination of V(D)J gene segments during T cell development in the thymus. Yet, the arbitrariness underlying this process can also produce autoreactive T lymphocytes. The thymus has developed several control mechanisms to simultaneously establish T cell immunity against non-self elements and impose self-tolerance. Particularly important in the choreography of T cell selection are thymic epithelial cells (TECs), which represent a key component of the thymic stromal microenvironment. TECs are typically subdivided into functionally distinct cortical (cTEC) and medullary (mTEC) lineages (1). While cTECs primarily mediate T cell lineage commitment and positive selection, mTECs fine-tune the negative selection of autoreactive thymocytes or promote their deviation into the T regulatory cell lineage (2). It is conceptually accepted that cTECs and mTECs differentiate from thymic epithelial progenitors (TEPs) present within the embryonic and postnatal thymus (2). Deficits in the function of TECs arise with aging, cytoablative regimens and infection, leading to a lower naïve T cell output. These thymic failures are pertinent in the elderly and patients undergoing bone marrow transplantations (BMT), contributing to their poor T-cell responses to new pathogens or predisposing to autoimmunity (3). Thus, the preservation of a regular thymic function also depends on the maintenance and differentiation potential of bipotent or lineage restricted TEPs.

In this review, we focus on critical changes in the molecular traits of TECs that occur during the first weeks of the murine postnatal life, and integrate how these alterations might precede events coupled with thymic involution.

THE BUILD-UP OF TEC MICROENVIRONMENTS

The initiation of TEC development coincides with the onset of thymus organogenesis, which starts around day 9-10 of the murine embryonic gestation (E9-10) (4). The expression of Forkhead box protein N1 (Foxn1) in the ventral area of the common thymus and parathyroid primordium marks a critical step in TEC specification (5). Still, Foxn1 expression needs to be continuously maintained during the differentiation of c/mTEC, wherein it imposes a complex genetic program that confers them the capacity to support distinct stages of thymopoiesis (6). TEPs formed during early thymus ontogeny constitute the primordial building blocks for the establishment and maintenance of c/mTEC microenvironments (7–9). Our comprehension about the mechanisms underlying TEC differentiation has considerably advanced with the identification of distinct populations containing bipotent or lineage-restricted progenitor activity (10–21) [further detailed below and reviewed in (22, 23)]. These studies led to the proposal of different refined models of TEC differentiation, whereby TEPs traverse through transitional stages that share a closer or distinct relationship with cTEC- or mTEC-unipotent precursors, prior to the commitment in mature c/mTEC subsets [reviewed in (2, 24, 25)]. Yet, it remains unclear the trajectories and molecular elements governing the differentiation of TEC progenitors into mature c/mTEC lineages.

The expansion and functionalization of c/mTEC compartment during early postnatal stages generates a supportive microenvironment that increases thymopoiesis, reaching its peak during young adulthood. Thereafter, T cell production progressively declines with aging, becoming residual in the aged thymus (26). During these periods, TECs undergo concomitant alterations in their composition and differentiation program. Although the density of TECs based on flow cytometry analysis might be underestimated (27), the number of TECs vigorously expands during postnatal life and early adulthood, followed by a progressive decline with age (28, 29). Changes in the size of TEC microenvironment appears to relate with the function of the thymus. While a reduction in the TEC compartment below a certain threshold restrains thymopoiesis (30, 31), the expansion of the thymic epithelial niche, for example *via* transgenic expression of Foxn1 or Cyclin D1, increases T cell generation (32, 33). Along this line, the frequency of cycling TECs is elevated during fetal life, progressively declines during the postnatal life and become a rare fraction in the aged mouse thymus (28). Transcriptomic analysis revealed that the expression of cell-cycle regulators is downregulated in TECs as early as 1 month (34). Moreover, the enforced expression of cMyc in TECs promotes the expansion of the TEC compartment, *via* the engagement of a genetic program akin to the one found in embryonic TECs (35). These results suggest that the loss in the proliferative rate of TECs, together with other alterations such as changes in cell survival and rate of differentiation, may contribute to a reduction in the size of TEC compartments with age. In the next sections, we outline specific cellular and molecular alterations that take place in c/mTEC during early postnatal life, and conjecture how those changes may anticipate subsequent functional losses in the capacity of TECs to sustain regular thymopoiesis in the long-term.

THE ASSEMBLY OF FUNCTIONALLY DEDICATED CTEC AND MTEC COMPARTMENTS

The first weeks of the postnatal life marks a period of intense turnover and functional diversification in the TEC niche, wherein key mature subsets in tolerance induction are generated or expanded (23). During this period, the changes in the cellularity and functionality of cTECs appear to unfold concomitant with the expansion and diversification of mTECs (11, 12, 36–38). This leads to a conspicuous inversion in the cTEC/mTEC ratio within the first 2 weeks after birth, which correlates with the intensification of thymopoiesis (11, 12, 28). In this regard, the consequent rise in the number of positive and negative selection events, will impose an increase demand on TEC compartments. Given that mature cTECs and mTECs have a limited life-span, the maintenance and specialization of their microenvironment seem to depend on the continual differentiation of their progenitors. These functional requirements are in part met by a symbiotic relationship with thymocytes (discussed further below) that stimulate specific proliferative and differentiation programs in TECs (39).

It remains surprising how little we know about the molecular program that underlies the differentiation of cTECs. Despite these gaps, several studies highlight that cTECs undergo molecular and functional changes during neonatal and puberty periods. In particular, cTECs downregulate the expression of key thymopoietic factors, such as Dll4 and IL-7, during the first weeks of postnatal life, which result from continual lymphoepithelial interactions (37, 38, 40, 41). These quantitative and qualitative disruptions in cTECs appear to anticipate the bona fide hallmarks that characterize TECs in the involuted thymus. In contrast to cTECs, our understanding of the cartography of mTEC differentiation is more complete (22). This process depends on reciprocal signals provided by several types of hematopoietic cells (1). These lymphoepithelial interactions, commonly referred as thymic crosstalk, engage specific members of the tumor necrosis factor receptor superfamily (TNFRSF), including receptor activator of NF-κB (RANK), CD40 and lymphotoxin β receptor (LTβR), in mTECs and their progenitors, leading to the activation of a nuclear factor kappa B (NF-κB)-dependent maturation program [reviewed in (1, 22)]. The cooperative action of TNFRSF members is not only important for the expansion of mTEC niches but also for their functional diversification. Upon the initial subdivision in

mTEC^{low} and mTEC^{high} (42), the discovery of Autoimmune regulator (Aire)-, Ccl21- and forebrain embryonic zinc finger-like protein 2 (Fezf2)-expressing cells revealed that mTECs harbors a variety of functionally distinct mature subsets (1, 22). Although Aire$^+$ and Fezf2$^+$ cells emerge during embryonic life (1, 22), their abundance significantly increases in the first weeks of life. In this regard, RANK-mediated signaling is essential to the expansion of Aire$^+$ mTECs, whereas CD40 also contributes to this process (43, 44). Although LTβR signaling was initially coupled to the development of Aire$^+$ (45) and Fezf2$^+$ lineages (46), subsequent studies indicated its involvement in the architecture of postnatal medullary compartment (47). Aire and Fezf2 regulate the capacity of mTECs to express large sets of non-overlapping tissue restricted antigens (TRAs), which are randomly organized in patterns of gene expression at the single cell level (48–50) and are reported to decrease their levels with age (51–53). In this regard, an earlier study underscore the importance of Aire expression in mTECs during neonatal period (54), which corelates with their capacity to control the generation of a unique population of T regulatory cells (55). It remains to be determined whether Aire expression during this temporal window particularly impacts on the quantity or quality of TRAs expression by mTECs.

The role of mTECs in tolerance induction extends beyond their promiscuous gene expression capacity. CCL21-producing cells represent a prototypical example of alternative roles of mTECs. CCL21-expressing mTEC represent a subset of mTEC^{lo} and control the migration of positively selected thymocytes towards the medulla (56, 57). CCl21$^+$ cells emerge during embryogenesis and their numbers also undergo a marked increase during the first weeks of life (57). Recent single cell RNA sequencing analysis suggests that Aire- and Ccl21a-expressing mTEC subsets do not share a direct lineage relationship (58). Moreover, the discoveries that Aire$^+$mTECs differentiate into Post-Aire cells (59, 60) further extended our view on the heterogeneity within thymus medulla. Post-Aire mTECs shutdown the expression of Aire, certain TRAs, CD80 and MHCII, while acquiring traits of terminally differentiated keratinocytes (61, 62). Two reports identified a highly differentiated mTECs that share molecular traits with tuft cells found at mucosal barriers. Fate-mapping analysis suggests that this subset can develop *via* an AIRE-dependent and AIRE-independent pathway (63, 64). Although their complete functional relevance remains elusive, tuft-like mTECs appear to regulate the development of invariant NKT cells and ILCs (63, 64). Future studies may uncover new specialized mTEC subsets and their role in imposing the limits of tolerance, or alternative processes in thymus biology.

THE THYMIC EPITHELIAL CELL PROGENITOR RESERVOIR

The diversification of TECs during the first weeks of life is dictated by the intricate balance between the rate of proliferation and differentiation of mature subsets. The rapid turnover of TEC microenvironments, with an estimated replacement time of one to two weeks to mTECs (28, 59), implicates the requirement for a regular generation of mature TECs from their upstream progenitors. One possibility is that bipotent TEPs continually produce lineage-committed precursors lacking long-term self-renewal capacity. Alternatively, and not mutually exclusive, the abundance of bipotent TEPs might decrease with age, being the maintenance of cortical and medullary epithelial niches assured by downstream compartment-restricted precursors. In the last years, several studies provide evidence for the existence of an arsenal of subsets enriched in purported bipotent TEC progenitors in the postnatal thymus (10, 13–15). One approach has employed *in vitro* 2D-clonogenic (10) or spheroids (13) assays to respectively isolate TEC progenitors that reside within EpCAM$^+$Ly51$^+$cTECs or EpCAM$^-$ cells, which were expanded *in vitro* and revealed the capacity to give rise to c/mTEC. Nonetheless, a more recent study indicate that cells isolated from EpCAM$^-$derived spheroids represent mesenchymal progenitors (65). Other methodologies resolved bipotent progenitor activity within defined subsets of UEA-1$^-$MHII^{lo} Sca-1$^+$ TECs (14) and MHCII^{hi} Ly51$^+$Plet1$^+$ cTECs (15). Both strategies employ reaggregate organ cultures (RTOCS) to determine the precursor-product lineage relationship to mature cells. Despite the advances, it remains to be determined the physiological contribution of these cells to the TEC microenvironment in the adult thymus. Thus, we still lack experimental evidence that demonstrates the existence of bona-fide bipotent TEC progenitors in the postnatal thymus, and their identification at the single cell level.

Downstream of TEC progenitors, complementary studies documented how mTEC compartments evolved from bipotent TEP and mTEC-restricted precursors (mTEPs), including mTEC-restricted SSEA-1+ and podoplanin+ (PDPN) mTEPs (16, 18). Fate-mapping studies show that the adult mTEC network arise from fetal- and newborn-derived TEPs expressing beta5t (β5t), a prototypical cTEC marker. Yet, the contribution of β5t+ TEPs to the adult mTEC niche decreases with age (19, 20), suggesting that the maintenance of the adult medullary epithelium is assured by mTEPs. Although bipotent TEPs might lose the expression of some traits found in the embryo (e.g. β5t), it is also possible that the abundance and/or the self-renewal properties of bipotent TEPs and/or lineage-restricted progenitors decline with time. Supporting this view, the clonogenic activity of purported bipotent TEPs that reside within the cortex decrease with age (10) and Cld3,4$^+$SSEA1$^+$ mTEC-restricted cells become rare in the adult thymus (16). Given that the numbers of embryonic TEPs dictates the size of functional TEC microenvironments (30), we infer that the loss in the TEC network that takes place with age may result from the decrease in the bioavailability and self-renewal capacity of TEPs early in life.

The advent of single cell RNA sequencing (scRNAseq) analysis have also contributed to our understanding of the heterogeneity and dynamic of TEC progenitors. This approach has emerged as a new unbiased method to identify novel subsets, providing a valuable platform to analyze their developmental

trajectories and determine their relationships with progenitor subsets identified by conventional methodologies. In this regard, new clusters termed "pre-Aire mTEC 1 and 2" (66) appear to present molecular traits similar to the ones found in podoplanin+ (PDPN) mTEPs (18). A subsequent study identified a novel cluster of "intertypical TECs" (51) that harbors traits akin to the ones found in podoplanin+ (PDPN) mTEPs (18), UEA-1^{-}MHII^{lo}Sca-1^{+} (14) and MHCII^{hi} Ly51^{+}Plet1^{+} (15) TECs. Since "intertypical TECs" are further segmented in distinct 4 subclusters, it would be interesting to determine if they associate to a particular bipotent or unipotent subset. Moreover, scRNAseq analysis reveal the existence of a previously unrecognized cluster of "perinatal cTECs". Interestingly, this subset harbors cells with a highly proliferative status and their abundance declines with age (51). Moreover, the combination of scRNAseq and fate mapping analysis revealed that β$5t^{+}$ TEPs acquire senescent-like properties with age, potentially explaining their failure to contribute to mTEC lineage beyond the neonatal stage (19, 20). Together, these findings indicate that the integration of multiple experimental approaches provides a more complete strategy to resolve the intricacies of the TEC compartment. Future studies should attempt to identify specific markers to resolve the newly characterized populations at a single level.

CONCLUDING REMARKS

The aforementioned studies underscore that the period between birth and early adulthood is a time of intense alterations in TEC microenvironments, which prepares them to the highly demand role of choreographing the selection of growing number of T cell precursors. In this sense, it is remarkable to appreciate the synchronous coordination between TEC differentiation and the requisites imposed by T cell development. Yet, the erosion of the pool of TEC progenitors seem to accompany the generation of specialized subsets with key roles in tolerance induction. We reason that an in-depth molecular analysis of TEC differentiation during early postnatal may provide insights on how TEC niches are maintained, and can be repaired in the aged thymus. Despite recent advances, it remains unclear how changes in the bioavailability of TEPs impact on the maintenance of TEC microenvironment across life, and ultimately on thymic output. Another unexplored area pertains to the physiological causes underlying the presumed age-dependent decrease and/or senescence of TEPs. Knowledge in these areas will not only permit to comprehend the basic principles that governs thymic function, but also target pathways for the treatment of disorders coupled to dysfunctional thymic/T cell responses.

AUTHOR CONTRIBUTIONS

NA and RP wrote the manuscript. All authors contributed to the article and approved the submitted version.

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