THYMUS FUNCTION AND AGING: A FOCUS ON THYMIC EPITHELIAL CELLS

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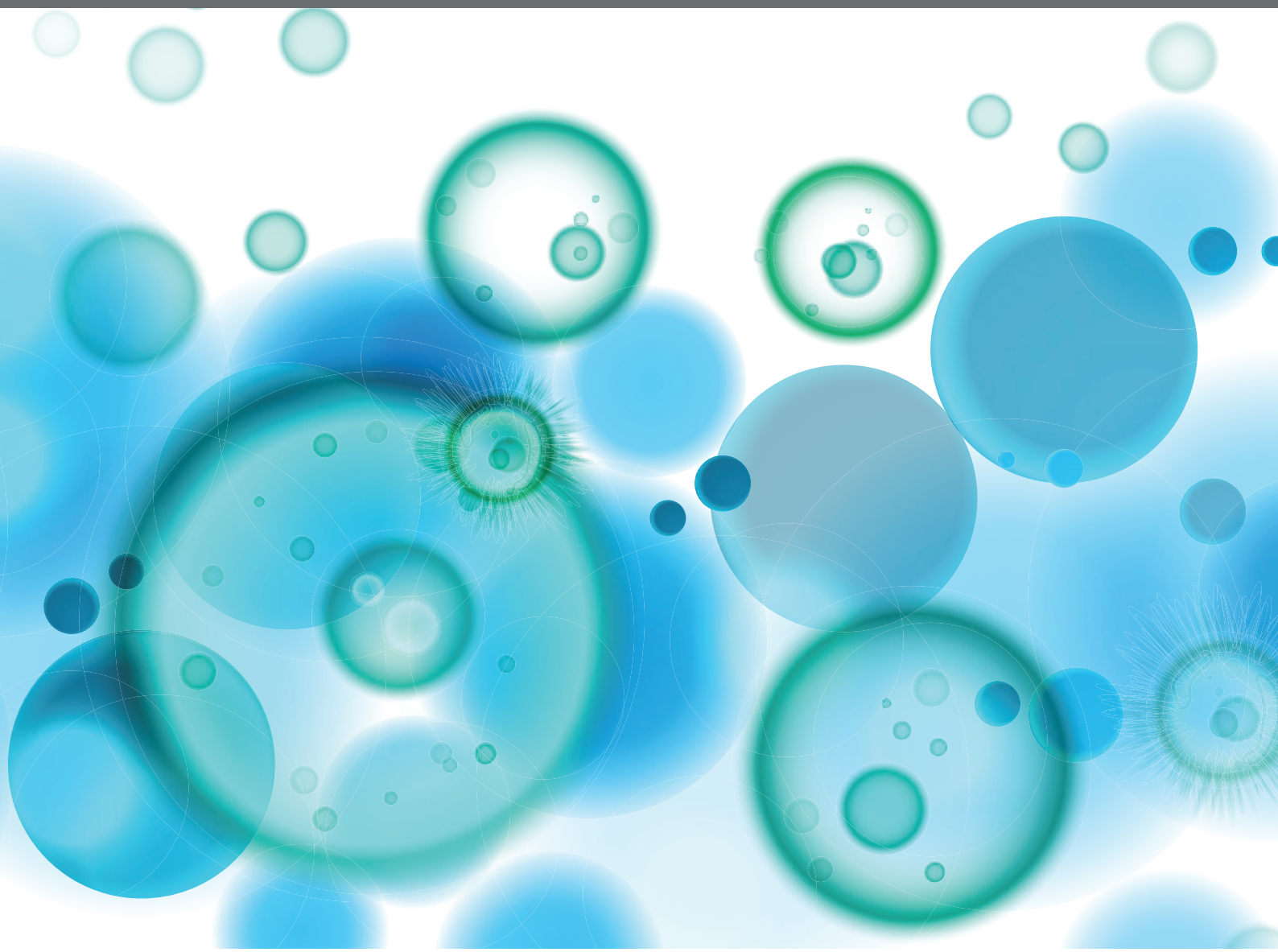

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THYMUS FUNCTION AND AGING: A FOCUS ON THYMIC EPITHELIAL CELLS

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Table of Contents

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Editorial: Thymus function and aging: A focus on thymic epithelial cells

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Editorial on the Research Topic
Thymus function and aging: a focus on thymic epithelial cells

The thymus is an unique organ in its ability to support the maturation of phenotypically and functionally distinct T cell sublineages and innate immune cells, which carry out multiple tasks to keep the organism healthy. Entering the thymus, lymphocyte precursors interact with the thymic stromal compartment mainly built of different thymic epithelial cells (TECs) and other non-lymphoid cells comprising the thymic microenvironment for T cell development (1). From the enormous variety of produced T lymphocytes, only a minor part can survive a rigorous checkpoint control and selection during this crosstalk (2). However, some physiological factors, including aging, stress, and pregnancy, as well as medical procedures such as thymectomy and chemo/radiotherapy, can harm the thymus function, which is associated with the decline of immune function and the risk of tumors and infectious and autoimmune diseases.

In this Research Topic are collected the efforts of many research groups in trying to overcome the thymus aging/injury problem by applying different regenerative or thymus replacement strategies. The basis of these is the epithelial compartment, in particular, the thymic epithelial stem cells (TESCs) as the target cells to stimulate thymus recovery *in vivo* or for growing thymus-replacing organoids *in vitro* (3, 4). Several research groups have described TESCs in the embryonal (5) and adult (6–8) mouse thymus, which were identified as the bipotent TEC progenitors differentiating into cortical (c) and medullary (m) TEC lineages (5–8). Progress in the identification of different TEC subtypes as well as other subtypes of thymic cells in the last several years was significant, especially with applying the single-cell RNA-sequencing technology (9–11).

T cell development in the thymus depends on Notch signalling (12) induced by the interaction of Notch1, present on immigrant cells, with a Notch ligand, delta-like ligand (Dll) 4, on the thymic epithelial cells. Hirano et al. propose a hypothesis that in the thymic

environment of ancestral vertebrates, where the thymus first appeared, primarily functions Dll1 and Notch2. The authors confirmed that Dll1 cooperates with Notch2 in T cell development in the murine thymus. Their results support the hypothesis that Dll1 regulates T cell development *via* Notch1 and/or Notch2 in the thymus of cartilaginous fishes. In the authors' opinion, during the evolutionary process, Dll4 replaces Dll1 in the induction of thymic Notch signalling, constituting an environment in the thymus suitable for immigrant cells bearing Notch1.

Initial studies, recently confirmed by genetic approaches, have extended the role of Notch signalling to the epithelial compartment of the thymus, showing that active Notch contributes to TEC development during embryonic life. García-León et al. showed that *in vivo* Notch activation is not confined to embryonic TECs, but Notch signalling, likely mediated through the Notch1 receptor, is induced as well in postnatal TECs mainly located in the medulla (mTECs). In both human and mouse thymus, numbers of mTECs showing Notch activation increased significantly with age, suggesting a conserved role for Notch in postnatal TEC homeostasis during aging. TEC-specific abrogation of Notch signalling disrupted the medullary thymic microenvironment and accelerated thymus atrophy. These data uncover a new role for Notch1 signalling in the control of adult mTEC homeostasis.

Differentiating the human pluripotent stem cells towards thymic endoderm, Sun et al. identified a new population of $FOXN1^{+}EPCAM^{+}CD90^{+}$ triple-positive TEC progenitors. They confirmed the existence of similar cells in cultures of neonatal human TECs. Also, they showed that a subset of primary neonatal human TECs co-express a marker of mesenchymal cells CD90 and a TEC marker EPCAM that reflect the presence of a mesenchymal program in human TECs. This program was more expressed in cTECs. Their results reveal that human TECs possess a hybrid gene expression program comprising epithelial and mesenchymal elements.

Proper T cell function is paramount to health and homeostasis. However, it is unclear whether the thymic ability to support incoming progenitors is affected by aging and the associated thymic involution. Mohtashami et al. compared the ability of progenitor T cells to home to the thymus of young and old mice and determined whether progenitor T cells can help support T cell regeneration in a clinically relevant model of hematopoietic stem cell transplants (HSCT). They demonstrated that the adoptive transfer of *in vitro*-generated pro-T cells in aged mice accelerated thymic reconstitution after chemotherapy and gamma irradiation compared to HSCT alone. Accelerated T cell recovery was also observed in both old and young mice receiving both pro-T cells and HSCT.

A critical part of the processes associated with central tolerance occurs in the thymic medulla. It depends on the presence of various types of dendritic cells (DCs), B cells, and highly specialized mTECs. Cooperation between these cells is required to remove autoreactive T cells efficiently. This crosstalk is relevant not only during thymus organogenesis and T cell development but also promotes the recovery of the thymus functionality after injuries. Březina et al. in their review paper, highlight the current knowledge concerning the pathways by which self-antigens are presented in the thymus and how they lead to the establishment of tolerance. They also examined and discussed the possible molecular mechanisms underpinning cooperative antigen transfer. Finally, they discussed the current results related to distinct preferences of DC subsets in acquiring thymic epithelial cell-derived antigens.

Shichkin and Antica discuss cellular architecture and molecular factors essential for correct thymic function relating to T cell positive and negative selection and generation of naïve T cells. The authors summarize the current understanding of the development and function of TECs and other stromal cell populations, the signalling and transcriptional pathways underlying the intrathymic cell interaction, and T cell development concerning developing new strategies for restoring thymic function after damage. The authors accentuate populations of intrathymic stem cells (SCs), including epithelial SCs, mesenchymal SCs, and lymphoid progenitor cells. The particular focus is on their radioresistance and, thus, possible contribution to thymus recovery after injury with irradiation or chemotherapy.

Rosichini et al. analyzed signals involved in the crosstalk between TECs and hematopoietic cells. The authors' primary focus is on how T cell signals regulate TEC function. The authors also discuss the relevance of these pathways in restoring thymic function and T cell immunity in experimental models and in the clinical setting.

Lagou et al. propose a fresh insight that chemotherapy-induced thymic involution, which is characterized by the extensive obliteration of the sensitive TEC compartment, can cause long-term defects in thymopoiesis and the establishment of diverse T cell pools of cancer survivors patients. Such delayed recovery of the T cell adaptive immunity may result in the prolonged disturbance of the cancer immunoediting mechanisms and lead to the development of persistent and mortal infections, inflammatory disorders, autoimmune precursor lesions, and second primary malignancies.

Finally, Iaiza's et al. are focused on the involvement of long non-coding RNAs (lncRNAs) in the acquisition of malignant traits by neoplastic TECs, and describes the possible use of these molecules as targets for the design of novel therapeutic approaches specific for TECs. Furthermore, they discuss the involvement of lncRNAs in myasthenia gravis-related thymoma.

In summary, we anticipate that the articles in this *Frontiers Research Topic* will provide researchers with a valuable resource for understanding how we can improve the current and develop new strategies for thymus recovery/replacement and translate them to clinics.

Author contributions

VS, MPF, IS, and MA wrote the editorial and invited authors to participate in the collection. All authors contributed to the article and approved the submitted version.

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Conflict of interest

Author Valentin Shichkin was employed by the company OmniFarma.

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

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CD90 Marks a Mesenchymal Program in Human Thymic Epithelial Cells *In Vitro* and *In Vivo*

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Thymic epithelium is critical for the structural integrity of the thymus and for T cell development. Within the fully formed thymus, large numbers of hematopoietic cells shape the thymic epithelium into a scaffold-like structure which bears little similarity to classical epithelial layers, such as those observed in the skin, intestine or pancreas. Here, we show that human thymic epithelial cells (TECs) possess an epithelial identity that also incorporates the expression of mesenchymal cell associated genes, whose expression levels vary between medullary and cortical TECs (m/cTECs). Using pluripotent stem cell (PSC) differentiation systems, we identified a unique population of cells that co-expressed the master TEC transcription factor *FOXN1*, as well as the epithelial associated marker EPCAM and the mesenchymal associated gene CD90. Using the same serum free culture conditions, we also observed co-expression of EPCAM and CD90 on cultured TECs derived from neonatal human thymus *in vitro*. Single cell RNA-sequencing revealed these cultured TECs possessed an immature mTEC phenotype and expressed epithelial and mesenchymal associated genes, such as *EPCAM*, *CLDN4*, *CD90* and *COL1A1*. Importantly, flow cytometry and single cell RNA-sequencing analysis further confirmed the presence of an EPCAM+CD90+ population in the CD45- fraction of neonatal human thymic stromal cells *in vivo*. Using the human thymus cell atlas, we found that cTECs displayed more pronounced mesenchymal characteristics than mTECs during embryonic development. Collectively, these results suggest human TECs possess a hybrid gene expression program comprising both epithelial and mesenchymal elements, and provide a basis for the further exploration of thymus development from primary tissues and from the *in vitro* differentiation of PSCs.

Keywords: human thymic epithelial cells, epithelial and mesenchymal components, primary cells culture, pluripotent stem cell differentiation, CD90/Thy1, cell identity

INTRODUCTION

The thymus is a haematopoietic organ where T cells develop, and central tolerance is established. The capacity to regenerate a functional analogue of this organ *in vitro* would provide an accessible and tractable experimental platform to study T cell development, and to gain a greater understanding of how tolerance is established and how it is undermined, the latter leading to autoimmunity (1, 2). Previously, we and others have used human pluripotent stem cells (PSCs) to generate thymic endodermal progenitor cells that, in principle, have the potential to differentiate into functional cortical or medullary thymic epithelial cells (c/mTECs) capable of supporting T cell differentiation (3–6). However, to date, the most advanced differentiation protocols have not been able to generate functional TECs from hPSCs *in vitro*. Instead, functional differentiation has only been achieved following transplantation into immunodeficient mice (3, 4, 6). A confounding factor in recreating the thymic epithelium *de novo* is the number of cell types that have a role in its genesis, coupled with insufficient knowledge concerning the origins and characteristics of TECs.

In distinction to other epithelial organs, functional development of the thymic epithelium relies on the influx of hematopoietic cells, which rapidly enlarge the thymus (7–9). Along with this, the thymic epithelium undergoes a drastic morphological change; the epithelial primordium is transformed from a tight cluster of epithelial cells into a scaffold-like structure that is interspersed with large numbers of T cell progenitors (10, 11). These changes coincide with the specification of cortical and medullary thymic epithelial cells (cTEC and mTECs), both of which are thought to derive from a common TEC progenitor (12–15). cTECs and mTECs are distinguished from each other by their location, functionality, and repertoire of gene expression. Classically, the pattern of cytokeratin expression has been used to distinguish mTECs and cTECs; TEC progenitors expressed both keratin 5 (KRT5) and KRT8, whose expression is subsequently restricted to mTECs and cTECs, respectively (16). Additionally, mTECs are also distinguished from cTECs by the former's expression of KRT14. Apart from keratin expression, cTECs and mTECs also develop distinct epithelial phenotypes. Immunofluorescence analysis shows that human thymic primordium at embryonic week 7 expresses high levels of EPCAM, a marker that is retained in mTECs at embryonic week 15 but substantially downregulated in cTECs (7). Similarly, the tight junction-forming proteins claudin 3 (CLDN3) and CLDN4 are highly enriched in mTECs (17). These observations indicate that TEC specification is coupled with morphological and molecular changes in typical epithelial characteristics, some of which may be important in the process of TEC differentiation from hPSCs.

Forkhead box protein N1 (FOXN1) is a master transcription factor that plays a critical role in the development of thymic epithelial cells (18). FOXN1 is detected mid-week 6 of human development, with its expression restricted to a site within the third pharyngeal pouch that marks the presumptive thymus primordia, in distinction to the presumptive parathyroid which is marked by GCM2 (glial cells missing transcription factor 2); both tissues develop from the third pharyngeal pouch (7, 19, 20). However, studies using Foxn1 deficient mice indicate that initial thymic commitment is Foxn1 independent, but that Foxn1 is required for specification of cTEC and mTEC from TEC progenitors (18) and Foxn1-null thymic primordium is unable to support hematopoietic colonization (21). In the context of hPSC differentiation *in vitro*, currently available methods direct differentiation to a stage where *FOXN1* expression is detectable, but cells fail to undergo further functional commitment (3, 5, 6). We previously generated *FOXN1*:GFP hPSC reporter lines that are a valuable tool for further dissecting the molecular regulation of human TEC development and for the isolation and examination of FOXN1+ cells (5).

In this study, we examined the characteristics of hPSC-derived thymic progenitors and neonatal thymic epithelial cells cultured under the same serum free conditions. Experiments using single cell RNA-sequencing confirmed that CD90 (also known as THY1), a gene often associated with mesenchymal cells, is expressed by human TECs and that this expression reflects a broader underlying mesenchymal gene expression program. Indeed, we show FOXN1+ TECs and TEC progenitors isolated from *in vivo* and *in vitro* sources co-expressed EPCAM and CD90. Further analysis revealed that human TECs expressed a cohort of mesenchymal markers, suggesting that TECs acquired an identity with characteristics of both epithelial and mesenchymal cell types. These findings provide biological insight into human thymic epithelial cell identity and a basis to further explore thymic development from pluripotent stem cells.

MATERIALS AND METHODS

Thymic Endoderm Differentiation From Human PSC

Work related to pluripotent stem cell lines was conducted in accordance with RCH Human Research Ethics Committee 33001A. Two *FOXN1*:GFP human PSC reporter lines (MEL1 and HES3) were used to generate FOXN1+ epithelial cells following our previously published protocol with modifications (5). Human PSCs were cultured with a standard E8 medium (Gibco)-based feeder-free cell culture system as described defined (22). At day 0, cells were harvested and deposited into each well (3×10^3cells/well) of a 96-well round-bottom nonadherent plate (Nunc) and briefly centrifuged to promote cell aggregation in to embryoid bodies (EBs). Differentiation was set up using our new chemically defined serum- and albumin-free (CD-SAF) medium (**Table 1**), supplemented with 100 ng/ml Activin A for 5 days for endoderm induction. On day 5, medium was replaced with only the CD-SAF medium without additional supplements. On day 7, EBs were transferred to gelatin-coated (0.1%) 96-well flat-bottom adherent plates (BD Falcon). From day 14, medium was supplemented with 40 ng ml^{-1} human keratinocyte growth factor (KGF; Peprotech, 100-19). Analyses of these cultures were performed between day 30 to day 60. In all instances PSC cultures and differentiations were maintained at 37°C, in a 5% CO_2/air environment.

TABLE 1 | Chemically defined serum- and albumin-free cell culture medium.

Items	Stock	Final	For 500 ml	REF/Cat#	Supplier
ITS-X (E)	100X	1X	5ml	777ITS032	InVitria
Polyvinyl alcohol	10%	0.1%	5ml	P8136-1KG	SIGMA
Methyl cellulose	10%	0.1%	5ml	M7027-250G	SIGMA
AA2P (L-Ascorbic acid 2-phosphate)	10 mg/L	50 ug/ml	2.5 ml	A8960-5G	SIGMA
Glutamax	100 X	1X	5 ml	35050-061	Gibco
NEAA	100 X	1X	5 ml	11140-050	Gibco
Lipid Concentrates		1/500	1ml	11905-031 (100ML)	Gibco
Embryo MAX Nucleosides	100X	1X	5ml	ES008-D	Millipore
Pen/Strep		1/200	2.5ml	15140-122 (100ML)	Gibco
IMDM/F12 media 1:1 mix			Up to 500 ml		Gibco

Neonatal Human Thymus Tissue Collection

Neonatal thymus tissues were obtained from Melbourne Heart Tissue Bank at The Royal Children's Hospital (RCH) from pediatric patients in accordance with the policies and ethics of RCH and Melbourne Children's Heart Tissue Bank. Samples were collected from infants, younger than one-year-old, who were diagnosed with congenital heart defects and underwent cardiac surgery. Tissue collection for research purposes was obtained under the human ethics approval (HREC 38192) following informed consent by a parent or guardian.

Thymic Stromal Cell Collection

Neonatal thymus tissue was mechanically disrupted to release thymocytes. Briefly, the thymus tissue was cut into pieces of approximately 0.5 cm^3, and then, using the plunger of a 20-ml disposable syringe, pressed against the membrane of a 40-µm cell strainer sitting in a sterile 6-cm tissue-culture plate with DME medium. Thymocytes were flushed through the membrane using cold DMEM medium. This process was repeated 4 times to dislodge blood cells. Then, the thymus stroma was minced into small pieces using surgical scissors. The minced thymic tissues were transferred to a Falcon tube and further dissociated using Collagenase Type 1 (2 ng/ml in IMDM medium, Worthington-biochem) at 37 °C for 4-5 hours. The cell suspension was then centrifuged, and collagenase buffer aspirated. The cell pellet was washed with cold PBS. Thymic cells were finally resuspended in the CD-SAF medium containing 10 ng/ml KGF and 10 µM Rock inhibitor Y-27632 (Stemcell Technologies, 72304) and plated onto Geltrex pre-coated 6-well cell culture plates. For analysis of fresh human thymic stromal cells, the cell solution was then passed through the cell-strainer cap of a FACS tube to ensure a single-cell suspension.

Culture of Neonatal Thymus-Derived Stromal Cells

During the first two weeks, the CD-SAF medium containing 5 ng/ml KGF was replenished every 3 days. At one week after plating, epithelial colonies consisting of human thymic cells with a polygonal shape emerged. After two weeks, the cell culture was passaged at a 1:2 ratio. Briefly, cells were washed once with PBS and then dissociated using prewarmed TrypLE (1X, ThermoFisher) for 5 minutes at 37°C. Then, a 1-ml Gilson pipette was used to physically dissociate the cells by repeatedly pipetting the cell solution. The cell solution was then diluted in PBS, to neutralize TrypLE, transferred to a 15 ml Falcon tube, and centrifuged for 3 minutes at 4C. Following removal of the supernatant the cell pellet was resuspended in the CD-SAF medium containing 5 ng/ml KGF and 10 µM Rock inhibitor Y-27632 and the cells then transferred to fresh Geltrex pre-coated plates. The CD-SAF medium containing 5 ng/ml KGF was replenished every 3 days. From this passage, human thymic epithelial cell cultures were passaged weekly at a 1:2 splitting ratio.

Flow Cytometry Analysis and Cell Sorting

Characterization of adherent cultures required dissociation into single cells by incubation with prewarmed TrypLE-select™ at 37 °C. Incubation time varied with the type of cell culture; 5 minutes for neonatal human thymus-derived TEC cultures and 10-15 minutes for and PSC-derived FOXN1+ cultures. Conjugated monoclonal mouse anti-human antibodies: CD104-APC (1:50, clone 422325, Invitrogen), EPCAM-PeCy7 (1:200, clone 12c2, BioLegend), EPCAM-BV421(1:50, clone 9C4, BioLegend), CD90-PE (1:100, clone 5e10, Biolegend), CD90-BV421 (1:50, clone 5e10, Biolegend) were diluted in FACS wash buffer (PBS supplemented with 5% fetal bovine serum) and incubated with cells for 20 minutes on ice. The cell suspension was washed twice with FACS wash solution to remove unbound antibodies and resuspended in FACS wash solution containing 1 µg/ml propidium iodide. Cell surface staining was examined using a Becton Dickenson (BD) LSRFortessa Cell Analyzer. Flow cytometry data were analyzed using the FlowLogic program (7.2.1, DataNova). Alternatively, cell purification was performed using a BD FACSaria FUSION or Influx cell sorter based on cell surface staining or the expression of a fluorescent reporter. Cells were collected using a 5ml FACS tube containing 0.5ml cold fetal calf serum.

RNA Sequencing

RNA was isolated using the ISOLATEII RNA micro-Kit (Bioline, BIO-52075) as described by the manufacturer. Library preparation and sequencing was performed by sequencing facility at the Victorian Clinical Genetic Services (VCGS) in Melbourne. Library was sequenced on by the Illumina Novaseq-6000 system for 20 million reads per sample. STAR aligner was used to map bulk sequencing data with the GRCh38-3.0.0 genome. Sequencing data was processed using the RNAsik

pipeline (23). The mapped count files generated were uploaded onto Degust (https://degust.erc.monash.edu) to perform differential gene analysis using the Voom method (24). Data related to genes deemed to be statistically significant were exported as a count matrix for further analyses with R version 3.6.1 to generate heatmaps and other visualizations. Pathway analyses and gene enrichment of selected genes was completed *via* Metascape analysis (25).

Single Cell RNA-Sequencing

Single cell suspension samples were prepared at 1,000,000 cells/ml with viability at approximate 90%. RNA extraction and library preparation were performed by the Victorian Clinical Genetics Service following 10x Genomics's Cell Preparation Guide (https://www.10xgenomics.com). Sequencing was performed with the Illumina Novaseq-6000 system with a target of 50,000 read depth for 6000 cells. FASTQ files generated from sequencing were used with the 10x genomics software cell ranger (version 6.0.2) to map reads to the human reference genome version GRCH38-3.0.0. This generated an output including the information related to each cell's barcoding, matrix including counts and features information.

Standard single cell RNA sequencing analysis was completed on RStudio (R version 3.6.1) with the Seurat package (version 4.0.1). Data preprocessing was completed for quality control purposes. Cells with less than 200 genes and more than 10,000 genes were excluded along with those that expressed more than 25% mitochondria, more than 30% ribosomal and more than 1.5% mitoribosome expression. Following normalization (NormalizeData, scale factor 10,000), integration across samples was completed with canonical correlation analysis (CCA). All genes within the original matrix were used to identify anchors that were then used for integration. This allowed for the identification of thymic epithelial cell identities, as we integrated our monolayer cells from primary human thymus samples (HTS) and hPSC-derived FOXN1+ cells with the published human thymus cell atlas (26). Data of uncultured primary cells was derived from the epithelial cell subset instead of total thymic cells, which was annotated by Park et al. in their original study. This annotated matrix specific to epithelial cells excludes conventional mesenchymal cells/fibroblasts, which is publicly available through Zenodo.org (https://zenodo.org/record/3711134#.YgBTaC_aGCR). This annotated thymic epithelial cell matrix was used for analysis in **Figure 3** for a direct comparison with *in vitro* derived human TECs and in **Figure 5** for identification of TECs expressing CD90. The integrated matrix of gene expression across samples was used for data scaling and cell cycle genes were regressed within the same step. The number of dimensions (n=20) was selected along with a resolution of 0.5 for cell clustering. FindMarkers, FeaturePlot and DotPlot analysis of cluster-specific genes was used to identify cluster identity. Rmagic (version 2.0.3) was used to uncover epithelial and mesenchyme signatures in **Figure 4** and **Figure S4** within the integrated samples with default parameters (27). Standard parameters within the package were used.

Data Availability

RNA-sequencing data is available in the public GEO data repository with the identification number GSE196005.

RESULTS

PSC Differentiation Identifies CD90 Expression as a Marker of FOXN1+ Thymic Endodermal Progenitor Cells

We previously described *FOXN1*:GFP PSC lines that enable the identification and purification of endodermal epithelial cells committed to the TEC lineage (5). Studies with these lines showed that *FOXN1*:GFP+ cells were marked by co-expression of EPCAM and CD104 (known as ITGB4). We have reproduced this result with feeder-free hESC cultures using a newly optimized PSC differentiation medium, chemically defined, and serum- and albumin-free (designated CD-SAF medium), based on our previously developed APEL medium (28). Previous analyses showed that these cultures contained cells that were PDGFRα+ and EPCAM-, suggestive of a fibroblastic or mesenchymal cell type. In order to further investigate this possibility, we examined cells for the expression of another widely used mesenchymal associated marker, CD90 (29). Unexpectedly, in addition to the presence of an EPCAM-CD90+ population, these cultures also contained FOXN1+ cells that co-expressed EPCAM and CD90. Indeed, FOXN1+ TEC progenitor cells could be divided into two sub-populations based on CD90 expression (**Figure 1A**).

To investigate the significance CD90 expression within the FOXN1+ cell population, we characterized cells representing the FONX1+CD90+ and FOXN1+CD90- fractions using RNA-sequencing. Principle component analysis showed that these fractions clustered separately, indicative of underlying differences in their gene expression profiles (**Figure 1B**). Further analysis also confirmed that surface expression of CD90 was faithfully reflected in the expression of *CD90* at the transcript level (**Figure 1C**). Consistent with flow cytometry analysis, *FOXN1* expression was comparable between the CD90+ and CD90- populations. Although not reaching statistical significance, the expression levels *EPCAM* and *CD104* suggested a possible difference in the expression of these two markers between the FONX1+CD90+ and FOXN1+CD90- populations. Indeed, we found that CD90- cells showed significantly higher levels of *CLDN4*, a gene whose expression is associated with mTECs (17). Conversely, neither of the two populations expressed the cTEC marker *LY75* (CD205) (30). We also found that both FONX1+CD90+ and FOXN1+CD90- populations expressed the NOTCH ligands *JAG1*, *JAG2*, *DLL1*, but not *DLL4*. Interestingly, this analysis also showed a consistent trend of increased expression of individual collagen genes in the CD90+ fraction, such *COL1A1*, which is also a well-established marker of cells undergoing mesenchymal transition from epithelial cells (**Figure 1C**) (31). Collectively, these results indicate that CD90 marked a subpopulation of hESC-derived FOXN1+EPCAM+ TEC progenitor cells.

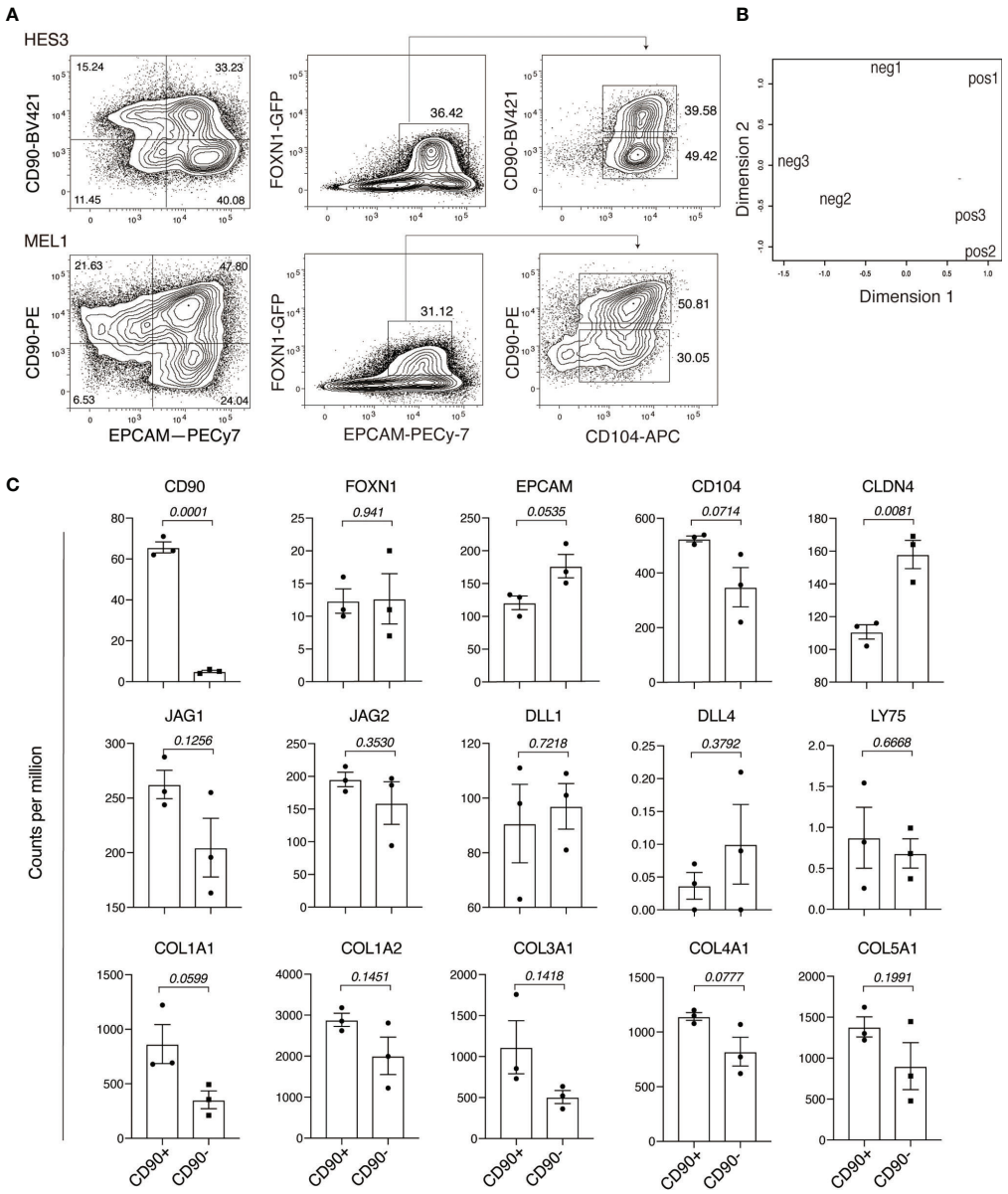

FIGURE 1 | Human pluripotent stem cell differentiation identifies a FOXN+EPCAM+CD90+ population. **(A)** Flow cytometry analysis of PSC-derived endodermal cultures for the expression of CD90, EPCAM, *FOXN1*:GFP and CD104. Data shows representative results of two independent PSC lines: HES3 and MEL1. **(B)** RNA-sequencing analysis of GFP+EPCAM+CD90+ (pos) and GFP+EPCAM+CD90- (neg) fractions showing the relationships between individual samples representing each population in a multi-dimensional scaling (MDS) plot. **(C)** Histogram representation of the expression of thymic associated genes, NOTCH ligand genes and collagens. The Y axis shows expression in counts per million (CPM) for GFP+EPCAM+CD90+ (CD90+) and GFP+EPCAM+CD90- (CD90-) populations. p values are of the comparison between CD90 positive and CD90 negative populations. Data is shown as the mean+/- SEM for biological replicates n = 3. Statistical significance was calculated using an unpaired t test, p values are indicated for each individual graph.

Derivation of Epithelial Cells From Neonatal Human Thymus in Chemically Defined Medium

Given that PSC-derived TEC progenitors generated *in vitro* represent an artificial system, we sought to examine the expression EPCAM, CD90, CD104 and FOXN1 on epithelial cells cultured from dissociated neonatal human thymus. In these experiments, we used the same CD-SAF medium employed above in order to aid the direct comparison between PSC and primary tissue-derived cell types. Thymic stromal cells were isolated from pediatric thymus tissue by physical separation followed by collagenase treatment. The cell suspension was subsequently seeded onto Geltrex-coated tissue culture plates in CD-SAF medium supplemented with 10 ng/ml keratinocyte growth factor (KGF).

One week later, we observed the emergence of colonies that comprised cells displaying a polygonal epithelial morphology (**Figure 2A**). These cells could be passaged weekly for more than 4 weeks. Over this time a single confluent well at day 0 routinely gave rise to 6 confluent wells by day 30. We further characterized *in vitro* cultured neonatal thymus derived epithelial cells by flow cytometry and RNA sequencing analysis. Flow cytometry analysis showed that pediatric thymus-derived stromal cells expressed the TEC-associated surface markers EPCAM and CD104, and that this expression was maintained over at least 3 consecutive passages (**Figure 2B**). Interestingly, nearly all cells expressed CD90, and its expression levels were not substantially different between subpopulations separated on the basis of EPCAM and CD104 expression (**Figure 2C**).

Neonatal Thymus Derived Epithelial Cells Express CD90 and EMT-Associated Genes

Next, we performed RNA-sequencing analysis of the above subpopulations based on their expression of EPCAM and CD104 (**Figure 2B** and **Figure S1A**). At the global level, principal component analysis and differentially expressed gene analysis indicated that each sorted fraction, representing EPCAM+CD104+, EPCAM-CD104- and EPCAM-CD104+ cells, was clearly separated from the other (**Figures S1B, C**). The expression levels of *EPCAM* and *CD104* transcripts correlated with the surface marker profile of each fraction, validating the integrity of the sorting strategy (**Figure 2D** and **Figure S1A**). Notably, *FOXN1* transcripts were effectively restricted to EPCAM+CD104+ cells. Moreover, the expression pattern of the recently identified TEC-associated marker *PDPN* (32) was similar to that of *CD104*. We found that EPCAM-CD104- double negative cells did not express the TEC-associated keratin genes *KRT5*, *KRT8* or *KRT14* whereas EPCAM+CD104+ double positive cells expressed the highest levels of these three keratins. Conversely, EPCAM-CD104- cells exclusively expressed the mesenchymal cell marker *PDGFRB*, suggesting a phenotype of conventional fibroblasts. In addition, *CD90* expression appeared to be progressively upregulated across the series EPCAM+CD104+, EPCAM-CD104+, EPCAM-CD104-, which pattern was also observed with the expression of mesenchymal associated transcription factors *SNAI2* and *ZEB1* (**Figure 2D**). Given the association of CD90 expression with mesenchymal cells, and the inverse correlation between its expression and that of EPCAM, we explicitly examined the expression of genes associated with the mesenchymal transition and cell polarity in the three distinct cell fractions. This analysis suggested a gradation of gene expression whereby the EPCAM-CD104- cells possessed stronger mesenchymal characteristics than the EPCAM-CD104+ and EPCAM+CD104+ populations (**Figure 2E** and **Figure S1D**). Conversely, EPCAM +CD104+ cells expressed higher levels of genes associated with epithelial polarity than the other two populations. In general, mesenchymal characteristics were corelated with the levels of *CD90* transcript (**Figure 2E**). Collectively, these results suggest that expression of CD90 marks a mesenchymal like program within neonatal thymus-derived epithelial cells, the degree of which is less pronounced than the conventional CD90+EPCAM-CD104- mesenchymal-like population associated with these cultures.

Single Cell RNA-Sequencing for Neonatal Human Thymus-Derived Epithelial Cells *In Vitro*

To examine the potential relationship between distinct populations of neonatal thymus derived epithelial cells we further characterized these cultures by single cell RNA-sequencing analysis. In this experiment, we analyzed samples that were derived from four independent donors (Donor 18-21), which contained varying proportions of the subpopulations marked by expression of EPCAM and CD104 (**Figure S2A**). Using conical correlation analysis (CCA function in Seurat), we integrated our cells with primary neonatal TECs from the human thymus cell atlas, the latter serving as a reference for the identities of cultured TECs (26) (**Figure S2B**). Therefore, this analysis allowed a direct comparison between the *in vitro* cultured thymic stromal cells and primary neonatal human TECs. Our results showed that *in vitro* cultured cells from the four donors showed a similar pattern of cell clustering to each other and also contained a limited number of populations that were present in primary neonatal human TECs (**Figure 3A**).

Our results showed that *in vitro* cultured cells contained two basic cell types distributed across 8 clusters (**Figure S2B**); clusters 0-4 and 6-7 were epithelial cells that expressed E-cadherin (*CDH1*) and cluster 5 comprised mesenchymal cells that expressed platelet derived growth factor receptor beta (*PDGFRB*) (**Figure 3B**). *EPCAM* was weakly expressed in the *CDH1*+ epithelial population whilst nearly all cells expressed *CD90* (**Figure 3B**). The *CDH1*+ epithelial population also expressed TEC-associated keratins (*KRT5, KRT8 and KRT14*), that were rare in the *PDGFRB*+ mesenchymal population (**Figure S2C**), consistent with our results of RNA-sequencing of the sorted cell fractions (**Figure 2D**). Although overall, cultured cells expressed the *KRT8* transcript, expression of other classical cTEC markers *LY75* and *PBSM11* was not detected. This contrasts with primary TECs, where expression of both *LY75* and *PBSM11* was clearly detected in cluster 7, suggesting a cTEC cluster that was effectively absent from cultured TEC populations (**Figure S2C**). Cluster 6 contained cells that expressed *CLDN4* and *AIRE*, suggestive of a mature mTEC phenotype (**Figure S2C**). As with the cTEC population encompassed by cluster 7, the mature mTEC population contained within cluster 6 was also only present in primary cell populations (**Figure S2C**). However, *CLDN4*+ cells were also found in cluster 4, which contained both primary and cultured cells. Within this cluster we detected a rare LY6D+ population that contained FOXN1+ cells (**Figure 3B**). In addition to *CLDN4*, cluster 4 was enriched for the expression of the mTEC associated gene *CD24*, a marker that was also broadly expressed at lower levels throughout the culture cell populations (**Figure 3B**). Collectively, our analysis suggested that cultured thymus derived epithelial cells possessed a phenotype that resembled immature mTECs.

In addition to cluster 4 marked by *CLDN4* and *CD24*, primary and cultured thymus derived cells also contributed to cluster 0 (**Figure 3C**). Differentially expressed gene analysis showed that

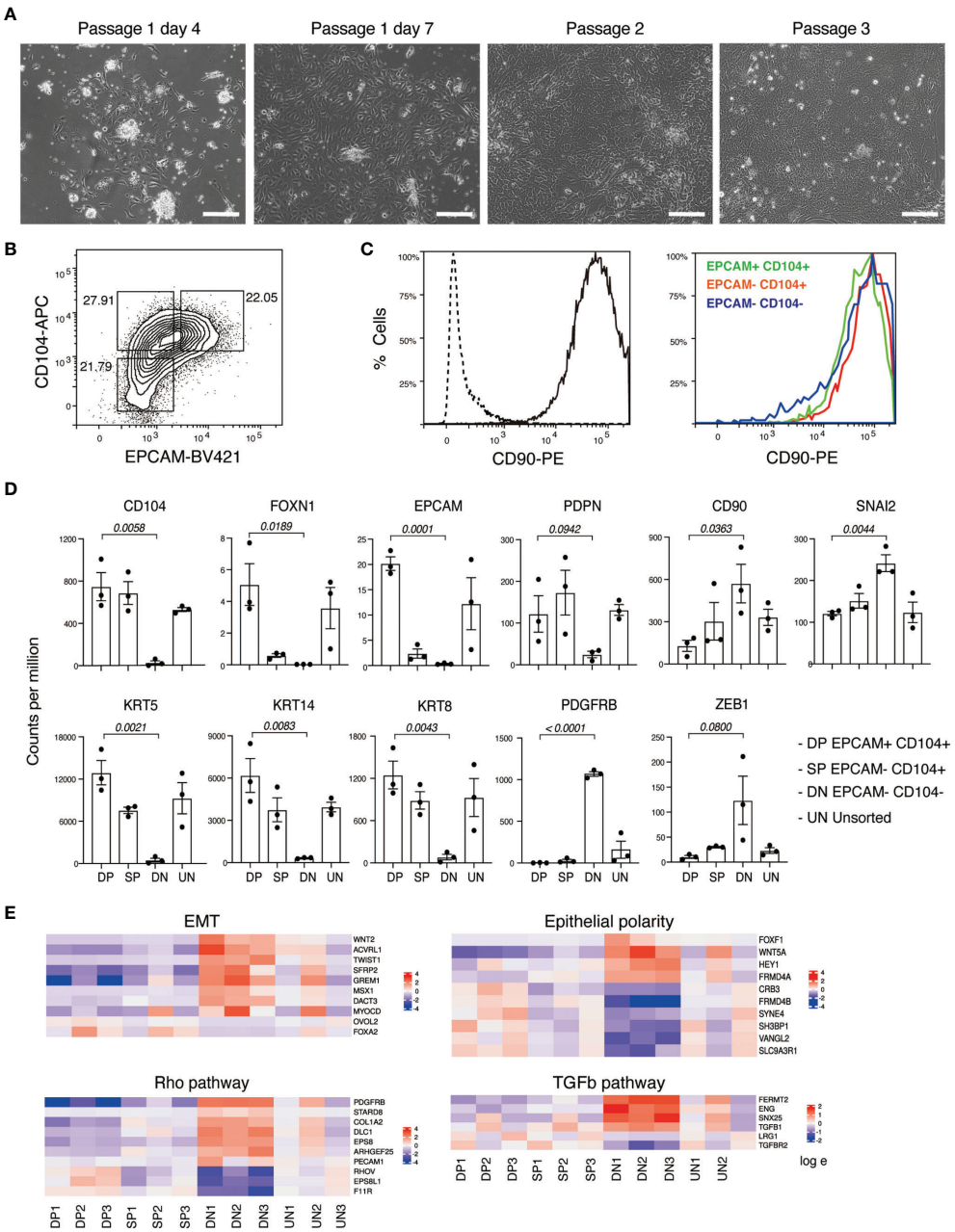

FIGURE 2 | Derivation and characterization of neonatal human thymus-derived monolayer cell cultures. **(A)** Bright field images showing freshly derived and passaged monolayer adherent cells from neonatal human thymus. Sale bar, 100 mm. **(B)** Flow cytometry analysis for CD104 and EPCAM expression on neonatal thymus-derived monolayer cell cultures. **(C)** Histogram representation of flow cytometry analysis for CD90 expression of neonatal thymus-derived monolayer cell culture (left) and of indicated fractions in **(B)** identified based on CD104 and EPCAM expression. **(D)** Histogram representation of gene expression levels of TEC associated genes including keratins in each of the four indicated populations measured in counts per million (from RNA-sequencing analysis). The indicated p values relate to the comparison of the EPCAM+ CD104+ double positive (DP) with EPCAM- CD104- double negative (DN) populations. Data shown in +/- with biological replicates n=3. Statistical significance was calculated with an unpaired t test **(E)**. Heatmap representation of the log fold change in the expression levels of genes found to be statistically significant (p value < 0.05) related to EMT, epithelial polarity, Rho and TGFβ signaling in the four indicated fractions. DP, EPCAM+ CD104+ double positive; DN, EPCAM- CD104- double negative; SP, EPCAM-CD104+ double positive; UN, unsorted sample.

this cluster contained cells that expressed genes encoding integrins, including *ITGB4*, *ITGA3* and *ITGA6*, and laminins, including *LAMB3* and *LAMA3*. Few primary TECs were found in clusters 1, 2 and 3, suggesting these clusters comprised cells that were generated under our specific culture conditions. Differentially expressed gene analysis and gene ontology analysis showed cluster 1 and 3 were enriched with genes responsible for cell cycle and division, whereases cluster 4

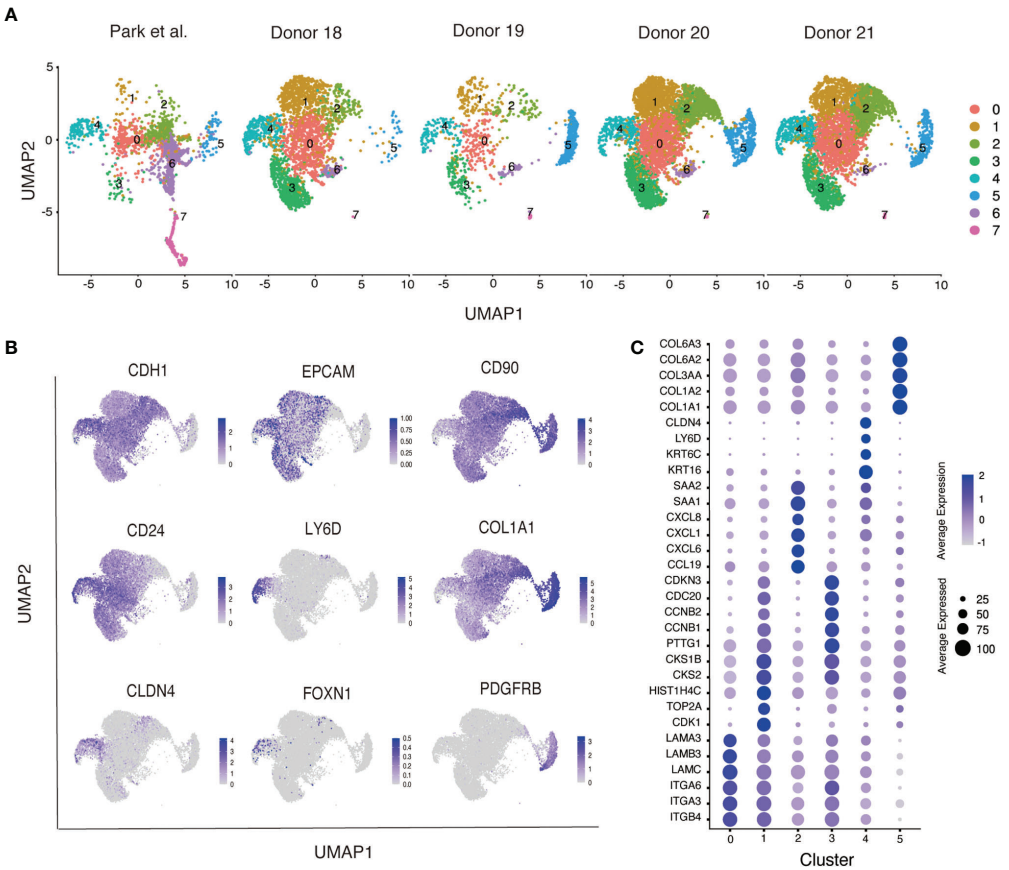

FIGURE 3 | Singe cell RNA-sequencing of neonatal human thymus-derived monolayer cell cultures. **(A)** Uniform Manifold Approximation and Projection (UMAP) analysis of single cell RNA-sequencing analysis showing cells from the four donors (Donor 18-21) integrated with primary neonatal human thymic epithelial cells from the Human Thymus Cell Atlas (26). Samples were separated by their original sample identity and grouped by cell type clustering. **(B)** Feature plots of key genes expressed in monolayer cultured neonatal human thymic cells. **(C)** Dot plot representation of cluster specific genes in the monolayer cultured neonatal human thymic cells. Distinct classes of integrins, laminins, cell cycling, chemokines and their ligands (CXCLs and CCLs), mTEC associated keratins, collagens, and serum amyloids (SAAs) are identified specific to respective clusters. Color intensity in each dot represents the average expression. Dot size represents the percentage of cells expressing that gene in its respective cluster.

expressed genes associated with immune functions, such as chemokines receptor/ligands (*CCL19*, *CXCL6*, *CXCL1* and *CXCL8*). Collectively, our flow cytometry and RNA-sequencing analysis revealed that neonatal TECs cultured *in vitro* contained a heterogeneous collection of cells that expressed genes that are associated with epithelial cell state and a restricted set of mTEC genes.

Single Cell RNA-Sequencing Analysis Identifies a Mesenchymal Signature of Human Thymic Epithelial Cells

To further explore the relationship between epithelial and mesenchymal gene signatures within *in vivo* human TECs populations, we independently analyzed epithelial cell subpopulations in the human thymus cell atlas (26). This single cell RNA-sequencing dataset includes human TECs from different development time points ranging from week 7 embryos to 40-year-old adults. Following the standard Seurat pipeline, our analysis revealed the co-clustering of early- and mid-embryonic development time points, whereas neonate, adolescent and adult cells formed individual clusters (**Figures S3A, B**). In particular, medullary and cortical compartments within these cells were further specified using classical cTEC markers (*LY75* and *PSMB11*) and mTEC markers (*KRT14*, *CLDN4*, *AIRE* and *FEZF2*) (**Figures S3C, D**) (16, 17, 30, 33). As with the original analysis performed in Python by 26, we also identified cTEC and mTEC subpopulations at different developmental time points (**Figure S3D**).

Next, we investigated the association of epithelial/mesenchymal gene expression signatures with human TECs using the MAGIC program, an imputation analysis tool that has been validated to study gene-gene interactions in the epithelial-to-mesenchymal transition (EMT) (27). First, we tested our analysis by examining the relationship between the expression of known functional TEC genes with markers of medullary (*CLDN4*) and cortical (*LY75*) identity (**Figure 4A**).

This analysis showed that the cTEC functional genes, *PSMB11* and *PRSS16*, were enriched in the *LY75*-high population, whilst the mTEC functional genes, *AIRE* and *FEZF2*, were restricted to a *CLDN4*-high population. These results support the application of MAGIC as a valid tool for studying gene-gene interactions in the context of thymic epithelial cell identity within this dataset.

We further applied MAGIC to investigate TEC subpopulations and their relationship to epithelial and mesenchymal programs. We focused on three TEC development stages: pre-hematopoietic colonization (early embryonic) (**Figure S4A**), hematopoietic colonization (mid embryonic) (**Figure S4B**) and fully functional thymus (neonate) (**Figure 4B**). We examined multiple epithelial and mesenchymal genes and their association to cTECs and mTECs, defined by the expression of *LY75* and *CLDN4*, respectively. Our results showed that *EPCAM* and *CDH1* (E-cadherin), established epithelial cell surface markers, were upregulated in concert with increasing expression of *CLDN4* (**Figure 4B**). Conversely, the EMT-related genes *FN1* (fibronectin) and *CD90* were strongly associated with *LY75*. Similarly, we found that the intracellular signaling molecules, *SMAD2* and *SMAD3*, were associated with *LY75*, suggesting a potential activity of the TGFβ cell signaling pathway in cTECs, a pathway known to be important in the generation of mesenchymal phenotypes from epithelial cells (34). Interestingly, TECs that expressed both *LY75* and *CLDN4* transcripts, potentially representing cells at the cortical medullary junction, possessed a hybrid expression pattern that contained both epithelial and mesenchymal associated genes (**Figure 4C**). These results highlight that cTECs and mTECs show distinct patterns of epithelial and mesenchymal gene expression, with the former having a more pronounced mesenchymal gene signature, including expression of CD90.

In neonatal thymus, we also detected TECs that were triple positive for EPCAM, CD90 and FOXN1 (**Figure 5A**). Single cell RNA-sequencing analysis also showed the expression of *CD90* on TECs marked by *PDPN* (32). To confirm the expression of CD90 on human TECs, we analyzed neonatal thymus samples by flow cytometry. This analysis showed that approximately 5% of the CD45- non-hematopoietic thymic stromal population were CD90 +EPCAM+ double positive cells (**Figure 5B**). This result was reproduced with neonatal human thymus samples from another five independent donors (**Figure S5**). Collectively, these results confirm the expression of CD90 on a subset of human TECs and suggest that human TECs exhibit a hybrid program of gene expression that has elements of mesenchymal and epithelial cell states.

Mouse Thymic Epithelial Cells Did Not Express CD90 (Thy1)

Since CD90 was originally identified as a specific thymocyte antigen in the mouse, we also surveyed its expression by re-analyzing a mouse thymus single cell RNA-sequencing dataset (35) (**Figure S6**). We annotated mouse thymus cell identities including TECs (*Epcam, Foxn1, Ly75* and *Cldn4*), thymocytes (*Ptprc* and *Cd3e*), myeloid cells (*Ptprc*, *Mpo*, *Cd52*, *Itgam* (CD11b)), endothelial cells (*Pecam1* and *Cdh5*), conventional mesenchymal cells (*Pdgfra* and *Col1a2*) and parathyroid cells (*Gcm2*). We found that Epcam was a faithful marker for TECs, covering almost all Foxn1+ cells in the developing thymus. Within the TEC population, *Ly75* and *Cldn4* further subdivided epithelial cells into two major compartments as cTECs and mTECs. Interestingly, unlike the human, mouse TECs rarely expressed *Thy1* (CD90) and its expression was restricted to thymocytes and a small fraction of mesenchymal cells. These results suggest a phenotypic difference of TECs between human and mouse, highlighting at least one mechanism of TEC-thymocyte interactions that is not conversed between the two species.

DISCUSSION

In this study, we provide evidence that human TECs possess a hybrid gene expression program comprising both epithelial and mesenchymal genes. Flow cytometry and gene expression profiling analysis identified CD90 as a potential marker of cells that possessed a mesenchymal-like program within cTEC populations. By developing a chemically defined serum free culture medium, we were able to derive TEC-like cells from human ESCs and neonatal human thymus, both of which provide a platform for studying TEC biology.

Our analysis showed expression of CD90 in multiple contexts of human TECs, including freshly isolated and cultured neonatal TECs (**Figures 2C**, **5B**), as well as human ESC-derived FOXN1+ TEC progenitor cells (**Figure 1A**). CD90, originally called THY1 (thymocyte differentiation antigen 1), is a specific surface marker of developing thymocytes in the mouse but not in the human (36). More broadly, CD90 has been shown to mark mesenchymal cells, including those that have transitioned from an epithelial state. In the latter case, cells of this phenotype also upregulate genes encoding extracellular matrix proteins, such as COL1A1 (34). In our PSC derived thymic endodermal cultures, we found that CD90 was expressed on EPCAM- non-epithelial cells, a cell type we had previously shown also expressed the mesenchymal marker PDGFRA (5). Unexpectedly, in the current study, we identified a distinct FOXN1+EPCAM+CD104+ population that expressed CD90 (**Figure 1A**). We confirmed the existence of a similar population of cells within neonatal TECs by both single cell RNA-sequencing analysis and flow cytometry (**Figure 5**). Indeed, analyses performed in the 1970s and 1980s suggested CD90 expression on cultured TECs and on the human cortical epithelium (37–39). More recently, Campinoti et al. identified the expression of CD90 on various TEC populations and also speculated that this expression may indicate an underlying hybrid epithelial/mesenchymal phenotype (40). Complementing our work showing expression of CD104 (integrin beta 4 subunit), Campinoti et al. found that CD104's sole pairing subunit CD49f (integrin alpha 6 subunit) was also expressed in human TECs. Their study taken in conjunction with our own work strongly argues that human TECs have an unconventional epithelial phenotype that includes mesenchymal-like characteristics. Indeed, our single cell transcriptomic analysis revealed that CD90 is more strongly associated with cTECs throughout

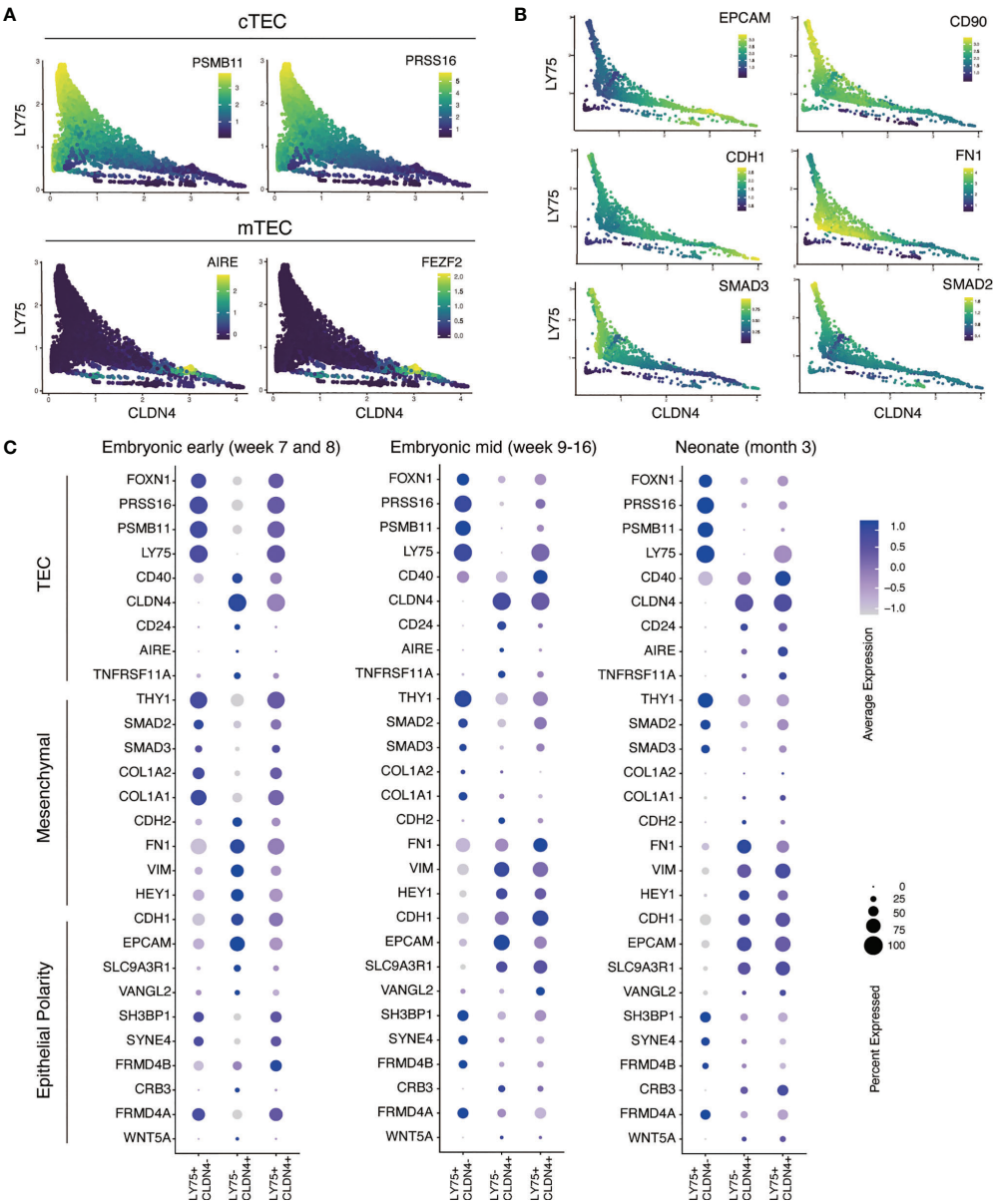

FIGURE 4 | Single cell RNA-sequencing analysis of epithelial and mesenchymal gene expression in primary human cTECs and mTECs. **(A)** Validation of MAGIC program for gene-gene association analysis with established cTEC and mTEC markers and functional genes. Scatter plots show the distribution of cells associated with CLDN4 expression representing mTECs (X axis) and LY74 representing cTECs (Y axis). Color intensity represents the level of gene expression as indicated by color key. **(B)** Scatter plots showing the MAGIC imputed values calculated for epithelial and mesenchymal gene expression associated with cTECs and mTEC genes in neonate human TECs. **(C)** Dot plot representation of the expression epithelial, mesenchymal and TEC genes in primary human TECs reported in the human thymic cell atlas (26). Color intensity in each dot represents the average expression. Dot size represents the percentage of cells expressing that gene in its respective cluster.

embryonic development (**Figures 4B** and **Figure S4**), suggesting that the outer cortical structure involves cells with a more pronounced mesenchymal signature. It is tempting to speculate that the open scaffold structure of the cortex, that results from its dramatic enlargement following hematopoietic colonization (7), could play a causative role in driving the mesenchymal characteristics of resident TEC populations. By contrast, medullary TECs, which are subject to a structurally distinct environment with fewer interceding blood cells, possess a more epithelial-like phenotype. These observations may give additional clues to further understanding in thymic epithelial cell identities and could provide novel insights into culturing techniques to derive human TECs *in vitro*.

The above observations suggest that cTECs and mTECs expressed distinct genes associated with different epithelial cell identities. Cell identity is defined by location and the repertoire of

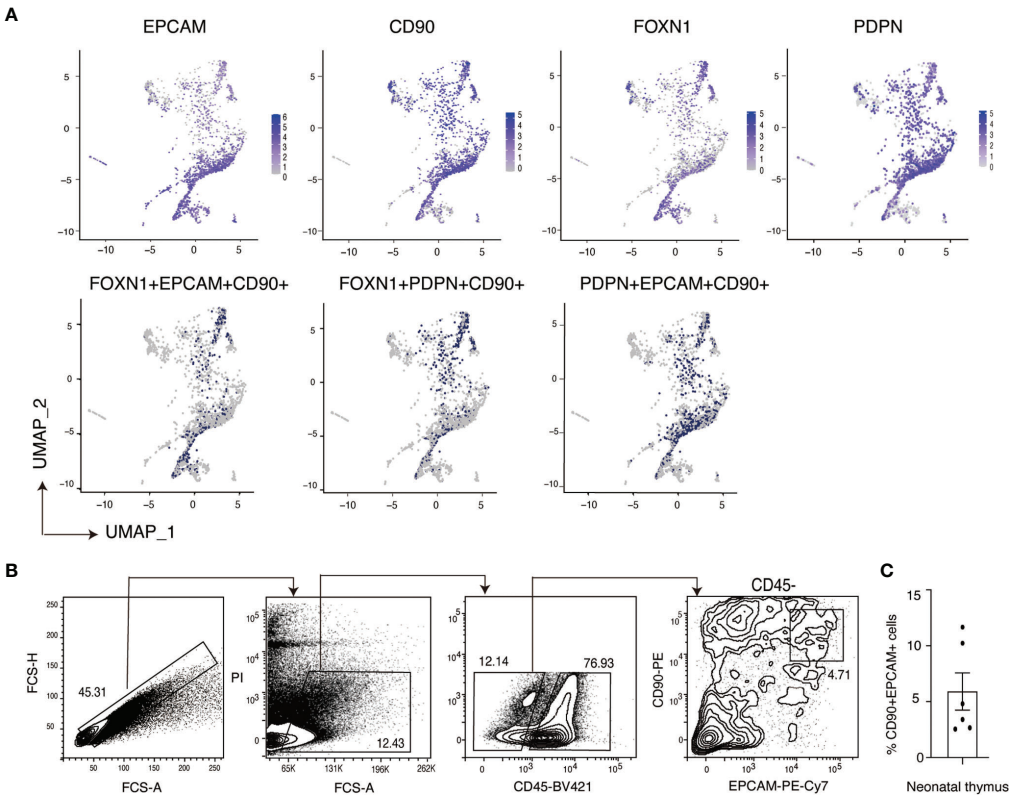

FIGURE 5 | Identification of EPCAM+CD90+ cells in primary neonatal human TECs. **(A)** Feature plot representation of single cell RNA-sequencing analysis of human neonatal human thymic epithelial cells expressing EPCAM, CD90, FOXN1 and PDPN separately and triple positive populations expressing these genes. **(B)** Representative flow cytometry plots (Donor 19) showing the expression of EPCAM and CD90 on primary neonatal human TECs. Non-thymocyte cells (middle) are enriched by an FCS-A gate (left). Thymic stromal cells (right) are enriched from the CD45- population. **(C)** Quantification summary of the frequency of CD90+ EPCAM+ double positive cells within the CD45- thymic stromal cell population of six human thymus donors. Data is shown as the mean +/- SEM. Flow cytometry results contributing to the data pots in **(B)** are shown in **Figure S5**.

expressed genes - two parameters that directly determine a cell's functionality. cTECs and mTECs in a fully functional thymus are believed to originate from the same bipotent progenitor population (12–14). During development, changes in location can precipitate changes in gene expression, and thus modulate cell identity. Epithelial cells frequently form a continuous layer, known as tissue epithelium, in which each cell is tightly connected to neighboring cells to create a defined axis and cell polarity (41). These cells share common characteristics in gene expression, such as the expression of EPCAM, by which they maintain the epithelium integrity and epithelial cell identity. However, our results suggest that epithelial identity may be influenced by changes in location that accompany organ morphogenesis and acquisition of functionality. The expression of mesenchymal associated genes in cTECs suggests that epithelial cells that participate in development-regulated migration events can adopt mesenchymal-like characteristics, and thus, possess a less pronounced epithelial phenotype (**Figure 4**). This phenomenon has been documented in other developmental systems; during liver development, EPCAM expression is maintained during hepatoblast differentiation towards cholangiocytes but is lost as cells form hepatocytes (42). Additionally, kidney epithelial cells also retain some mesenchymal characteristics that are potentially carried over from their immediate mesenchyme precursors that condense over the ureteric bud during renal development (43). Interestingly, it has been speculated that the retention of these characteristics might make kidney epithelial cells prone to undergoing an EMT under stress or inflammatory conditions (44, 45). These examples suggest that transitions across different epithelial and mesenchymal states is a common property of many cell systems and may present opportunities to manipulate cell phenotypes to create new cell types with new identities and functionality.

To minimize the influence of serum on the epithelial cell phenotype, we developed a chemically defined serum-free medium that permitted derivation of epithelial cells from the neonatal thymus and pluripotent stem cells (**Figures 1**, **2**). This chemically defined medium avoids inherent risks of reproducibility often associated with serum products, including fetal calf serum and human serum sourced albumin (46, 47). As such, this medium provides a stable cell culture system to identify downstream biological consequences of defined treatments or experimental conditions. As indicated by the expression of *CLDN4* and *CD24* but not *LY75*, we found that using KGF as a sole growth factor promoted an immature mTEC like phenotype

in PSC-derived FOXN1+ cells and in neonatal thymus-derived monolayer epithelial cells (**Figures 1C**, **3B**). Nevertheless, these mTEC-like cells did not express functional genes, such as *AIRE* and *FEZF2*. We speculate that this might be due to the lack of certain components in the culture that can drive functional differentiation of mTECs, such as lymphoid hematopoietic cells and conventional thymic mesenchymal cells (48, 49). As such, the addition of hematopoietic factors such as stem cell factor and interleukin 7, mesenchyme factors including fibroblast factor FGF2, as well as PDGF, may support the growth or survival of these auxiliary cell types. In addition, expression of functional TECs genes of *AIRE* and *FEZF2* might be induced from our immature mTECs by additional factors to activate key pathways, such as the lymphotoxin and the RANK signals (1, 50). Therefore, future experiments could examine these variables to promote the assembly of an artificial human thymus organ culture.

DATA AVAILABILITY STATEMENT

The data presented in the study are deposited in the GEO repository, accession number GSE196005. Data is publicly released and accessible via this identifier: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE196005.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Royal Children's Hospital Human Research Ethics Committee 33001A for work related to human pluripotent stem cell lines. Human thymus tissue collection for research purposes was obtained under the human ethics approval (HREC 38192) at the Royal Children's Hospital following informed consent by a parent or guardian. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

Conceptualization, SS and ES. Methodology SS, JL, and ES. Investigation, SS and JL. Reagents Acquisition AP, EP, and IK. Formal analysis, SS, JL, and HTN. Supervision, AE, ES, and MR. Writing-original draft, SS and ES. Writing-review & editing, all authors. Funding acquisition, AE and ES. All authors contributed to the article and approved the submitted version.

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Dll1 Can Function as a Ligand of Notch1 and Notch2 in the Thymic Epithelium

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T-cell development in the thymus is dependent on Notch signaling induced by the interaction of Notch1, present on immigrant cells, with a Notch ligand, delta-like (Dll) 4, on the thymic epithelial cells. Phylogenetic analysis characterizing the properties of the Dll4 molecule suggests that Dll4 emerged from the common ancestor of lobe- and ray-finned fishes and diverged into bony fishes and terrestrial organisms, including mammals. The thymus evolved in cartilaginous fishes before Dll4, suggesting that T-cell development in cartilaginous fishes is dependent on Dll1 instead of Dll4. In this study, we compared the function of both Dll molecules in the thymic epithelium using *Foxn1-cre* and *Dll4*-floxed mice with conditional transgenic alleles in which the *Dll1* or *Dll4* gene is transcribed after the cre-mediated excision of the stop codon. The expression of Dll1 in the thymic epithelium completely restored the defect in the *Dll4*-deficient condition, suggesting that Dll1 can trigger Notch signaling that is indispensable for T-cell development in the thymus. Moreover, using bone marrow chimeras with *Notch1*- or *Notch2*-deficient hematopoietic cells, we showed that Dll1 is able to activate Notch signaling, which is sufficient to induce T-cell development, with both the receptors, in contrast to Dll4, which works only with Notch1, in the thymic environment. These results strongly support the hypothesis that Dll1 regulates T-cell development *via* Notch1 and/or Notch2 in the thymus of cartilaginous fishes and that Dll4 has replaced Dll1 in inducing thymic Notch signaling *via* Notch1 during evolution.

Keywords: delta-like 1, delta-like 4, Notch1, Notch2, thymus, phylogenesis

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INTRODUCTION

The Notch system—highly conserved from invertebrates to mammals—regulates lineage specification during organogenesis in various cell types (1, 2). These signals travel between adjacent cells *via* the specific interaction of the Notch receptors with their ligands, belonging to the delta-like and jagged protein families. Their specific binding results in the proteolysis of Notch and the movement of the

Notch intracellular domain (NICD) into the nucleus, where the active fragment of Notch functions as a scaffold protein with the DNA-binding protein, RBPJ, and transcriptional activators. It is an essential component of signal transduction.

During the differentiation of hematopoietic cells, only the T-cell lineage requires a specialized environment in the thymus, where the immigrant cells receive Notch signaling induced by the interaction of Notch1 on the immigrant cells and the Notch ligand Delta-like 4 (Dll4) on the thymic epithelial cells (3–5). Evidently, within the four essential factors, namely, Ccl25, Cxcl12, Scf, and Dll4, Dll4 provides the key stimulus that determines the fate of T cells in a *Foxn1*-deficient background (6). Moreover, as the expression of Dll4 is maintained by the three-dimensional structure of thymic epithelial cells, their monolayer cultures lose Dll4 expression and the ability to support T-cell development *in vitro* (7, 8). Thus, Dll4 defines the thymus as the site of T-cell development.

The expression of Dll4 in thymic epithelial cells is induced by Foxn1, a transcription factor essential for thymic development (9–11), *via* its interaction with the enhancer region of the *Dll4* locus (12) that is shared with Foxn4 in endothelial cells (13). The phylogenetic significance of these transcription factors in thymic development has been analyzed in detail (9–11). Interestingly, Foxn1 is expressed alone in the mammalian thymic epithelium, while it is co-expressed with Foxn4 in the thymus of cartilaginous fishes, inducing a characteristic structure that supports B-lymphopoiesis. Therefore, the thymic environment appears to have changed during evolution (10, 11). Moreover, sex hormones regulate the expression of Dll4. Steroid administration causes thymocyte death and thymic atrophy, and conversely, sex steroid ablation increases thymopoiesis. This could be explained by the fact that sex hormones characteristically suppress the expression of Dll4 in thymic epithelial cells and that sex steroid ablation increases the expression of Dll4, resulting in efficient T-cell development in the thymus (14). Thus, the expression of Dll4 in thymic epithelial cells may be a clinical target to improve the T-cell supply from aged thymuses.

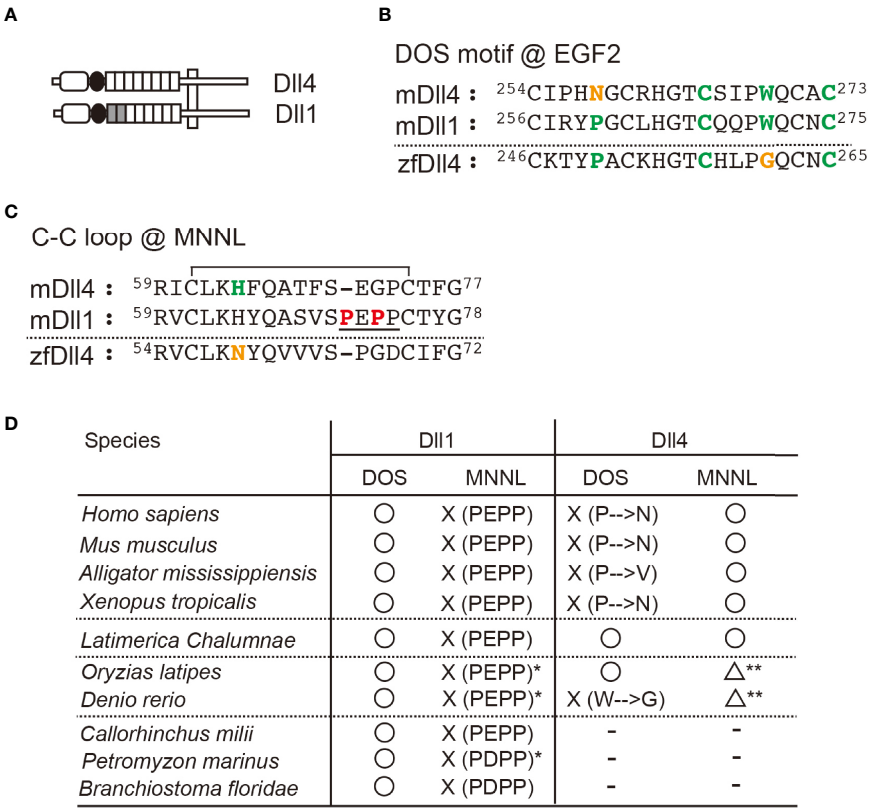
It is important to note that the *Dll4* gene is absent in the early jawed vertebrates (cartilaginous fishes), where only the *Dll1* gene is present (9). This is consistent with the presence of the *Dll1* ortholog gene transcript in the epithelium of the thymus-like structure in the gills of lamprey larvae (15). Therefore, when the thymus first appeared in early jawed vertebrates as a site of T-cell development, Dll1, and not Dll4, may have predominated the thymic environment. In mice, Notch1 is an essential partner of Dll4 for T-cell development in the thymus, but Notch2 is also detected in hematopoietic progenitors immediately after their thymic migration (16). Therefore, it is unclear why thymic immigrants lacking Notch1 cannot receive Dll-mediated Notch signaling *via* Notch2 (17). In some cases, a specific combination of Notch and Notch ligands may function selectively in a context-dependent manner (18). However, whether Notch2 can mediate Notch signaling and contribute to T-cell development in the thymus has not been examined.

We have previously revealed the physiological significance of Dll4 in murine T-cell development in the thymus (4, 16). Dll1 is scarcely detected in the thymic environment (19, 20) and dispensable for triggering T-cell development in the thymus (19). Moreover, the superiority of Dll4 over Dll1 for T-cell induction has been shown (21). We attributed the functional characteristics of Dll4 to the mobility of the loop structure within the module at the N-terminus of Notch ligand (MNNL) domain at the tip of the ligand and showed that the DOS motif observed in the first/second epidermal growth factor (EGF)-like repeat present in Notch ligands—except Dll4—augments the activity of Dll4 using their chimeric molecules (21). Therefore, Dll family members bind to Notch and trigger signaling differently based on their structural features. In this study, we showed the phylogenetic interrelationships of the *Dll1* and *Dll4* homologous genes and discussed the emergence and evolution of both the genes based on the properties of the MNNL and first/second EGF-like repeat regions that characterize the Dll molecules. Furthermore, we demonstrated that Dll1, which likely functions as a Notch ligand during thymus emergence, can support T-cell development in thymic epithelial cells with both Notch1 and Notch2, whereas Dll4 only works with Notch1, in our experimental model.

RESULTS

The *Dll4* Gene Identified in Coelacanth Shares Distinctive Characteristics With the *Dll1* and *Dll4* Genes in Terrestrial Organisms

Dll1 and *Dll4* are conserved in bony fish and terrestrial organisms, including mammals. These Notch ligands share structural characteristics, but mammalian Dll4s do not retain the DOS motif necessary for the binding of Dll1 to Notch1 due to the substitution of Pro in the motif to Asn at the second EGF-like repeats (**Figures 1A, B**) (2, 16). On the other hand, the N-terminal MNNL region of murine Dll4 that contains a loop structure with a wide range of motion directly contributes to binding with Notch1 (22). In contrast, that of murine Dll1 loses the ability to move widely because of its rigidity due to the sequential presence of unique prolines (**Figure 1C**) (21). Therefore, Dll molecules seem to bind to Notch1 in different regions. To examine the characteristics of Dll1 and Dll4 molecules during evolution, we sorted the genes of Dll family members from the National Center for Biotechnology Information (NCBI) database according to their homologies and formed a phylogenetic tree (**Supplementary Figure 1**). This analysis indicated that the *Dll4* gene first emerged in bony fishes, while the *Dll1* genes were identified from amphioxus (*Branchiostoma floridae*) and vertebrates, which is consistent with a previous report (9). In the *Dll4* genes, the DOS motif maintained in many bony fishes (23 species, including medaka fish, **Figure 1D**) is different from the one in terrestrial organisms, with some exceptions (zebrafish, arowana, and Japanese pufferfish) that have another substitution in the DOS motif (Trp to Gly, **Figure 1D**). However, one of the critical residues at the interface of the MNNL region (22), His, was substituted with Asn in all bony fishes (**Figure 1D**), suggesting that the



D

Species	Dll1		Dll4	
	DOS	MNNL	DOS	MNNL
Homo sapiens	○	X (PEPP)	X (P-->N)	○
Mus musculus	○	X (PEPP)	X (P-->N)	○
Alligator mississippiensis	○	X (PEPP)	X (P-->V)	○
Xenopus tropicalis	○	X (PEPP)	X (P-->N)	○
Latimerica Chalumnae	○	X (PEPP)	○	○
Oryzias latipes	○	X (PEPP)*	○	△**
Denio rerio	○	X (PEPP)*	X (W-->G)	△**
Callorhinchus milii	○	X (PEPP)	-	-
Petromyzon marinus	○	X (PDPP)*	-	-
Branchiostoma floridae	○	X (PDPP)	-	-

FIGURE 1 | Characteristic features of Dll1 and Dll4. **(A)** Schematic structure of Dll1 and Dll4. The MNNL and DSL domains are represented by an open rectangle with round corners and a filled circle, respectively. The EGF-like repeat is shown by square, and the first and second repeats retaining the DOS motif in Dll1 are filled. Both Notch ligands are present on the cell membrane (vertical square). **(B, C)** Amino acid (AA) sequence comparison of the DOS motif in the second EGF-like repeat **(B)** and the C–C loop in MNNL domain **(C)** between murine (m) Dll4, Dll1, and zebrafish (zf) Dll4. Numbers on the AA sequences represent the position from the N-terminus. The AAs in the DOS motif (**B**, bold green) and their substitution (**B**, bold orange) are labeled. Similarly, histidine in the C–C loop **(C)**, contributing to the direct binding with Notch (**C**, bold green) and its substitution (**C**, bold orange), is also labeled. The C–C loop in mDll1 contains a characteristic proline-rich AA region (**C**, underlined with unique prolines, bold red). Line over the sequence represents the disulfide bridge between cysteine residues (61st to 74th in mDll4). **(D)** Characterization of the conservation of DOS motif (DOS) and the predicted functionality of MNNL domain (MNNL) in Dll1 and Dll4 homologs in various species. ○, functional; X, non-functional; △, attenuated (predicted); –, absent. *One of the orthologs retains PEPP or PDPP residues. **The histidine residue (bold green in **C**) at the C–C loop pf MNNL domain is replaced with asparagine (bold orange in **C**).

binding ability of MNNL of Dll4 in bony fishes to Notch is likely to be reduced. In contrast, the *Dll1* genes are highly conserved with the DOS motif and unique Pro residues in the C–C loop of the MNNL region (**Figure 1D**).

The *Dll4* gene found in coelacanth—the famous lobe-finned fish—surprisingly encodes both the DOS motif and His in the MNNL region, which is different from those in terrestrial organisms and bony fishes (**Figure 1D**). We previously showed that a murine Dll4-derived chimera with Dll1-derived first and second EGF-like repeats containing the DOS motif exhibited stronger activity to trigger Notch signaling than the original Dll4 (21). Therefore, Dll4 in coelacanths should induce a stronger Notch signal than in other species. It is understood that ray- and lobe-finned fish that evolved into tetrapods share a common ancestor, and coelacanths have shown a slow rate of molecular and morphological evolution (23). As Dll4 in coelacanths is predicted to show intermediate characteristics between those of tetrapods and bony fishes, it was estimated that the *Dll4* gene first appeared in a form similar to the coelacanth one in the common ancestor of lobe- and ray-finned fishes and changed to its respective forms in terrestrial organisms and bony fishes during evolution.

The thymus, a primary lymphoid organ essential for T-cell development, emerged in jawed vertebrates approximately 500 million years ago (9). As the *Dll4* gene has never been identified in cartilaginous fish (*Callorhinchus milii*, **Figure 1D**), Dll1 is likely to function as a Notch ligand on the thymic epithelium in gnathostomes ancestors. However, it is unclear whether Dll1 can function as a Notch ligand on the thymic epithelium and support T-cell development in the thymus, and which Notch receptor actually interacts with Dll1 in thymic immigrants.

Dll1 Can Support the T-Cell Development in the Murine Thymus

To explore the ability of Dll1 to trigger Notch signaling in the thymic epithelium, we used conditional transgenic (Tg) mice, in which one copy of the *Dll1* or *Dll4* gene was transcribed by the CAG

promoter after Cre-dependent gene deletion of floxed *GFP* cDNA at the *Rosa26* locus (hereafter referred to as iD1 and iD4 Tg) (21). In these Tg mice, we were able to detect the expression of GFP in EpCAM$^+$ PDGFRα$^-$ thymic epithelial cells derived from the fetal murine thymus (**Figure 2A**), indicating that the CAG promoter substantially transcribed the inserted gene cassette containing *Dll1* or *Dll4* cDNA in the *Rosa26* allele. A small difference in fluorescence intensity could be due to the difference between *Dll1* and *Dll4* cDNA sequences because one copy of either of the cDNAs was inserted into the same site of the *Rosa26* locus. After breeding *Foxn1-Cre* and *Dll4-floxed* mice, consistent with GFP expression, exogenously expressed HA-tagged Dll4 or Dll1 was

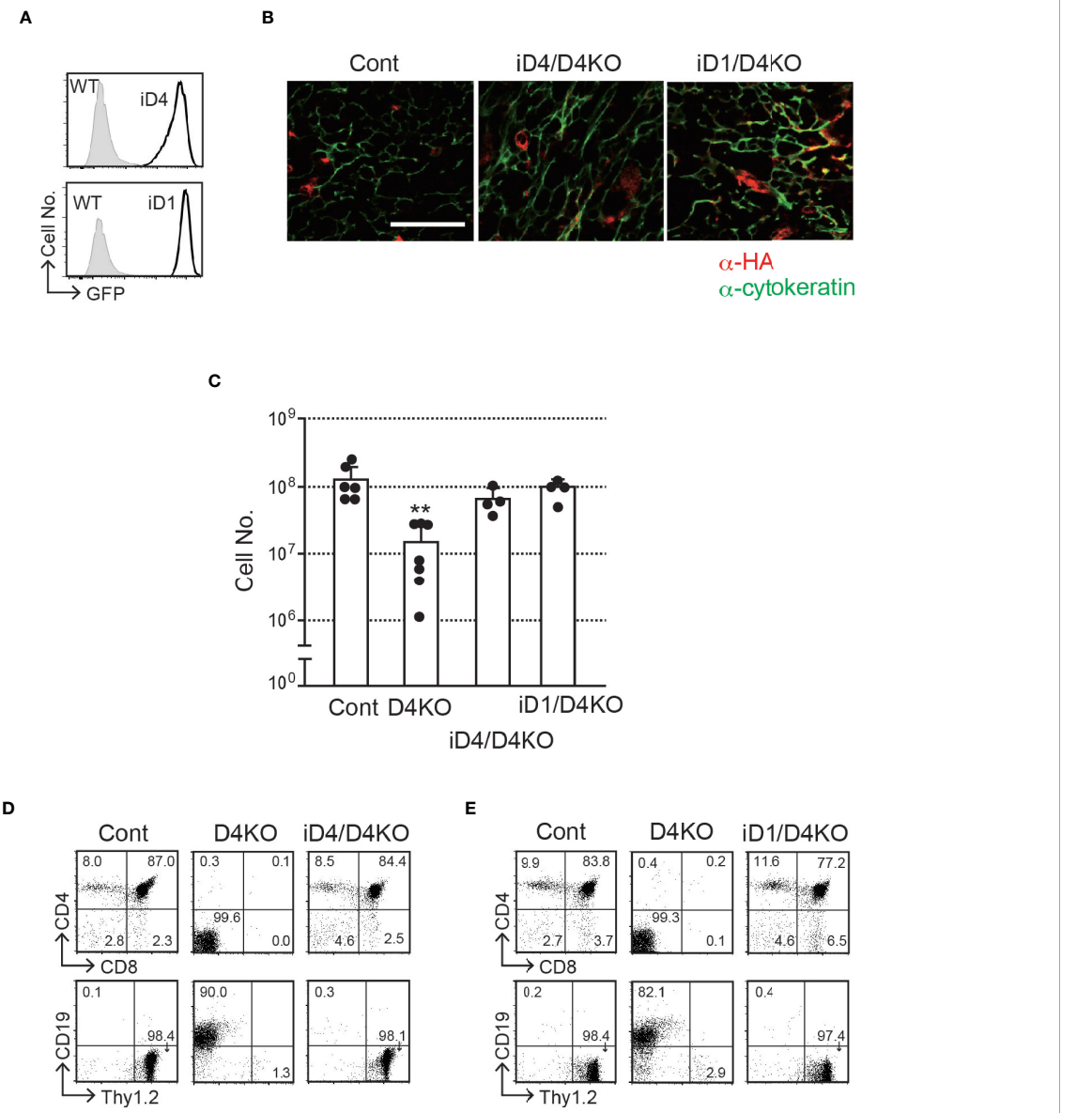


FIGURE 2 | T-cell development in the thymus with epithelial cells expressing exogenous Dll4 or Dll1 in *Dll4*-deficient background. **(A)** GFP expression, transcripts driven by CAG promoter at the *Rosa26* locus of iD1 and iD4 mice, was detected in EpCAM$^+$PDGFRα$^-$ epithelial cells obtained from fetal (E15.5) thymus using flow cytometry. Open histograms indicate GFP expression of *Rosa26*$^{floxedGFP\text{-}Dll4}$ (iD4) or *Rosa26*$^{floxedGFP\text{-}Dll1}$ (iD1) mice, and filled histograms indicate the intrinsic fluorescence of the identical cell population of control (WT) mice. **(B)** Representative results of immunofluorescence microscopy analysis of the thymus from *Dll4*$^{f/f}$ (Cont), iD4*Dll4*$^{f/f}$FoxN1-Cre (iD4/D4KO), or iD1*Dll4*$^{f/f}$FoxN1-Cre (iD1/D4KO) mice stained with anti-HA (red) and anti-cytokeratin (green) antibodies are shown. Intense and widespread red staining (anti-HA Ab) were nonspecific staining. Scale bar: 50 μm. **(C)** Thymic cellularity (mean ± SD) of 8- to 12-week-old *Dll4*$^{f/f}$ (Cont, n=6), *Dll4*$^{f/f}$FoxN1-Cre (D4KO, n=7), iD4*Dll4*$^{f/f}$FoxN1-Cre (iD4/D4KO, n=4), or iD1*Dll4*$^{f/f}$FoxN1-Cre (iD1/D4KO, n=4) mice. **p<0.01 by Student's *t*-test. **(D, E)** Flow cytometric analysis of thymocytes from the mice shown in panel **(B)** was performed. Thymocytes were stained with mAbs against surface molecules as indicated. Numbers in the profiles indicate the relative percentages for each quadrant. Results represent more than three independent experiments **(A, B, D, E)**.

detected in cytokeratin$^+$ thymic epithelium (**Figure 2B**). Thus, we examined the effect of exogenous expression of Dll4 or Dll1 on T-cell development under endogenous *Dll4*-deficient thymic conditions in which T-cell development has been completely impaired (4, 5). The reduction in total cell numbers observed in the *Dll4*-deficient thymic lobe was completely reversed by the expression of exogenous Dll4 or Dll1 (**Figure 2C**). Consistent with the cell numbers, efficient T-cell development was detected with exogenous Dll4 or Dll1 without endogenous Dll4 in the thymic epithelium (**Figures 2D, E** and **Supplementary Figure 2**). Thus, Dll1 can function as a Notch ligand to support T-cell development in the thymus. However, it remains unclear which Notch receptor binds to exogenous Dll1 and mediates signal transduction in T-cell progenitors in these Tg mice.

Both Notch1 and Notch2 Are Detected on the Common Lymphoid Progenitors in the Murine Bone Marrow

In mammals, of the four Notch receptors identified, three—Notch1, Notch2, and Notch3—have been detected in the blood cells (24). The phenotypes of conditional KO mice demonstrated that Notch1 and Notch2 mainly contribute to the development of hematopoietic cells (3, 25). Here, we confirmed the expression of Notch receptors on pre-thymic T-cell progenitors in the murine bone marrow (**Figure 3**). As a population that includes hematopoietic stem cells, weak but detectable expression of Notch1 and high expression of Notch2 were detected in lineage marker-negative c-kit$^+$Sca1$^+$ (KSL) cells. During differentiation toward the lymphoid lineage, the Notch1 expression increased, but that of Notch2 decreased, and both receptors were clearly detected in the common lymphoid progenitors (lineage marker-negative c-kit^{low}Sca1^{low}IL-7R$^+$). These profiles were consistent with those obtained in a similar population of the murine fetal liver (26). We did not observe Notch3 and Notch4 expression in these progenitors. In addition, both Notch1 and Notch2 are co-expressed in the earliest stage of T-cell progenitors in the thymus (early T-cell progenitors) (16, 27, 28). These results suggest that thymic immigrants express both Notch1 and Notch2 on their surfaces and receive Notch signaling *via* both receptors. We have reported that signal transduction from the active intracellular fragment of Notch1 or Notch2 is sufficient for the initiation of T-cell lineage development (29) and that Notch2 complements Notch1 to mediate inductive signaling for T-cell development in pro-T stages (27). Therefore, we expected that both Notch1 and Notch2 could contribute to the T-cell development in the thymus, especially with exogenous Dll1 that preferentially stimulates Notch2-mediated signal transduction in other cell types (18, 19).

Both Dll1 and Dll4 Interact With Notch1 in the Thymus

To reveal functional Notch receptors for exogenous Dll4 and Dll1 on T-cell progenitors, we prepared BM chimeras in iD1/iD4 Tg mice with *Notch1*- or *Notch2*-deficient bone marrow cells and

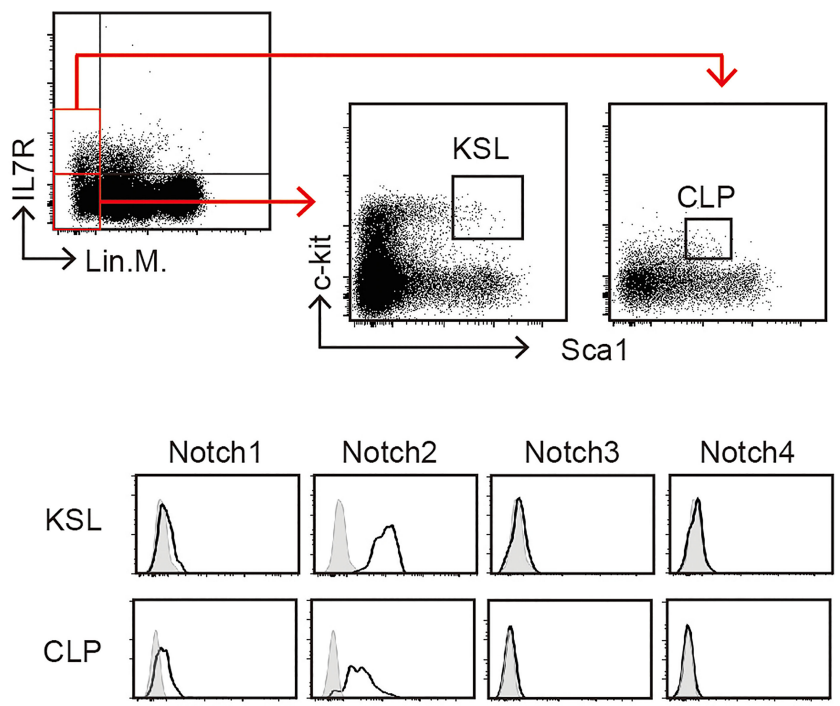


FIGURE 3 | Expression of Notch receptors on immature hematopoietic cells in the bone marrow. Bone marrow (BM) cells of C57BL/6 mice were subdivided into KSL (lineage markers$^-$, c-kit$^+$, and Sca1$^+$) and CLP (lineage markers$^-$, c-kit^{low}, and Sca1^{low}) populations (upper panels) and analyzed for Notch expression using flow cytometry (lower panels). Open histograms indicate staining with mAbs recognizing Notch1, Notch2, Notch3, or Notch4. Filled histograms indicate staining with control hamster IgG. These profiles represent at least three independent experiments.

examined their T-cell development in the thymus. *Notch2*-deficient BM cells were prepared from *Notch2*$^{f/f}$ mice (23) with *Cre/ERT2* knock-in allele in the *Rosa26* locus (30) after tamoxifen administration and then transferred into irradiated iD1 or iD4 Tg mice with a *Dll4*-deficient background (**Supplementary Figure 3A**). Six weeks later, T-cell development in the thymus with *Notch2*-deficient T-cell progenitors was completely rescued by exogenous Dll4 and Dll1 and was comparable to that in WT mice (**Figures 4A, B**). In BM chimeras with *Notch2*-deficient BM cells, CD21^{high}CD23^{-} splenic marginal zone B (MZB) cells disappeared selectively (**Figure 4C**), which is consistent with previous reports using mice with B-cell-specific deletion of *Notch2* (25) or systemic disruption of *Dll1* (19). These results suggest that both Dll1 and Dll4 can trigger Notch signaling *via* Notch1 in the thymus.

Only Dll1 Can Induce T-Cell Development With Notch2 in the Thymus

Next, we prepared BM chimeras with *Notch1*-deficient BM cells. However, systemic depletion of *Notch1* affects the survival of mice, and it is difficult to obtain *Notch1*-deficient BM cells. Thus, we performed sequential transplantation of the BM (**Supplementary Figure 3B**). First, irradiated WT host mice (CD45.2^{+}) were reconstituted with BM cells derived from *Notch1*$^{f/f}$ mice (31) with *Cre/ERT2* knock-in allele in *Rosa26* locus; then, they were treated with tamoxifen. After a week of the last tamoxifen treatment, *Notch1*-deficient BM cells were secondarily transferred into iD1/iD4 mice with a *Dll4*-deficient background. In that case, it was difficult to control the efficiency of thymopoiesis reconstitution. Therefore, we used GFP^{+} BM cells as an internal control at the first transplantation and evaluated T-cell development relative to the GFP^{+} control in secondary BM chimeras. In these experiments, the majority of *Notch1*-deficient BM cells differentiated into CD19^{+} B-lineage cells in the thymus under control and *Dll4*-deficient conditions (**Figure 5A**). Moreover, similar developmental patterns were observed in iD4 mice, indicating that Dll4 does not support Notch2-mediated T-cell development in the thymus (**Figure 5A**). In contrast, *Notch1*-deficient BM cells were able to differentiate into T-lineage cells, including CD4/CD8 double-positive (DP) and single-positive (SP) cells, in the thymus of iD1 mice, but not into B-lineage cells—the default phenotype in the absence of Notch signaling (**Figure 5A**). These phenotypes were also confirmed in the inguinal lymph nodes (**Supplementary Figure 4**). However, T-cell development of *Notch1*-deficient cells supported by Dll1 may be less efficient than that of WT cells because, in some cases, the ratios of the number of the DP cells derived from *Notch1*-deficient cells were lower than those from the GFP^{+} internal control (**Figure 5B**). We observed spontaneous differentiation of Thy1.2^{+} T-lineage cells in the *Dll4*-deficient thymus. This phenotype would be caused by the inefficient differentiation of B cells in the sequential BM transplantation experiments. In some cases, GFP^{+} cells did not efficiently differentiate into SP cells, which might be due to the excess expression of GFP. These results suggest that Dll1 on the epithelial cells, but not Dll4, interacts with Notch2 on the immigrant cells in the thymus and retains its superiority over Dll4 for induction of T-cell development *via* Notch2.

Pairing of Dll1/Notch2 Is Present in the Thymus of Elephant Sharks

After estimating the time of emergence of the two Dll molecules, we formed a phylogenetic tree of *Notch1* and *Notch2* genes to determine when the Notch receptors evolved to comprise multiple molecules (**Supplementary Figure 5**). Like with the emergence of the *Dll4* gene in coelacanths and bony fishes, the *Notch2* gene was first recognized in cartilaginous fishes and has been passed on to coelacanths and bony fishes. On the other hand, the *Notch1* genes, like the *Dll1* genes, were identified in all vertebrates, including lampreys and amphioxus. Notably, the *Notch1* gene in the elephant shark (*Callorhinchus milii*) contains shorter amino acid residues (1950 aa) than other *Notch1* homologs (2531 aa, mouse; 2508 aa, coelacanth; 2437 aa, zebrafish) and 18 EGF-like repeats (typically 36 in other species) in the extracellular region. These characteristics are unlikely to be sufficient for ligand binding. In contrast, the *Notch2* homolog in elephant sharks seems functional, suggesting that only the Notch2-like receptor can be expressed as a functional receptor in thymic progenitor cells in elephant sharks. Therefore, in the thymus of elephant sharks, only the interaction between Dll1 and Notch2 is expected to transduce Notch signaling essential for T-cell development. In this study, we demonstrated that Dll1 triggers Notch signaling *via* Notch2 to induce the development of mature T cells, and it is the only kind of Dll-mediated Notch signaling present in elephant sharks, where the thymus primordium was first observed.

DISCUSSION

The thymus is thought to have emerged in jawed vertebrates—the common ancestors of cartilaginous fishes and bony vertebrates. As the *Dll4* gene, which encodes the essential Notch ligand for T-cell development in bony vertebrates, has never been found in the elephant shark (*C. milii*), likely, Dll1 emerges first as a Notch ligand in the thymus. In this study, we showed the potential of Dll1 to support T-cell development with both Notch1 and Notch2, whereas Dll4 preferentially cooperates with Notch1 in the murine thymus. In jawed vertebrates, before the coexistence of the two Notch receptors was established, Dll1 may have had advantages in triggering Notch signaling.

We confirmed that the *Dll4* gene in the coelacanth encodes a Notch ligand with distinctive features in two regions, DOS and MNNL, which are required for murine Dll1 and Dll4 to bind Notch, respectively. Therefore, like the murine Dll4-based chimera with the Dll1-derived DOS motif (21), the coelacanth Dll4 should act as a hyperactive Notch ligand, as the murine Dll molecules only have one or the other motif. On the other hand, most Dll4 molecules in bony fishes seem to lose the functional MNNL but retain the DOS motif, which resembles that of murine Dll1. This information raises the possibility that the hyperactive Dll4, which emerged in the common ancestor of lobe- and ray-finned fishes, weakened its activity during evolution to tetrapods and bony fishes *via* different mechanisms. Gain-of-function mutations of Notch receptors induce malignant transformation in various cell types (32); thus, limiting the intensity of Notch signaling to a certain

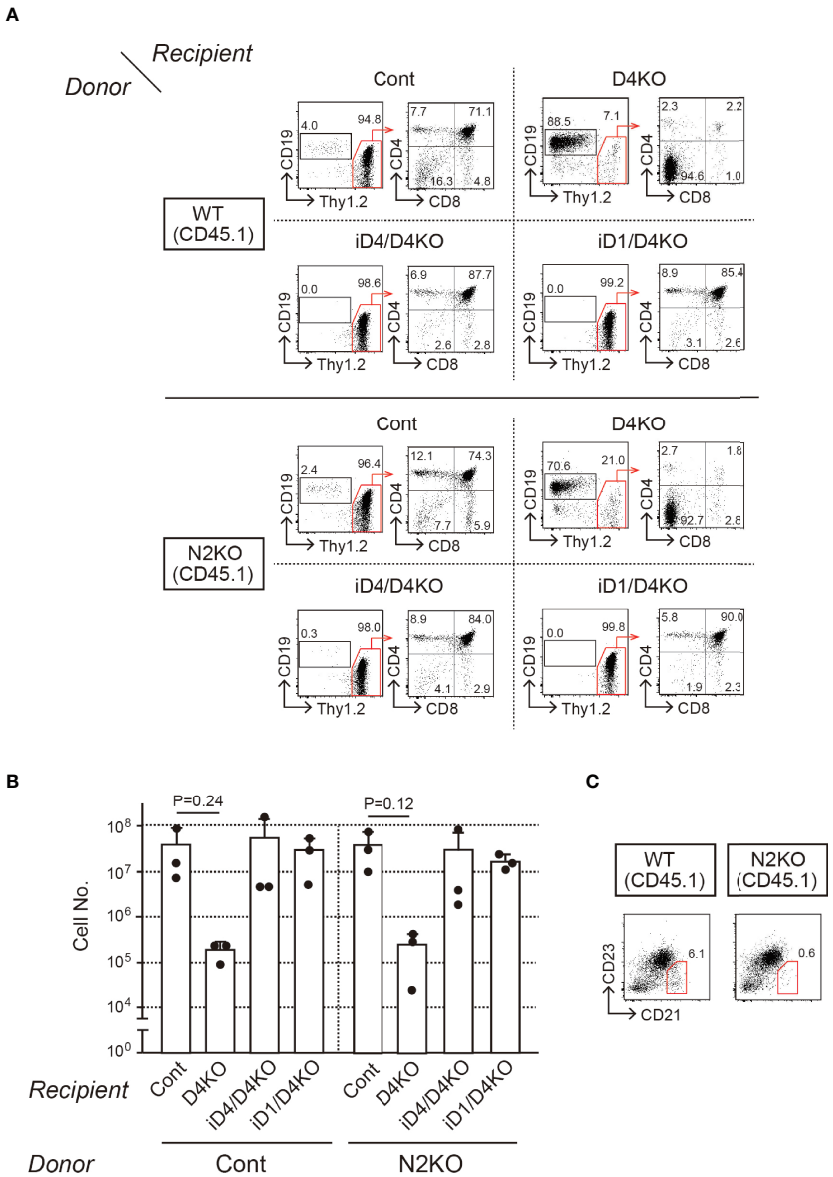


FIGURE 4 | T-cell development from *Notch2*-deficient BM cells in the thymus of iD4 and iD1 mice. Age-matched control (WT) or *Notch2*-deficient (N2KO) BM cells were obtained from *Notch2*$^{f/f}$ or *Rosa26*^{CreERT2}*Notch2*$^{f/f}$ mice (CD45.1) 1 week after the administration of tamoxifen. BM chimeric mice were prepared in irradiated (6 Gy) C57 BL/6 mice (CD45.2) with *Notch2*-deficient BM and analyzed 4 weeks after the reconstitution (**Supplementary Figure 3A**). **(A)** Flow cytometric analysis was performed using the thymocytes from BM chimeric mice with control (WT) or *Notch2*-deficient (N2KO) BM cells (*Donor*) in *Dll4*$^{f/f}$ (Cont), *Dll4*$^{f/f}$FoxN1-Cre (D4KO), iD4*Dll4*$^{f/f}$FoxN1-Cre (iD4/D4KO), or iD1*Dll4*$^{f/f}$FoxN1-Cre (iD1/D4KO) mice as the recipients (*Recipient*). Numbers in the profiles indicate the relative percentages, in CD45.1$^+$ cells (left panels, CD19 vs. Thy1.2) and CD45.1$^+$Thy1.2$^+$ cells (right panels, CD4 vs. CD8), for each quadrant or fractions. **(B)** Thymic cellularity (mean ± SD) of BM chimeric mice in *Dll4*$^{f/f}$ (Cont, n=3), *Dll4*$^{f/f}$FoxN1-Cre (D4KO, n=3), iD4*Dll4*$^{f/f}$FoxN1-Cre (iD4/D4KO, n=3), or iD1*Dll4*$^{f/f}$FoxN1-Cre (iD1/D4KO, n=3) mice is shown. There are no statistically significant differences found between control and *Notch2*-deficient BM cells by Student's *t*-test. Each closed circle indicates the number of thymocytes (CD45.1) in each mouse. **(C)** Representative CD21/CD23 profiles in the donor-derived B cells (CD45.1$^+$B220$^+$) obtained from the spleen of BM chimeric mice with control (WT) or *Notch2*-deficient (N2KO) BM cells in *Dll4*$^{f/f}$ mice are shown. The red polygons and numbers in the profiles indicate the MZB cell fraction and their frequencies. Results represent three independent biological replicates **(A, C)**.

range may be advantageous. Interestingly, the proximity between the appearance of the *Dll4* gene and the precise beginning of the coexistence of *Notch1* and *Notch2* seems to be related to the fact that Dll1 and Dll4 cooperate with Notch1/Notch2 and selectively with Notch1, respectively. Subsequently, the combination of Dll4 and Notch1 to induce stable Notch signaling is preferentially utilized, as seen in the induction of T-cell development (21).

Based on several findings regarding the significance of Notch receptor–ligand interactions in the development of various organs, it is clear that Dll4 binds to Notch1, whereas Dll1 does

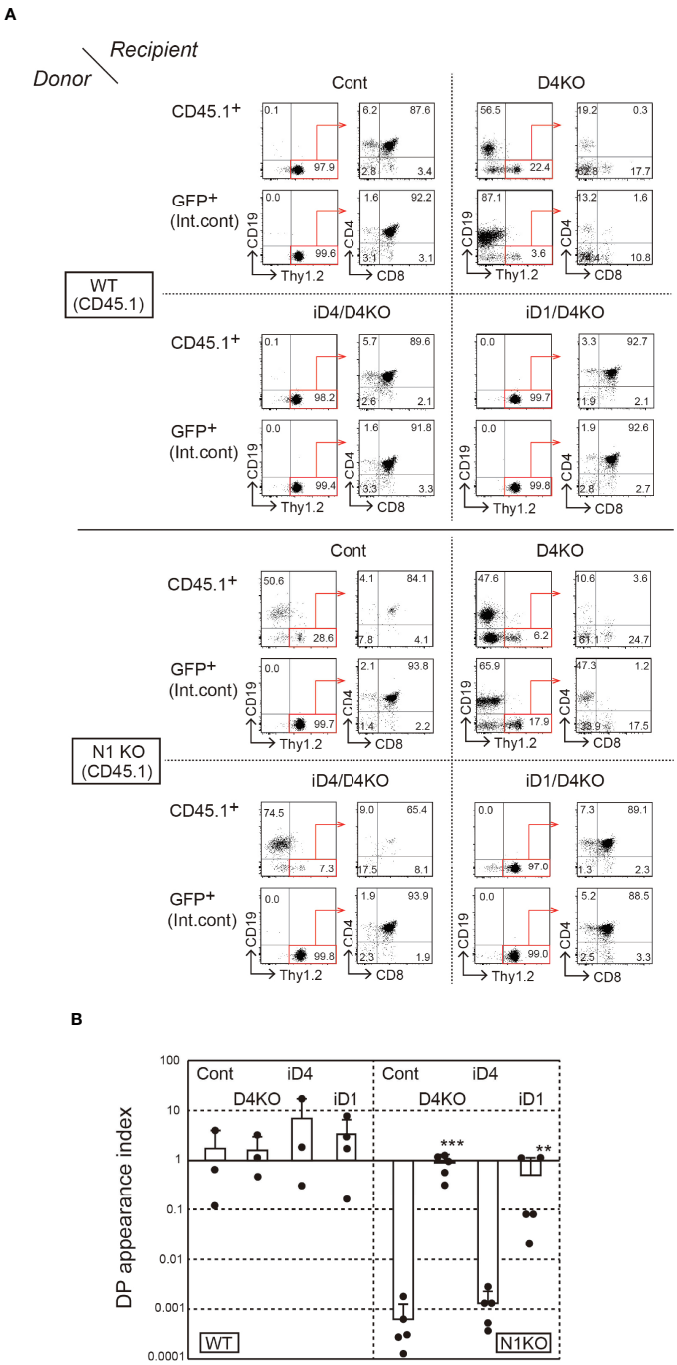


FIGURE 5 | T-cell development from *Notch1*-deficient BM cells in the thymus of iD4 and iD1 mice. The primary BM chimeras were prepared in irradiated WT (CD45.2) mice with BM cells from *Notch1*$^{f/f}$ (WT, CD45.1) or *Rosa26*^{CreERT2}*Notch1*$^{f/f}$ (N1KO, CD45.1) mice and GFP Tg mice. The control and *Notch1*-deficient BM cells were obtained from the primary BM chimeras after the administration of tamoxifen and serially transferred into *Dll4*$^{f/f}$ (Cont), *Dll4*$^{f/f}$FoxN1-Cre (D4KO), iD4*Dll4*$^{f/f}$FoxN1-Cre (iD4/D4KO), or iD1*Dll4*$^{f/f}$FoxN1-Cre (iD1/D4KO) mice. Four weeks after the reconstitution, thymocytes were analyzed (**Supplementary Figure 3B**). **(A)** Flow cytometric analysis was performed using the thymocytes from the secondary BM chimeric mice. Numbers in the profiles indicate the relative percentages, in CD45.1$^+$ or GFP$^+$ cells (internal control, Int. cont) (left panels, CD19 vs. Thy1.2) and CD45.1$^+$Thy1.2$^+$ or GFP$^+$Thy1.2$^+$ cells (right panels, CD4 vs. CD8), for each quadrant or fractions. Results represent at least three independent biological replicates. **(B)** The efficiencies of the appearance of CD4$^+$CD8$^+$ (DP) thymocytes derived from control (WT) or *Notch1*-deficient (N1KO) BM cells were examined. DP appearance index was calculated as the ratio of CD45.1$^+$/GFP$^+$ DP thymocytes and CD45.1$^+$/GFP$^+$ B220$^+$ B cells in lymph node (mean ± SD; WT as donor; Cont, n=3; D4KO, n=3; iD4/D4KO, n=3; iD1/D4KO, n=4; N1KO as donor; Cont, n=5; D4KO, n=5; iD4/D4KO, n=5; iD1/D4KO, n=5). The data were collected from three independent experiments. Each closed circle indicates the index in each mouse. **p < 0.01, ***p < 0.001 by Mann–Whitney *U*-test.

not distinguish between Notch1 and Notch2 and functions equally well with both (18). During vascular development, Dll1-mediated Notch1 activation is essential for the maintenance of arterial identity during late-stage arteriogenesis in mouse fetuses (13.5 days of gestation, E13.5) (33). Dll1 and Notch1 are also important for somitogenesis (34, 35). In both cases, Dll4 could not completely compensate for the loss of Dll1, suggesting a functional difference between Dll1 and Dll4 (36–38). In contrast, as both Dll1 and Notch1 are necessary for retinal development and Dll4 could substitute for Dll1 function, there was functional redundancy in the retina (37–39). In this study, the exogenous expression of Dll1 in the thymic epithelium in place of Dll4 supported T-cell development with similar cellularity, suggesting functional overlap between them. Thus, their functional differences were context dependent and might be due to the difference in the threshold amount of Notch signaling required. Reportedly, both Dll1 and Notch2 are essential for the appearance of marginal zone B (MZB) cells in the spleen, indicating that Dll1 cooperates with Notch2 to transduce Notch signaling (19, 25). In the spleen, Dll1 is expressed on stromal cells in the follicles and encounters the precursors of MZB cells or MZB cells that express Notch2 to activate Notch signaling that determines or maintains their cell fate (40). In contrast, Dll4 functions with Notch1 at an early stage (around E8.5) for the specification of arterial fate during vascular development in mice. In our study, neither endogenous nor exogenous Dll4 function with Notch2. Therefore, it was suggested that Dll1 can interact with both Notch1 and Notch2, in contrast to Dll4 that only acts as a functional ligand for Notch1 in the thymus. Significant contribution of the Notch2–Dll4 interaction has not been reported in lineage specifications of other organs, too. We have previously shown that interaction between Dll4 and Notch2 *in vitro* is clearly detected and other Notch–Dll combinations using Notch1- or Notch2-expressing cells stained with soluble form of the extracellular regions of Dll1 or Dll4 (21). Thus, dysfunction between Notch2 and Dll4 seems to be observed only *in vivo*, and there would be some unknown mechanisms underlying their inefficient interaction.

Using *in vitro* cultures with a monolayer of stromal cells, it was shown that Dll1 but not Dll4 induces Notch signaling *via* Notch2 that is sufficient for the specification of T-lineage cells (17). However, Dll1-mediated Notch2 signaling was not sufficient to drive T progenitors into the DP stage, and BM progenitor-derived T-lineage cells arrested their differentiation at the DN3 stage because of the impaired expression of the TCR β chain. In this study, the transition from DN to DP stage was completely restored, and mature SP T cells were also observed in iD1 mice with *Notch1*-deficient BM progenitors. These results suggested that Dll1-mediated Notch2 signaling can support the expression of the TCR β chain necessary for differentiation into DP and SP stages. This discrepancy may reflect the high capacity of the native thymic environment to support T-cell development. However, the efficiencies of the appearance of DP thymocytes derived from *Notch1*-deficient BM progenitors in iD1 mice were, in some cases, less than those derived from Notch1-bearing WT BM progenitors. In addition, stage-specific deletion of Notch receptors revealed that Notch1 is the main transducer of Notch signaling in DN2b/DN3 stages, while Notch2 has minor cooperative effects on Notch target genes (27). These differences can be attributed to the lower expression of Notch2 at the DN3 stage than that of Notch1 (16). Therefore, the downstream impact of the Dll1 and Notch2 interaction may not be identical to that of Dll4 and Notch1 in the thymus.

In conclusion, we showed here that Dll1 was supporting T-cell development with ancient Notch receptors when the thymus emerged and was replaced by Dll4 to trigger Notch signaling *via* Notch1 during their evolution. The latter combination might have some functional advantages in inducing T-cell development in the thymus.

MATERIALS AND METHODS

Phylogenetic Analysis

We performed an evolutionary analysis using the maximum likelihood method. The evolutionary history was inferred using the maximum likelihood method and the JTT matrix-based model (41), and the tree with the highest log likelihood (−17,502.39 for *Dll*, −55,656.23 for *Notch*) is shown. The percentage of trees in which the associated taxa were clustered together is shown next to the branches. Initial trees for the heuristic search were obtained automatically by applying neighbor-join and BioNJ algorithms to a matrix of pairwise distances estimated using the JTT model and then selecting the topology with a superior log likelihood value. The tree was drawn to scale with branch lengths measured as the number of substitutions per site. This analysis involved 20 *Dll* and 18 *Notch* amino acid sequences. We obtained a total of 937 *Dll* and 2,848 *Notch* positions in the final dataset. Evolutionary analyses were conducted using MEGA11 software (42, 43).

Mice

Dll4$^{f/f}$ Foxn1-cre mice have been described previously (4). These mice were bred with iD1 or iD4 transgenic mice that retained the CAG promoter-driven *floxed GFP* and *Dll1* or *Dll4* cDNA at the *Rosa26* locus (21). *Notch1$^{f/f}$ Rosa26^{CreERT2}* or *Notch2$^{f/f}$ Rosa26^{CreERT2}* mice with the CD45.1 allele (16, 25, 30, 31, 44) were maintained in our laboratory. To delete the floxed genes, we administered tamoxifen (100 mg/kg) to the mice by i.p. injection four times on separate days. One week after treatment, BM cells were obtained and used as a source of *Notch1*- or *Notch2*-deficient hematopoietic cells for transplantation. GFP transgenic mice were originally established by our group (45). All mice were maintained under specific pathogen-free conditions, and the animal experiments were approved by the Animal Experimental Committee (Tokai University, Kanagawa, Japan).

BM Transplantation

For BM reconstitution experiments with *Notch2*-deficient BM cells, semi-lethally irradiated (6 Gy) *Dll4$^{f/f}$* (Cont), *Dll4$^{f/f}$ FoxN1-Cre* (D4KO), iD4 *Dll4$^{f/f}$ FoxN1-Cre* (iD4/D4KO), or iD1 *Dll4$^{f/f}$ FoxN1-Cre* (iD1/D4KO) mice (CD45.2) were transplanted

intravenously with BM cells from age-matched *Notch2*$^{f/f}$ (1×10^7, CD45.1, represented as WT in the figure) or *Notch2*$^{f/f}$ *Rosa26*^{CreERT2} mice (1×10^7, CD45.1, represented as Notch2 KO), who had been administered tamoxifen 1 week before the experiments and analyzed at 6 weeks after reconstitution. For those with *Notch1*-deficient BM cells, semi-lethally irradiated C57BL/6 mice were transplanted with BM cells from age-matched *Notch1*$^{f/f}$ (5×10^6, CD45.1, represented as WT in the figure) or *Notch1*$^{f/f}$ *Rosa26*^{CreERT2} (5×10^6, CD45.1, represented as Notch1 KO) mice, with GFP-transgenic (5×10^6, CD45.2) mice as an internal control. After 4 weeks, the mice were administered tamoxifen, and 1 week after the treatment, BM cells were prepared for secondary transplantation into the recipient mice as described above.

Flow Cytometric Analysis

For flow cytometric analysis, the following monoclonal antibodies (mAbs) and reagents were used: BV650-CD4 and PE-Cy7-CD3 (BD Biosciences, Tokyo, Japan); PE/Cy7-CD4, BV510-CD4, APC-CD8, APC/Cy7-CD8, Alexa700-CD8, FITC-CD11b, PerCp/Cy5.5-CD21, PE-CD23, PE-CD45.1, PE-CD45.2, FITC-B220, APC-B220, Pacific Blue-Thy1.2, Pacific Blue-c-Kit, APC-IL7Rα, PE-EpCAM, FITC-Gr-1, PE/Cy7-Sca-1, PE-Hamster IgG, PE-Notch1, PE-Notch2, PE-Notch3, and PE-Notch4 (BioLegend, Tokyo, Japan); and PerCp/Cy5.5-CD4, PerCp/Cy5.5-CD19, PE-Thy1.2, APC-Thy1.2, APC-PDGFRα, and FITC-TER119 (Thermo Fisher Scientific, Tokyo, Japan). PE/Cy7-CD19 and APC/Cy7-CD45.1 (Thermo Fisher Scientific). Stained cells were measured using FACSVerse (BD Biosciences) or FACSFortessa (BD Biosciences). Data were analyzed using FlowJo software (BD Biosciences).

Immunohistochemistry

Immunohistochemical analysis was performed as previously described (4). Tissue sections of thymus were stained with FITC-anti-pan-cytokeratin (Sigma-Aldrich, Tokyo, Japan) and rabbit anti-HA Ab (Bio-Rad, CA, US) antibodies. Visualization was performed with Alexa594-anti-rabbit IgG antibody (Thermo Fisher Scientific). The stained slides were observed with LS880 (ZEISS).

Statistical Analysis

Statistical analyses, as indicated in the figure legends, were performed using GraphPad Prism software (version 5.0; GraphPad Software, Inc., San Diego, CA). Differences between the two groups were evaluated using Student's *t*-test for parametric samples and Mann–Whitney *U*-test for non-parametric samples. Statistical significance was set at $p<0.05$.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by the Animal Experimentation Committee of Tokai University.

AUTHOR CONTRIBUTIONS

KiH, HH, and KH designed and performed all the experiments, analyzed the data, and wrote the manuscript. YT, JI, MY, NN, and TS performed the experiments and supported data collection. MT performed the phylogenetic analysis. MO provided the GFP mice and supported the animal experiments. KA and SH supervised this study.

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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.2022.852427/full#supplementary-material

Supplementary Figure 1 | Phylogenetic analysis of *delta-like (Dll) 1* and *Dll4* genes. Homologs of *Dll1* and *Dll4* genes are sorted from the NCBI database in terms of their homology to murine *Dll1* and *Dll4* genes. The evolutionary history was inferred using the maximum likelihood method and the JTT matrix-based model (41). The tree with the highest log likelihood (-17502.39) is shown. The percentage of trees in which the associated taxa were clustered together is shown next to the branches. The tree was drawn to scale with branch lengths measured as the number of substitutions per site. The analysis involved 20 amino acid sequences. There were 937 positions in the final dataset.

Supplementary Figure 2 | The numbers of thymocyte subsets. Thymic cellularity (mean ± SD) of thymocyte subsets ($CD4^+CD8^+$, DP; $CD4^+CD8^-$, 4SP; $CD4^-CD8^+$, 8SP; $CD4^-CD8^-$, DN; $CD19^+$, CD19) derived from the mice in **Figure 2** were shown. **P<0.01, *P<0.05 by Student *t*-test.

Supplementary Figure 3 | Bone marrow (BM) chimera experiments. The experimental schemes for BM chimeras are shown for **Figures 4A** and **5B**.

Supplementary Figure 4 | Mature T cells from Notch1-deficient(Italic) BM cells in the lymph nodes of iD1 and iD4 mice. All data were obtained from inguinal lymph nodes of the BM chimera, as shown in **Figure 5**. **(A)** Flow cytometric analysis of inguinal lymph nodes from secondary BM chimeric mice. Numbers in the profiles indicate the relative percentage of $CD45.1^+$ or GFP^+ cells (internal control, Int. cont) (left panels, B220 vs. CD3) and $CD45.1^+CD3^+$ or GFP^+CD3^+ cells (right panels, CD4 vs. CD8) for each quadrant or fraction. The results represent at least three independent biological replicates. **(B)** The efficiency of $CD3^+$ T cells derived from control (WT) or *Notch1*-deficient (N1KO) BM cells was examined. The T cell appearance index was calculated as the ratio of $CD45.1^+/GFP^+$ $CD3^+$ T cells and $CD45.1^+/GFP^+$ $B220^+$ B cells in the inguinal lymph nodes (mean ± SD; WT as donor; Cont, n=3; D4KO, n=3; iD4/D4KO, n=3; iD1/D4KO, n=4; N1KO as donor; Cont, n=5; D4KO, n=5; iD4/D4KO, n=5; iD1/D4KO, n=5). Data were collected from three independent experiments. Each closed circle indicates the index for each mouse. **P<0.01, ***P<0.001 using Mann–Whitney *U* test.

Supplementary Figure 5 | Phylogenetic analysis of *Notch1* and *Notch2* was performed as shown in **Supplementary Figure 1**. The tree with the highest log likelihood (-55656.23) is shown. This analysis involved 18 amino acid sequences with a total of 2848 positions in the final dataset.

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REVIEW
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Long Non-Coding RNAs in the Cell Fate Determination of Neoplastic Thymic Epithelial Cells

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Thymic Epithelial Tumors (TETs) arise from epithelial cells of the thymus and are very rare neoplasms comprising Thymoma, Thymic carcinoma, and Thymic Neuroendocrine tumors that still require in-depth molecular characterization. Long non-coding RNAs (lncRNAs) are emerging as relevant gene expression modulators involved in the deregulation of several networks in almost all types of human cancer, including TETs. LncRNAs act at different control levels in the regulation of gene expression, from transcription to translation, and modulate several pathways relevant to cell fate determination under normal and pathological conditions. The activity of lncRNAs is strongly dependent on their expression, localization, and post-transcriptional modifications. Starting from our recently published studies, this review focuses on the involvement of lncRNAs in the acquisition of malignant traits by neoplastic thymic epithelial cells, and describes the possible use of these molecules as targets for the design of novel therapeutic approaches specific for TET. Furthermore, the involvement of lncRNAs in myasthenia gravis (MG)-related thymoma, which is still under investigation, is discussed.

Keywords: thymoma, thymic carcinoma, thymic epithelial tumors (TETs), ncRNAs (non coding RNAs), miRNA - microRNA, lncRNA - long noncoding RNA, MALAT1, myasthenia gravis

INTRODUCTION

The thymus is the primary lymphoid organ located at the level of the anterior mediastinum. The thymus plays an essential role in educating and enabling the maturation of prelymphocytes into mature T lymphocytes, which are involved in the adaptive immune response. Thymic epithelial-reticular cells (TECs) play a major role in the maturation of T lymphocytes. However, TECs may

undergo neoplastic transformation, resulting in certain types of tumors, such as thymic epithelial tumors (TETs) (1).

TETs are relatively rare neoplasms in middle-aged or elderly adults that represent 0.2-1.5% of all cancers and comprise thymoma, thymic carcinoma (TC), and thymic neuroendocrine tumors (2). TETs are characterized by wide variability and heterogeneity in their malignant behavior. In 30% of cases, TETs are asymptomatic; however, in 40% of cases, local symptoms, such as chest pain, cough, dyspnea, and hoarseness, are displayed while in the remaining 30% of cases, systemic symptoms, with superior vena cava syndrome (SVC), and in the most aggressive forms, weight loss, ensue. To date, the etiology of TETs has not been established and the risk factors remain unclear (3).

TETs classification has remained controversial subject for many years. After different classification approaches [Bernatz et al. in 1961, Rosai and Levine in 1976, Marino and Muller-Hermelink, in 1985 (4)], Rosai and Sobin published a new classification in the World Health Organization (WHO) series in 1999, dividing thymic tumors into three major subgroups based on the morphology of epithelial cells and the percentage of epithelial and lymphocyte populations: type A, type B, and type C (thymic carcinoma). Type A thymomas are tumors with a component of spindle-oval EC but lack lymphocytes, whereas type B thymomas are characterized by large EC with dendritic or plump (epithelioid) morphology, forming networks where lymphocytes are attracted. Notably, the combination of these two morphologies has been designated as type AB (5). Thymic carcinoma (type C) is a rare malignancy, representing less than 1% of thymic tumors, and is characterized by cytological atypia, more aggressive behavior, and local and distant metastases (liver, lymph nodes, or bones) (6).

Recently, a new WHO classification of thoracic cancers was established, which includes new diagnostic criteria and rare entities, such as hyalinizing clear cell carcinoma (7).

Interestingly, thymoma is strongly associated with various paraneoplastic syndromes (PNS), such as myasthenia gravis (MG), red cell aplasia, polymyositis, systemic lupus erythematosus, Cushing syndrome, and syndrome of inappropriate antidiuretic hormone secretion (8).

Thymomas are associated with MG in 30-50% of cases, and thymoma occurs in 10-15% of cases of MG (9). MG (myos = muscle, asthenos = weakness, gravis = severe) is an autoimmune disease that affects the neuromuscular junction (NMJ) of the skeletal muscle, causing muscle weakness of different severity, several complications such as myasthenia crisis, and in some cases, acute respiratory paralysis (10–13). MG can develop at any age, particularly in young women (>30 years) and older men (>60 years) (14).

Thymoma-associated myasthenia gravis (TAMG) is frequently reported in adults and is characterized by alterations in thymus function (14). The association between MG and thymoma is due to the dysregulation of positive and negative selection of T cells in the thymus (15). For example, in cortical thymoma, the lack of medullary epithelial cell function and defects in the autoimmune regulator complex (AIRE), which is responsible for negative selection, leads to the production of autoreactive T cells specific for acetylcholine receptors (AChR) that are exported to the periphery, where autoreactive T cells stimulate and activate B cells to produce antibodies against AChR (14, 16). Although the required treatment for thymoma-associated MG is tumor removal, remission is not inevitable. Moreover, removal of thymoma in non-myasthenic patients does not prevent the subsequent onset of MG. Patients with thymoma have been observed to develop antibodies against AChR and symptoms of myasthenia after the resection of the tumor (17).

MOLECULAR PATHWAYS IN TETs AND MG

Although the etiology of TETs is still poorly understood, advanced next-generation sequencing (NGS) techniques have recently allowed the mapping of gene mutations and epigenetic alterations occurring in thymic tumors.

One of the most frequently mutated genes in TETs is *GTF2I*, whose mutation is specifically associated with types A and AB (78%), but is less frequent in more aggressive histological types, such as thymic carcinomas (8%) (18). The overexpression of EGFR and HER2 and mutations in KIT, IGR-1, and neurotrophin receptors have been recently demonstrated in some cases, as reviewed by Scorsetti et al. (6). Gain-of-function mutations in HRAS and NRAS and loss-of-function mutations in TP53 are less common, but are considered founder mutations (19).

Thymic carcinoma is characterized by loss of chromosome 16q, mutations in epigenetic regulatory genes (*BAP1*, *ASXL1*, *SETD2*, *SMARCA4*, *TET2*, *DNMT3*A, and *WT1*), and anti-apoptotic genes (*BCL2* copy number gains) (8, 20).

Notably, gene expression profiling in TGCA study revealed four molecular subtypes, represented respectively by type B (subtype 1), TC (subtype 2), AB (subtype 3), and a mix of types A and AB (subtype 4). TGCA study also revealed four distinct molecular clusters using PARADIGM analysis. In particular, the upregulation of TP53 and downregulation of oncogenes, such as MYC/Max, MYB, and FOXM, characterize the A-like cluster, while the downregulation of TP53 and upregulation of MYC/Max, MYB, FOXM1, and E2F1 in AB-, B-, and C-like clusters are consistent with the high aggressiveness of B3 and TC tumors. Furthermore, this study highlighted that types A, AB, B, and TC are not a continuum of diseases, but are instead distinct biological entities (5, 19), Recently, metaplastic thymomas were reported to harbor the YAP1-MAML2 translocation, whereas 6% of pretreated types B2 and B3 and a combined TC and B3 thymoma (but not in thymoma and "pure" TCs) may be associated with KMT2A-MAML2 translocation (7).

Alterations in inflammatory and thymus function, which occur in thymic neoplasms, and mutations in the *AIRE* gene locus promote the development of autoimmune diseases, such as MG (16). EOMG is associated with HLA-DR3, HLA-B8, and other autoimmune risk genes, whereas LOMG is weakly associated with HLA-DR2, HLA-B7, and mLA-DR-B1*15:0 (14). Aneuploidy and intratumoral overexpression of genes

that have a similar sequence to autoimmune targets (CHRNA1, RYR3, and NEFM) are common in patients (19).

In addition to mutations in protein-coding genes, alterations in ncRNA molecules have been reported to significantly impact the initiation, progression, and response of TETs and MG to therapy, as described in the next section.

IDENTIFICATION OF ncRNAs IN TETs AND MG

Despite being considered "junk" for a long time, ncRNAs have emerged as functionally relevant in nearly all physiological and pathological cellular processes (21, 22). These new discoveries have been aided by powerful high-throughput approaches, such as next-generation sequencing (NGS), transcriptome studies, molecular network analyses, and artificial intelligence-guided prediction of ncRNA function (23).

ncRNAs play a role in many biological, physiological, and developmental processes, including several diseases and tumors. ncRNAs are produced by transcription from different genomic regions and post-transcriptional maturation and modification. ncRNAs can be divided into two classes according to their length (24): small non-coding RNAs (ncRNAs) and lncRNAs (usually > 200 nt).

In recent years, ncRNAs have been found to play an important role in gene regulation at different levels. Several studies have demonstrated the involvement of ncRNAs in transcriptional regulation, RNA maturation, chromatin remodeling, post-transcriptional RNA regulation, and modification (25). Dysregulation of ncRNAs is involved in many human diseases and in tumor initiation and progression (26). Regulatory ncRNAs can be divided into two classes: circular and linear.

Circular RNAs (circRNAs) are a class of covalently closed RNA molecules and are thus more stable than linear RNAs. The expression patterns of circRNAs are cell type-, tissue-, and developmental stage-specific (27, 28). Depending on their localization, circRNAs can exert different functions, including acting as microRNA (miRNA) sponges, modulating the activity of RNA-binding proteins (RBPs), or acting as protein scaffolds, which can be translated into polypeptides owing to the presence of internal ribosome entry sites (IRES) or m^6A modifications (27, 29). Many circRNAs are differentially expressed in several cancer types compared with their untransformed counterparts and are related to tumor growth, metastasis, and therapy resistance (30, 31). Interestingly, several studies have demonstrated that circRNAs are differentially expressed in thymoma and MG (32, 33), and have highlighted their important role as biomarkers for the diagnosis of this disease (34).

Another major group of regulatory ncRNAs is represented by linear ncRNAs, comprising small ncRNA (18-200 nt) and lncRNAs (>200 nt) (35).

Among small ncRNAs, miRNAs play a predominant role in post-transcriptional regulation, binding to specific mRNA targets and causing their degradation or translation inhibition. miRNAs are single-stranded RNAs with an average length of 22 nucleotides that are derived from hairpin-structured precursors (36). Several miRNAs are altered in different human diseases, including cancer. The roles of miRNAs in thymus differentiation, development, and involution have been extensively described by Cron et al. (37). Moreover, their relevance in the treatment, diagnosis, and prognosis of TETs and MG is emerging (38).

Several research groups have identified miRNAs that are differentially expressed between thymic tumors and normal samples, as well as between thymic carcinoma and thymoma and histotype classes (39–41). In particular, Bellissimo et al. found that miR-145-5p was epigenetically downregulated in thymic carcinoma cells (42), confirming its well-known tumor suppressor role (43). Similar to circRNAs, miRNAs are excellent biomarkers for cancer diagnosis found in the serum of patients with TETs and MG (44–49).

LncRNAs IN TETs

Although miRNAs represent one of the most studied biomarkers involved in thymic tumorigenesis among all ncRNA classes, the study of lncRNAs is becoming more relevant. Based on their localization, lncRNAs can bind to genes, transcripts, miRNAs, and proteins that regulate different cellular processes, such as gene expression, transcription, and post-transcriptional regulation, through different mechanisms of action. Further, lncRNAs can act as guides of chromatin modification complexes, including DNA methyltransferase and histone-modifying enzymes, on specific genomic loci that induce activation or inhibition of target genes in *cis* or *trans* (50); function as decoys for transcription factors and other effectors, impairing their regulatory activity (50); serve as modular scaffolds, and bind and drive two or more physically distant proteins into specific genomic regions to regulate gene expression (51); act as sponges of miRNAs, sequestering them and preventing their ability to promote degradation or repression of target genes (52, 53); and influence splicing (54) and the stability of mRNAs, regulating their post-transcriptional expression (55). Therefore, according to their mechanisms of action, lncRNAs are involved in the regulation of various biological processes, including cellular survival, proliferation, differentiation, apoptosis, invasion, and metastasis. Consequently, aberrant lncRNA expression is tightly correlated with cancer development (56).

Deregulation of lncRNAs in TETs

Similar to many types of human tumors, such as breast cancer (57), lung cancer (58), colorectal carcinoma (59), ovarian cancer (60) and prostate cancer (61), dysregulated lncRNAs in TETs may contribute to tumor onset and progression. Different studies have identified a large spectrum of lncRNAs in thymic epithelial tumors using high-throughput sequencing technologies. In this context, most of the identified lncRNAs act as miRNA sponges, playing oncogenic or tumor suppressor roles. An example of this type of regulation is represented by lncRNA LOXL1-AS1, miR-525-5p, and the HSPA9 gene network. Data from TCGA study showed that high expression of LOXL1-AS1 and downregulation of miR-525-5p correlated with poor prognosis in TET (62).

Similar to other cancer types (63, 64) in thymic tumors, miR-525-5p acts as a tumor suppressor, inhibiting cell growth and invasion, and inducing apoptosis by repressing the target gene, *HSPA9*. *HSPA9* is upregulated in thymoma and thymic carcinoma and correlated with poor patient survival. The positive association between LOXL1-AS1 and HSPA9, which is consistent with the downregulation of miR-525-5p, was confirmed by *in vitro* experiments. The silencing of LOXL1-AS1 promotes thymic tumor progression by acting as a sponge of miR-525-5p and increasing the expression of HSPA9 (62). Similar to LOXL1-AS1, another network is represented by the interaction between lncRNA LINC00174, miR-145-5p, and miR-145-5p predicted target genes involved in thymic tumorigenesis (65). In this study, the upregulation of lncRNA LINC00174 in frozen tissue samples of thymoma compared to its normal counterparts was identified. LINC00174 is negatively associated with miR-145-5p, a well-known tumor suppressor of miRNA downregulation in TETs (39, 42), and positively correlated with miR-145-5p predicted targets (**Figure 1**). The inhibition or overexpression of miR-145-5p modulates LINC00174 expression and its associated genes (65). Notably, the poor prognosis of TET patients, characterized by high expression of LINC00174 and its associated genes and low expression of miR-145-5p, suggests an oncogenic role of LINC00174 in TETs. According to these data, LINC00174 silencing impairs cell growth and proliferation, cell migration, and lipid metabolism. Similar to LINC00174, MALAT1 can act as a sponge for miR-145-5p in TET. MALAT1 is a well-known oncogenic lncRNA that regulates different biological processes, such as cell proliferation, apoptosis, angiogenesis, invasion, and metastasis, and contributes to cancer development (66–68). Using a luciferase assay, the interaction between miR-145-5p and MALAT1 was demonstrated in the thymic cancer cell line, IU-TAB1. In this thymic tumor context, the downregulation of MALAT1 increased miR-145-5p expression and led to a reduction in cell proliferation and an increase in the apoptosis rate compared to that observed in the control. Additionally, the combination of MALAT1 silencing and miR-145-5p overexpression induces a synergistic effect, suggesting that MALAT1 may regulate the thymic cancer phenotype by inhibiting miR-145-5p (69). Recently, the expression of MALAT1 has been a focus in our studies where the relationship between lncRNA MALAT1 and METTL3, a methyltransferase enzyme that catalyzes the N6-methyladenosine (m^6A) modification, were described in the thymic carcinoma cell line, TC1889. Of note, the expression of lncRNAs can be regulated by m^6A modifications (70), and the downregulation of METTL3 leads to increased localization of MALAT1 in nuclear speckles and decreased m^6A modification of MALAT1 lncRNA (71), which probably impinges on its functional activity. In the past, we observed a similar delocalization of MALAT1 in nuclear speckles with consequent altered splicing (72) due to the presence of mutant p53 protein in breast cancer cells. Another interesting lncRNA-miRNA-target network in the

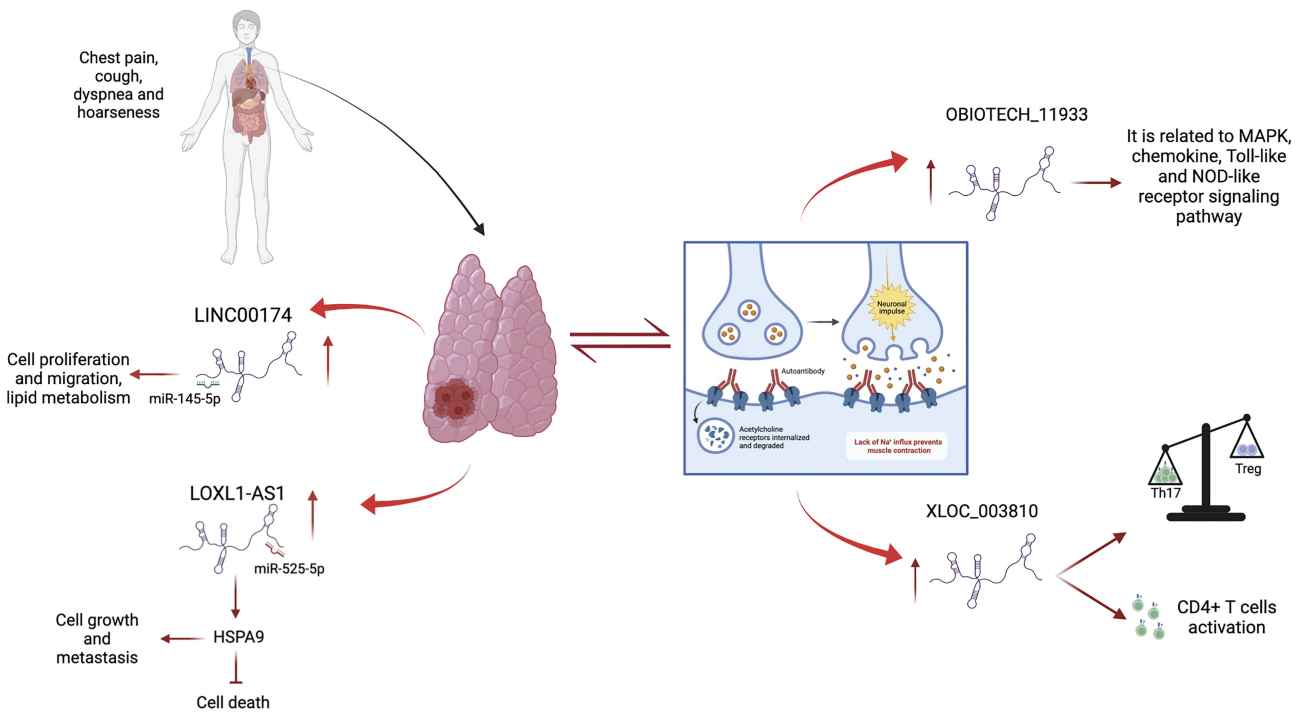

FIGURE 1 | Contribution of lncRNAs in TETs and MG. LINC00174 and LOXL1-AS1 are up-regulated in thymoma and thymic carcinoma, where they act as sponges of miR-145-5p and miR-525-5p, respectively, promoting cell growth, metastasis, migration, and lipid metabolism and inhibiting cell death. LncRNAs are deregulated in MG, an autoimmune disease strongly associated with thymoma. Oebiotech_1193 and XLOC_003810 are overexpressed in MG patients and are involved in inflammatory pathways.

control of thymoma progression was reported by Yang et al. (73). The lncRNA RP11-424C20.2 regulates the expression of the *UHRF1* gene (ubiquitin-like containing PHD ring finger 1) by sponging miR-378a-3p; the RP11-424C20.2/UHRF1 axis is strongly associated with a better outcome in thymoma patients, which is related to the different types of infiltrating immune cells, such as B cells, macrophages, CD8+ and CD4+ cells, neutrophils, and dendritic cells. The role of UHRF1 is well established; it is an epigenetic modifier that regulates immune infiltration and the tumor immune microenvironment through its interaction with DNA histone deacetylase genes (73, 74). Therefore, RP11-424C20.2 expression can influence the prognosis of patients with thymoma by regulating the expression of UHRF1 *via* miR-378a-3p sponging (73).

Owing to this recent evidence, the identification of altered lncRNAs in TETs and the characterization of their role in the promotion of tumorigenesis could provide new potential therapeutic targets relevant for the treatment of TETs.

LncRNAs as a Predictive Factor of Patient Prognosis in TETs

Studies on the profiles of lncRNAs expressed in thymoma tissue samples have revealed that altered expression of specific lncRNAs may correlate with overall or disease-free survival. For example, Su et al. (75) identified a panel of lncRNAs that predicts the recurrence of thymic epithelial tumors. They analyzed a cohort of 114 TET patients from TCGA study and identified four lncRNAs, ADAMTS9-AS1, HSD52, LINC00968, and LINC01697, which are significantly related to recurrence-free survival (RFS). These lncRNAs can be used to divide TET patients into high-risk and low-risk groups, respectively, with shorter and longer RFS. Based on ROC analysis, these lncRNAs represent a better prognostic model for the RFS of patients than the WHO classification and Masaoka stage. Although these lncRNAs constitute a good factor of discrimination between different TETs subtypes and their associated stages, the trial had some limitations: few samples from TCGA, absence of evidence of their predictive power in other types of cancer, and biological characterization of their role in TETs (75). Furthermore, the altered expression of ADAMTS9-AS1 (one of the four RFS-related lncRNAs) with five other lncRNAs, namely AFAP1-AS1, LINC00324, VLDLR-AS1, LINC00968, and NEAT1, was detected in another study by RNA-seq and profiling expression analysis in 25 thymoma patients and 25 healthy individuals (76). These lncRNAs are involved in the development of different types of cancers and regulate several biological processes and molecular pathways. For example, ADAMTS9-AS1 induces cell migration and proliferation in colorectal carcinoma, affecting β-catenin expression (77); LINC00968 reduces drug resistance and invasion of tumor cells in breast cancer (78); AFAP1-AS1 increases epithelial-mesenchymal transition by impairing RhoC, ROCK1, p38MAPK, and Twist1 signaling pathways in osteosarcoma (79); LINC00324 inhibits the NOTCH pathway, regulating apoptosis and cell proliferation in papillary thyroid cancer (80); VLDLR- AS1 modulates the expression of genes involved in fat loss in cancer cachexia, acting as a sponge of hsa-miR-1224-3p (81) and finally, NEAT1, by sponging miR-193b-3p, activates cyclin D, promoting cell proliferation in cervical cancer (82). Notably, the differential expression of these lncRNAs in TETs affects the disease-free survival of patients. In particular, the high expression of ADAMTS9-AS1 and low expression of LINC00324 are correlated with the worst prognosis of patients. Similar to other types of cancer, the expression of these lncRNAs was found to be correlated with the deregulation of miRNA clusters and target genes involved in the regulation of tumorigenic signaling pathways, including PI3K/Akt, FoxO, HIF-1, and Notch, supporting their oncogenic role in the tumorigenesis process (76). Moreover, Gong et al. found that AFAP1-AS1, LINC00324, and VLDLR-AS1 were associated with the RFS of patients with TETs (83).

According to these data, bioinformatic analysis performed for different types of TETs (A, B, AB, and TC) revealed that different competitive endogenous RNA (ceRNA) networks were significantly associated with the overall survival of individuals. The two most important lncRNAs in this ceRNA network were LINC00665 and NR2F1-AS1. The association between their expression and patient prognosis aligns with their biological function (84). LINC00665 binds to mRNAs MYO10 and WASF3 through the miRNAs, hsa-miR-140 and hsa-miR-3199. LINC00665 is upregulated in lung cancer, regulates cellular proliferation and invasive ability in lung adenocarcinoma, and is a predictive factor of this tumor (85). NR2F1-AS1 can indirectly interact with FBN1, GALNT16, HAND2, and MCAM through miR-140, miR-139, and miR-141. NR2F1-AS1 leads to the impairment of osteosarcoma, acting as a sponge of miR-483-3p and increasing FOXA1 gene expression (86).

Based on these recent studies, profiling analysis of lncRNA expression can be used as a potential and innovative strategy for the detection and follow-up of thymic epithelial tumors (**Table 1**).

LncRNAs IN MG

The alteration of lncRNA expression could play a prominent role in distinguishing thymomatous and non-thymomatous MG and clarifying the molecular mechanisms underlying its pathogenesis. In this context, by using lncRNA and mRNAs microarray analyses, Luo et al. (87) identified an aberrant expression of different lncRNAs between MG patients with thymoma and healthy controls, and MG patients without thymoma and normal individuals. In the first case, lncRNAs upregulated in MG patients with thymoma were associated with different regulatory pathways that contribute to thymic cancer progression and immune cell proliferation, such as cell response to interferon-γ, positive regulation of cytokine production, chemokine receptor binding, and regulation of smooth muscle cell proliferation. In particular, the most upregulated lncRNA in MG patients with thymoma is Oebiotech_11933, an lncRNA related to the MAPK, chemokine, and Toll-like receptor signaling pathways (87–89). In the second case, although altered lncRNAs in MG patients without thymoma revealed

TABLE 1 | LncRNAs deregulated in TETs.

lncRNAs	Expression	Biological function	Prognostic clinic value	References
LOXL1-AS1	Upregulated in thymoma and thymic carcinoma	LOXL1-AS1 acts as a sponge for miR-525-5p, increasing HSPA9 expression.	High levels of LOXL1-AS1 and HSPA9 are associated with poor prognosis	**Wang et al.** (62)
LINC00174	Upregulated in thymoma and thymic carcinoma	LINC00174 acts as a sponge for miR-145-5p	High levels of LINC00174 and low level of miR-145-5p are associated with poor prognosis	**Tito et al.** (65)
MALAT1	Upregulated in thymic carcinoma	MALAT1 acts as a sponge for miR-145-5p. MALAT1 localization is m^6A-dependent and is involved in c-MYC induction	High levels of MALAT1 are associated with poor prognosis	**Tan et al.** (69) **Iaiza et al.** (71)
RP11-424C20.2	Upregulated in thymoma	RP11-424C20.2 acts as a sponge for miR-378a-3p, increasing UHRF1 expression	High levels of RP11-424C20.2 and UHRF1are associated with better prognosis	**Yang et al.** (73)
AFAP1-AS1 LINC00324 VLDLR-AS1	Upregulated in thymoma	They are involved in the regulation of cell proliferation	High levels of AFAP1-AS1 and low levels of LINC00324 and VLDLR- AS1 are associated with poor disease-free survival	**Ji et al.** (76)
LINC00665	Upregulated in thymoma	LINC00665 acts as a sponge for miR-140 and miR-3199, increasing MYO10 and WASF3	High levels of LINC00665 are associated with poor overall survival	**Chen et al.** (84)
NR2F1-AS1	Upregulated in thymoma	NR2F1-AS1 acts as a sponge for miR-140, miR-139 and miR-141, increasing FBN1, GALNT16, HAND2, and MCAM expression	High levels of NR2F1-AS1 are associated with poor overall survival	**Chen et al.** (84)

their association with the same cellular pathways in MG patients with thymoma (i.e., positive regulation of cytokine production and chemokine receptor binding), they showed a lower association with cell response to interferon-γ. These data highlight that the discrimination between MG patients with or without thymoma may depend on the presence of altered lncRNAs involved in the regulation of IFN-γ expression (90). Additionally, these lncRNAs have been observed to function by regulating the transcription of genes in *cis* or *trans* (87). Consistent with this study, Ke et al. found another lncRNA, XLOC_003810, which is highly expressed in MG-associated thymoma patients, and revealed an increase in activated CD4+ T cells compared to that in control samples. *In vitro* experiments using thymic mononuclear cells demonstrated that the overexpression of XLOC_003810 leads to an increase in CD4+ T cells and production of the inflammatory cytokines IFN-γ, TNF-α, and IL-1β. In contrast, the downregulation of XLOC_003810 caused the opposite results. Consequently, as the activation of CD4+ T cells and inflammatory cytokines plays an important role in the development of thymoma-associated MG, XLOC_003810 lncRNA could contribute to the pathogenesis of these cellular pathways (91). The study by Niu et al. supports the role of XLOC_003810 in MG with thymoma. XLOC_003810 affects the balance between T helper 17 (Th17) and T regulatory cells (Tregs) (92). T helper cells are active in the adaptive immune response against antigens and pathogens, and Tregs have suppressive potential, preventing autoimmune diseases (93). The overexpression of XLOC_003810 leads to higher levels of Th17 cells than Tregs, which, on the contrary, increases upon silencing of this lncRNA. This association is also evident in MG-T patients and is characterized by an increase in CD4+ T cells and Th17 cells and a decrease in Treg cells (92). As the number of Tregs increases in MG-T patients upon immunosuppressive treatment (94) and the number of Th17 cells correlates with the severity of the disease (95), the alteration of XLOC_003810 expression could enhance the imbalance in the Th17/Treg ratio, favoring the pathogenetic mechanism. Moreover, the discrimination between patients with MG with or without thymoma is also determined by the different hypomethylation and hypermethylation levels associated with the aberrant expression of lncRNAs. The presence of DNA methylation sites has been observed in three immune-related lncRNAs, namely AC004943.1, FOXG1-AS1, and WT1-AS, in (MG-T) patients. DNA methylation is an epigenetic modification catalyzed by DNA methyltransferase enzymes that promote the silencing of gene expression. In this context, tissue samples of thymoma patients with MG are characterized by lower methylation levels of these lncRNAs than those without MG. Consequently, MG-T patients showed a higher expression of these immune-related lncRNAs that correlate with their involvement in pathogenesis, regulating different biological processes, such as transmission at the neuromuscular junctions, cell cycle, actin and Ras GTPase binding, and herpes simplex virus 1 infection associated with MG development (96). Although the lncRNA, MALAT1, is a known oncogene that promotes thymic cancer development, as described previously, it plays a protective role in MG (**Figure 2**). Compared to healthy individuals, lower expression of MALAT1 has been observed in MG patients, together with higher expression of miR-338-3p, an oncogenic miRNA that directly targets MSL2, a gene involved in chromatin organization and DNA damage response. A previous study revealed the interaction between MALAT1, miR-338-3p, and MSL2 by luciferase assay, demonstrating that the silencing of MALAT1 leads to an increase in miR-338-3p expression, reducing MSL2 protein levels (97). The downregulation of MALAT1 in MG patients suggests its involvement in the inhibition of T lymphocytes, suggesting that it could be a specific target for MG treatment. Finally, different lncRNAs are

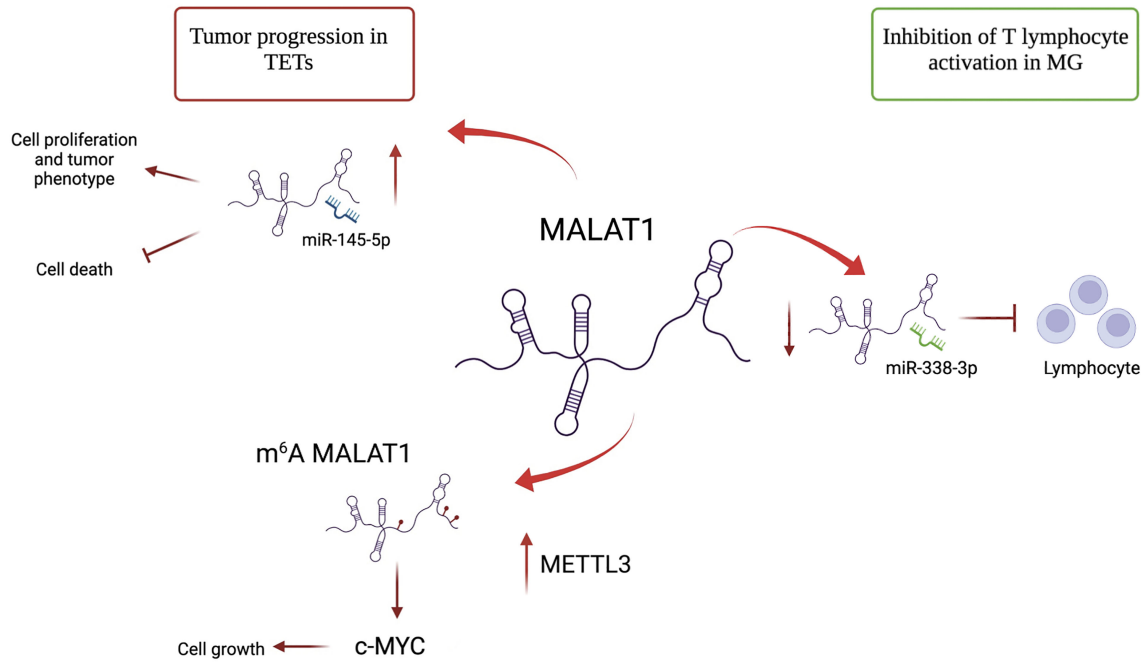

FIGURE 2 | Different roles of lncRNA MALAT1 in TETs and MG. MALAT1 has an oncogenic role in TETs. It promotes cell proliferation by acting as a sponge of miR-145-5p. MALAT1 is m^{6}A-modified due to METTL3 overexpression in TET and induces c-MYC protein, further contributing to proliferation. In MG, MALAT1 plays a protective role by binding mir-338-3p and avoiding T lymphocyte activation.

involved in the regulation of hydrolase, phosphorylase, and dephosphorylase enzyme activities, which affect the activation of T cells during selection in the thymus, promoting MG development (98).

Based on the evidence, a large spectrum of lncRNAs can regulate different signaling pathways that contribute to the development of associated MG-thymoma. As a result, they could be used as biomarkers to distinguish between two types of MG, namely MG with or without thymoma. Moreover, the data suggest the possible use of these pathogenesis-related molecules as therapeutic targets (**Table 2**).

CONCLUDING REMARKS

Recent advances in next-generation sequencing technologies have enabled the study of the role of ncRNAs in the development and progression of cancer. Particularly, the aberrant expression of the most studied groups of ncRNAs, such as miRNAs, circRNAs, and lncRNAs, is associated with tumorigenesis, highlighting the role of ncRNAs as oncogenes or tumor suppressors. In this review, we sought to provide an overview of lncRNA regulation in the initiation and progression of TET and MG. Many lncRNAs identified in these diseases play an oncogenic role, acting as

TABLE 2 | LncRNAs deregulated in Myasthenia Gravis.

lncRNAs	Expression	Biological function	Prognostic clinic value	References
XLOC_003810	Upregulated in myasthenia gravis associated with thymoma	XLOC_003810 increases CD4+ T cell activation and inflammatory cytokines, such as IFN-γ, TNF-α, and IL-1β XLOC_003810 regulates Th17/Treg balance	CD4+ T cells are activated, and inflammatory cytokines are significantly expressed in the thymic tissue of MG patients with thymoma. Positive correlation between XLOC_003810, which promotes the shift in Treg cells toward Th17 cells, and the clinical severity of the disease in MG-T patients.	**Hu et al.** (91) **Niu et al.** (92)
AC004943.1 **FOXG1-AS1** **WT1-AS**	Upregulated in myasthenia gravis associated with thymoma	They are involved in the regulation of transmission of neuromuscular junctions, cell cycle, actin, Ras GTPase binding, and herpes simplex virus 1 infection.	The higher expression of these lncRNAs was correlated with a lower DNA methylation level and influence the prognosis of thymoma	**Zhuang et al.** (96)
MALAT1	Downregulated in myasthenia gravis associated with thymoma	MALAT1 acts as a sponge of miR-338-3p, reducing MSL2 expression levels	Low expression of MALAT1 in MG patients compared with controls, suggesting that it inhibited T lymphocyte activation and the protective effect in the pathogenesis of MG	**Kong et al.** (97)

sponges of tumor suppressor miRNAs and consequently regulating many cellular pathways that contribute to the cancer phenotype. The identification of aberrant expression of lncRNAs and studies on their inhibition or overexpression allow us to understand their contribution to the thymic cancer phenotype and suggest specific targeted therapies. Various lncRNAs differentially expressed in tumor vs. normal tissues in patients with TET are potential powerful biomarkers for the detection and follow-up of diseases.

The role of the lncRNA, MALAT1, which has several opposing functions, is particularly intriguing. In thymoma and thymic carcinoma, MALAT1 regulates cell proliferation by acting as an miR-145-5p sponge and contributing to c-MYC induction, following its change in subnuclear localization due to METTL3 methylation. In contrast, MALAT1 has a protective role in MG, acting as an miRNA sponge and inhibiting T lymphocyte activation. Although several lncRNAs have been identified to date, the function and expression of many lncRNAs in TETs and MG pathogenesis and progression remain unclear. Therefore, further studies on these ncRNAs are necessary. Moreover, the development of novel lncRNA-directed therapeutic strategies could represent a promising and powerful approach for the management of TET and MG.

AUTHOR CONTRIBUTIONS

AI and CT designed and wrote the manuscript. SM, GF, MM, and FF reviewed and edited the manuscript. FG, AS, EG, AF, EM, VP, FV, and GB conceptualized the study and provided feedback regarding the content of the manuscript. All authors approved the final version of the manuscript.

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Abrogation of Notch Signaling in Embryonic TECs Impacts Postnatal mTEC Homeostasis and Thymic Involution

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Notch signaling is crucial for fate specification and maturation of thymus-seeding progenitors along the T-cell lineage. Recent studies have extended the role of Notch signaling to thymic epithelial cells (TECs), showing that Notch regulates TEC progenitor maintenance and emergence of medullary TECs (mTECs) in fetal thymopoiesis. Based on immunohistochemistry studies of spatiotemporal regulation of Notch activation in the postnatal thymus, we show that *in vivo* Notch activation is not confined to fetal TECs. Rather, Notch signaling, likely mediated through the Notch1 receptor, is induced in postnatal cortical and medullary TECs, and increases significantly with age in the latter, in both humans and mice, suggesting a conserved role for Notch signaling in TEC homeostasis during thymus aging. To investigate the functional impact of Notch activation in postnatal TEC biology, we used a mouse model in which RPBJκ, the transcriptional effector of canonical Notch signaling, is deleted in epithelial cells, including TECs, under the control of the transcription factor Foxn1. Immunohistochemistry and flow cytometry analyses revealed no significant differences in TEC composition in mutant (RPBJκ-KO^{TEC}) and wild-type (WT) littermate mice at early postnatal ages. However, a significant reduction of the medullary region was observed in mutant compared to WT older thymi, which was accompanied by an accelerated decrease of postnatal mTEC numbers. Also, we found that organization and integrity of the postnatal thymic medulla critically depends on activation of the canonical Notch signaling pathway, as abrogation of Notch signaling in TECs led to the disruption of the medullary thymic microenvironment and to an accelerated thymus atrophy. These features paralleled a significant increase in the proportion of intrathymic non-T lineage cells, mostly B cells, and a slight decrease of DP thymocyte numbers compatible with a compromised thymic function in mutant mice. Therefore, impaired Notch signaling induced in embryonic development impacts postnatal TECs and leads to an accelerated mTEC degeneration and a premature thymus involution. Collectively, our data have uncovered a new role for Notch1 signaling in the

control of adult mTEC homeostasis, and point toward Notch signaling manipulation as a novel strategy for thymus regeneration and functional recovery from immunosenescence.

Keywords: thymus, notch, thymic epithelial cells, premature degeneration, thymic involution

INTRODUCTION

T lymphocytes, unlike the rest of blood cell lineages derived from multipotent hematopoietic progenitor/stem cells (HPCs), develop in a specialized organ distinct from the bone marrow or the embryonic liver; *i.e.* the thymus (1). Thymic epithelial cells (TECs) are the specific components of the thymus microenvironment that provide unique inductive signals for keeping early thymic progenitors on track to T-cell differentiation (2–4). Two molecularly and functionally distinct TEC subsets are sequentially involved in T-cell development, cortical (c) TECs and medullary (m) TECs, which are located at the thymus cortex and medulla, respectively. cTECs impose T-cell commitment and induce the differentiation, expansion and positive selection of developing thymocytes, by providing continuous activation of the evolutionary conserved Notch signaling pathway (5–7) through the expression of the nonredundant Delta-like 4 (DLL4) Notch ligand (8, 9). Notch is a family of transmembrane receptors (Notch1 to Notch4 in mammals) with a major role in the regulation of critical processes such as cell fate specification, differentiation and proliferation/apoptosis in multiple cell lineages. Upon interaction with a membrane-bound specific ligand (Delta-like or Jagged in mammals), the intracellular domain of Notch (ICN) is proteolytically cleaved and released, entering the nucleus where it behaves as a transcriptional regulator of downstream genes, activating a particular genetic program (10, 11). In the thymus, progenitors that interact with TECs in the cortex activate the T-cell maturation program and then migrate to the medulla where mTECs promote their terminal differentiation and participate in central tolerance induction (12–14).

Despite its unique and crucial function in generating self-restricted and self-tolerant functional T cells throughout life, the thymus is the first organ to undergo aged-related involution. This is an evolutionary conserved process beginning as early as birth and no later than the onset of puberty in humans and mice (15). Thymic involution mainly results from the degeneration of the epithelial component of the thymic stroma and is characterized by dramatic reductions in thymus size and TEC numbers, the expansion of adipocytes and fibroblasts, and the disorganization of the thymic architecture, leading to diminished thymocyte numbers and reduced naïve T cell output (16–18). These features characterize as well the thymic involution process induced under physiological stress conditions such as infection, pregnancy, and cancer treatments (reviewed in 18). While several molecular mechanisms have been proposed to be involved in stress-induced acute thymic atrophy, the underlying mechanisms of chronic age-related involution remain less clear. Recent studies have documented many changes of TEC biology throughout life, revealing a surprisingly dynamic population with a high turnover (17). Therefore, understanding how TEC maintenance and regeneration are regulated in the adult thymus is of critical relevance for understanding thymic involution.

cTECs and mTECs arise early in ontogeny from a common thymic epithelial progenitor cell (TEPC) originated in the thymic primordium derived from the embryonic third pharyngeal pouch endoderm (19). This bipotent TEPC was identified in the fetal thymus (20–22) and its existence has been confirmed in the adult thymus (23–26), although the physiological contribution of bipotent TEPC to adult TEC generation remains controversial (27). In the embryo, differentiation of TEPCs into cTEC and mTEC lineages and development of a functional thymus is critically controlled by the transcriptional regulator Foxn1 (23, 28), which is induced in TEPCs by signals provided by other thymic components, including developing thymocytes (29–31). However, how cTEC/mTEC lineage specification and differentiation from the TEPC is induced has been a matter of intense debate. Studies showing that fetal TEPCs exhibit features and markers associated with the cTEC lineage (32, 33), support a serial progression model of TEC differentiation, in which cTEC lineage represents a default pathway, whereas mTEC specification from the common TEPC requires additional specific cues (34). The potential mechanisms controlling this mTEC specification step and the emergence of separate mTEC- and cTEC-restricted progenitors have remained poorly understood, although independent evidence has begun to emerge suggesting that the Notch pathway may be involved. In fact, signaling provided by the DLL1 Notch ligand induces maturation of fetal mTECs leading to the organization of medullary areas in a FTOC (35), while mice deficient in Jagged2 have thymi with reduced medullary areas (36). In the adult thymus, however, TEC-specific overexpression of active Notch leads to inhibition of mTEC lineage development and reduced TEC cellularity (37), indicating that Notch expression by TECs might be temporally regulated. Recently, two groups have provided genetic evidence that Notch signaling plays a crucial role at multiple embryonic stages during TEC development, but may be dispensable in postnatal life (38, 39). Importantly, they showed that Notch activation is required for maintenance/expansion of the undifferentiated TEPC and mTEC-restricted progenitor pools, and also for mTEC fate induction (38), while once the mTEC lineage was specified, further mTEC development was independent of Notch activity. Accordingly, repression of the Notch pathway was shown mandatory for progression of early mTECs to the mature mTEC stage (39), a fact that concurs with the downregulation of Notch activation in TECs after birth (35, 37). Collectively, these data have revealed a critical role of Notch as a potent regulator of TEPC homeostasis and mTEC lineage fate during

fetal thymus development, although Notch function in the epithelial compartment of the postnatal thymus remains to be investigated. This is an important issue, regarding the hypothetical contribution of TEPC to adult TEC turnover (17, 23–27), which may impact the dynamics of thymus involution and its consequences to immunosenescence.

In this study, we have approached the potential contribution of Notch to postnatal TEC biology using two complementary strategies. First, we performed quantitative immunohistochemistry and confocal imaging approaches of *in situ* thymus Notch signaling (40) and provide evidence of a spatiotemporal regulation of *in vivo* Notch activation in both human and mouse postnatal TECs. Then, we made use of an *in vivo* genetic model of *Foxn1*-controlled conditional inactivation of Notch signaling in murine epithelial cells, including TECs, and reveal that lack of Notch signaling accelerates age-dependent loss of mTEC numbers and affects medulla integrity in the postnatal thymus. Therefore, we suggest a key role for Notch signaling in the control of postnatal mTEC homeostasis and age-dependent thymic involution.

MATERIALS AND METHODS

Human and Mouse Thymus Samples

Human thymus biopsies were obtained from male and female Caucasian pediatric patients aged 3-days to 15-years undergoing corrective cardiac surgery, after informed consent was provided, and in accordance with the Declaration of Helsinki and to the procedures approved by the Spanish National Research Council Bioethics Committee.

Animal studies were reviewed and approved by the Animal Experimentation Ethics Committee of the Comunidad de Madrid, in accordance with the recommendations of the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (ETS 123). Mice were kept under specific pathogen-free conditions and used according to institutional regulations. C57BL/6J mice were obtained in-house from the departmental breeding facility. C57BL/6J *RBPjκ*$^{fl/fl}$ conditional knockout mice generated by Prof. Tasuku Honjo (41) and C56BL/6J *Rosa26*^{loxPLacZ} reporter mice (Jackson Laboratory) were obtained from Dr. Jose Luis de la Pompa (CNIC, Madrid). The transgenic B6D2F1/J Foxn1-Cre line containing seven copies of the Cre transgene under the control of the *Foxn1* promoter has been previously generated (42), and heterozygous mutants (*Foxn1*$^{Cre/+}$) were kept as a colony. Mice homozygous for a conditional deletion of RBPjκ specifically in epithelial cells were obtained by crossing *Foxn1*$^{Cre/+}$ heterozygous to *RBPjκ*$^{fl/fl}$ homocygous mice, followed by backcrossing of resultant *Foxn1*$^{Cre/+}$ *RBPjκ*$^{fl/+}$ F1 heterozygous to *RBPjκ*$^{fl/fl}$ homocygous mice (**Supplementary Figure 1A**). Further selection of *Foxn1*$^{Cre/+}$*x RBPjκ*$^{fl/fl}$ mice was performed by PCR genotyping (**Supplementary Material**). These mice, referred to as RBPjκ-KO^{TEC}, displayed Cre-mediated *RBPjκ* deletion exclusively in epithelial cells, including TECs, but not in other thymic cells. Mouse gender was not considered in any experiment. *Foxn1*$^{+/+}$*x RBPjκ*$^{fl/fl}$ littermates were used as wild-type (WT) controls. Selection of crossed mice was performed by PCR genotyping of genomic DNA obtained by proteinase K (Sigma) digestion of 3 weeks-old mouse ear discs tissue, as described in **Supplementary Material**.

Immunohistochemistry and Confocal Microscopy

Tissue samples were fixed in 4% paraformaldehyde/phosphate-buffered saline (PBS) [PFA/PBS, Sigma-Aldrich] and paraffin-embedded (Paraplast Plus, Sigma-Aldrich). Serial 8 µm sections were obtained from formalin-fixed paraffin-embedded (FFPE) slides that were mounted on poly-lysine-coated slides (SuperFrost UltraPlus, Thermo Fisher Scientific). Deparaffinised, rehydrated FFPE tissue slides were properly blocked as previously described (40). Tissue antigens were retrieved by boiling in sodium citrate (10 mM, pH 6.0) and endogenous peroxidase activity was quenched using 1% H2O2 100% methanol. For blocking of non-specific antibody binding sites, samples were incubated for 1h in blocking solution (3% bovine serum albumin, 20 mM MgCl2, 0.3% Tween 20, 5% fetal bovine serum in PBS), and permeabilized slides were incubated in blocking solution containing primary antibodies (**Supplementary Table 1**). Background and nonspecific staining was determined by incubating with Ig isotype-matched controls (**Supplementary Figures 2, 3**). Before addition of secondary antibodies, tissue endogenous biotin was quenched with Avidin/Biotin blocking solutions (Vector Laboratories). For Jag1 signal detection, tissue slides were incubated for 1 hour at RT with a horseradish peroxidase (HRP)-coupled anti-rabbit IgG secondary antibody (DAKO) and the signal was amplified using a Cyanine-3 Tyramide Signal Amplification (TSA-Cy3) Kit (NEL 744, 25 Perkin Elmer). For Notch1, Notch3, Notch4 and cleaved Notch1 (ICN1) signal detection, biotinylated anti-rabbit IgG secondary antibody (Vector Laboratories) was added before signal amplification with an Avidin/Biotin-HRP complex (Elite Vectastain ABComplex Kit, Vector Laboratories) and TSA-Cy3 Kit. For pan-cytokeratine (pCK) signal detection, Alexa Fluor dye-conjugated secondary antibodies were used (Thermo Fisher Scientific). The ABC-amplified signal was developed by adding Alexa Fluor 488- or Alexa Fluor 555-conjugated streptavidin (Thermo Fisher Scientific). Nuclei were stained with Topro3 (Thermo Fisher Scientific) and slides mounted with Fluoromount-G (SouthernBiotech).

Images were acquired using an LSM510 or an LSM900 laser scan confocal microscope (Zeiss) coupled to an Axio Imager.Z1 or an Axiovert 200 or an Axio Imager 2 (Zeiss) microscope using the following magnifications (Zeiss): 10× Plan-Neofluar (numeric aperture [NA] 0.3), 25× Plan-Neofluar [oil (NA 0.8)], 40×Plan-Neofluar [oil (NA 1.3)], 40xPlan-Apochromat [oil (NA 1.3)] and 63×Plan-Apochromat [oil (NA 1.4)]. Images were processed using ImageJ. Brightness and contrast were adjusted equally in samples and controls when needed. For defining nuclear (Hes1, ICN1 and Topro) regions of interest (ROIs), Otsu algorithm was used to select positive cells by intensity threshold (43) For defining pCK^{+} ROIs, Li algorithm

(44) was used (**Supplementary Figure 3**). A median filter at 0.2 μm was used to remove noise before creating the selections.

Quantitative analyses of $Hes1^+$ or $ICN1^+$ cell numbers in thymus cortical or medullary regions was performed by using image thresholding (45). As TECs, and particularly cTECs, form an extensive network of finely branched cell processes, numbers of individual TECs in this network are difficult to define (46). Therefore, no quantitative measurements of TEC frequencies, especially of $Hes1^-$/$ICN1^-$ TECs, in the cortex *vs* the medulla could be performed. Rather, total numbers of $Hes1^+$/$ICN1^+$ nuclei within pCK^+ ROIs were calculated relative to total Topro area or to pCK^+ area defined in the thymus cortex or the medulla (**Supplementary Figure 3**). To this end, pCK^+ ROIs were first defined as described above, and then used to create binary masks. Both nuclear (Hes1 or ICN1) and pCK binary masks where then processed on Image J's "Image Calculator" using the logic operator "AND". During image processing, a particular pixel intensity level (the threshold) is automatically defined by algorithms. Then, the number of pixels within the threshold is used to make a selection of ROIs, which exclusively contain the pCK-specific signal. The ROI is then used to calculate the total pCK^+ area of TECs (in μm) and the number of $Hes1^+$/$ICN1^+$ nuclei within. Every cell out of the pCK ROI, including thymocytes positive for Notch activation markers, are systematically excluded and thus not considered in the analysis

Histomorphometric measurements of thymic cortex and medulla (**Supplementary Figure 4**) were also performed in ImageJ by ROIs using Jag1 and/or Topro intensity level threshold (43).

Hematoxilin/Eosin and β Galactosidase (LacZ) Staining

Skin samples were fixed in 4% paraformaldehyde (PFA)/PBS solution (Sigma-Aldrich) and embedded in paraffin. Deparaffinised tissue slides were incubated for 3 min. in Harry's hematoxilin (Sigma), washed and quickly differentiated (10 to 15 sec) in acid alcohol solution (0.5% HCl; 70% ethanol). Next, they were incubated for 9 min. in 0.5% (w/v) Eosin solution (Sigma) and sequentially dehydrated in graded ethanol series. Tissue slides were briefly incubated in xylene, mounted with Entellan mounting medium (Merck, Millipore), and analyzed with an optical microscope (DM2500; Leica) equipped with a CCD camera (DFC420; Leica), with Leica Application Suite software (version 4.3.0).

For β-galactosidase staining, thymic samples were fixed in 0.125% glutaraldehyde/PBS solution, washed (0.02% Nonidet-P40, 0.11% sodium deoxycholate, and MgCl2 2mM in phosphate buffer 0.1M, pH 7.3) and stained with X-gal staining solution (washing buffer supplemented with potassium ferricyanide 5mM, potassium ferrocyanide 5mM and 1mg/ml of X-gal resuspended in N,N-dimethyformamide). Samples were then washed, fixed in 4% PFA/PBS and paraffin-embed. Sections (8 μm) were mounted on poly-lysine-coated slides (SuperFrost Ultra Plus, Thermo Scientific) and deparaffinised as specified earlier. Cell nuclei were stained with Nuclear Fast Red (Vector Labs), sequentially dehydrated in graded ethanol series and xylene, and mounted with Entellan mounting medium (Merck, Millipore).

Flow Cytometry

For flow cytometry TEC analysis, thymus samples from either RBPjκ-KO^{TEC} or *Foxn1*$^{+/+}$*x RBPjk*$^{fl/fl}$ control littermates, no separated by gender and aged from 0.5- to 12-months, were dissociated in RPMI medium (1.25 mg/ml collagenase D (Roche) following three digestion steps of 15 min at 37° C. Isolated cells were then diluted in RPMI1640 medium with 10% FBS (Gibco) containing DNaseI (Roche; 0.05 mg/ml). After filtering cell suspension through 70 μm cell strainer (Filcon) to remove clumps, flow cytometry was performed using a sequential gating strategy (**Supplementary Figure 5**) on cells stained with DAPI (Beckman Coulter) to exclude dead cells, anti-CD45-FITC (eBioscience) and anti-TER-119-FITC (Biolegend) mAb, to exclude hematopoietic and erythroid-lineage cells. Anti-MHCII-PECy7 (eBioscience) and anti-EpCAM-APCCy7 (Biolegend) was used to electronically gate TECs. EpCAM-gated TEC cells were then analyzed for reactivity with the anti-Ly51-PE (eBioscience), and UEA-1 biotinilated (Vector Labs) cTEC and mTEC-specific mAbs, respectively, developed using Streptavidin-APC (Biolegend).

For thymocyte flow cytometry, Ficoll-Hypaque (Lymphoprep, Axis-Shield PoC AS)-separated thymus cell suspensions were stained with the following mAbs: anti-CD8-FITC (Life Technologies), anti-CD4-PE (BD Biosciences), anti-CD19-PE (eBioscience), anti-CD90 (Thy1) (Biolegend), anti-CD11b-FITC (BD Biosciences), anti-B220-PE-Cy5 (Life Technologies), anti-NK1-APC (BD Biosciences). Anti-Ly5.1 and anti-Ly5.2 mAbs (BioLegend) were used in adoptive transfer experiments. Acquisition and analysis was performed in a FACSCanto II (BD Biosciences). All flow cytometry data were analyzed using FlowJo Version 10.0.7.2.

Adoptive Cell Transfer

For the generation of BM chimeric mice, cell suspensions were isolated by Fycoll-Hypaque from BM samples obtained from femurs of 9 weeks-old RBPjκ-KO^{TEC} ($Ly5.2^+$) mice, and BM cells ($5x10^6$) were resuspended in 100μl of sterile PBS and injected i.v. into 8 weeks-old C57BL/6 ($Ly5.1^+$) hosts (n=4) subjected to lethal irradiation (10 Gys) the day before. Recipient mice were euthanized 4 months post-transplantation and thymus reconstitution by $Ly5.2^+$ cells was analyzed by flow cytometry. As control, BM cells from *Foxn1*$^{+/+}$*x RBPjk*$^{fl/fl}$ WT littermates ($Ly5.2^+$) were injected into C57BL/6 ($Ly5.1^+$) irradiated hosts (n=2).

Statistics

Statistical analysis was performed with GraphPad Prism 7.0 Software. The normal distribution of the data was tested using the Shapiro–Wilk normality test. When comparing two means of normal data, statistical significance (p) was determined by the unpaired two-tailed Student's t-test. When comparing two-means of non-normal data, statistical significance (p) was determined by the unpaired Mann-Whitney test. When comparing more than two groups of normal data, one-way ANOVA was used, and for no normal data Klustal-Wallis was

used. When comparing groups of two independent variables, two-way ANOVA was used. In all cases, the a-level was set at 0.05. Data in graphs are presented as mean ± SEM.

RESULTS

Notch Signaling Is Active *In Vivo* in Human and Mouse Postnatal TECs

Detailed analyses of Notch activation in postnatal TECs are scarce in mice and remain to be performed in humans. We approached this issue by three-color immunohistochemistry and quantitative confocal microscopy of several postnatal human (≤ 6-years) and mouse (≤ 5-months) thymus samples labelled with a mAb recognizing the well-established target of canonical Notch signaling Hes1 (13), together with a TEC-specific anti-pCK mAb mix, and with Topro3 for nuclear staining. General examination of representative thymus sections stained with anti-Hes1 and Topro3 revealed a continuous pattern of nuclear Hes1 expression throughout the whole human thymus, which seemed more prominent at the medulla and was similar in the mouse postnatal thymus. Detailed analyses aided by the co-staining with anti-pCK, allowed the identification of $Hes1^+$ pCK^+ TECs at the thymus cortex and medulla in both species (**Figures 1A, C**). $Hes1^+$ cells lacking the pCK TEC maker, characterized in previous studies as developing thymocytes (40), were also identified distributed throughout the inner cortex in both human and mouse thymi; while, as shown previously (40), $Hes1^+$ thymocytes seemed less abundant at the medulla, suggesting that Hes1 expression at the medulla occurs mostly in TECs. (**Figures 1A, C**). A significant fraction of such $Hes1^+$ mTECs, which displayed the highest Hes1 expression levels, was found accumulated in Hassal's corpuscles (HCs) in the human thymus (**Figure 1A**). Therefore, these results indicate that Notch activation is conserved in postnatal cTECs and mTECs. Quantitative confocal analyses based on thresholding image approaches (45; **Supplementary Figure 3**), confirmed that measurable numbers of $Hes1^+$ nuclei were distributed within the cortical and medullary pCK^+ areas analyzed in both human and mouse postnatal thymus samples, with $Hes1^+$ cells being more abundant at the medulla in both species (**Figures 1B, D**). However, no frequencies of $Hes1^+$ cTECs *versus* mTECs could be establish by this approach, as TECs, and particularly cTECs, have a complex morphology and display a high intrathymic cellular density (46), making it difficult to identify individual TECs and to define TEC numbers within particular ROIs. Collectively, these analyses provide the first direct evidence that Notch signaling is active *in vivo* in the human postnatal thymus, in TECs located both at the cortex and the medulla, and confirm that activation of Notch is also induced after birth in the mouse thymus, pointing to a conserved role for Notch signaling in postnatal TEC biology.

The Notch1 Receptor Mediates *In Vivo* Activation of Notch Signaling in Human Postnatal mTECs

While murine fetal TECs express several Notch receptors (35, 38, 39), genetic evidence has been provided that Notch1 is the receptor responsible for Notch activation in mouse embryonic TECs (39). To begin to decipher which Notch receptor/s is responsible for *in vivo* Notch signaling in the human thymus, we analyzed *in situ* Notch receptor expression in tissue sections of human postnatal thymus labeled with the anti-pCK mAb in combination with a mAb specific for either Notch1, Notch3 or Notch4. Immunohistochemistry and confocal microscopy showed that, as expected from previous studies (40), Notch1 is broadly expressed by pCK-negative thymocytes distributed mostly throughout the cortex. In addition, Notch1 was expressed by a minor population of pCK^+ cTECs and by a significant number of mTECs (**Figure 2A**). Notch3 displayed an expression pattern similar to Notch1, and was significantly expressed by cortical thymocytes, but only by few cTECs, while substantial numbers of mTECs coexpressed pCK and Notch3 (**Figure 2A**). In contrast, Notch4 expression was essentially confined to a non-epithelial pCK^- population located at medulla, which has previously been characterized as dendritic cells (47), although rare $Notch4^+$ mTECs could be identified as well (**Figure 2A**). Therefore, as shown before for mouse fetal TECs (39), Notch1 may be the preferential receptor that mediates Notch signaling *in vivo* in human postnatal TECs in both cortex and medulla, with a possible contribution of Notch3 in mTECs.

To directly investigate the contribution of Notch1 to *in vivo* activation of Notch signaling in human postnatal TECs, we performed immunohistochemistry and confocal microscopy, using a mAb against the active intracellular form of Notch1 (ICN1) in combination with anti-pCK antibodies. These analyses confirmed Notch1 activation *in situ* in the human postnatal thymus, revealing nuclear expression of ICN1 in cells distributed throughout both the cortex and the medulla (**Figure 2B**). As shown before (40), we found that significant numbers of cells expressing active Notch1 in the cortex were pCK-negative hematopoieitic cells, although $ICN1^+$ cTECs were also identified, while cells that display Notch signaling at the medulla seemed to be mostly pCK^+ mTECs (**Figure 2B**). Quantitative analyses of imaging data allowed to measure significant numbers of $ICN1^+$ nuclei within the pCK^+ cortical and medullary areas (**Figure 2C**), supporting that both cTECs and mTECs activate Notch1 *in vivo*. Collectively, the observed ICN1 expression pattern suggests that the Notch1 receptor contributes significantly to *in vivo* activation of Notch signaling in human postnatal TECs.

Activation of Notch Signaling Increases With Thymus Age in Postnatal mTECs

In the course of our studies on *in vivo* activation of Notch signaling, we noticed a consistent heterogeneity of $ICN1^+$ cell numbers among human thymus samples at distinct postnatal ages from 1-month to 6-years. Considering that significant physiological changes occur in the human thymus during the first few years of life (15), we wanted to investigate the possibility that activation of Notch signaling could be regulated along time in the postnatal thymus. To this end, we performed quantitative immunohystochemistry and confocal microscopy of ICN1

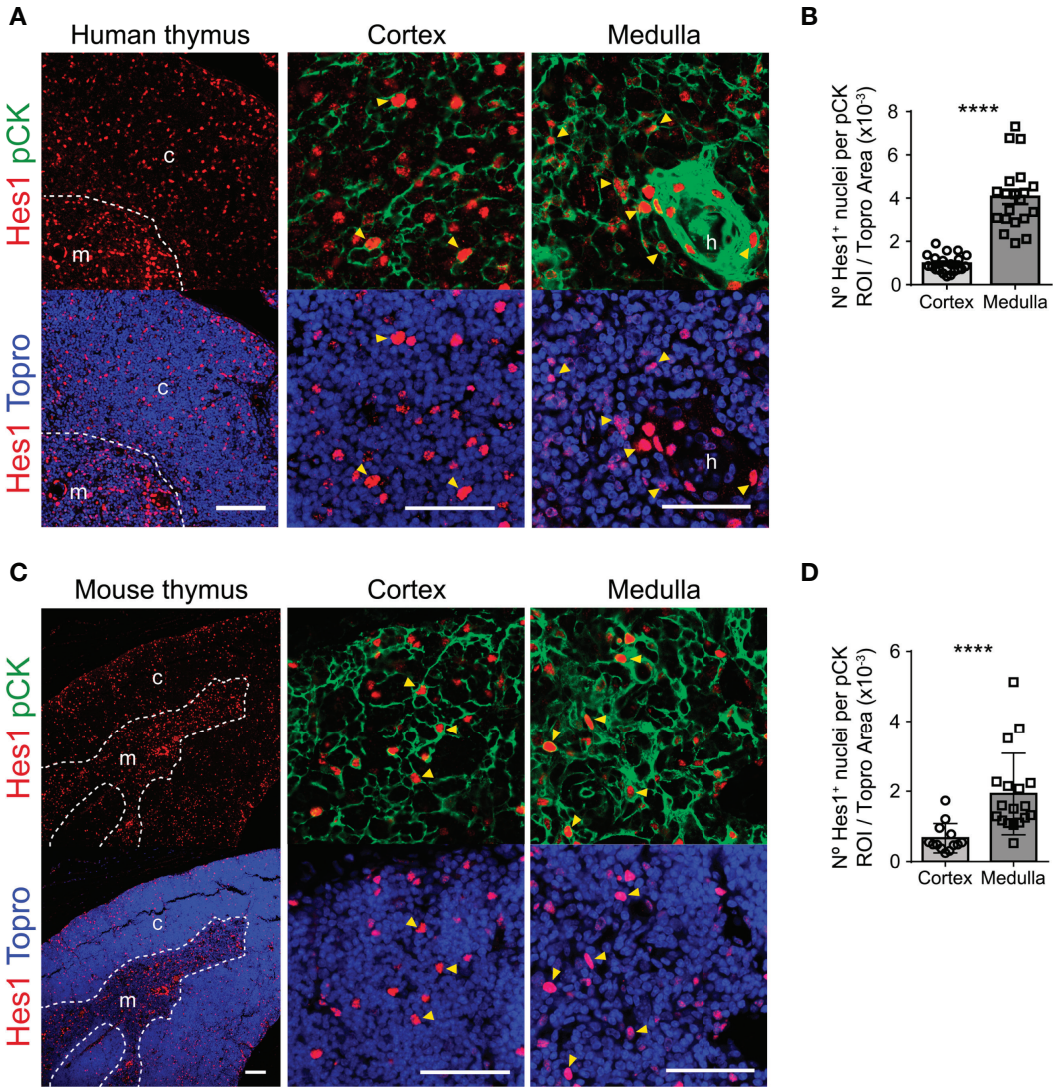

FIGURE 1 | Notch signaling is active *in vivo* in human and mouse postnatal TECs. Immunohistochemistry of the canonical Notch target Hes1 (red) in postnatal human (≤ 6-years) and mouse (≤ 5-months) thymus. TECs are characterized by expression of pCK (green). Topro3 shows nuclear staining (blue). **(A)** General view (scale bar: 100μm) of Hes1 expression in a representative human thymus sample (18-months), and detailed view (scale bar: 50μm) of Hes1 and pCK expression in the thymus cortex and medulla. Dotted line, corticomedullary junction; c, cortex; m, medulla. Arrowheads indicate Hes1 expression in TECs (pCK^+); h, Hassal's corspuscles. **(B)** Bar graphs show numbers of $Hes1^+$ nuclei within pCK^+ ROIs relative to total ($Topro^+$) cellular areas analyzed in the human thymus cortex and medulla. Data are shown as mean numbers ± SEM per field obtained from n= 8-10 different 63x images from sample, (n ≥ 2 independent human thymus samples aged ≤ 6-years). ****$p < 0.0001$. **(C)** General view (scale bar: 100μm) of Hes1 expression in a representative mouse thymus sample (5 months), and detailed view (scale bar: 50μm) of Hes1 and pCK expression in the thymus cortex and medulla. Dotted line, corticomedullary junction; c, cortex; m, medulla. Arrowheads indicate Hes1 expression in TECs (pCK^+). **(D)** Bar graphs show numbers of $Hes1^+$ nuclei within pCK^+ ROIs relative to total ($Topro^+$) cellular areas analyzed in the mouse thymus cortex and medulla. Data are shown as mean numbers ± SEM per field obtained from n= 10 different 63x images from sample, (n ≥ 2 independent mouse thymus samples aged ≤ 5-months). ****$p < 0.0001$.

expression in two groups of human thymus samples representative of early (≤1.5 years) and late (6-13 years) postnatal ages. The selected groups were expected to differ in age-dependent physiological features associated to thymic involution, as regression of the thymic epithelium can be observed early in life in humans, long before puberty (reviewed in 15). As current data in mice have shown that *in vivo* Notch activation during thymopoiesis is selectively induced in medullary-lineage TECs (38, 39), age-dependent Notch activation was specifically analyzed in the thymus medulla. We thus performed detailed image analyses of ICN1 and pCK expression in mTECs and found that activation of Notch1 signaling was more prominent in the medulla of late compared to early human postnatal thymi (**Figure 3A**). Although morphologically heterogeneous, mTECs are less dense than cTECs, and therefore more easily defined as individual cells

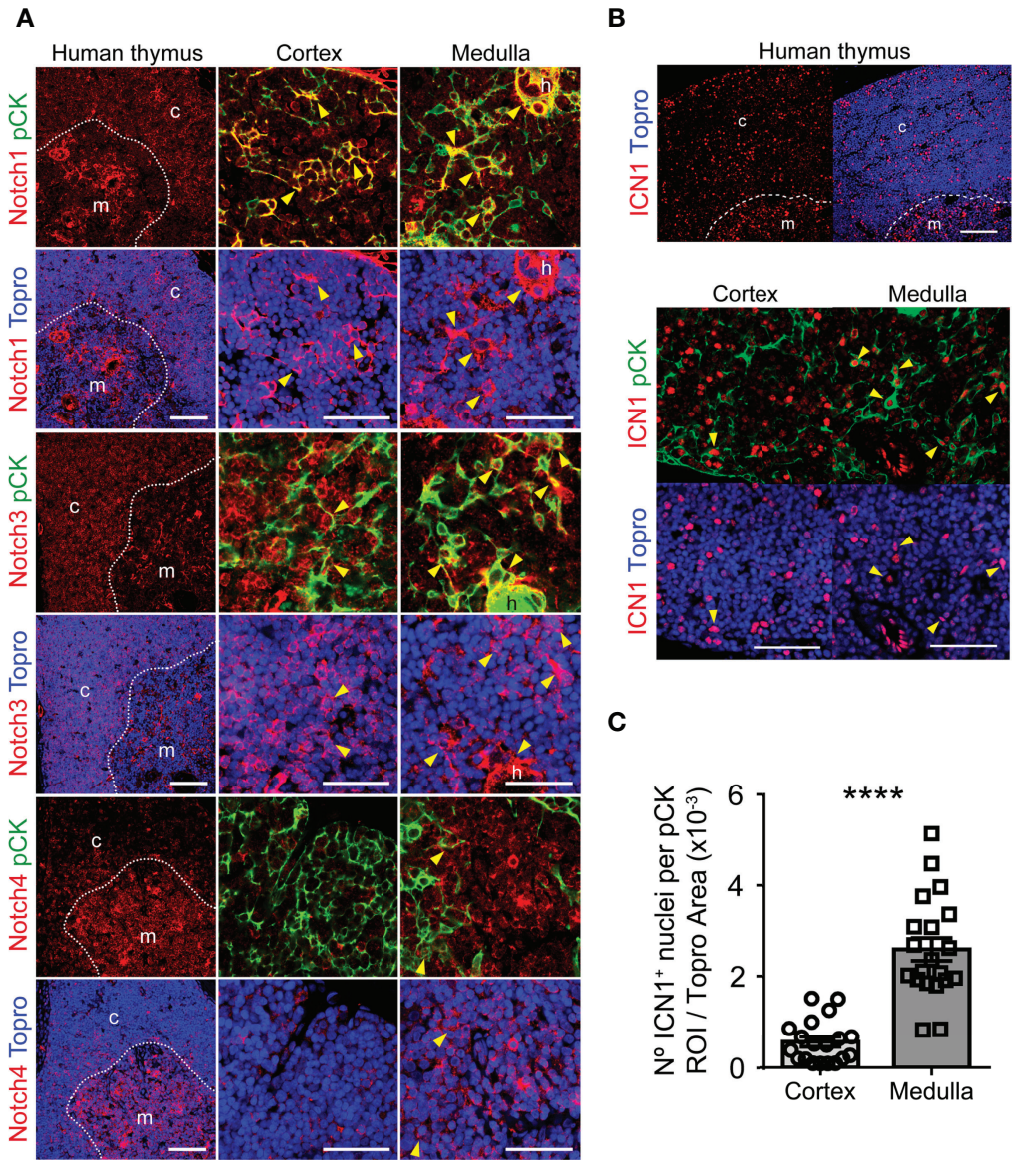

FIGURE 2 | Expression of Notch receptors and activation of Notch1 signaling in human postnatal thymus. **(A)** Immunohistochemistry of the indicated Notch receptors in the human postnatal thymus (≤ 18 months). TECs are characterized by pCK expression (green). Topro3 shows nuclear staining (blue). General view (scale bar: 100μm) of Notch receptor distribution in representative human postnatal thymus samples, and detailed view (scale bar: 50μm) of Notch receptor expression by TECs located at the cortex and medulla. Dotted line, corticomedullary junction; c, cortex; m, medulla. Arrowheads indicate Notch receptor expression by TECs (pCK^+). Images shown are representative of n ≥ 5 different 63x images from n = 2 independent human thymus samples. **(B)** Immunohistochemistry of active intracellular Notch1 (ICN1) (red) in representative human postnatal thymus samples (≤ 6-years). TECs are characterized by expression of pCK (green). Topro3 shows nuclear staining (blue). General view (scale bars: 100μm) of ICN1 expression (top) and detailed view (scale bars: 50μm) of ICN1 and pCK staining in human thymus cortex and medulla (bottom). Dotted line, corticomedullary junction; c, cortex; m, medulla. Arrowheads indicate ICN1 expression in TECs. **(C)** Bar graphs showing numbers of $ICN1^+$ nuclei within pCK^+ ROIs relative to total ($Topro^+$) cellular areas analyzed in human thymus cortex and medulla samples. Data are shown as mean numbers ± SEM per field obtained from n=10 different 63x images per thymus sample (n=3 independent thymus samples aged ≤ 6-years), ****$p < 0.0001$.

(46), allowing us to perform quantitative measurements of pCK^+ cells expressing nuclear ICN1, as shown in **Supplementary Figure 3B**. These analyses revealed that numbers of mTECs expressing ICN1 increased 50% on average in the late compared to the early human postnatal thymus (**Figure 3B**), supporting an age-dependent activation of Notch1 signaling in mTECs. Then, we investigated whether this progressive increase of mTECs expressing active Notch1 could be observed in mice. To this end, we performed quantitative analyses of *in situ* Notch1 activation in mTECs from mice aged 2-weeks to 9-months

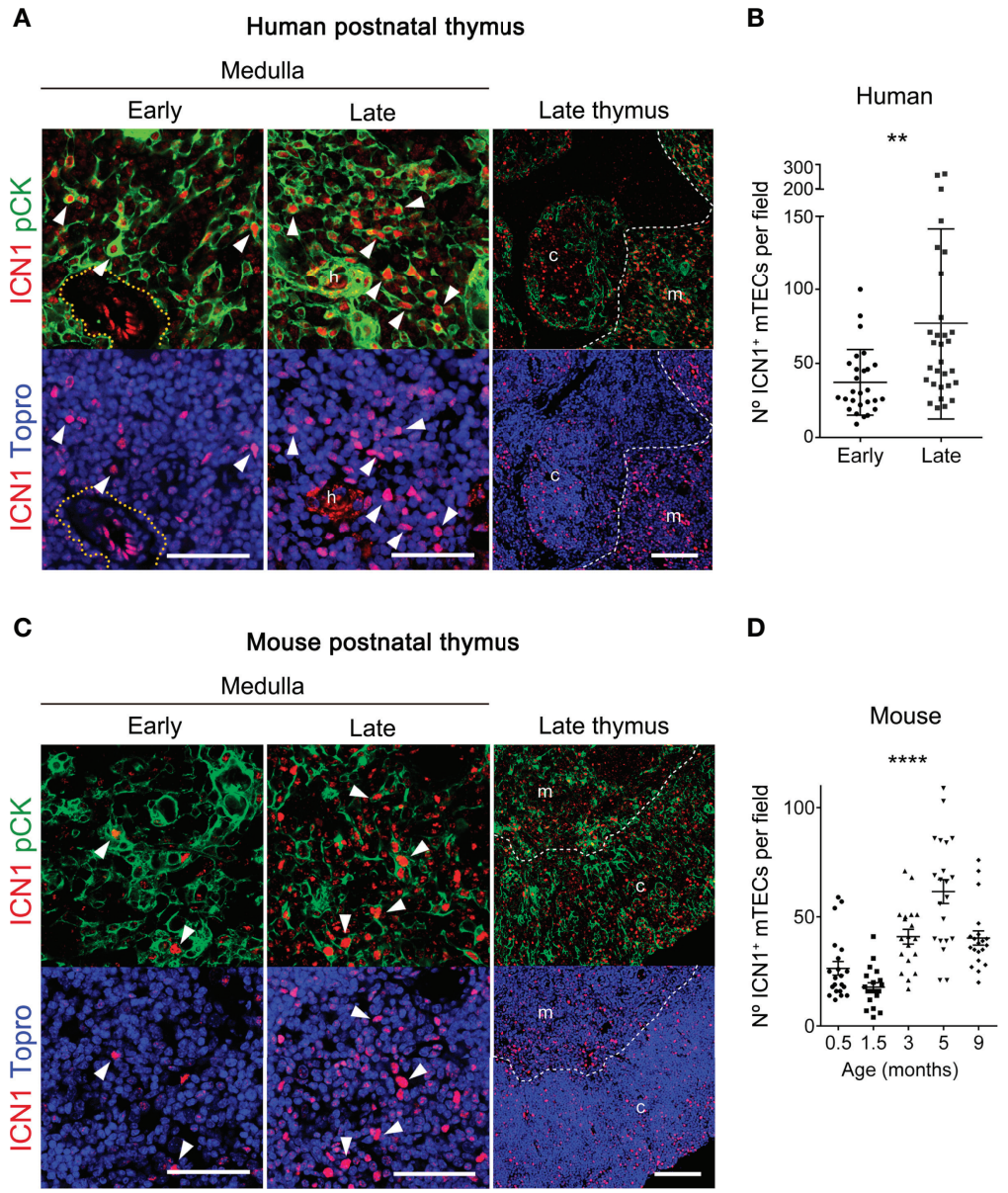

FIGURE 3 | Notch1 signaling increases with thymus age in postnatal mTECs. **(A, C)** Immunohistochemistry of active intracellular NOTCH1 (ICN1, red) and pCK (green), with nuclei in blue (Topro3) either in human thymus samples representative of early (≤ 1.5-years) and late (6-13 years) postnatal ages **(A)**, or in mouse early (≤ 4-weeks) and late (3-9 months) postnatal thymi **(C)**. General views (scale bar: 100μm) of ICN1 and pCK expression in representative late thymus samples are shown on the right. Detailed views (scale bars: 50μm) of ICN1 and pCK expression at the early and late thymus medulla are shown in the left and middle panels, respectively. Dotted line defines the perivascular space; * indicates endothelial cells expressing ICN1; h, Hassal's corspuscles. Arrowheads indicate ICN1 expression in pCK^+ TECs. Images are representative of n ≥ 10 different images from independent sample (n ≥ 3 thymus samples). **(B)** Numbers of $ICN1^+$ pCK^+ mTECs in human thymus samples representative of early (≤1.5-years) and late (6-13 years) postnatal ages labelled as in **(A)**. Data are shown as mean numbers ± SEM per field obtained by counting $ICN1^+$ pCK^+ medullary cells from n ≥ 10 different 63x images per thymus sample (n= 3 independent thymus samples from each group of age), $^{**}p < 0.01$. **(D)** Numbers of $ICN1^+$ pCK^+ mTECs in thymus samples of mice aged from 0.5- to 9-weeks, labelled as in B). Data are shown as mean numbers ± SEM per field obtained by counting $ICN1^+$ pCK^+ medullary cells from n=10 different 63x images per thymus sample (n= 2-3 independent thymus samples from each group of age), $^{****}p < 0.0001$.

(**Figures 3C, D**). The results showed a slight, but not significant, decrease in the numbers of murine mTECs that expressed active Notch1 during the first weeks of life from 0.5 to 1.5 months of age (**Figure 3D**), coincident with the period of neonatal thymus growth (17). However, $ICN1^+$ mTEC numbers increased significantly by 3 months, and up to 4-fold by 5 months (**Figure 3D**), confirming a highly significant age-dependent upregulation of Notch1 activation in postnatal mouse mTECs.

Collectively, the observed age-associated activation of Notch1 signaling in the postnatal thymus of both humans and mice suggests a conserved role for Notch1 signaling in the biology of postnatal mTECs.

Foxn1-Controlled *RBPjκ* Deletion Abrogates Canonical Notch Activation in Postnatal TECs

To better understand the contribution of the Notch pathway to postnatal mTEC biology, we next analyzed the impact of impaired Notch activation in TECs, by using a conditional loss-of-function mouse model, in which canonical Notch signaling was selectively abolished in epithelial cells by crossing Foxn1-Cre mice (42) to the *Rbpj*$^{fl/fl}$ conditional knockout mouse line (41) (**Supplementary Figure 1A**). Transgenic Cre expression in Foxn1-Cre mice parallels endogenous *Foxn1* expression in epithelial cells and can be detected as early as E10.5 in the thymus primordium (42). Crossing Foxn1-Cre mice to the Rosa26^{loxPlacZ} reporter strain has revealed Foxn1 protein expression at E11.5, while *Foxn1*-controlled β-galactosidase reporter expression detected by LacZ staining is induced at E12.5 (42), and can be observed in the postnatal thymus as well (**Supplementary Figure 1B**). Therefore, RBPjκ in Foxn1-Cre x *Rbpj*$^{fl/fl}$ homozygous mice (hereafter referred to as RBPjκ-KO^{TEC}) might not be abolished before E11.5-12.5, which corresponds to a time in development when TEC progenitors have been established and their progeny has contributed to an initial thymus primordium. At later stages, emerging TECs and skin epithelial cells (42), will be unable to activate the canonical Notch signaling pathway in mutant mice (41). Confirming Notch abrogation in skin epithelial cells, RBPjκ-KO^{TEC} mice developed macroscopic cutaneous lesions, which were evident at 8-months, when animals showed clear signs of disease including numerous lesions at the face, footpad, tail and ventral skin (**Supplementary Figure 1C**). Microscopic examination of these lesions revealed a clear disorganization of the skin with signs of inflammation, leukocyte infiltration, hair follicle hyperproliferation, and the generation of keratin cysts (**Supplementary Figure 1D**), consistent with previous observations in distinct mouse models of Notch-deficient skin epithelium (48, 49).

Having confirmed the loss of Notch activation in the skin of RBPjk-KO^{TEC} mice, we next investigated specific abrogation of Notch signaling in mutant postnatal TECs (≥ 5-months), as compared to *Foxn1*$^{+/+}$*x RBPjk*$^{fl/fl}$ WT littermate controls. To this end, we performed comparative immunohistochemistry of Hes1 expression as readout of canonical Notch activation. Consistent results showed a prominent expression of Hes1 in the medulla of WT postnatal thymi, which was drastically reduced in RBPjk-KO^{TEC} mutant thymi, confirming abrogation of Notch signaling (**Figure 4A**). Detailed examination of the cortical and medullary TEC niches (**Figure 4B**) confirmed that, as shown above (**Figures 1**, **3**), TECs that display Notch signaling *in vivo* represent a conspicuous population in the medulla of WT thymi, and Hes1^{+} TECs were also detected in the WT cortex (**Figures 4C, D**). Quantitative measurements of Hes1^{+} nuclei distributed within pCK^{+} ROIs (**Supplementary Figure 3**) revealed a significant reduction of nuclei expressing Hes1 in both the cortex and the medulla of mutant RBPjκ-KO^{TEC} thymi compared to WT thymi of mice aged 3-months (**Figure 4C**), and a similar reduction was maintained in mice of 5-9-months (**Figure 4D**), which was consistently more significant in the medulla than in the cortex (**Figures 4C, D**). Collectively, these results confirmed that *Foxn1*-controlled abrogation of RBPjk impairs canonical activation of the Notch pathway in a substantial population of mTECs and also in a subset of cTECs in the postnatal thymus of RBPjκ-KO^{TEC} mutant mice.

Foxn1-Controlled Abrogation of Notch Signaling Leads to an Accelerated Loss of Postnatal mTECs

To investigate the impact of the specific abrogation of Notch signaling in the TEC compartment of the postnatal thymus, we next performed flow cytometry to analyze the TEC composition of thymi isolated from mutant RBPjκ-KO^{TEC} mice and *Foxn1*$^{+/+}$*x RBPjk*$^{fl/fl}$ WT littermates at different postnatal ages. To this end, cell suspensions from collagenase-dissociated thymi were analyzed for expression of EpCAM and MHC-class II (MHC-II) TEC markers after electronic exclusion of hematopoietic and erythroid-lineage cells by gating off CD45^{+} and Ter119^{+} cells (**Supplementary Figure 5**). Absolute and relative cell counts of EpCAM^{+} cells revealed no significant numerical differences of total TECs between RBPjκ-KO^{TEC} and WT thymi at early (4-weeks) postnatal ages, while TEC proportions decreased significantly in late (8-months) postnatal thymi of RBPjκ-KO^{TEC} mutant mice (**Figure 5A**). As we found that active Notch is expressed *in vivo* in mTECs in an age-dependent manner, we assessed whether the observed decrease of TEC numbers in aged mutant mice was the result of a preferential loss of mTECs. Thus, we then quantified cTECs and mTECs among EpCAM^{+} TECs by FACS analyses based on expression of the specific Ly51 and UEA1 markers, respectively (**Figure 5B**). No significant differences were observed in the proportions of either cTECs or mTECs in RBPjκ-KO^{TEC} compared to WT thymi at 4-weeks of age, while relative mTEC numbers decreased significantly in thymi from 8-months-old RBPjκ-KO^{TEC} mice compared to *Foxn1*$^{+/+}$*x RBPjk*$^{fl/fl}$ control littermates (**Figures 5B, C**). Therefore, TEC-specific loss of Notch signaling results in a marked decrease in the proportions of TECs in late but not early RBPjκ-KO^{TEC} mutant thymi, which results in a preferential reduction of mTECs. To further assess the kinetics of mTECs loss, we performed quantitative flow cytometry analyses of cTEC and mTEC numbers in mutant and WT littermates aged from 2- to 26-weeks. We found no significant differences in relative TEC numbers between the two groups at young postnatal ages (2- and 4-weeks) However, TEC proportions decreased markedly at 9-weeks in RBPjκ-KO^{TEC} compared to WT littermate mice, and this decrease progressed steadily to 26-weeks (**Figure 5D**). Importantly, we found that WT mice also displayed a progressive age-dependent decrease of relative TEC numbers, as previously reported (17), although loss of TECs in mutant mice followed accelerated kinetics compared to WT littermates (**Figure 5D**). Therefore, impaired Notch signaling in TECs results in a marked acceleration of TEC number loss in the

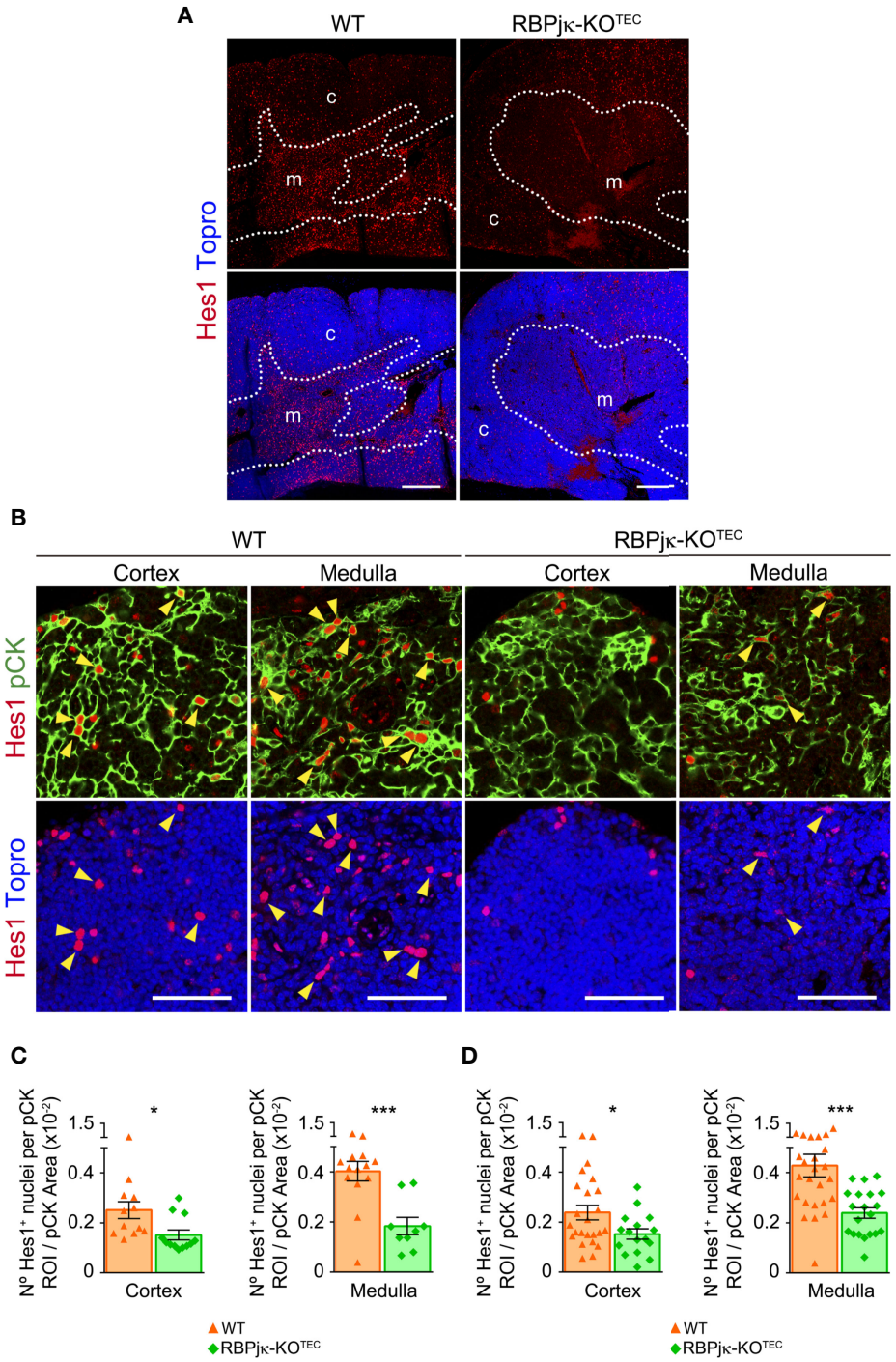

FIGURE 4 | Abrogation of canonical Notch signaling in postnatal TECs of RBPjκ–KO^{TEC} mice. **(A)** General view of Hes1 expression (red) in postnatal thymi (5-months) of mutant RBPjκ–KO^{TEC} and WT *Foxn1*$^{+/+}$*x RBPjk*$^{fl/fl}$ littermate mice. Topro3 shows nuclear staining (blue). Scale bar: 200μm. c, cortex; m, medulla. Dotted line, corticomedullary junction. **(B)** Immunohistochemistry of Hes1 (red), and pCK (green) with nuclei in blue (Topro3) in the cortex and medulla of postnatal thymi (5-months) from WT and RBPjκ–KO^{TEC} mice. Arrowheads indicate Hes1 nuclear expression. Scale bars: 50μm. Images are representative of n ≥ 10 images per sample (n ≥ 3 independent thymus samples). **(C, D)** Bar graphs show numbers of Hes1^{+} nuclei within pCK^{+} ROIs relative to total pCK^{+} cellular areas analyzed in the thymus cortex and medulla of mutant RBPjκ–KO^{TEC} and WT *Foxn1*$^{+/+}$*x RBPjk*$^{fl/fl}$ littermate mice aged 3-months **(C)** or 5-9-months **(D)**. Data are shown as mean numbers ± SEM per field obtained from n ≥ 10 different 63x images per sample (n=4 independent samples). *p < 0.05; ***p < 0.00.

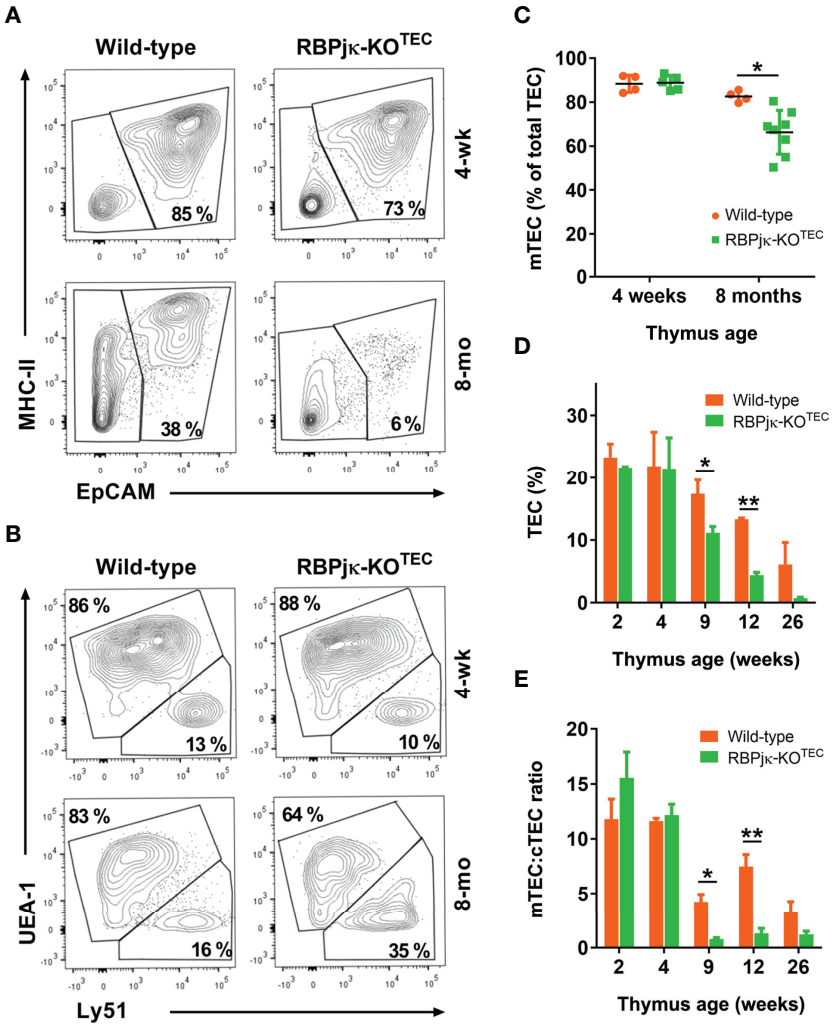

FIGURE 5 | *Foxn1*-controlled abrogation of canonical Notch signaling leads to an accelerated age-associated loss of postnatal mTECs. **(A)** Representative flow cytometry MHC-classII and EpCAM expression analysis of CD45- and Ter-119-depleted cell suspensions of collagenase/dispase-treated young (4-weeks) and old (8-months) thymi from RBPjκ–KO^{TEC} mutant and *Foxn1*$^{+/+}$*x RBPjk*$^{fl/fl}$ littermate WT mice (n ≥ 8). **(B)** Representative flow cytometry analysis of UEA1 and Ly51 expression on gated EpCAM+ TECs in **(A)**, (n ≥ 4). **(C)** Relative numbers of UEA1^{+} mTECs among EpCAM^{+} TECs from RBPjκ–KO^{TEC} and *Foxn1*$^{+/+}$*x RBPjk*$^{fl/fl}$ WT thymi of the indicated ages, analyzed as in B). Data are shown as mean percentages ± SEM (n ≥ 4 thymus samples per age). **(D)** Relative numbers of EpCAM^{+} TECs present in cell suspensions obtained as in **(A)** from thymi of *Foxn1*$^{+/+}$*x RBPjk*$^{fl/fl}$ WT and RBPjκ–KO^{TEC} mutant mice aged 2- to 26-weeks. Data are shown as mean percentages ± SEM (n ≥ 3 thymus samples per age). **(E)** Ratio of mTEC:cTEC proportions among total EpCAM^{+} TECs from WT *Foxn1*$^{+/+}$*x RBPjk*$^{fl/fl}$ and mutant RBPjκ–KO^{TEC} thymi at the indicated postnatal ages. Data show mean values ± SEM (n ≥ 4 thymus samples per age). *p<0.05; **p<0.01. p values were calculated using a two-tailed t-test.

postnatal thymus. Independent quantification of relative cTEC and mTEC numbers revealed a preferential decrease of mTECs along age in both WT and mutant mice, which led to a significant reduction of the mTEC:cTEC ratio in both mouse groups by 9-weeks (**Figure 5E**). The mTEC:cTEC ratio was maintained to minimal levels up to 26-weeks in RBPjκ-KO^{TEC} thymi, and the decrease was less pronounced in the thymus of WT littermates (**Figure 5E**). Therefore, our results indicate that in both RBPjκ-KO^{TEC} and WT mice, the observed age-associated decrease of postnatal TEC numbers can be attributed to a preferential loss of mTECs. However, abrogation of Notch signaling in RBPjκ-KO^{TEC} mutant mice leads to an accelerated loss of postnatal mTECs, suggesting that Notch activation regulates mTEC homeostasis in postnatal life.

Abrogation of Canonical Notch Signaling in TECs Leads to a Reduced and Disorganized Postnatal Thymic Medulla and Accelerates Thymic Involution

Age-dependent mTEC loss occurs in normal thymus as part of the thymic involution process (17). It is thus possible that Notch signaling may contribute to the control of mTEC homeostasis and age-dependent thymus involution in postnatal life. As thymic regression results in loss of thymic structure and disorganization

of thymic architecture (reviewed in 18), we next performed histomorphometric analyses aimed at establishing detailed comparisons between the cortical and medullary compartments of postnatal thymi from RBPjκ-KO^{TEC} mice and *Foxn1*$^{+/+}$*x RBPjk*$^{fl/fl}$ WT littermates. Expression of the Notch ligand Jag1, which is selectively expressed on TECs located at the medulla (40), was used to define the medullary microenvironment (**Supplementary Figure 4A**). Cortical and medullary area measurements by confocal microscopy revealed no significant differences in size and morphology of the cortex and medulla of young (0.5-months) thymi from RBPjκ-KO^{TEC} mice, as compared with WT littermates. However, a significant reduction of the medullary area was evident at 3 and 5 months of age in RBPjκ-KO^{TEC} thymi (**Figure 6A**). Compared to the WT thymic medulla, the mutant medulla appeared disorganized and composed of small discrete islets (**Figure 6A**), suggesting that TEC-specific abrogation of Notch signaling leads to the disruption of the medullary thymic microenvironment. Accordingly, histomorphometric measurements of cortical and medullary areas revealed a significant decrease of the average medulla to cortex area ratio of RBPjκ-KO^{TEC} thymi compared to WT thymi from the 3- and 5-months-old mice analyzed

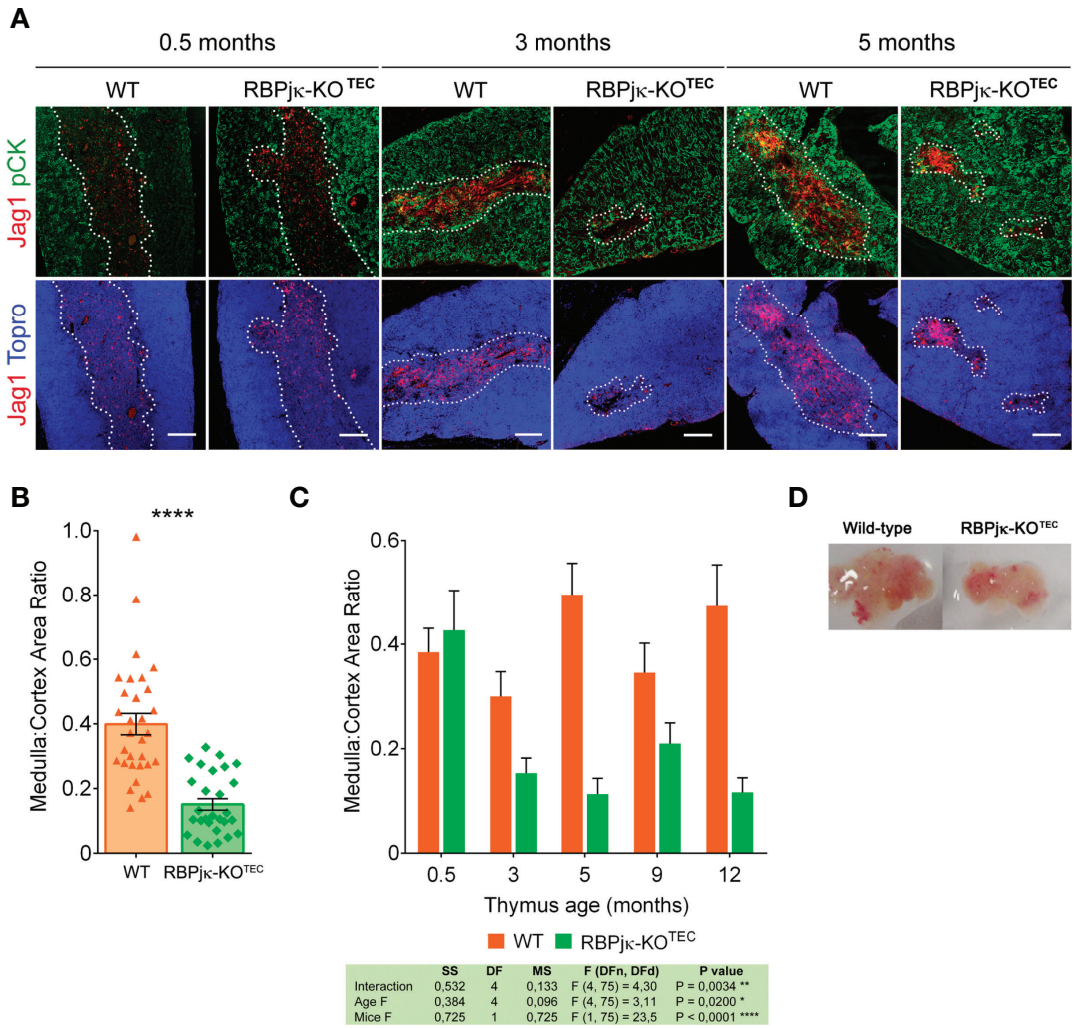

FIGURE 6 | Abrogation of Notch signaling in TECs results in thymic medulla disruption and thymus atrophy. **(A)** Immunohistochemistry of thymi from mutant RBPjκ–KO^{TEC} and WT *Foxn1*$^{+/+}$*x RBPjk*$^{fl/fl}$ littermate mice at 0.5-, 3- and 5-months of age. TECs are characterized by expression of pCK (green), Jag1 Notch ligand (red) expression marks medullary TECs, and Topro3 shows nuclear staining (blue). Images are representative of n ≥ 10 images per sample (n ≥ 3 thymus samples per age). Scale bar: 200μm. **(B)** Ratio of medulla: cortex area measurements derived from histomorphometric analysis (**Supplementary Figure 4**) of postnatal thymi from WT *Foxn1*$^{+/+}$*x RBPjk*$^{fl/fl}$ and mutant RBPjκ–KO^{TEC} mice in A). Coexpression of pCK and Jag1 (confined to the medulla) was used to calculate medullary areas. Cortical areas were identified as pCK^{+} Jag1^{-} and nuclear staining by Topro3 (blue) defined total thymic area. Data are shown as mean area ratios ± SEM obtained from n ≥ 10 images per sample (n ≥ 3 thymus samples per age). p values were calculated using a two-tailed t-test. **(C)** Ratio of medulla: cortex area measurements derived from histomorphometric analyses as in **(B)** of thymi obtained from WT *Foxn1*$^{+/+}$*x RBPjk*$^{fl/fl}$ and mutant RBPjκ–KO^{TEC} mice at the indicated ages. Data are shown as mean area ratios ± SEM obtained from n ≥ 10 images per sample (n ≥ 3 thymus samples per age). Two-way ANOVA table summarizing the statistical analysis is shown *p<0.05; **p<0.01. **(D)** Thymus atrophy in RBPjκ–KO^{TEC} mice mutant mice at 3-months of age.

(**Figure 6B**). Importantly, kinetic studies based on histomorphometric measurements of postnatal thymi at increasing ages, from 0.5- to 12-months, revealed that the significant reduction of the medulla to cortex area ratio observed in RBPjκ-KO^{TEC} thymi at 3-months of age was progressive along life (**Figure 6C**). Also, macroscopic examination revealed that the observed medulla reduction correlated with postnatal thymus atrophy in mutant mice that was evident by 3-months (**Figure 6D**). Collectively, these data indicate that maintenance of the anatomical organization and integrity of the postnatal thymic medulla critically depends on the activation of the canonical Notch signaling pathway in mTECs.

The above findings showing a reduced and disorganized medulla in RBPjκ-KO^{TEC} postnatal thymi is consistent with the possibility that specific abrogation of Notch activation in TECs results in a premature thymic involution and leads to an impaired thymus function. To investigate this possibility, we analyzed T-cell development and thymic output in RBPjκ-KO^{TEC} and WT *Foxn1*$^{+/+}$*x RBPjk*$^{fl/fl}$ aged mice by flow cytometry. We found that thymocyte numbers were equivalent in young WT and RBPjκ-KO^{TEC} mice (not shown), but decreased significantly in mutant compared to WT mice along life, to up to 70% by 12-months (**Figure 7A**). The observed thymocyte decrease paralleled a weak but significant reduction of the CD4$^+$CD8$^+$ double positive (DP) thymocyte subset in RBPjκ-KO^{TEC} mice (**Figures 7B, C**). This decrease could be attributed to a homeostatic defect in mTECs (18) and associated paracrine signaling axes (46), which may indirectly affect cortical epithelial cell function. Alternatively, it may directly result from a defective function of cTECs in mutant mice. In addition to the DP cell loss, we observed a marked increase of non-T lineage (Thy1$^-$) cells in mutant mice compared to WT littermates, which accounted for up to 20% of total thymic cells at 12-months (**Figure 7D**). Flow cytometry analyses using lineage-specific markers identified B cells as the major non-T cell type accumulating in the adult mutant thymus, but NK cells and myeloid cells were also significantly increased (**Figure 7E**). As increased frequencies of thymic B cells is a feature associated with thymic involution in aged mice (18), our results suggest that a defective thymic microenvironment rather than an intrinsic functional defect of developing thymocytes is responsible for the observed expansion of non-T lineage cells in RBPjκ-KO^{TEC} thymi. To assess this possibility, we performed adoptive transfer experiments consisting on intra-venous injection of total hematopoietic cells isolated from the BM of either RBPjκ-KO^{TEC} or WT *Foxn1*$^{+/+}$*x RBPjk*$^{fl/fl}$ Ly5.2$^+$ littermates into lethally-irradiated C57BL/6J Ly5.1$^+$ normal mice. Flow cytometry analyses of cells recovered from the thymus of host mice at 4 months post-transplant revealed no differences in the reconstitution efficiency of BM progenitors from either WT or mutant mice, as indicated by the equivalent proportions of Thy1$^+$ T-lineage cells and DP, double negative (DN) and single positive (SP) subsets present in the host thymi (**Figure 7F**). Therefore, we can exclude an intrinsic functional defect of T-cell progenitors derived from RBPjκ-KO^{TEC} mutant mice. Based on our results, we concluded that *Foxn1*-controlled impaired activation of canonical Notch signaling leads to an accelerated loss of mTECs accompanied by disruption of the medulla integrity in the postnatal thymus, which concurs with an aberrant increase in the proportion of thymic non-T lineage cells and a decrease in DP thymocyte numbers, compatible with a premature thymic involution.

DISCUSSION

We have studied the potential contribution of the Notch pathway to postnatal TEC biology using two complementary strategies. First, we analyzed Notch activation *in situ* in the human postnatal thymus by performing quantitative immunohistochemistry and confocal imaging. Our results show for the first time that Notch activation is regulated *in vivo* in the human thymic epithelium in a spatio-temporal manner. We found that Notch signaling, mediated in particular through the Notch1 receptor, is induced *in situ* in postnatal human TECs mostly located at the medulla, and this activation pattern is conserved in the mouse. Importantly, numbers of mTECs showing Notch activation increase significantly with age in both human and mouse postnatal thymi, suggesting a conserved role for Notch signaling in TEC homeostasis during aging. To further investigate this possibility, we made use of an *in vivo* genetic model of *Foxn1*-controlled conditional inactivation of Notch signaling in murine epithelial cells. The model revealed that impaired Notch signaling in mutant TECs leads to an accelerated age-dependent decrease of postnatal mTECs that results in the disruption of the medullary thymic microenvironment and in an accelerated thymus atrophy.

The observation that Notch signaling is activated *in situ* in the epithelial compartment of the postnatal thymus was somehow unexpected, as preliminary studies in mice (35–37), recently confirmed by genetic approaches, pointed to a role of Notch signaling limited to embryonic stages of TEC development, while Notch activation has been shown to be downregulated afterwards disappearing in postnatal TECs (38, 39). Accordingly, Notch signaling critically regulates mTEC-lineage fate specification of embryonic TEC progenitors, but further mTEC development is dependent on repression of Notch activation (39), a process that may rely on HDAC3 function (37). These results seem in conflict with our finding that Notch is active *in vivo* in postnatal TECs; particularly, in a significant population of TECs located at the medulla. However, an important question is whether such mTECs with active Notch are immature or fully mature mTECs. While our current results cannot give a definitive answer to this question, the first possibility seems very likely considering that, during embryonic TEC development, Notch signaling is critical not only for mTEC specification, but also for maintenance/expansion of the pool of undifferentiated TEPC and mTEC-restricted progenitors (38, 39). Considering that both TEPC and mTEC progenitors have been identified in the adult murine thymus (23–27), an attractive explanation for our results would be that expression of active Notch in the postnatal thymus is restricted to the TEPC and/or mTEC progenitor pools (50), thus controlling the high turnover of mTECs and their

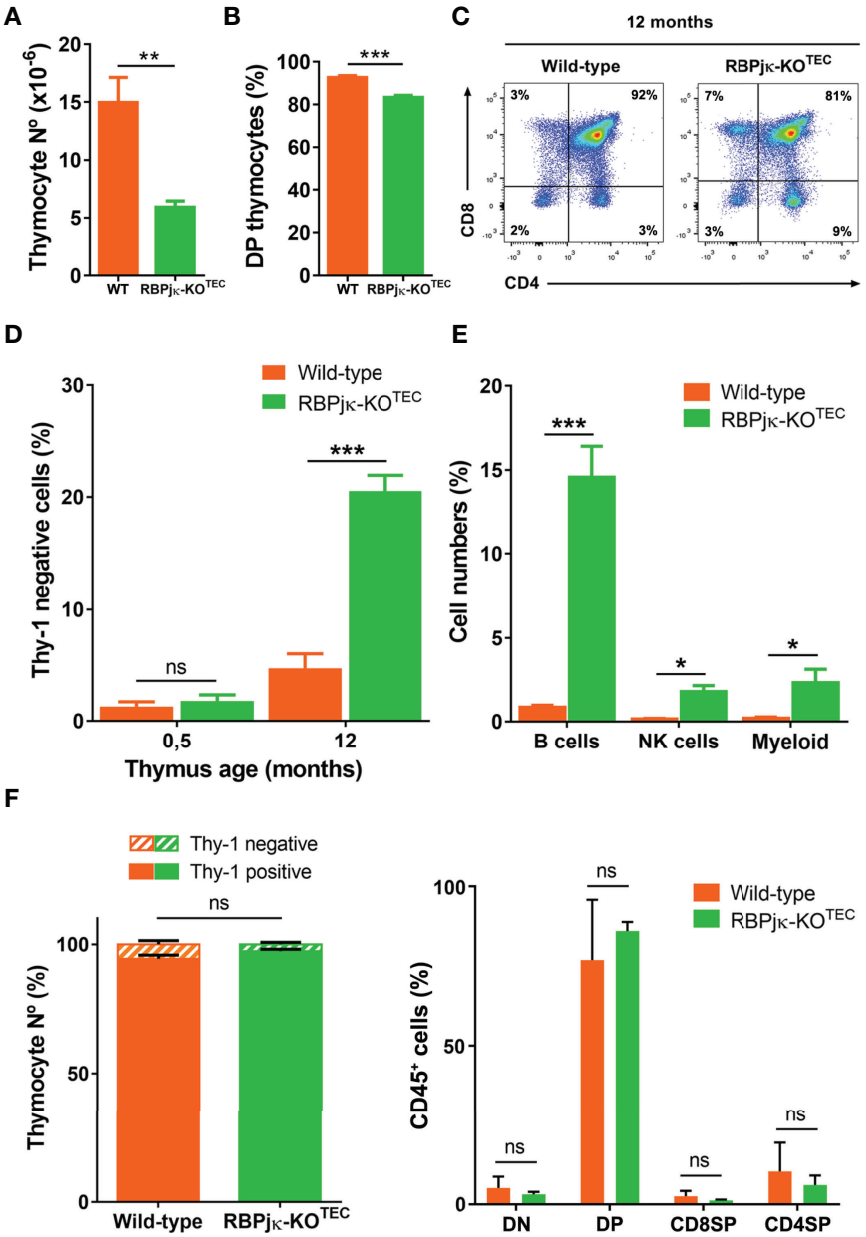

FIGURE 7 | Abrogation of canonical Notch activation in TECs results in premature thymic dysfunction. **(A)** Absolute numbers of total thymocytes isolated from 12-month-old WT *Foxn1*$^{+/+}$*x RBPjk*$^{fl/fl}$ or mutant RBPjκ–KO^{TEC} thymi. Data show mean numbers ± SEM, (n ≥ 4). **(B)** Percentages of CD4^{+}CD8^{+} DP thymocytes among Thy1^{+} thymic cells from 12-month-old WT *Foxn1*$^{+/+}$*x RBPjk*$^{fl/fl}$ or RBPjκ–KO^{TEC} mice. Data show mean percentages ± SEM, (n ≥ 4). **(C)** Representative flow cytometry analysis of CD4 and CD8 expression on gated Thy1^{+} thymocytes from 12-months-old WT *Foxn1*$^{+/+}$*x RBPjk*$^{fl/fl}$ and RBPjκ–KO^{TEC} thymi, (n ≥ 4). **(D)** Percentages of thymic cells lacking Thy1 obtained from WT *Foxn1*$^{+/+}$*x RBPjk*$^{fl/fl}$ or RBPjκ–KO^{TEC} thymi of the indicated ages. Data are shown as mean percentages ± SEM, (n ≥ 4). **(E)** Percentages of B, NK and myeloid cells among total thymus cells from 12-month-old WT *Foxn1*$^{+/+}$*x RBPjk*$^{fl/fl}$ or RBPjκ–KO^{TEC} mice. Data show mean percentages ± SEM, (n ≥ 4). **(F)** Percentages of either total Thy1^{+} T- and Thy1^{-} non-T-lineage cells (left) or DN, DP and CD4^{+} and CD8^{+} SP thymocytes (right) reconstituting the thymus of WT C57BL/6J (Ly5.1^{+}) mice transplanted with BM cells from WT *Foxn1*$^{+/+}$*x RBPjk*$^{fl/fl}$ or RBPjκ–KO^{TEC} (Ly5.2^{+}) mice. Data are shown as mean percentages ± SEM, (n=3). *p<0.05; **p<0.01; ***p<0.001. p values were calculated using a two-tailed t-test ns, not significant.

maintenance and regeneration in the adult thymus (17). In fact, it is known that the TEC compartment has an extensive cell division in fetal and neonatal life, but postnatal TEC proliferation decreases significantly by 4 weeks (17), while medullary TECs display relatively high turnover rates also during the postnatal stage. An alternative possibility is supported by the finding that mTECs that display active Notch signaling accumulate in the postnatal human thymus in HC, a structure derived from

terminally differentiated mTECs, suggesting that Notch activation could be induced in mature mTECs.

Considering the high developmental and functional heterogeneity revealed for the TEC compartment (14, 51), generation of conclusive results on the exact maturation stage of postnatal mTECs that activate Notch signaling *in vivo* demands further studies. Nonetheless, an interesting finding of our work is that postnatal mTECs activate Notch signaling in an age-dependent manner in both humans and mice, as revealed by quantitative analyses. Comprehensive kinetics in mice showed that numbers of mTECs with active Notch signaling increased by 3-months of age, immediately after achievement of maximal thymic cellularity and coincident with the initiation of thymic involution (17). It is thus possible that activation of Notch signaling is upregulated at early postnatal ages to counteract the loss of mTECs associated with thymic involution (17). Supporting such a role, our loss-of-function genetic approach has shown that abrogation of canonical Notch signaling results in decreased proportions of TECs, mostly of mTECs, during postnatal life, while normal mTEC numbers were found during the first month of life. These findings concur with the results shown by Blackburn and coworkers using a distinct Foxn1-Cre x Rbpjfl/fl mouse model (38), in which mTEC generation is impaired in embryonic life, but mTECs proportions were normalized at week 8 after birth. Given that transgenic Cre expression in FoxN1-Cre mice parallels endogenous Foxn1 expression in epithelial cells (E11.5), and Foxn1-controlled expression is induced one day later as indicated by β–galactosidase expression (42), Notch signaling could not be abolished before E12.5 in mutant mice, which corresponds to a time in development when TEC progenitors have been established and their progeny has contributed to an initial thymus primordium. Thus, a relatively late timing of RBP-Jκ deletion could result in reduced numbers rather than total loss of mTEC progenitors that would be able to recover normal numbers of mTECs in mutant thymi early after birth. Importantly, we show that, after mTEC numbers are normalized, abrogation of Notch signaling in mutant mice results in a further age-dependent dramatic loss of mTECs. Whether mTEC loss results from the impaired maintenance/expansion or the enhanced mortality of mTECs and/or mTEC progenitors remains to be determined; but itconcurred with a marked disorganization of the thymic medulla architecture, and a significant reduction in thymus size, together with diminished thymocyte numbers, decreased proportions of DP thymocytes and the accumulation of intrathymic B cells. As all these features are associated with age-dependent thymic involution (16–18), we concluded that abrogation of Notch signaling in postnatal TECs may accelerate thymus aging and impaired thymus function (49). Accordingly, DP thymocyte frequency is a readout of thymus functionality that correlates inversely with thymus involution and mTEC loss (18), and has been associated with apoptosis susceptibility of thymocytes (52). Although we cannot establish whether DP thymocyte loss is directly dependent on the homeostatic defect in mTECs, it is posible that defective mTECs located at the corticomedullary junction, where accumulation of $ICN1^+$ TECs was observed, could impact viability of recently selected DP thymocytes migrating from the cortex to the medulla. Alternatively, a defective mTEC paracrine signaling axis may indirectly affect cortical epithelial cell function (46), or defective DLL4 expression on mutant cTECs (53) may affect DP thymocyte generation.

Understanding how mTEC maintenance and regeneration are regulated in the adult thymus downstream of Notch signaling is of critical relevance for understanding thymic involution, but the effectors involved in such Notch-mediated function remain to be identified. In this regard, it is worth noting that Myc and cyclin D1, two well-known downstream targets of Notch signaling have been shown to contribute to TEC growth and to promote a dramatic increase of thymus size upon ectopic expression in TECs (18, 54, 55). Notably, as described for Notch activation (38, 39) Myc transcription declines in TECs during embryonic development, and minimal levels have been described after birth, suggesting that regulation of Myc function is required to limit thymic growth in adult mice. As Myc expression in adult TECs drives proliferation and results in thymic regeneration (54), it is possible that Notch signaling controls mTEC maintenance and thymic involution through Myc. An important question is how Notch signaling is temporally regulated to control mTEC maintenance and thymus homeostasis. To answer this question, we have to consider that spatio-temporal regulation of Notch ligand expression defines particular Notch signaling microenvironments in the thymus (40, 56). Manley and coworkers have shown tan Notch1 signaling in TEC development begins soon after the onset of Foxn1 expression, when Jag1 and DL4 Notch ligands are expressed (39). Notch1 could also be the receptor mediating Notch signaling in postnatal mTECs, given the coincident patterns of Hes1 and active intracellular Notch1 (ICN1) expression observed in both human and mouse postnatal thymi. Although we cannot ignore the expression of Notch3 in human mTECs, this receptor could be upregulated following Notch1-mediated signaling as reported in thymocytes (56). In the postnatal thymus medulla, a possible source of Notch1 ligand would be other mTECs, which express Jag1 (40), though Notch ligand presented by developing thymocytes could induce Notch1 activation as well. In this regard, it is important to note that crosstalk between developing thymocytes and TECs in one of the mechanisms that control TEC development and likely thymus involution (16–18). TECs depend on the presence of thymocytes for their differentiation and organization (57, 58), and they reciprocally provide the signals that regulate T lymphocyte generation (59). Therefore, Notch activation could be negatively regulated in mTECs during thymopoiesis once a given cellular density of SP thymocytes has been reached at the medulla. In this regard, recent results by Blackburn's group provided evidence of a cross-regulatory relationship between Notch and Foxn1, the master regulator of TEC differentiation that is required to maintain the postnatal thymic microenvironment in a dosage-sensitive manner (60–62), suggesting a Foxn1-mediated repression of Notch activity that could be reinforced *via* its direct ligands (38). Conversely, Foxn1 downregulation during thymus involution (60–62) could trigger Notch activation to counteract mTEC loss and thymus aging.

While further studies are required to reach a full understanding of mechanisms controlling postnatal mTEC turnover and thymic involution, our results point toward manipulation of Notch signaling as a novel and promising strategy for thymus regeneration during aging.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Spanish National Research Council Bioethics 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 Animal Experimentation Ethics Committee of the Comunidad de Madrid (PROEX 002.16/21).

AUTHOR CONTRIBUTIONS

MLT conceptualized, designed and supervised the study, wrote the manuscript and acquired funding. MG-L, MM, CC, and JA collected and processed the samples, performed the experiments, analyzed the data, and prepared the figures. SŽ and GH developed the animal models and supervised the study. MG-L drafted the first version of 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.2022.867302/full#supplementary-material

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Signaling Crosstalks Drive Generation and Regeneration of the Thymus

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Optimal recovery of immune competence after periods of hematopoietic insults or stress is crucial to re-establish patient response to vaccines, pathogens and tumor antigens. This is particularly relevant for patients receiving high doses of chemotherapy or radiotherapy, who experience prolonged periods of lymphopenia, which can be associated with an increased risk of infections, malignant relapse, and adverse clinical outcome. While the thymus represents the primary organ responsible for the generation of a diverse pool of T cells, its function is profoundly impaired by a range of acute insults (including those caused by cytoreductive chemo/radiation therapy, infections and graft-versus-host disease) and by the chronic physiological deterioration associated with aging. Impaired thymic function increases the risk of infections and tumor antigen escape due to a restriction in T-cell receptor diversity and suboptimal immune response. Therapeutic approaches that can promote the renewal of the thymus have the potential to restore immune competence in patients. Previous work has documented the importance of the crosstalk between thymocytes and thymic epithelial cells in establishing correct architecture and function of thymic epithelium. This crosstalk is relevant not only during thymus organogenesis, but also to promote the recovery of its function after injuries. In this review, we will analyze the signals involved in the crosstalk between TECs and hematopoietic cells. We will focus in particular on how signals from T-cells can regulate TEC function and discuss the relevance of these pathways in restoring thymic function and T-cell immunity in experimental models, as well as in the clinical setting.

Keywords: immune reconstitution, thymus, T cells, immune-senescence, thymic epithelial cells

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INTRODUCTION

Optimal immune recovery after periods of hematopoietic insults is key to reestablish patient immune competence and sustain response to vaccines, pathogens and tumor antigens. This is particularly relevant for patients receiving high doses of chemotherapy or radiotherapy, for instance, associated with the conditioning regimen employed in preparation to hematopoietic cell

transplantation (HCT). These patients experience profound and prolonged periods of lymphopenia, which can be associated with an increased risk of developing life-threatening infections and, in cancer patients, tumor relapse. In fact, infections and relapse have been inversely correlated with the degree of immune reconstitution and account for greater than 50% of mortality after allogenic HCT (allo-HCT) (1–5).

The thymus represents the primary organ responsible for the maturation and differentiation of a broad pool of *naïve* T cells capable of recognizing an extremely large array of pathogens and tumor antigens. The process of T-cell development involves the migration of bone marrow-derived T-cell progenitors through the thymus and requires physical contact between developing thymocytes and the supporting thymic stromal microenvironment which consists of thymic epithelial cells (TECs), macrophages, endothelial cells (ECs), fibroblasts and dendritic cells (DCs) (6–8). Multiple developmental pathways, including Notch, Sonic Hedgehog, and WNT coordinate this complex hierarchical process (9–12). Despite its crucial role in generating T cells, thymic function may be profoundly impaired by acute insults, such as that caused by infections, stress, chemotherapy and radiotherapy. Delayed or defective recovery of thymic function has been associated with adverse clinical outcomes in patients receiving allo-HCT (13–18). Thymic function progressively declines with age, a well-known physiological process known as thymic involution (19). Age-associated thymic involution limits the recovery of thymic function after acute insults and significantly contributes to the decline of T-cell receptor (TCR) diversity in older individuals (20, 21). As a direct consequence, older patients are more prone to bacterial and viral infections and, possibly, to tumor antigen escape. The identification of clinical strategies that can restore thymic function and enhance immune reconstitution represent a major clinical need.

Through the mechanistic understanding of the molecules and pathways driving the maintenance of thymic function and its recovery after insults, several potential regenerative targets have been identified. They include growth factors (such as bone morphogenetic protein 4, stem cell factor, kit ligand and keratinocyte growth factor), the modulation of hormones (such as the inhibition of sex steroids and the use of growth hormone, insulin-like growth factor-1 and ghrelin), cytokines (such as interleukin (IL)-7, IL-12 and IL-21), chemokines (such as CXCL12/CXCR4) and the adoptive transfer of preformed T-cell progenitors, as well as *ex vivo* expanded thymus-derived endothelial cells (22). However, at present, none of these approaches is approved as a standard therapy to enhance thymic function and immune reconstitution.

THYMIC CROSSTALK REGULATES TISSUE MAINTENANCE AND ITS REGENERATION

Thymic crosstalk, a set of reciprocal regulations between thymocytes and the thymic environment, is critical to orchestrate thymocyte and TEC development, as well as to start thymic recovery after periods of stress or immunological injuries (23). Thymic epithelium represents a predominant stromal cell population within the thymus, which is classically divided into two subsets based on their spatial distribution and specialized function: cortical TECs (cTECs) and medullary TECs (mTECs) are responsible for positive and negative selection of thymocytes, respectively (10).

cTECs are critical for fate commitment, expansion, and positive selection of the developing thymocytes. On the other hand, mTECs are involved in the negative selection and maturation of thymocytes (2–4). mTECs can be further divided based on the expression of MHCII and additional molecules, such as CD40 and CD80/86. Within thymic microenvironment, while innate lymphoid cells (ILCs), endothelial cells and fibroblasts are mostly resistant to damage (24–26), thymic epithelium is particularly sensitive to the effects of chemotherapy and radiotherapy, with the $MHCII^{high}$ mTEC subset representing the population most sensitive to insults, likely due to the high proliferative rate of these cells (27, 28).

TECs play a fundamental role in the development and selection of T cells providing key thymopoietic signals, including Interleukin-7 (IL-7), Notch-ligand Delta Like 1 and 4, as well as self-peptide–MHC complex. On the other hand, the maturation and maintenance of TECs is closely dependent on instructive signals provided by the bone marrow-derived lymphoid component. Indeed, thymocyte-derived signals are indispensable for the appropriate development and spatial organization of cTEC and mTEC subsets during late fetal development and adult life as revealed through the use of different genetic mouse models (29–31). *Tcra* KO and Zeta-chain-associated protein kinase 70 (*Zap70*) KO mice, in which thymocyte development is blocked at the double positive (DP) stage, showed severely impaired thymic medulla organization (32, 33). Similarly, *Recombination activating gene (Rag)*1 KO and *Rag2* KO mice, in which thymocyte development is arrested at the double negative (DN) 3 stage, showed impaired medulla formation. Transgenic mice expressing high copies of the human CD3 epsilon molecule, which display a block at the DN1 stage of differentiation, showed impaired cortical thymic function and disrupted thymic architecture (34, 35). Importantly, transplantation of T-cell depleted bone marrow cells in severe combined immunodeficiency (SCID) mice, restored thymic architecture organization (36). In addition, the transfer of mature T cells into SCID mice promoted the recovery of the medullary epithelial structure, providing evidence that the regenerative signals on thymic epithelium can be instructed by both progenitor and mature T cells (37).

Interestingly, data suggesting that the infusion of mature T cells can boost thymic and immune recovery come also from clinical studies in which patients received allo-HCT followed by the adoptive transfer of donor T cells. Vago et al. demonstrated that the transfer of donor T cells genetically engineered to express the Herpes Simplex Virus thymidine kinase suicide gene (a safety switch system to be activated in case of graft-versus-host disease, GvHD) induced improved thymic function,

as demonstrated by increased levels of T-cell receptor excision circles (TRECs) and recent thymic emigrants (RTEs) (38). Using chest tomography scans, this study also demonstrated that patients infused with modified T cells showed enlargement of active thymic tissue when compared to pre-transplant levels (38). In addition, recent observations collected at our center suggested that patients receiving donor T-cells genetically modified with the inducible Caspase 9 suicide gene showed rapid recovery of thymic function evaluated by the quantification of TRECs in patient peripheral blood (39). Data on enhanced immune recovery after the infusion of mature T-cells in patients, come also from studies in which the adoptive transfer of virus-specific T-cells generated a broad enhancement of the T-cell immunity (40, 41). The beneficial effect of the infused mature T cells on thymic function is likely to be transient in nature, but sufficient to provide regenerative signals, which result in faster recovery of thymic structure and accelerated immune reconstitution post damage. Nevertheless, it remains to be explored the long term persistence of these effects. The characterization of the underlying mechanisms of such effects could be of valuable importance to reveal pathways crucial for the regeneration of the human thymus that can be exploited to develop immune boosting therapies.

REGENERATIVE PATHWAYS

In this review, we will analyze the signals involved in the crosstalk between TECs and T-cells, looking beyond the process of thymocyte maturation and exploring how signals from T cells can regulate TEC function (**Figure 1**). Although several pathways (including Notch and Hedgehog) are known to have pivotal roles in T-cell and TEC development, we will highlight crosstalk signals described to regulate thymic function and T-cell immunity postnatally in experimental models, as well as in the clinical setting.

RANKL

Receptor activator of nuclear factor kappa B ligand (RANKL) is a TNF superfamily member encoded by *Tnfsf11* gene in mouse (42). Although a soluble form of RANKL (sRANKL) exists, this factor is expressed as a type II transmembrane protein whose ectodomain specifically interacts with its cognate receptor RANK (encoded by Tnfrsf11a). Thus, RANK-RANKL signaling is mostly mediated by the physical interaction of different cell types. RANK stimulation results in both canonical and non-canonical NF-kB signaling, together with MAPK activation (43). These events lead to the upregulation of genes involved in proliferation, survival and differentiation, thus resulting in pleiotropic effects on human physiology. First identified as a key component of bone metabolism, RANKL was then characterized as a crucial mediator in both organ development and immunity (44). In fact, despite having normal splenic architecture, *Tnfrsf11a* KO mice show null lymph-nodes organogenesis, while *Tnfsf11* KO mice show reduced thymic size and block of thymocyte maturation between DN3 and DN4 stage of differentiation (45, 46).

Within the thymus, RANK is expressed by subsets of mTECs residing in both $Aire^+$ and $Aire^-$ subpopulations (47, 48). On the other hand, RANKL is mostly provided by CD4 SP thymocytes and LTi cells, while CD8 SP thymocytes and invariant natural killer T (iNKT) cells contribute for the presentation of RANKL to a lesser extent (48–50). In this context, cell-cell interactions are of paramount importance in controlling central tolerance and T-cell production, as RANK signaling stimulates $Aire^+$ mTEC maturation in concert with CD40 and LTα pathways (47, 51). Importantly, $Aire^+$ $mTEC^{HI}$ cells are also the primary cell population responsible for the production of osteoprotegerin (OPG) in the thymus, a soluble decoy receptor for RANKL encoded by *Tnfrsf11b* (52). OPG binding to RANKL inhibits its interaction with RANK. In fact, thymus tissues from *Tnfrsf11b* KO mice show increased mTEC cellularity (50, 53).

Besides its roles in thymic physiology, RANK-RANKL pathway is also implied in thymic regeneration upon immunological insults. In fact, RANKL is upregulated in CD4 thymocytes and LTi cells during thymus recovery in mice exposed to sublethal total body irradiation (SL-TBI) (54). On the other hand, a recent report demonstrated that increased CD4 T-cell-mediated RANK signaling in the thymus causes enhanced generation of mTEC. This results in an imbalance of cTEC and mTEC proportion, eventually leading to defective thymopoiesis (55). For its pivotal role in health and disease, the administration of RANKL or RANKL partial agonists has been exploited in mouse models reproducing particular clinical conditions, such as psoriasis and ischemic stroke (54, 56–58). Furthermore, in mouse models of HCT, sRANKL exogenous administration drives TEC regeneration, as demonstrated by increase in cellularity of thymic epithelial progenitor cells, cTEC and mTEC subsets (54). sRANKL-treated mice also showed early homing of lymphoid progenitors in the thymus and T-cell reconstitution (54). Moreover, Desanti et al. showed that stimulation of mTEC progenitors with RANK agonistic antibodies resulted in CD40 upregulation, thus suggesting a role in mTEC maturation (59).

CD40L

CD40L is a transmembrane protein and a tumor necrosis factor (TNF) superfamily component playing key roles in both innate and adaptive immunity (60). CD40L is expressed by activated T and B cells, basophils, monocytes, NK and mast cells and signals through physical interaction with its cognate receptor CD40 (61). The latter is a transmembrane costimulatory receptor firstly identified on B cells as a factor responsible for their activation and proliferation (62). In subsequent studies, CD40 was also reported to be expressed by activated T cells, DCs, fibroblasts, epithelial and endothelial cells (60, 63–66). CD40 signaling drives upregulation of co-stimulatory molecules, cytokine production and cross-presentation of the antigen in DCs, thus

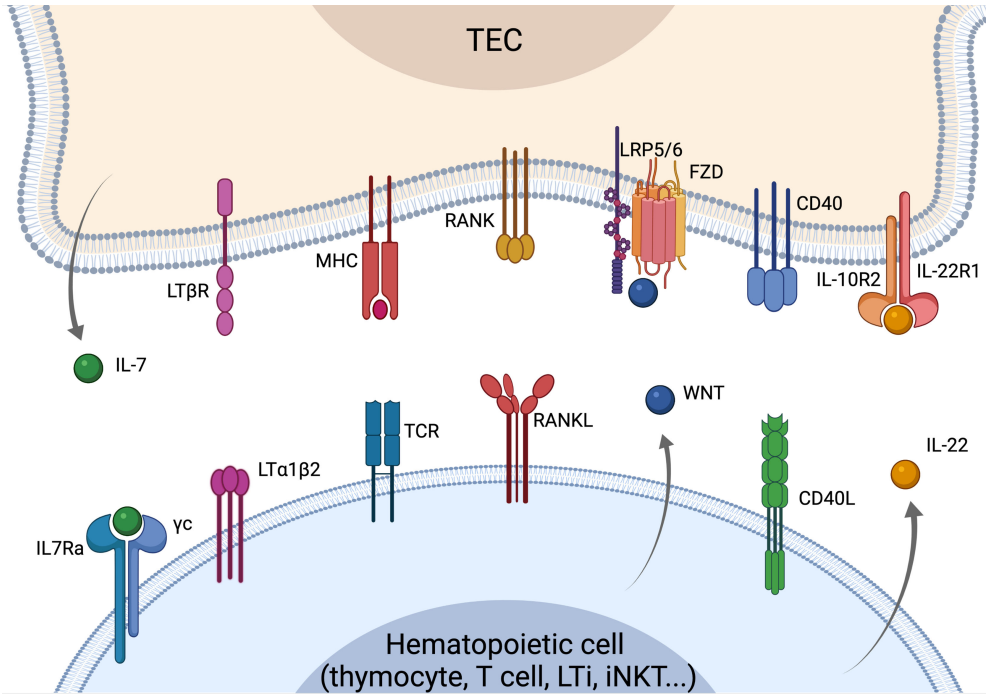

FIGURE 1 | Overview of the crosstalk signals driving TEC development, differentiation and regeneration. Recover of thymus function is strictly dependent on the crosstalk signals between TECs and cells of the hematopoietic compartment. LTα and RANKL are mostly provided by SP thymocytes leading to mTEC maturation and differentiation. RANKL is overexpressed by CD4 thymocytes and LTi cells upon insults and can drive the recovery of thymus function. WNT signaling regulates TEC proliferation and homeostasis, while CD40L is involved in TEC maturation and proliferation. LTi- and T-cells-derived IL-22 is key in sustaining TEC proliferation and thymus recovery upon insults. IL-7 is mostly produced by cTEC acting as a key mediator of thymocyte maturation and proliferation. Elements of the figure were generated using Biorender.com.

promoting DC-mediated T-cell activation (60, 67). Moreover, it was shown that CD8 and CD4 T cells directly communicate through CD40-CD40L interaction and this pathway is indispensable for the generation of CD8 T-cell memory (66).

As CD40-CD40L axis plays a crucial function in antigen presenting cell (APC) regulation, several studies investigated the role of CD40 signaling within the thymus in the context of T-cell development and selection, and self-tolerance induction (68). Here, similarly and in synergy with RANKL, CD40L stimulates mTEC maturation in the postnatal thymus, with both *Cd40* KO and *Cd40lg* KO mice showing a reduction in mTECs without affecting cTEC compartment (27, 51). On the other hand, Dunn et al. produced transgenic mice expressing CD40L cDNA under the control of the proximal *lck* promoter (69). These mice carrying *Cd40lg* overexpression in thymocytes showed alterations in organ architecture, with an abnormal mTEC proportion and reduction in thymus cortex (69).

Within the thymic medulla, RANKL and CD40L are upregulated in CD4 single positive (SP) thymocytes, this finding suggesting a key role of CD4 SP in regulating mTEC maturation and homeostasis (50, 59, 70). However, flow-cytometry analyses highlighted a great heterogeneity within CD4 SP population, with $CD25^{-}CD4^{+}TCR\beta^{high}$ thymocytes showing the highest RANKL positivity during the early SP stage ($CD69^{+}$), while being mostly $CD40L^{+}$ in subsequent maturation steps ($CD69^{-}$) (59). This temporal regulation of TNF family ligands expression in thymocytes is paralleled by a RANKL-dependent CD40 upregulation in mTECs, eventually leading to mTEC proliferation and maturation (59).

LYMPHOTOXIN-α

Lymphotoxin-α (LTα) is another member of TNF superfamily that was originally identified as a soluble factor secreted by lymphocytes having cytotoxic effects on tumor cells (71, 72). Subsequent studies showed that, besides its soluble homotrimer (LTα3) form, LTα could associate with the transmembrane protein LTβ resulting in the membrane-bound heterotrimer LTα1β2 (73). The latter signals through cell-cell interactions with its cognate receptor LTβR, resulting in both canonical and non-canonical activation of NF-kB pathway (74, 75). This signaling has several implications in immunity including the regulation of lymphoid organ development. In fact, both *Lta*, *Ltb* and *Ltbr* KO mice show similar phenotypes lacking lymph nodes and Peyer's patches, and abnormal splenic architecture (76–78).

Besides activated T and B cells, LTα1β2 is also expressed by NKs and type 3 ILCs (ILC3). On the other hand, LTβR is mainly expressed by epithelial and endothelial cells among macrophages and DCs (72).

Within the thymus, LTβR is expressed by the entire stromal compartment, especially by TECs, while LTα1β2 is mostly provided by single positive thymocytes (70, 79). Here, LTβ or LTβR deficiency leads to aberrant mTEC development and altered medulla organization (79–82). In particular, it was shown that LTα/LTβR signaling mediated by mature thymocytes is indispensable for the generation of terminally differentiated mTECs, as demonstrated by involucrin expression (83).

Besides its role in steady state, LTα is also important during insult recovery, as demonstrated by the fact that both *Lta* and *Ltbr* KO mice show impaired thymic recovery in *in vivo* models of HCT (47, 84). Upon SL-TBI, LTα1β2 upregulation is induced in radio-resistant LTi cells leading to thymic recovery through the stimulation of TEC proliferation and survival (54). On the other hand, LTα/LtβR signaling is also implied in T-cell progenitors homing and mature T-cells egress from the thymus in both steady state and HCT settings (84–86). For these reasons, LtβR agonistic antibody administration following HCT has been evaluated in mouse models, leading to an increase in thymic output and immune reconstitution (85).

INTERLEUKIN-7

IL-7 is a stromal-derived, non-redundant cytokine having a key role in regulating immunity and immune reconstitution (87). The active form of human IL-7 is a glycoprotein of 25 kDa that is mainly produced within the lymphoid organs and that signals through the IL−7 receptor (IL−7R) (2). The latter is a hetero −dimer consisting of IL−7Rα and the common cytokine receptor γ−chain (γc). Triggering of the receptor mediates anti−apoptotic and co−stimulatory proliferative signals, mostly on T- and B-cell lineages (88). In the thymus, IL-7 is primarily produced by TECs and fibroblasts (22). Using a IL-7 reporter mouse, it has been shown that TECs expressing high levels of IL-7 reside within a subset of cTECs defined as $CD205^{+}Ly51^{+}CD40^{low}$ (89). Cooperatively with Notch1, IL-7 provides proliferative signals to DN and DP thymocytes (90) and also sustains the recombination of the T-cell receptor γ−chain (TCRγ) locus (87). On the other hand, besides receiving maturation signals throughout their development, thymocytes control mTEC gene expression and differentiation, thus regulating the formation of a proper thymic microenvironment architecture (50, 70). For instance, thymocytes can downregulate *Il7* expression by TECs in a negative feedback fashion (91). In fact, lymphopenic *Rag2 Il2rg* double KO mouse strain shows a markedly increased proportion of $IL−7^{+}$ TECs compared to WT mice (91). IL-7R-deficient mice show defective thymic microenvironment, especially in corticomedullary structure, and reduced mTEC development (92, 93). While this phenotype is most likely due to a failure of the crosstalk normally provided by IL-7-dependent thymocytes and other cells of the hematopoietic lineage, a possible direct impact of IL-7 on thymic stromal cells is currently unknown. Interestingly, as discussed above, Vago et al. observed that serum levels of IL-7 peaked after every infusion of donor T-cells in transplanted patients, this suggesting that mature T-cells may induce IL-7 production, although the underlying mechanism is still largely obscure (38).

In the periphery, IL-7 has a key role in T-cell homeostatic proliferation and its production is tightly regulated, as the levels of IL-7 in the peripheral blood increase during lymphopenia remaining high until T-cell pool returns to steady state conditions (18, 94, 95). Given its crucial role in T-cell homeostasis, exogenous administration of IL-7 has been tested in several clinical conditions (87). In the context of HCT, IL-7 administration drives both CD4 and CD8 T-cell expansion, and this phenomenon is accompanied by an increase of TCR repertoire diversity (96). Most recently, IL-7 administration has been used in a murine model of age-related lymphopenia. Aged mice were subjected to IL-7 treatment and both numbers of CD4 and CD8 *naïve* T-cells in spleen and lymph nodes rose to levels similar to those observed in adult mice (97).

INTERLEUKIN-22

IL-22 is a monomeric cytokine released as a 179 amino acid monomeric protein (98). As IL-7, IL-22 is a non-conventional cytokine targeting stromal rather than hematopoietic compartment. In fact, the main targets of IL-22 are epithelial cells and fibroblasts within the thymus, liver, kidneys, lung and pancreas (99). On the other hand, the main contributors for IL-22 production are αβ and γδ T-cells, as well as ILCs, although fibroblasts, neutrophils and macrophages are also reported as secondary sources of IL-22 (99–104).

While systemic expression of IL22 is low during steady state, its production is induced upon negative stimuli, such as tissue injury and inflammation (99). During these pathologic conditions, IL-22 exerts controversial effects, being involved in both epithelial tissue regeneration and upregulation of different inflammatory mediators, including TNF, IL-6 and LPS-binding protein (105–107).

Within the thymus, IL-22 is involved in stromal regeneration following insults. In fact, IL-22 upregulation occurs in thymus-resident lymphoid tissue inducer (LTi) cells in mice exposed to SL-TBI (107). In turn, IL-22 production acts directly on mTEC compartment, providing proliferation and survival signals to the damaged tissue (107). Besides endogenous IL-22 production in injured thymus, recent findings demonstrated that exogenous administration of IL-22 could also promote faster thymic recovery. In fact, murine models of HCT showed that donor-derived T-cells are a major contributor for IL-22 production upon transplantation, leading to TEC proliferation and thymus recovery (108, 109). Moreover, exogenous IL-22 administration accelerates thymic regeneration after insults (107, 109).

Although IL-22 administration is currently being evaluated for the treatment of several conditions, only few trials are

exploring the infusion of IL-22 or their agonists in the HCT setting (NCT02406651, NCT04539470). While these studies are primarily focused on acute GvHD treatment or prevention, the recent results herein reviewed suggest the possibility to use IL-22 in restoring thymic function during the first period after the transplant.

WNT

WNT-signaling plays an important role during thymic development and in the maintenance of its function in adult life (11). In humans, 19 different WNT family members have been identified along with 15 WNT receptors and coreceptors. WNT regulates the stabilization of β-catenin which, in the absence of any WNT signaling, is degraded in a cytoplasmatic "destruction complex" consisting of glycogen synthase kinase 3β (GSK), adenomatous polyposis coli (APC), axis inhibition protein (AXIN) and casein kinase (CK). After the binding of WNT to a member of the Frizzled receptor family and its coreceptors low-density lipoprotein-receptor related proteins (LRP) 5 and 6, the β-catenin is no longer degraded leading to its accumulation, activation and translocation to the cell nucleus where it regulates downstream transcription factors of the TCF/LEF family. The crucial role of WNT in the thymus has been demonstrated in several genetic models. *Tcf-1* KO mice showed altered T-cell differentiation with a partial block at the double negative and immature single positive stages (110). Mice carrying a constituency active form of β-catenin in TECs show altered thymic organogenesis, reduced TEC proliferation and loss of TEC identity (111). The inhibition of WNT signaling through the forced expression of the canonical WNT inhibitor DKK1 leads to loss of TEC progenitors and thymic degeneration (112). Downregulation of WNT signaling has been also linked to the age-associated involution of the human thymus (113).

While stromal cells, such as TECs, are the major producers of WNT family members, cells of the hematopoietic lineage can also express WNTs. WNT proteins, such as WNT4 and WNT5b, expressed by TECs and thymocytes sustain the proliferation of TECs, which is partially achieved by increasing the expression of the key thymopoietic factor FoxN1 (114–116). Upregulation of FoxN1 expression represents a major step towards the regeneration of thymic function. Previous studies demonstrated that FoxN1 and its downstream genes are upregulated during the endogenous process of thymic reconstitution after sublethal dose of radiation (24). Importantly, induction of FoxN1 expression alone is sufficient to reverse thymic involution and regenerate the organ in mice (117). Together, these data demonstrate that the levels of FoxN1 tightly control thymic regeneration and the identification of factors regulating its expression could have a strong rationale for thymic boosting approaches. Whether mature T-cells can express members of the WNT family and induce the upregulation of FoxN1 in TECs when transferred *in vivo* would represent an interesting regenerative approach to investigate.

CONCLUSIONS

Several strategies have been proposed to restore thymic function after injuries and insults. Among these, the administration of chemokines and growth factors have been explored in several preclinical mouse studies displaying very promising results. However, when transferred to the clinic, the same strategies have shown modest regenerative potential. Until now, increasing thymic function and T-cell production remains a major challenge for the treatment of several conditions, especially in the early phase following HCT. Besides the HCT setting, boosting thymic function is of paramount importance for the treatment of other T-cell deficiencies associated with pathological, as well as physiological conditions. Thymic involution is a well-known phenomenon associated with a progressive decline of thymic size and output with age which paralleled with a decrease in immune surveillance in the elderly (118–120). Therapeutic approaches that can promote thymic function in older individuals can increase peripheral T-cell diversity, enhance the immunity against pathogens and response to vaccines, and, possibly, reduce the risk of malignancy through better immune-surveillance mechanisms against transformed cells. As previously discussed, the work by Vago et al. demonstrated that the infusion of mature donor T cells can rejuvenate the thymus of adult transplanted patients (aged 17-66). Whether a similar approach can restore TEC functionality in older individuals, in which the residual thymic tissue is limited (121) remains an avenue to be explored. On the other hand, it is highly unlikely that the same approach can mediate beneficial effects in restoring thymic function and the process of T-cell development in patients with intrinsic genetic defects which alter TEC function, for instance as a consequence of FoxN1 deficiency in patients affected by the nude/severe combined immunodeficiency. In fact, these defects cannot be mitigated by changes in the hematopoietic compartment as suggested by the inefficacy of bone marrow transplantation in these patients (122).

AUTHOR CONTRIBUTIONS

MR, MC, IS, MF, FL and EV wrote, drafted, and edited the manuscript. All authors contributed to the article and approved the submitted version.

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REVIEW
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Mechanisms of Direct and Indirect Presentation of Self-Antigens in the Thymus

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The inevitability of evolution of the adaptive immune system with its mechanism of randomly rearranging segments of the T cell receptor (TCR) gene is the generation of self-reactive clones. For the sake of prevention of autoimmunity, these clones must be eliminated from the pool of circulating T cells. This process occurs largely in the thymic medulla where the strength of affinity between TCR and self-peptide MHC complexes is the factor determining thymocyte fate. Thus, the display of self-antigens in the thymus by thymic antigen presenting cells, which are comprised of medullary thymic epithelial (mTECs) and dendritic cells (DCs), is fundamental for the establishment of T cell central tolerance. Whereas mTECs produce and present antigens in a direct, self-autonomous manner, thymic DCs can acquire these mTEC-derived antigens by cooperative antigen transfer (CAT), and thus present them indirectly. While the basic characteristics for both direct and indirect presentation of self-antigens are currently known, recent reports that describe the heterogeneity of mTEC and DC subsets, their presentation capacity, and the potentially non-redundant roles in T cell selection processes represents another level of complexity which we are attempting to unravel. In this review, we underscore the seminal studies relevant to these topics with an emphasis on new observations pertinent to the mechanism of CAT and its cellular trajectories underpinning the preferential distribution of thymic epithelial cell-derived self-antigens to specific subsets of DC. Identification of molecular determinants which control CAT would significantly advance our understanding of how the cellularly targeted presentation of thymic self-antigens is functionally coupled to the T cell selection process.

Keywords: thymus, central tolerance, antigen presentation, thymic epithelial cells, dendritic cells, cooperative antigen transfer

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INTRODUCTION

The immune system is considered to be one of the most complex entities in the body. It generates various specialized cells which primarily detect and eliminate pathogens to protect the host. This process of immune "self-nonself discrimination" is a fundamental attribute of a healthy immune system (1). Since T cell antigen receptors (TCRs) are generated by random somatic recombination without regards to a target, i.e. self or nonself-specific, T cells that express a potentially dangerous self-reactive TCR are either removed through the process of negative selection (recessive tolerance)

or diverted into thymic regulatory T cells (Tregs), the lineage of cells with the propensity to downregulate inflammatory responses (dominant tolerance) (2–4). These processes, which together are generally classified as central tolerance, are operational in the thymus and robustly limit the self-reactive repertoire within the T cell population (5, 6). One of the key molecules of central tolerance is the Autoimmune regulator (AIRE). AIRE has been determined to be a transcriptional regulator that promotes the "promiscuous", or "ectopic" expression of thousands of tissue-restricted self-antigens (TRAs), specifically in medullary thymic epithelial cells (mTECs) (7).

A critical part of the processes associated with central tolerance occurs in the thymic medulla and depends on the presence of various types of dendritic cells (DCs), B cells, and highly specialized non-hematopoietic antigen presenting cells (APCs) known as mTECs. These cells participate in recessive and dominant tolerance *via* cell autonomous antigen presentation (6). Recent data also suggests that cooperation between these cells in an unidirectional antigen transfer i.e., mTECs to DCs, which we will refer to as cooperative antigen transfer (CAT), is required for the efficient induction of T cell tolerance and Treg selection (8, 9). However, while CAT represents an important physiological pathway for imposing T cell tolerance, until recently, it was unclear how many cell subsets of mTECs and DCs participate in this process. In addition, it was not known how distinct DC subsets are recruited to mTECs resulting in efficient CAT, whether these cells interact in a stochastic or deterministic fashion and perhaps the most importantly, the specific roles of these cells in the establishment of tolerance.

In this review, we will highlight the current knowledge concerning the pathways by which self-antigens are presented in the thymus and how they lead to establishment of both recessive and dominant tolerance. We will also examine and discuss the possible molecular mechanisms underpinning CAT. Finally, we will draw attention to the current model of CAT which proposes distinct preferences of DC subsets in the acquisition of thymic epithelial cell-derived antigens.

DIRECT ANTIGEN PRESENTATION

The mechanisms of central tolerance are based on the premise that developing thymocytes (either $CD4^+$ or $CD8^+$) gauge their level of autoreactivity *via* the interactions of their TCRs with self-peptide-MHC (pMHC) complexes presented on the surface of thymic APCs (6). However, it is quite striking how developing thymocytes are able to see an entire collection of host self-antigens in an anatomically confined thymic space remained enigmatic over a long period of time. In the late 1980's, researchers unexpectedly found that some cell types were able to express seemingly irrelevant tissue specific genes (10, 11). This phenomenon was referred to as "ectopic gene expression" and led to the proposal that thymus cells can create a "patchwork quilt" of self-antigens which they present to developing T cells (12). These self-antigens are classified into three main groups: (i) antigens that exhibit a ubiquitous expression pattern; (ii) antigens which are specifically expressed by particular cell subtypes under certain conditions (such as those expressed by class-switched B cells); and (iii) antigens whose expression is limited to only one or up to a few anatomical places outside the thymus (7, 13–15). The latter category of self-antigens represent tissue-restricted antigens (TRAs) whose expression has been attributed to a rare population of thymic stromal cells, mTECs (13). The specifics of TRAs expression are very different from those of standard gene expression in peripheral tissues: (i) TRAs, whose production is tightly regulated, are expressed by a single mTEC in a stochastic manner (only 1-3% of all mTECs express a given TRA at any given time) (16, 17); (ii) TRA genes are often expressed from a single-allele using alternative transcriptional start sites (18); (iii) sex-related genes are expressed by mTECs irrespective of gender (16, 19); (iv) TRAs contain several development-related genes that are expressed by mTECs with no connections to the developmental status of the organism (13). These attributes enable mTECs to express a broad repertoire of self-antigens that are needed for proper T cell selection.

Recently, RNA sequencing technology has helped determine that mTECs express more than 18,000 genes, which represent approximately 85% of the protein-coding genome (20, 21). Compared to other cell types from different tissues, the number of genes typically range from 12,000 to 14,000 (i.e., 60- 65% of coding genome) (22). Remarkably, there are approximately 4,000 genes in mTECs regulated by AIRE (7, 21). Thus, a set of mTEC-dependent TRAs can be expressed in an AIRE-dependent or AIRE-independent manner. While the regulation of AIRE-independent promiscuous gene expression is still not completely understood, the transcription factor Family Zinc Finger 2 (FEZF2) was suggested to play a complementary role in mediating immune tolerance to AIRE-independent TRAs (23). Also, as mentioned above, any TRA at any given time is expressed only by 1-3% of mTECs and one mTEC is able to co-express approximately 100-300 TRAs (16, 17, 24). Correspondingly, it was postulated that 200-500 mTECs are sufficient to cover the entire TRA repertoire (22). This suggests that TRA expression is in the thymus controlled by the rules of "ordered stochasticity", where the initial co-expression pattern of TRAs is stochastic, but then is highly regulated by a coordinated set of events (24).

The previously mentioned process, in which the recognition of epitopes derived from TRAs by self-reactive T cells leads to their deletion or conversion to Treg-lineage was described in classical studies that employed neo-self-antigen technology. Using mouse models in which the expression of hen egg lysozyme (HEL) or membrane-bound chicken ovalbumin (mOVA) was driven by the rat insulin promotor (RIP), and thus expressed in an AIRE-dependent manner, it was described that *Aire* knockout (KO) mice possessed an increased number of neo-self-antigen specific (TCR-HEL or OT-II, respectively) $CD4^+$ T cells, suggesting a role of $AIRE^+$ mTECs in clonal deletion (25, 26). Also, using tetramer enrichment technology, it was shown that polyclonal T cells which are specific for particular TRAs are modestly increased in *Aire* KO mice (14).

Using mTEC-specific neo-self-antigen models along with TCR transgenic systems, *Aschenbrenner et al.* suggested that AIRE-expressing mTECs also play a crucial role in Tregs generation (27). This was confirmed for organ specific Tregs which required AIRE-dependent expression of TRAs as well (19, 28). The importance of AIRE itself in shaping the Tregs repertoire was implied by deep sequencing of the complete TCRα genes in Tregs and conventional T cells (Tconv) that were isolated from *Aire* KO mice. This experiment showed that in the absence of AIRE, TCR sequences which were usually found among a Treg lineage could be detected in the repertoire of Tconv cells (29). However, other studies presented evidence that AIRE is essential for the generation of Tregs, mostly during the neonatal period of life (30–32).

Even though the mechanisms of central tolerance have been extensively studied, there is still a paucity of information detailing the mechanism controlling the decision-making process between clonal deletion and Tregs generation. The simplest models used to illustrate the specifics of the mechanism have been based on the fact that high-affinity interaction leads to clonal deletion, while weaker interactions have resulted in Tregs generation (6). This is in agreement with studies that have used T cell transgenic systems specific to neo-self-antigens which, however, exhibit a high affinity for TCR-pMHC interaction and are skewed to massive clonal deletion rather than Tregs deviation (25, 26). On the other hand, the MHC-tetramer technology which operates using natural TCR affinities provides evidence that the clonal deletion of TRA-specific thymocytes is far from being complete and is rather biased towards Treg selection (14, 33–35). Specifically, this phenomenon was described using MHCII tetramers specific to neo-self-antigens, whose expression is restricted to either all (ubiquitous antigens) or various tissues (TRA-like expression pattern). It was shown that the recognition of ubiquitous antigens led to a massive deletion of antigen-specific T cells, whereas the recognition of TRA-like antigens predominantly promoted the diversion to Treg lineage (14, 34). This observation opened the question of whether different types of APCs play a non-redundant role in mediating clonal deletion or Tregs selection.

It has been known for more than a decade that the thymic population of APCs is heterogeneous in its nature since it includes cells of hematopoietic and non-hematopoietic origin. In recent years, the robustness of single-cell RNA sequencing (scRNAseq) has not only yielded a vast amount of information about thymic APC heterogeneity (36–40) but have provided a set of new markers to distinguish these APC subsets. Historically, thymic epithelial cells (TECs) have been divided into two major populations: mTECs and cortical TECs (cTECs) (41). Recently, combining lineage tracing technology with scRNAseq, it was revealed that mTECs are highly heterogeneous and comprise of multiple populations that have different molecular and functional characteristics. These include immature and $CCL21^+$ mature $mTEC^{Low}$, $AIRE^+$ $mTEC^{High}$, corneocyte-like mTECs (Post-Aire mTECs), and tuft cell-like mTECs (36, 42–44). Even though there are publications describing the roles of particular mTEC-subtypes, such as the attraction of single positive (SP) thymocytes to the medulla ($CCL21^+$ $mTEC^{Low}$) (45), modulation of Type 2 immune responses (tuft cell-like mTECs) (36, 46), or production of pro-inflammatory cytokines (Post-Aire mTECs) (47), the exact function of specific TEC-subtypes in mechanisms of clonal deletion or Tregs selection is largely unknown. The data which has been compiled so far suggests that the $AIRE^+$ $mTEC^{High}$ subset, by presenting peptides derived from TRAs, plays a non-redundant role in Tregs generation, whereas the other mTEC-subpopulations predominantly participate in clonal deletion due to the presentation of ubiquitous antigens (14, 34, 35). Because the direct MHC-dependent interaction between developing T cells and mTECs is required for proper medullary organization, assessing the exact function of TEC-subtypes in tolerance would require the development of models that target MHC expression in particular TECs subpopulations. So far, this aim was partially achieved with $AIRE^+$ mTECs where the MHCII transactivator, C2TA, was knocked down by *Aire* promotor-driven shRNA. C2TAkd mice showed a moderate increase in $CD4^+$ T cells suggesting the role of mTECs in clonal deletion. Interestingly, the introduction of MHCII deficient bone marrow to this system further increased the number of $CD4^+$ T cells suggesting that mTECs and DCs play non-redundant roles in clonal deletion (48). Further analysis comparing the unique TCRα sequences of $CD4^+$ T cells from C2TAkd and mice with MHCII deficient bone marrow revealed that even though mTECs were able to perform clonal deletion, their relative contribution to this process was minimal compared to bone marrow-derived DCs (8). Since such a non-redundant role of mTECs and DCs has also been shown for Tregs selection, it indicated the functional dichotomy of epithelial and DC cellular networks involved in the establishment of central tolerance.

Historically three major conventional subtypes of DC have been described within the thymus: plasmacytoid DCs (pDC), classical DC Type 1 (cDC1), and classical DC Type 2 (cDC2) (49). These major DC subtypes, commonly expressing CD11c marker, are delineated by their expression of lineage specific surface markers and transcription factors. The cDC1 population is defined by the expression of the chemokine receptor, XCR1, and requires the transcription factors BATF3 and IRF8 (50), whereas cDC2 subset expresses SIRPα and partially requires the transcription factor IRF4 (51). In general, the function of DCs in central tolerance was first determined in CD11c-Cre-DTA mice in which their genetic ablation led to impaired clonal deletion of T cells and development of severe autoimmunity (52), demonstrating their indispensable role in establishment of tolerance. Along with the conventional DC-subtypes mentioned above, the thymus also accommodates other DC-like subsets, such as monocyte-derived DCs (moDC) or cells that resemble activated or migratory DCs which are present in peripheral lymphoid and non-lymphoid tissues (53–55). These cells are characterized by increased expression of CCR7 and have been described as activated DCs (aDC) (39, 54).

The function of cDC1 has been mostly attributed to the cross-presentation of mTEC-derived self-antigens to developing T cells

(described in detail in the next chapter). Also, the previously mentioned CCR7$^+$ aDC derived from XCR1$^+$ cDC1 have been shown to be particularly important in this process (54). On the other hand, the activated DCs also change their displayed self-peptidome, through changes in proteasome subunits, phagosome enzymes, and autophagy proteins. Thus, aDC have the potential to tolerize developing T cells to self-antigens that are associated with their activation (56). This should be particularly important during inflammation in the immune periphery when DCs are activated to protect the host from the development of autoimmune reactions towards self-molecules that are associated with DC-activation (54). In contrast to cDC1, cDC2 has been shown to originate in the periphery, and thus capable of presenting antigens acquired in peripheral tissues (57). This was first postulated by *Bonasio et al.* showing that OT-II thymocytes were selectively deleted in the thymus after intravenous injection of OVA-loaded exogenous DCs (58). More recently, the specific population of trans-endothelial DCs was described to be responsible for delivering and presenting peripheral blood-borne antigens to the thymus for clonal deletion (59). Interestingly, the positioning and function of these cells was shown to be dependent on CX3CR1 expression which also marks the specific population of DCs previously associated with presentation of intestinal-derived microbial antigens in the thymus (60). This data suggests that CX3CR1$^+$ cDC2 cells are responsible for the delivery of peripheral antigens to the thymus for T cell clonal deletion (53, 59, 60). In addition, the thymic population of pDC was also shown to be involved in mediating central tolerance since adoptively transferred OVA-loaded pDCs migrated to the thymus and promoted the deletion of OT-II thymocytes. Interestingly, the migration of pDC into the thymus was shown to be dependent on the CCR9/CCL25 axis, which is also important for migration of cells into intestinal tissues (61). This suggests that in addition to cDC2, pDCs could also be responsible for presentation of peripheral antigens in the thymus.

The thymus also accommodates a population of B cells that seem to be "licensed" for antigen presentation, the phenomenon in which the thymic microenvironment plays an indispensable role (62, 63). The thymus contains a population of class-switched B cells that express AIRE and thus can present some of the AIRE-dependent antigens to thymocytes (62). It has also been shown that self-specific B cells in the thymus can acquire antigens *via* B cell receptor- (BCR) mediated endocytosis and promote tolerance by presenting these antigens to thymocytes (64). Moreover, class-switched B cells may also play an important role in driving tolerance to unique B cell antigens and such tolerization of T cells would be crucial during the adaptive immune response in the periphery (15, 63, 65). Given that thymic B cells and their role in antigen presentation were reviewed at length (66), we will primarily discuss antigen distribution and presentation in mTECs and thymic DCs.

Taken together, the thymus is a unique place, where the vast majority of antigens derived from the host's own tissues is presented to mediate clonal deletion or Treg conversion of self-reactive T cells. A large proportion of these antigens are directly presented to thymocytes by a unique population of AIRE$^+$ mTEC^{High}. Moreover, thymic populations of hematopoietic APCs also participate in central tolerance mechanisms by direct presentation of antigens that cannot be presented by mTECs, such as blood-borne antigens, antigens derived from microbiota, or B cell specific antigens.

INDIRECT PRESENTATION OF TISSUE-RESTRICTED ANTIGENS

As described in the previous chapter, mTECs are a critical cellular source of self-antigens in the thymus. However, the total number of mTECs is quite limited (approx. hundreds of thousands per thymus in a young mouse (67, 68)). Moreover, each individual mTEC presents a distinct set of TRAs that constitutes a mere fraction of this TRA pool (16). In addition, mTECs were recently found to be highly heterogeneous, comprised of cell subsets, some of which weakly displayed or were incapable of producing or presenting TRAs (38). Another frequently discussed issue is the fact that antigen processing and presentation often differs in mTECs and peripheral APCs, which begs the question of how closely mTEC-centered central tolerance mimics antigen presentation in the periphery, and thus ensures the scope and stringency of negative selection (69). This concept has led researchers to consider whether mTEC-autonomous production and presentation of self-antigens is sufficient to establish a fully operational immune tolerance or if such a task is beyond their collective capacity and discretion.

Nearly two decades ago, *Gallegos and Bevan* provided insight into this issue. They convincingly showed that thymic clonal deletion of OVA-specific CD4$^+$, and to some extent CD8$^+$ T cells, is dependent on antigen presentation by bone marrow-derived (BM) APCs. Since the expression of the membrane bound OVA (mOVA) antigen under RIP was directed exclusively to mTECs, the authors concluded that mOVA must be transferred to BM APCs and displayed in the context of their MHC molecules for the efficient deletion of cognate T cells (70). Given that developing T cells reside in the medulla for 4-5 days as they rapidly move to scan pMHCs on a variety of APCs (67, 71), such antigen transfer from mTECs to BM APCs can significantly reinforce the establishment of central tolerance. This new phenomenon which "sealed the gaps" in mTEC-driven tolerance was referred to as indirect antigen presentation (70).

The original data obtained with the RIP-mOVA model was complemented by another transgenic system, where OVA was produced only in those mTECs which expressed AIRE, e.g. Aire-OVA knock in (Aire-OVA-KI) mice (72). In sharp contrast to the *Gallegos and Bevan* study, where the deletion of mOVA-specific OT-II T cells was found to be completely dependent on the indirect presentation by BM APCs, direct presentation of OVA by mTECs was sufficient to delete OTII T cells in the Aire-OVA-KI model. The explanation for this discrepancy is likely due to the fact that in the RIP mOVA model, the mOVA is expressed predominantly by mTEC^{Low}, whereas the expression

of OVA in Aire-OVA-KI mice is restricted to the $mTEC^{High}$ subset whose antigen presentation capacity is robust (72). Since $mTEC^{Low}$ are poorly presenting APCs, their presentation of mOVA is presumably insufficient to induce a proper clonal deletion and/or deviation of OT-II T cells to Tregs without the support of DCs. This hypothesis is supported by the evidence from *Hinterberger et al.* that showed that a reduction in the expression of MHCII on mTECs leads to the impaired selection of OVA-specific T cells, regardless of DCs depletion (48). On the other hand, indirect presentation was repeatedly shown to be dependent on AIRE, since it upregulates the expression of several chemokines which attract DCs to the vicinity of AIRE-expressing mTECs (72–74). AIRE also supports indirect antigen presentation by suppressing CTLA-4 expression in mTECs, hence keeping the key costimulatory role of CD80/86 molecules on BM APCs for agonist selection of Tregs uncompromised (75). In fact, the deviation of T cells into Tregs was found to be dependent on AIRE in both modes of antigen presentation (72). This is consistent with the observation that for the agonist selection of Tregs, the presentation of TRAs by APCs residing in the medulla of the thymus is absolutely necessary (76) which is in contrast to the requirements for clonal deletion of T cells that appears to be much less dependent on a functional medullary microenvironment (74, 77, 78).

Using two-photon microscopy of *ex vivo* thymic slices from RIP mOVA mouse, it was recently shown that most of the mOVA specific $CD8^+$ OT-I T cells were activated by BM APCs through indirect antigen presentation, while the activation of $CD4^+$ OT-II T cells was found to be equally dependent on both direct and indirect mOVA presentation. In contrast to the RIP mOVA system, RIP OVA^{HI} mice which produced an intracellular form of OVA under RIP, showed a much higher activation of OT-II T cells by BM APCs than OT-I T cells (79). Thus, it seems that subcellular localization of OVA predicates its predominant indirect presentation on MHCI or MHCII molecules. Importantly, this study also suggested that when polyclonal T cell repertoire is considered, an indirect antigen presentation played a primary role in the deletion of $CD4^+$ T cells. In addition, there was evidence that an indirect presentation was, in general, as crucial as the direct presentation of antigens by mTECs in both $CD4^+$ and $CD8^+$ T cell tolerance (79).

The indispensable role of indirect antigen presentation in the context of the polyclonal T cell repertoire was ascertained by TCRα sequencing of BM chimeras that exhibited partial or full MHCII deficiency on mTECs and BM APCs, respectively (8). It has been found that TCR specificities sensitive to indirect presentation generally do not overlap with those specificities engaging mTECs. Furthermore, BM APCs were found to be crucial not only for clonal deletion but for the generation of Tregs where approximately 30% of unique Treg TCR specificities were dependent on MHCII presentation by BM APCs. Moreover, a vast array of TCR sequences that were either deleted or deviated into Tregs by BM APCs turned out to be dependent on AIRE. Paradoxically, these T cell clones could not be deleted or transformed into Tregs by direct antigen presentation (8). A logical explanation for this observation relies on the fact that mTECs and BM APCs possess different antigen processing machinery that results in the presentation of distinct peptides from a particular TRA (80–82). Hence, indirect presentation not only raises the number of cells which present TRAs, it also extends the repertoire of T cell clones affected by the processes of central tolerance. In support of these results, other studies have reported a requirement for indirect presentation to delete or deviate into Tregs those T cells which engage certain AIRE-dependent TRAs, namely proteolipid protein (PLP) (35, 83), interphotoreceptor retinoid binding protein (IRBP) (33), or prostate-specific antigen MJ23 (9).

As previously stated, the routine use of scRNAseq by us and others has led to the exploration of thymic BM APC heterogeneity (39, 53). Even though it has been repeatedly shown that thymic DCs are those BM APCs which participate in indirect antigen presentation, their relative contribution to this process remains unclear (8, 9, 28, 79, 83–86). Importantly, all thymic DC subsets are capable of obtaining antigens from mTECs (53, 87). Nevertheless, while pDC, cDC2, and moDC are also known to present the antigens acquired from outside of the thymus (59–61), cDC1 and aDC seem to establish central tolerance primarily through indirect presentation of mTEC-derived antigens (8, 54, 88). Indeed, cDC1 and aDC are localized to the medulla in proximity to AIRE-expressing $mTEC^{High}$ (39, 74). Moreover, the cooperation of cDC1 with mTECs was found to be indispensable for keeping the process of tolerance establishment operational, since autoimmune manifestations are much more profound in mice that are deficient in both cDC1 and mTECs compared to mice deficient only in the mTEC or cDC1 cell compartment (89). Although cDC1 deficient mice do not display differences in their overall frequency of Tregs compared to WT mice (89), their Treg repertoire was found to be aberrant, mainly in respect to those clones which recognized AIRE-dependent TRAs (8). Nevertheless, recent experiments with cDC1 deficient *Batf3* KO mice have shown that merely 2% of clonally deleted T cells and 12% of generated Tregs were completely dependent on the cDC1 lineage (88). Another report which used *Batf3* KO mice as a model, also showed a negligible role of cDC1 in clonal deletion of $CD8^+$ T cells (90) which, given the robust cross-presentation capability of cDC1 (91), was surprising. On the other hand, thymic cDC2 revealed an efficient cross-presentation of mTEC-derived antigens to $CD8^+$ T cells in *ex vivo* thymic slices (79), which indicated their contribution to the deletion of self-reactive cytotoxic T cells. It is of note that several studies have provided evidence that Tregs are generated by cDC2 and not by cDC1 (9, 86, 92). Notably, it was observed that a CCR7 deficient thymus displayed a reduction in the cDC1 lineage which lead to an enhanced Treg selection by cDC2 that expressed low levels of MHCII (92). However, this result is puzzling in the context of recently described $CCR7^+$ aDC subsets which are marked by high MHCII expression with several transcriptomic analyses showing that these subsets are molecularly fully equipped for Treg generation (39, 53, 54). Moreover, a recently published study from our lab showed that moDC can enhance Treg generation in

the thymus under inflammatory conditions *via* the acquisition of mTEC-derived antigens (53). Thus, at this juncture, while it seems that each thymic DC subset can contribute to indirect antigen presentation, the level of their contribution to recessive versus dominant tolerance in respect to the accompanying physiological circumstances requires further clarification.

It was shown recently that the abrogated phagocytic activity of BM APCs led to impaired deletion of $CD8^+$ T cells (93). The authors of this study proposed a model that illustrated the clearance of self-reactive T cells by BM APCs preventing their escape from clonal deletion and subsequent autoimmune manifestations in immune periphery. This process was found to be dependent on the expression of phosphatidylserine and the scavenger receptor, TIM-4, on apoptotic cells and phagocytes, respectively. Thus, it seems that rapid phagocytosis, besides indirect antigen presentation, represents an essential capability of BM APCs to establish central tolerance. In fact, clonal deletion was found to be most efficient when T cells engaged indirectly presented antigen on the same BM APC which also phagocytosed such a T cell (93). However, based on the current knowledge, we propose that the observed breakdown of central tolerance in phagocytosis-deficient thymus is likely caused by deficiencies in indirect antigen presentation (83, 88) (see **Figure 1** highlighting physiological benefits of indirect antigen presentation).

COOPERATIVE ANTIGEN TRANSFER

In 1994, *Bruno Kyewski´s* group reported that thymic DCs acquire antigens which are produced by thymic epithelium (94). This original finding gained importance ten years later when *Gallegos and Bevan* showed that indirect presentation of mTEC-derived antigens by thymic DCs is crucial for the maintenance of central tolerance (70). Hence, it became obvious that the transfer of antigens from mTECs to DCs, referred to as "Cooperative antigen transfer" (CAT) (69), is a prerequisite for indirect antigen presentation. According to our data and the results of others, all currently described DC subsets participate in CAT (53, 83, 87, 95). However, given that their heterogeneity is determined by a distinct gene expression profile (39, 53), each subset might employ a distinct mechanism to achieve it. Theoretically, CAT can be mediated by cell contact-independent and several cell contact-dependent mechanisms, namely via: i) exosomes, ii) trogocytosis, iii) gap junctions and iv) endocytosis/phagocytosis, and was shown that it involves antigens with nuclear, cytosolic or membrane localization (83). Regarding the cell contact-independent mechanism, it was reported that human mTECs secrete exosomes which contain TRAs when cultured *in vitro* (96). However, it has been repeatedly shown using transwell assays that exosomes do not

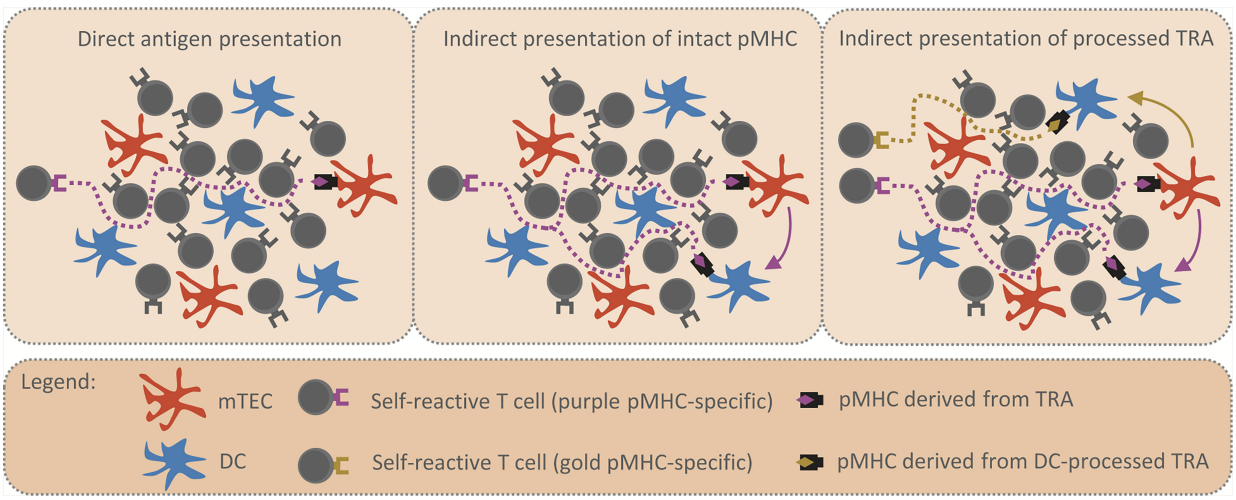

FIGURE 1 | Reinforcement of central tolerance by indirect presentation. The schemes depict model situations in which self-reactive T cells (purple and golden TCR) migrate through the thymic medulla to engage their cognate TRA (purple and golden rhombus) presented by mTEC (in orange) or DC (in blue) and undergo the processes of central tolerance. Possible migration pathways of self-reactive T cells are visualized by the dotted lines. The first scheme (left panel) displays the situation where TRA is presented directly by a single mTEC, thus, there is a low probability that a self-reactive T cell will encounter the mTEC and be tolerized. In the second scheme (middle panel), the intact pMHC shown in the left scheme is transferred from mTEC to DC (purple arrow). The antigen presentation is enhanced, since the same TRA is presented both directly and indirectly by mTEC and DC, respectively. The third scheme (right panel) captures the situation in which the TRA is transferred to (golden arrow) and subsequently processed by DC. Since the antigen processing machinery of DC is distinct from that of mTEC, a DC-processed pMHC complex (golden rhombus) is recognized by a self-reactive T cell of different specificity (golden TCR) from the original (purple TCR). Therefore, indirect presentation not only enhances antigen presentation in the medulla (middle panel), it also broadens the repertoire of T cell clones subjected to processes of central tolerance (right panel).

serve as a source of TRAs. Indeed, CAT requires and is dependent on a cell-cell contact (88, 95, 97).

Trogocytosis is a process in which two cells exchange portions of their plasma membranes (98). For example basophils were shown to obtain intact pMHCII complexes from DCs through trogocytosis, and thereby served as APCs, even though they did not express antigen presenting machinery genes, including those encoding MHCII (99). By the same token, trogocytosis has been suggested to drive CAT of intact pMHCII molecules in the thymus ensuring their rapid presentation to T cells (83, 97). Paradoxically, while pMHCII molecules are localized to lipid rafts, these membrane microdomains were found to be dispensable for operational CAT (95). In our latest study, we took advantage of a $Foxn1^{Cre}Confetti^{Brainbow2.1}$ model in which we directly compared the transfer of membrane-bound CFP with cytosolic RFP or YFP from mTECs to DCs (87). Strikingly, the efficiency of the transfer of CFP was weak in comparison to cytosolic antigens. Additionally, in marked contrast with transfer of cytosolic antigens, the acquisition of CFP was negligible in all thymic DC subsets, except $XCR1^+$ aDC. Hence, we focused on $XCR1^+$ aDC and visualized the differences in their uptake of CFP, RFP, and YFP using imagestream. Notably, while RFP and YFP were strictly localized to the intracellular vesicles of $XCR1^+$ aDC, CFP was localized in their plasma membranes. Hence, this result indicates that the mechanism of CAT in the context of cytosolic antigens differs from that of membrane-bound molecules and suggests that $XCR1^+$ aDC utilize trogocytosis to perform CAT.

Since gap junctions manage to transport particles of molecular weight up to 1,8 kDa (100), transfer of small, cytosolic peptides might occur through this mechanism. Although all subsets of thymic DCs robustly acquire cytosolic, mTEC-derived antigens, $XCR1^+$ aDC were shown to also excel in this mode of CAT (54, 87). As previously mentioned, cDC1 and $XCR1^+$ aDC reside in permanent, close contact with $AIRE^+$ mTECs (39, 74). Hypothetically, in this niche gap junctions might be formed between mTECs and DCs to drive CAT. However, so far there has not been published evidence to support this hypothesis.

$AIRE^+$ mTECs exhibit a rapid turnover (101) and a tangible fraction matures into a senescent/apoptotic post-Aire mTEC (43). Although these mTEC subsets downregulate genes encoding antigen presenting machinery, according to a recent study, they retain high levels of TRA expression (38). Hence, apoptotic mTECs might serve as a reservoir of TRAs and act as an ideal phagocytosis substrate for DCs residing nearby. Indeed, experiments using a mouse strain which exhibit a knocked out scavenger receptor CD36 showed that cDC1 used this receptor to engulf apoptotic mTECs (88). An array of T cell clones whose clonal deletion/agonist selection relied on cDC1, also depended on functional CD36 and the lack of this receptor led to autoimmune manifestations. However, while CD36 seems critical for the establishment of central tolerance, cDC1 are endowed with yet another mechanism of CAT. A comprehensive study from *Bernard Malissen´s* lab unravelled the complex transcriptomic changes underpinning cDC1 homeostatic maturation into $XCR1^+$ aDC in the thymus. Interestingly, this process resembles the immunogenic maturation of peripheral DCs or their maturation within tumors (54, 102). Notably, the maturation of cDC1 within tumors is driven by the scavenging of apoptotic tumor cells and is at least partially dependent on another scavenger receptor, AXL (102). To some extent, mTECs resemble tumor cells, since their DNA is highly stressed due to the AIRE-mediated formation of DNA double-strand breaks (103, 104). Thus, hypothetically, AXL-mediated CAT might drive cDC1 maturation in the thymus. There seems to be a consensus that thymic maturation of cDC2 converges with that of cDC1 into aDC phenotype (54, 55). Interestingly another scavenger receptor, TIM-4, is expressed by thymic cDC2 (53). Since the absence of TIM-4 abrogates the uptake of apoptotic bodies by thymic DCs and causes the breakdown of central tolerance (93), we posit that this molecule is also one of the drivers of CAT. Correspondingly, antigen uptake by thymic cDC2 or cDC1 was shown to be completely inhibited after the administration of Cytochalasin D and NH_4Cl, inhibitors of phagocytosis (57).

With the exception of scavenger receptors expressed by DCs, chemokines and immune receptors expressed by mTECs are also considered to be critical molecular determinants of CAT. As mentioned in the previous chapter, mTECs express various chemokines in an AIRE-dependent manner which attract DCs of both cDC1 (XCL1) and cDC2 (CCL2, CCL8, CCL12) lineages to the vicinity of mTECs to facilitate CAT (73, 74, 105). In this context, we have recently shown that mTECs express Toll-like receptor (TLR) 9 whose signaling upregulates the expression of a set of AIRE-independent chemokines (53). This resulted in an enhanced migration of moDC to the thymic medulla, increased their potency for CAT, and in general, decreased the cellularity of thymic cDC1. Given that mice with the ablation of TLR9 signaling specifically in mTECs, displayed a decreased frequency and functionality of Tregs, it suggests that mTEC-produced chemokines which drive the enrichment of moDC in the medulla positively modulate agonist Treg selection. Recently, the checkpoint molecule, CTLA-4, expressed on the surface of mTECs was found to negatively affect the transfer of mTEC-derived MHCII molecules to cDC2 and more overtly to cDC1 (75). Since the silencing of CTLA-4 is AIRE-dependent event, AIRE also sustains CAT through this mechanism.

Finally, it has been postulated that adhesion molecules play a key role in CAT. Interestingly, thymic DCs exhibit a high expression of EPCAM, an adhesion molecule which is a standard epithelial cell marker (83, 95). Since it was observed in Foxn1eGFP knock-in mice, that those DCs which displayed a high positivity for mTEC-derived eGFP also possessed high levels of EPCAM, it was assumed that they acquired EPCAM along with eGFP from mTECs (83). Nevertheless, a recent study verified that $EPCAM^+$ DCs express mRNA levels encoding this molecule comparably to mTECs, arguing that thymic DCs themselves produce EPCAM (95). Interestingly, thymic DCs outcompeted splenic DCs in their competence to perform CAT *in vitro* (83, 95). Since splenic DCs lack the expression of

EPCAM (95), it is possible that the high expression of EPCAM by thymic DCs is a contributing factor to their efficient performance of CAT.

PREFERENTIAL PAIRING IN CAT

CAT has been described as a very complex process primarily because of the previously found heterogeneity of thymic APCs. Historically, CAT was shown as unidirectional process from mTECs to thymic APCs but specifically attributed to thymic DCs (53, 79, 83, 95). This unidirectionality advocates that CAT is a tightly regulated process (potential regulators were detailed above) that requires specific molecules to be expressed by both donors (TECs) and acceptors (thymic DCs), which also suggests that their differential expression affects the effectivity of CAT. This statement is supported by observations that distinct subtypes of DCs vary in their capacity to acquire TEC-derived antigens. Whereas CAT to cDC1 and cDC2 was reported to occur with the same efficiency, the transfer of antigens to pDC is fairly limited (53, 95). Notably, pDC were shown to be attracted to Hassall's corpuscles, the structures formed by Post-Aire mTECs, which due to the lower expression of MHCII and persistent production of TRAs are considered as a source of self-antigens for CAT (47, 106). Accordingly, it was shown that the homing of thymic pDC into Hassal´s corpuscles in the human thymus endows pDC with the ability to generate Tregs (107). These findings suggest a role of thymic pDC in CAT specifically from Post-Aire mTECs. We have recently documented that the thymic moDC could also be drawn to the proximity of Post-AIRE^{+} mTECs due to the enhanced expression of pro-inflammatory chemokines (40, 53). This "preferential pairing" between specific subsets of TECs and thymic DCs has also been suggested by others. Notably, *Perry et al.* used as a model antigen GFP expressed only by AIRE^{+} mTECs in the thymus (Aire-GFP mouse model). The BM chimeras of WT cells injected into the Aire-GFP mouse revealed the antigen transfer of GFP specifically to XCR1^{+} DCs and only limited transfer to SIRPα^{+} cDC2 (88). On the other hand, the OVA antigen from the RIP-mOVA mouse model, whose expression is enriched in mTEC^{Low} or Post-Aire mTECs was transferred to SIRPα^{+} cDC2 with higher efficiency than to cDC1 (72, 79). These observations let us to predict that distinct subsets of thymic DCs acquire antigens from distinct subsets of TECs.

Our recent publication aimed to test this prediction by using several *Cre* reporter mouse models in which the expression of fluorescent TdTOMATO (TdTOM) protein is enriched in different subsets of TECs (87). The crossing of previously characterized *Cre*-based models with *Rosa26*^{TdTOMATO} led to the generation of Foxn1^{Cre}Rosa26^{TdTOMATO} where TdTOM is expressed by all TECs (53, 108), Csnb^{Cre}Rosa26^{TdTOMATO} that restricts its expression to mTEC^{High} and their close progeny (36, 109), and Defa6^{iCre}Rosa26^{TdTOMATO} where TdTOM mimics the expression of AIRE-dependent TRA while its production is limited to a minority of AIRE^{+} mTEC^{High} and their progeny (110). Using linear regression correlations with the predominant expression of TdTOM in a certain population of TECs and TdTOM transfer to distinct subsets of thymic DCs, the study demonstrated that CAT is mediated predominantly by preferential pairing between mTEC^{Low} and cDC2, mTEC^{High} and XCR1^{+} and XCR1^{-} aDC, and Post-Aire mTECs and pDC. Interestingly, two populations of thymic DCs, XCR1^{+} cDC1 and moDC did not reveal any or showed a limited correlation despite their high participation in the process of CAT (87). Since the previously mentioned study from Perry et al. described the XCR1^{+} DCs as the only DC-subtype which was able to acquire the GFP antigen from mTEC^{High}, one can assume that this transfer was directed to mature XCR1^{+} aDC (88). On the other hand, as previously pointed out, because the XCR1^{+} aDC subset was shown to descend from XCR1^{+} cDC1 and their maturation in tumor tissues was shown to be dependent on antigen uptake, antigens expressed by mTEC^{High} may be indeed preferentially acquired by cDC1, which then initiates their maturation to aDC (54, 102). To verify such a scenario, the future identification of regulators of CAT and their subsequent genetic ablation will be necessary to test the prediction that XCR1^{+} aDC should not be generated in the absence of CAT from mTEC^{High} to XCR1^{+} cDC1 (102).

Remarkably, the situation with the thymic moDC population seems to be quite different. Using Foxn1^{Cre}Confetti$^{Brainbow2.1}$ mice and mixed BM chimeras where fifty percent of DCs express TdTOM, we recently showed that thymic moDC represent the major subtype which is responsible for the acquisition of antigens from multiple TECs or other DC-subsets (87). This probably reflects their enhanced migration capacity and phagocytic activity (53). Also, the fact that antigens could be transferred from one thymic DC to another thymic DC, challenges the dogma of exclusively unidirectional antigen transfer from TECs to DCs. Thus, it is clear that self-antigens produced by mTECs could be shared and presented to developing thymocytes by many distinct thymic DC-subtypes (87).

Having defined the main mechanistic framework of "preferential pairing" in CAT, the major question regarding the physiological consequences of this process in the central tolerance remain to be determined. As previously described, the CAT and subsequent indirect presentation of TEC-derived antigens to thymocytes help to overcome the recognizable limitations of mTEC-mediated tolerance, and thus extend the scope of self-antigen presentation in the thymus (6, 8, 48). As we have suggested, the recognition of ubiquitous antigen leads to T cell clonal deletion, whereas the recognition of TRA-like antigens generally promotes diversion to Treg lineage (14, 34). Thus, preferential pairing in CAT might underline the dichotomy of the selection process: either widening the scope of only Treg-generation or clonal deletion. This proposition is supported by the fact that XCR1^{+} DCs that preferentially acquire TRA-like antigens from mTEC^{High} are crucial for the generation of Tregs (8), whereas the SIRPα^{+} DC that acquire ubiquitous antigens from mTEC^{Low}, cTECs or other DC-subsets are more attributed to clonal deletion (14, 111). Thus the "preferential pairing" in CAT between specific subtypes of TECs and DCs can be viewed as a crucial process in discriminating between clonal deletion and Tregs selection and also

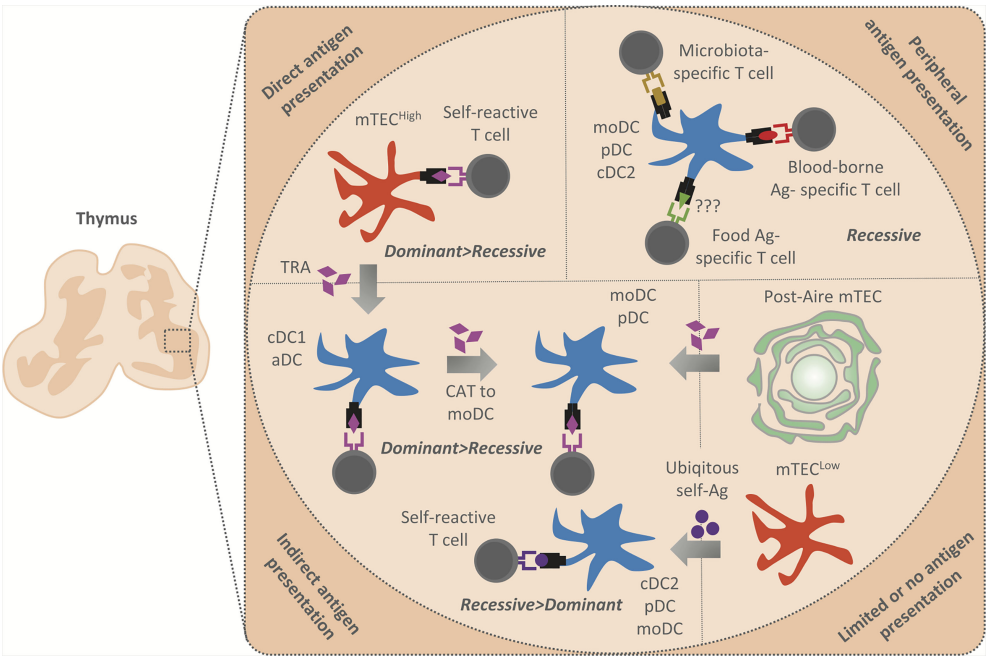

FIGURE 2 | Summary of antigen presentation modes in the thymic medulla. The antigens presented in the thymic medulla are of both intra and extrathymic origin. Their presentation leads to the establishment of both dominant (Treg induction) and recessive (clonal deletion) tolerance. Peripheral antigens, i.e. blood-borne antigens (Ag) (in red), microbiota antigens (in gold), and possibly food antigens (in green) are delivered into the thymus by moDC, pDC or cDC2, and presented by these APCs to establish recessive tolerance. Note that blood-borne antigens are presented in the cortico-medullary junction where an extensive vasculature is situated. TRA (in purple) are generated by mTEC^{High} which either present them directly to establish dominant and recessive tolerance or transferred to cDC1 and aDC in their vicinity by CAT (gray arrows). cDC1 and aDC then establish recessive and more effectively dominant tolerance through indirect TRA presentation. Since moDC strongly acquire antigens from other DC, we suggest that these cells acquire TRA from cDC1 or aDC to enhance the establishment of dominant and recessive tolerance. Post-Aire mTEC which are part of Hassal's corpuscles have limited antigen presentation capacity, however, maintain a high TRA expression. We suggest that Post-Aire mTEC serve as a reservoir of TRA for moDC and pDC, which seems to interact with them. Thus, TRA transfer from Post-Aire mTEC to moDC and pDC might lead to indirect presentation and establishment of both dominant and recessive tolerance. Finally, cDC2, pDC and moDC also acquire antigens from mTEC^{Low} which express a low amount of TRA and are limited in their antigen presenting capacity. Indirect presentation of antigens tranferred from the mTEC^{Low} subset is presumed to lead to the induction of recessive tolerance, since these antigens are ubiqitously expressed (in violet).

enabling the spreading of the antigens for both arms of central tolerance, recessive and dominant (see **Figure 2** summarizing modes of antigen presentation in the thymus).

CONCLUSION

In the last decade, we have witnessed significant growth in our understanding of the contribution of mTEC- and DC-cell autonomous versus mTEC-to-DC cooperative presentation of self-antigens to selection processes which underline the establishment of central tolerance. However, the question of whether and how the individual subsets of mTECs and DCs provide a functionally non-redundant contribution to the deletion of self-reactive clones or their conversion to Tregs remains unresolved. The major technical hurdle in this process is the absence of suitable organismal reagents which would permit the ablation of antigen presentation function in phenotypically defined individual APC subsets present in a thymic microenvironment. The cellular architecture of the medulla which, to certain extent, is the result of the interplay between cytokines and chemokines which regulate the recruitment, differentiation, maturation, and apoptosis of participating cell subsets and guide cell-cell interactions, inevitably generate an important framework within which selection processes must be thoroughly considered and intensively studied. The question of how TCR-pMHC affinity-based selection events are modulated within such a dynamic microenvironment is largely unknown. While many questions remain to be answered in order to understand the intricacies of T cell selection processes, primarily those concerning CAT (see **Box 1**), one thing is becoming clear. As illustrated by the existence of

BOX 1 | Questions to resolve.

How the spatial architecture of the medulla and its key structural features support CAT in respect to the distribution of various DC subsets in this microenvironment?

What soluble and cellular factors in the thymic medulla influence apoptosis of mTECs and thymic DCs serving as a substrate for CAT?

How the preferential localization of each particlar DC subset in the microenvironment of medulla, its cell mobility, phagocytic activity, and chemotactic ability contribute to its capacity to participate in CAT?

What are the molecular determinants regulating the preferential pairing of mTEC and DC subsets?

preferential partnership between specific subsets of TECs and DCs for CAT, despite the increasing complexity of our understanding how central tolerance operates, it seems that this process is largely deterministic. Having this in mind, it is reasonable to assume that in future, we will be able to decipher the principles of T cell selection and in turn apply them to various clinical therapeutic interventions. Revealing the molecular determinants which control and modulate presentation of self-antigens will be next important step towards a unified view of how the universe of self-antigens and its cellular distribution in thymus is functionally coupled to the T cell selection process.

AUTHOR CONTRIBUTIONS

JB, MV, and DF wrote and finalized the manuscript. All authors contributed to the article and approved the submitted version.

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REVIEW
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Key Factors for Thymic Function and Development

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The thymus is the organ responsible for T cell development and the formation of the adaptive immunity function. Its multicellular environment consists mainly of the different stromal cells and maturing T lymphocytes. Thymus-specific progenitors of epithelial, mesenchymal, and lymphoid cells with stem cell properties represent only minor populations. The thymic stromal structure predominantly determines the function of the thymus. The stromal components, mostly epithelial and mesenchymal cells, form this specialized area. They support the consistent developmental program of functionally distinct conventional T cell subpopulations. These include the MHC restricted single positive $CD4^+$ $CD8^-$ and $CD4^-$ $CD8^+$ cells, regulatory T lymphocytes ($Foxp3^+$), innate natural killer T cells (iNKT), and γδT cells. Several physiological causes comprising stress and aging and medical treatments such as thymectomy and chemo/radiotherapy can harm the thymus function. The present review summarizes our knowledge of the development and function of the thymus with a focus on thymic epithelial cells as well as other stromal components and the signaling and transcriptional pathways underlying the thymic cell interaction. These critical thymus components are significant for T cell differentiation and restoring the thymic function after damage to reach the therapeutic benefits.

Keywords: thymus, thymic epithelial cells (TEC), thymic microenvironment, thymus regeneration, T cells, intrathymic regulators, thymic stem cells

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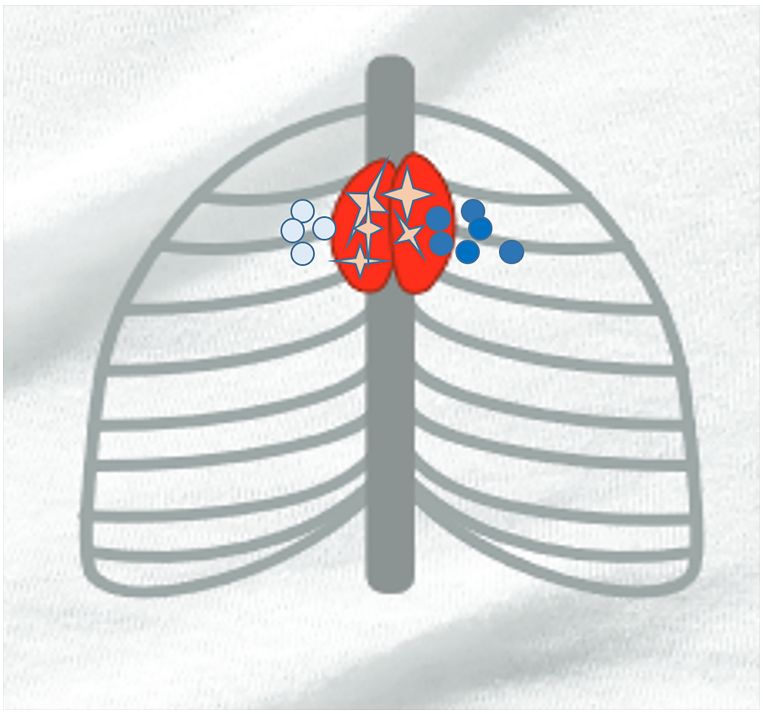
INTRODUCTION

The thymus controls the constant production of self-tolerant T lymphocytes throughout the whole life of the organism. This lymphoid organ consists of two lobes, each enveloped by connective tissue. The outer compartment of the lobes is the cortex, where early-stage thymocytes develop. The medulla is the inner compartment, where later thymocyte stages develop. The intersection of these regions is the cortical-medullary junction (CMJ), where blood vessels transport the hematopoietic progenitors from the bone marrow to the thymus and mature T lymphocytes from the thymus to

Abbreviations: cTECs, cortical Thymic Epithelial Cells; DCs, Dendritic Cells; DN, Double Negative; DP, Double Positive; ECs, Endothelial Cells; HSCs, Hematopoietic Stem Cells; IL, Interleukin; ILCs, Innate Lymphoid Cells; Fbs, Fibroblasts; LPCs, Lymphoid Progenitor Cells; MFs, Macrophages; MCs, Mesenchymal Cells; MSCs, Mesenchymal Stem Cells; mTECs, medullary Thymic Epithelial Cells; NK, Natural killer; SCF, Stem Cell Factor; SP, Single Positive; Sv, Sievert; TECs, Thymic Epithelial Cells; TEPS, Thymic Epithelial Progenitor Cells; TESCs, Thymic Epithelial Stem Cells; THGF, Thymocyte Growth Factor; TLPs, T Lymphocyte Progenitors; TLSCs, Thymic Lymphoid Stem Cells.



GRAPHICAL ABSTRACT |

the peripheral lymphoid organs, and here both, immature and mature T cells reside (1, 2). CMJ is the site of progenitor immigration and the mature single-positive (SP) thymocytes emigration. The CMJ is also a place where the committed progenitors of medullary thymic epithelial cells (mTECs), termed junctional TECs, can be found (3, 4). Each subcompartment of the thymus contains several subtypes of TECs as well as dendritic cells (DCs), mesenchymal cells (MCs), and endothelial cells (ECs) (4–12). Additionally, B cells, natural killer (NK) cells, fibroblasts (Fbs), and macrophages (MFs) are present in the thymus (4, 8, 11, 12). These cells collectively establish and maintain the thymic microenvironment, which supports the differentiation of T cells (4, 6, 7) (**Figure 1**).

In the medulla, mTECs and medullary fibroblasts (mFbs) form a reticular structure where SP thymocytes are located and where they develop tolerance to self-antigens presented mTECs and mFbs (4). The presence of DCs and B cells in the medulla also contributes to the induction of T cell tolerance (4, 11, 13, 14). Part of the thymic ECs is encircled by pericytes, specialized fibroblast-like cells that express actin and contractile like the smooth muscle cells (4). Besides stromal cells, a range of uncharacteristic cells structurally similar to the epidermal or ciliated epithelium, neuroendocrine, muscle, or nerve cells is also present in the thymus (4). These cells can represent the subpopulations of differentiated mTECs forming Hassall's corpuscles, neuroendocrine cell-like mTECs, and thymic tuft cells (4, 7). It is assumed that such a high diversity among mature mTECs might be the basis for their contribution to producing an assorted collection of self-antigens for the self-tolerance formation (7).

TECs are embedded in a 3D mesh structure. Together with MCs, TECs produce the thymic extracellular matrix (TECM), primarily composed of collagen type I and IV, fibronectin, and laminin (15, 16). TECM acts as a reservoir for soluble factors, that are essential for maintaining vital molecular pathways important for thymus organogenesis and T cell development (6, 16, 17). The thymus also hosts some tissue-specific progenitor/stem cell populations, especially thymic epithelial progenitor/stem cells (TEPCs/TESCs) (7, 18–27), mesenchymal stem cells (MSCs) (28–33), and lymphoid progenitor cells (LPCs) (10, 12, 34–36). At least part of LPCs probably is stem cells and resident radioresistant intrathymic stem cells (RTSCs) (37–45, 47–71).

The early studies identified TEC progenitors in murine embryonic thymic primordia and provided evidence that mTECs and cTECs share a common origin. These TEC progenitors might generate all known TEC subtypes *in vivo* and were sufficient to fully reconstitute the thymic epithelial microenvironment that supported normal T cell development (18–20). Later studies have reported that embryonic TEPCs expressing cortical markers can generate both cTECs and mTECs (21–23). Several groups have also reported the identification of bipotent TEPCs in adult mouse thymus (24–27). However, it remains unclear if the populations of fetal and adult TEC progenitors are the same, and that should be additionally studied.

Throughout the differentiation and maturation of T lymphocytes, which constitute over 95% of the thymus, there

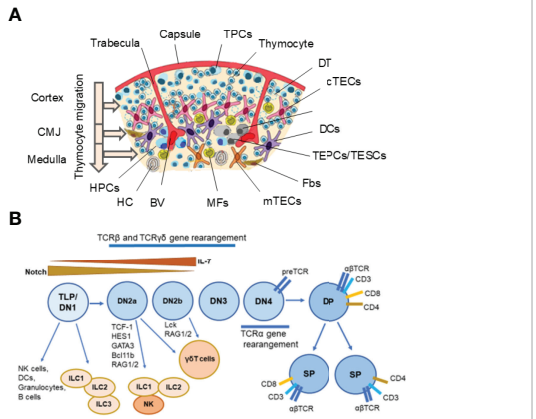


FIGURE 1 | Thymus cell architecture **(A)**, and T cell and innate lymphoid cell (ILC) development in the thymus **(B)**. The thymus consists of two lobes that are separated by connective tissue strands (trabeculae) in lobules. Each thymic lobule consisted of the cortex and medulla. The cortex contains $CD34^+$ uncommitted pluripotent hematopoietic precursor cells (HPCs) entering the thymus at the cortico-medullary junction (CMJ) and migrating to the capsule, committed double negative (DN) $CD4^-CD8^-$ T precursor cells (TPCs) located in the subcapsular region (DN1–DN4 stages), and immature double positive (DP) $CD4^+CD8^+$ (Pre-DP) cortical thymocytes migrating through the cortex and CMJ to the medullar zone. The medulla contains single positive (SP) $CD4^+$ and $CD8^+$ naïve thymocytes migrating to the periphery after maturing. Stromal-epithelial compartment of the thymus is submitted by minor populations of $EpCam^+$ ($CD326^+$) $Foxn1^+$ bipotent thymic epithelial precursor cells/thymic epithelial stem cells (TEPCs/TESCs), and mesenchymal stem cells (MSCs) located probably into the thymic parenchyma close to the CMJ region, as well as $EpCam^+CD205^+$ cortical thymic epithelial cells (cTECs) located in the cortex and $EpCam^+Air^+$ medullary thymic epithelial cells (mTECs) located in the medulla. The cortex and medulla also contain macrophages (MFs), fibroblasts (Fbs), and dendritic cells (DCs) that, together with cTECs and mTECs, participate in the differentiation, maturation, and positive and negative selection of thymocytes. T cell and ILC lineages diverge at the stages of early T precursors/double negative 1 (ETP/DN1) and the DN2-DN3 transition stage. Depending on the status of the TCR loci, the strength of Notch signaling and activities of E-Id proteins and Bcl11b, multipotent TLPs may develop conventional αβ T cells or acquire innate-like properties and give rise to thymic natural killer (NK) cells, DCs, granulocytes, B cells, one of three ILC subsets and invariant γδ T cells. Resident ILC progenitors have been suggested to originate from failed T cell development and locally maintain the mature ILC pool. BV, Blood Vessel; DT, Dead Thymocytes; HC, Hassall's Corpuscle. **(A)** modified from Shichkin and Antica, 2020 (9); the article is licensed under a Creative Commons Attribution 4.0 International License. **(B)** modified from Shin and McNagny, 2021 (138); the article is distributed under the terms of the Creative Commons Attribution License (CC BY).

are three critical activities with a significant influence on the development of each T cell bearing a unique T cell receptor (TCR): 1) the TCR α and TCR β gene rearrangement and expression; 2) the positive selection of T cells that can distinguish self-major histocompatibility complex (MHC); 3) negative selection eliminating T cells that are potentially autoreactive (13, 14). T cells that withstand the negative selection and recognize self-MHC finally become mature $CD4^+$ or $CD8^+$ SP non-autoreactive T lymphocytes and migrate to the periphery (14). As well as T cell maturation and differentiation are supported and directed by numerous cytokines forming an intrathymic cytokine network, their traffic inside the thymus is orchestrated by chemokines, chemokine receptors, and G protein-coupled receptors (GPCR) (6). Both chemokine and cytokine networks are maintained by stromal cells and TECs, including cortical and medullary TECs (6–8).

TECs provide most of the specialized thymic functions mediating different phases of T cell development. cTECs are essential for the thymocyte progenitors' commitment to T cells by providing the delta-like ligand Dll4 for Notch receptors (43–45), constitutively expressed by TLPs (43, 47, 48). Further, they drive the thymocyte expansion at several stages of development by providing different growth factors and critical cytokines, such as interleukin 7 (IL-7) (48–50) and stem cell factor (SCF), among others (6–8, 37, 51). cTECs also regulate the positive selection of T lymphocytes by delivering a unique set of peptides produced by β5t, a thymus-specific proteasome subunit (52). mTECs, on the other hand, expressing chemokines CCL19 and CCL21, promote the migration of positively selected thymocytes from the cortex to the medulla, where they regulate their negative selection and development of $Foxp3^+$ natural T regulatory cells ($Foxp3^+$ Treg), invariant γδ T cells, and invariant NKT cells (13, 14, 53, 54). mTECs also regulate the accumulation of DCs, one of the critical hematopoietic components of the thymic microenvironment, and their positioning in the medulla by secreting the XCL1 chemokine (55–57).

Therefore, developing a functional, self-tolerant T cell repertoire involves the communication between developing thymocytes and cTECs, mTECs, and other stromal and hematopoietic thymus components, many subtypes of which were recently additionally identified both in mice and human using modern single-cell RNA-sequencing (scRNA-seq) analysis (58–63). The role of these newly identified thymic cell subtypes in thymus function and development should be clarified to understand how this new knowledge may contribute to the *in vitro* thymus bioengineering reconstruction and *in vivo* thymus regenerative strategies. The review, in particular, discusses these issues.

THYMIC EPITHELIAL CELLS

The thymic function mainly depends on the TEC compartment of the stroma. While cTECs control T cell commitment and their positive selection, mTECs provide mechanisms to form the central tolerance of these T cells. The crucial role of mTECs in the T cell tolerance forming depends on several factors: the primary autoimmune regulator (Aire) regulating the expression of several tissue-restricted genes, and the Aire-independent mechanisms regulated, in particular, by Fezf2 (7, 51, 64, 65). The thymic epithelial component during embryogenesis and in the postnatal thymus are also maintained by TEPC/TESC-mediated cross-regulatory signaling between Notch and Foxn1 (66–70).

Numerous investigations indicate that cTECs and mTECs have a common epithelial progenitor during fetal (18–22, 70, 71)

and postnatal development (24–27). In the mouse fetal thymus, this precursor appears in the thymic primordium as early as generated from the third pharyngeal pouches (3PPs) (18, 21, 70, 71). Early experiments showed that in mice both cTECs and mTECs are generated from fetal TEPCs expressing surface determinants that are recognized by the mAbs MTS20 and MTS24 (18, 19), later identified as the Plet1 (placenta-expressed transcript-1) antigen (72). Three complementary studies have almost simultaneously reported that embryonic TEPCs expressing cortical markers CD205 and β5t, and expressing IL-7, can generate both cTECs and mTECs (21–23). Later, another study showed that embryonic CCRL1$^+$ cTECs also contain cells with mTEC potential (73). These studies led to the conceptual model that TEC progenitors exist within the cTEC niche prior to committing to the mTEC lineage – serial progression model (74). Thus, both TEC subsets arise from TEPCs/TESCs that express markers associated with mature cTECs, particularly CD205 and β5t (21, 23, 71). This fact suggests that fetal TEPCs are associated with the development of cTEC lineage but that for mTEC lineage specification, additional signals are essential (71). Since there is the shared expression of surface antigens between cTECs and the bipotent TEPCs, identifying the cTEC-restricted sublineage of TEPCs is still unsolved (7, 71). Furthermore, despite the further characterization of the bipotent TEPCs in the adult thymus (24–27), the phenotype of these cells yet remains to be specified.

Mouse TECs express the surface protein Plet1 that marks TEPCs/TESCs located in the thymic parenchyma at the CMJ (72). In the mature thymus, these TEPCs/TESCs additionally express Ly51 surface protein and also can generate both cTECs and mTECs (26). Moreover, Plet1$^+$ TEPCs/TESCs express CD326 (EpCAM) surface protein (26). Thus, in the adult mice, bipotent TEC progenitors are CD326$^+$UEA1$^-$Ly-51$^+$Plet1+MHC class II^{hi}, which comprises <0.5% of adult TECs (26). Human TECs do not express Plet1 but express CD326, and therefore, in combination with Foxn1, this marker was used for isolation of the human TEPCs/TESCs from the neonatal and postnatal thymus (75, 76).

Under the nonadhesive conditions, TEPCs in the mouse thymic cultures can form spheroid colonies, typical for cells with stem cell properties. These spheroid colonies (termed thymospheres) were EpCam$^-$ and Foxn1$^-$, and they generated both cTECs and mTECs (24). However, another group has reported that such thymospheres are formed by Foxn1$^-$ EpCam$^-$ MCs, and they have the potential to generate only adipocytes, but not TECs (31). Moreover, this study has shown that cells forming the thymospheres derive from the neural crest, and these structures can include bipotent TEPCs (31). These two studies were fulfilled with the mouse thymus, and there is still missing data concerning thymospheres of the human thymus, and therefore, they require further careful analyses. Since the existence of different TEPCs with self-renewing properties of stem cells remains discussed, additional studies are necessary to identify the earliest stages of TEC development in the embryonic and postnatal thymus that generate cTEC and mTEC lineages (71). cTECs and bipotent TEPCs share the expression of CD205, Ly51, and β5t. This sharing makes cTECs and TEPCs challenging to distinguish. However, recent data suggest that bipotent precursors have characteristics usually linked to cTECs before acquiring mTEC features (71). Key markers and pathways in TEC development from bipotent mouse TEPCs are presented in **Figure 2**.

The appearance of the earliest mTEC progenitors needs active Notch signaling in TEPCs, while further mTECs development is Notch independent, and continuing Notch activity supports the undifferentiated state of TEPCs (70). Moreover, Notch acts

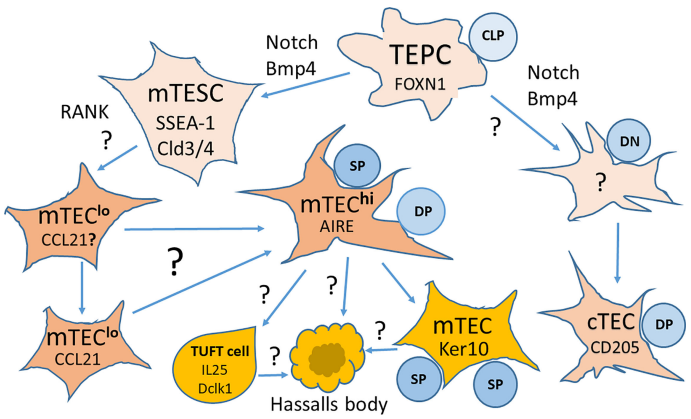


FIGURE 2 | Key markers and pathways in the development of thymic epithelial cells (TECs) from bipotent thymic epithelial progenitor cells (TEPCs). TEPCs differentiate into medullary and cortical thymic epithelial cell lineages (mTECs and cTECs, respectively) that are regulated by Foxn1 expression. mTEC development goes through an intermediate stem cell stage (mTESCs), expressing the stem cell marker SSEA-1, and requires Notch signaling for the formation of mature Aire expressing mTECs^{hi}. Other pathways to the differentiation of mTEC subsets are still a matter of intensive research. It is also yet not known whether cTEC development goes through a similar intermediate stage. CLP, Committed Lymphocyte Precursor; DN, Double Negative Thymocytes; DP, Double Positive Thymocytes; SP, Single Positive Thymocytes. Modified from Alawam et al., 2020 (71); the article is distributed under the terms of the Creative Commons Attribution License (CC BY).

before NF-κB signaling to regulate mTEC lineage progression (70). These data suggest the complex influence of Notch signaling on TECs development and function. However, the mechanisms of Notch signaling on TEC development have not yet been clarified. Thus, Notch is a potent controller of the TEPCs and mTECs performance during T cell development. Therefore Notch is highly relevant for strategies generation/regeneration of the functional thymic tissue both *in vitro* and *in vivo*.

A minor population of TECs, expressing claudin 3 and 4 (Cld3/4) and SSEA-1 (stem cell marker) have been termed mTEC stem cells due to their self-renewal capabilities and the ability to differentiate into mTECs but not cTECs (7, 77). mTEC stem cells are also characterized a low expression of β5t and CD205 and a high expression of RANK (receptor activator of NF-κB) and lymphotoxin β receptor (LTβR) (7, 71). The generation of mTEC stem cells is Foxn1/Relb-independent as mTEC precursors emerge in Relb-deficient mice (78). However, their expansion and differentiation partially depend on LTβR, RANK, CD40, and p52 signaling (79–85). As shown, LTβR and RANK receptors are involved in the NF-κB pathway activation and control the proliferation and maturation of mTECs through an Aire-dependent way and the crosstalk with positively selected self-reactive $CD4^+$ thymocytes (80, 81, 86–88). At this, lymphotoxin signaling is necessary for the expression of *Aire* and its downstream target genes. The failure of *Aire* induction in the thymus of lymphotoxin-deficient and LTβR-deficient mice contributes to autoimmunity against self-antigens normally protected by Aire (82). While only RANK signaling is essential for mTEC development during embryogenesis, cooperation between CD40 and RANK signals is required in postnatal mice (83). The RANK ligand (RANKL) produced by positively selected thymocytes is responsible for fostering thymic medulla formation, regulating the cellularity of mTECs by interacting with RANK and osteoprotegerin (84). Further expansion of the mature mTEC population requires autoantigen-specific interactions between positively selected $CD4^+$ thymocytes bearing autoreactive T cell receptor (TCR) and mTECs bearing cognate self-peptide - MHC class II complexes. This interaction also engages the CD40 on mTECs by CD40L induced on the positively selected self-reactive $CD4^+$ thymocytes (85). This antigen-specific TCR-MHC class II-mediated crosstalk between $CD4^+$ thymocytes and mTECs is pivotal for generating a mature mTEC population competent for ensuring the central T cell tolerance (88). Recent RNA-seq analysis of transgenic mouse models has shown that self-reactive $CD4^+$ thymocytes induce critical transcriptional regulators in $mTEC^{lo}$ and control the composition of $mTEC^{lo}$ subsets, including $Aire^+$ $mTEC^{hi}$ precursors, post-Aire and tuft-like mTECs (88). This interaction also upregulates the expression of tissue-restricted self-antigens, cytokines, chemokines, and adhesion molecules important for T cell development, and these interactions between self-reactive $CD4^+$ thymocytes and mTECs are critically essential to prevent multiorgan autoimmunity (88). In addition, histone deacetylase 3 (HDAC3) is an essential regulator of mTECs differentiation (89), as well as STAT3 signaling is vital for mTECs expansion and maintenance (90, 91).

A fresh look at TEC heterogeneity, especially mTECs, provides the scRNA-seq technology analyzing the transcriptome patterns in combination with conventional surface marker analysis. Though both of these approaches have shown the identity of the main TEC populations, many unknown TEC subtypes, in particular, thymus tuft cells, were identified by scRNA-seq in both mice (62, 92) and humans (63). In the recent study authors additionally identified a population of $Bpifa1^+$ $Plet1^+$ mTECs that was preserved during thymus organogenesis in mice, and these mTECs highly expressed tissue-resident adult stem cell markers (93). Depending on the levels of MHCII and CD80, mTECs are broadly subdivided into $mTECs^{lo}$ (*Cldn4*, lower levels of HLA class II) and $mTECs^{hi}$ (*Spib, Aire, Fezf2*, higher levels of HLA class II). The $mTEC^{lo}$ population includes the majority of mTECs. This population contains several subpopulations, including mTECs expressing a high level of the CCL21 chemokine and *Ccl21a* and *Krt5* genes ($CCL21^+$ mTECs called also mTECs I) and stages representing $Aire^+$ $mTECs^{hi}$ (mTECs II) expressing the *Aire* and *Fezf2* genes (88). Recent data received with the help of scRNA-seq combined with lineage tracing and recovery from ablation have identified inside the $mTEC^{lo}$ the short-living transit-amplifying cell population (TAC-TECs) that is the immediate precursor of Aire-expressing mTECs (94). These data also suggest that the TAC-TECs may also be the precursor of the *Ccl21a*-high mTEC population (94). However, yet it is unclear that $Aire^+$ and $Fezf2^+$ mTECs are developmentally related to $CCL21^+$ mTECs. $Aire^+$ $mTECs^{hi}$ population further differentiates into post-Aire mTECs (mTECs III) expressing *Pigr* and *Cldn3* genes and thymic tuft cells (mTECs IV) producing IL-25 (62) and expressing *Avil* and *Pou2f3* genes (88). Post-Aire mTECs also contain Hassall's bodies that contribute to the forming of thymic T cell tolerance as proposed (95). Finally, post-Aire mTECs become enriched for proteins classically associated with end-stage keratinocytes, such as involucrin (Ivl), Lekti, and a variety of different keratins, obtaining a corneocyte-like phenotype (7, 62, 71, 96–98). scRNA-seq analysis of the human thymus confirmed these four main mTEC subpopulations but added mTEC-myo and mTEC-neuro as two additional subpopulations presented in humans but absent in mice (63).

The essential feature that may be suitable to discriminate populations within $mTECs^{hi}$ is the expression of the *Aire* gene, which is essential for the efficient deletion of self-reactive T cells (71, 86). Further, mTECs express the transcription factor Fezf2, which is required to activate some Aire-independent genes (64, 65). A high co-expression level of both factors, Aire and Fezf2, has been demonstrated by the cells expressing molecules associated with antigen presentation (65, 99). The co-expression of Aire and Fezf2 is a feature of the mouse and human mTECs (64, 100). In addition to that, within the $mTEC^{lo}$ population, there has also been detected the Fezf2 expression, but without Aire expression (71, 81).

$Aire^+$ mTECs continue their development past the stages of Aire expression and become typically differentiated keratinocytes

(96, 97). These cells form well-known structures within the thymic medulla called Hassall's corpuscles that can be identified by simultaneous keratin 10 and involucrin expression (71, 95). During ontogeny, Aire$^+$ mTECs develop first due to the RANKL provision by DP thymocytes (83–85, 88). These Aire$^+$ cells can progress to Aire$^-$ cells that are characterized by lower levels of MHCII expression (7, 71, 96). However, post-Aire mTECs differ from other populations of mTECs MHCII^{lo} by the absence of CCL21 expression (7, 88, 97). A single-cell RNA sequencing analysis suggests the existence of two central populations of post-Aire mTECs presented by the keratin-10$^+$ involucrin$^+$ mTECs and the thymic tuft cells similar to the tuft cells originally described at mucosal sites (7, 62, 71, 101). Both types of tuft cells express *IL25*, *Trmp5*, *Dclk1*, and *IL17RB* genes (62, 101). The thymic tuft cell functions are still poorly understood. Since thymic tuft cells, unlike intestinal tuft cells, express high levels of MHCII (62, 101), it is possible that they have an active role in antigen presentation and thymic T cell selection (71, 92). Further, they might also regulate the innate immune networks, both thymic ILC and iNKT cells, within the thymus (17, 62, 102). Understanding how tuft cells and iNKT cells are connected to the intrathymic development of Tregs requires further studies. Although there is some evidence of DCLK1$^+$ tuft cell presence within the human thymus (62), it is not clear whether human and mouse thymic tuft cells express a similar array of receptors and secreted factors.

The described heterogeneity of mTECs can explain how the thymus medulla supports the development of different T cell lineages, including conventional αβT cells, Foxp3$^+$ Treg, invariant γδ T cells, and CD1d$^-$-restricted iNKT cells (13, 53, 58–61, 71, 103).

cTECs are functionally very heterogeneous and among them are the thymic nurse cells (TNCs) represented by large epithelial cell complexes in which single cTECs enclose viable thymocytes. This unique feature of cTECs has been described in mice, where about 10–15% cTECs form such complexes, including four to eight DP thymocytes (104). cTECs that form TNCs have increased CD205, CXCL12, TGFβ, TSSP, and VCAM-1 compared to the cTECs that are not part of TNC structures (71, 105, 106). Analysis of DP thymocytes within the TNCs shows that they are enriched for cells that have undergone secondary TCRα rearrangements, indicating that they may provide an environment that enables efficient positive selection (71, 105).

Many studies demonstrated that the transcription factor Foxn1, a master regulator of TECs specification during the early stage of thymus development, is involved in the mechanisms of thymic involution. The high levels of Foxn1 expression are required for TEC development and maturation; moreover, thymopoiesis is dependent on the stable Foxn1 expression, and the reduced levels of Foxn1 have been observed in the aged thymus (2, 67–69, 107, 108). In the postnatal thymus, Foxn1 levels progressively and age-related decrease leading finally to the collapse of the thymic microenvironment and the complete failure of T cell production (69). However, the thymic involution can be reversed by an increased re-expression of Foxn1 (109). This thymic renewal is in line with restoring the thymic epithelial composition, effective thymopoiesis, a decrease of naïve T cells number in the periphery, and expansion of the memory T cells (15, 46, 109, 110).

In addition to Foxn1, several molecules and signaling pathways essential for T cell development were identified in the postnatal thymus in several transcriptome studies (69, 110). In particular, Wnt4 was described as a possible expression controller of Foxn1 in the early stages of thymic development (110, 111). Its expression is decreased with age matching the downregulation of Foxn1 (2, 108, 110, 111). Bone morphogenic protein-4 (Bmp4) is produced by thymic Fbs and ECs and participates in the early morphogenesis of the thymus (112). Receptors for Bmp4, BMPR I and II, are expressed in the postnatal thymus mainly by TEPCs. Bmp4 signaling mediates transforming growth factor-beta (TGF-β) through activation of *Smad4* (113, 114). The disbalance of TGF-β signaling molecules can reduce the capacity of TECs to support T cell development and, in this context, contributes to thymic involution (1, 17, 107, 112). Thus, understanding the molecular mechanisms regulating the levels of Foxn1 and other essential transcriptional factors provides critical knowledge for the development of TEC restoring strategies in thymus-compromised patients. Further studies in this promising area of research are essential.

THYMIC MESENCHYMAL CELLS AND FIBROBLASTS

MCs are the leading producers of the thymic extracellular matrix. This matrix ensures a structural mesh microenvironment for T cell migration and provides the main reservoir of cytokines and growth factors essential for epithelial and lymphoid progenitors during their differentiation and maturation. MCs have been described in all tissues and organs, where they have various mechanical and metabolic functions (37). Nevertheless, bone marrow MCs are the most explored since they are part of the hematopoietic stem cells (HSCs) niche, where the mesenchymal stem cells (MSCs) reside (113, 114). It has been shown that they can coordinate tissue regeneration and regulate the immune response (115–118).

On the other hand, it is little known about thymic MCs (TMCs) and especially their stem/progenitor cells (TMSCs) in the thymus function and development. Previous studies of the thymic stroma in both mice and humans using flow cytometry and bulk RNA-seq technology identified only several phenotypically distinct TMC subtypes (28–30). Modern research using gene expression profiles at single-cell resolution has shown a high heterogeneity among TMCs. In particular, this approach allowed identifying the unique transcriptional fingerprints of 12 non-epithelial stromal subtypes, including endothelial cells, vascular mural cells, neural crest-derived cells, mesothelial cells, and fibroblasts. Moreover, among the fibroblast population, at least 11 distinct capsular and medullary subtypes

were identified, including capFb1a, capFb2b, and mFb1a as fibroblast subtypes with precursor potential and capFb3 to originate from mesothelial cells (63, 119).

TMCs contribute to the regulation of the TEC proliferation through the production of a set of cytokines, such as fibroblast growth factor (FGF) -7 and -10, insulin-like growth factor (IGF)-1, and -2, and retinoic acid (4, 28, 29, 115). Endosialin (CD248) positive TMCs play an essential role in revascularization during regeneration of the postnatal thymus after damage caused by infection (116). In contrast, fibroblast-specific protein 1 (FSP1) positive TMCs are required to preserve the mTEC compartment (4, 118, 119). The function of thymic MCs is similar to the function of other organ-specific MCs and includes the clearance of apoptotic cells in the thymus (29). Mouse TMCs that are negative for Foxn1 and EpCAM when cultured in nonadhesive conditions *in vitro* can form thymospheres, having adipocyte forming potential (31). TMCs can support the viability and differentiation of autologous thymocytes through direct contact, as has been shown in mouse co-cultures (117). On the other hand, there are indications of immunomodulating properties of human TMCs since they can reduce the proliferation of already activated thymocytes by 50%, as well as they can induce only a negligible proliferation of responding cells when tested in allogeneic co-cultures (29).

In the neonatal human thymus, some MCs are present that can be defined as trilineage stem cells, which include the previously postulated properties: i) attachment to the plastic surface, ii) expression of MSC-like surface markers, and iii) differentiation potential into osteogenic, chondrogenic, and adipogenic mesenchymal cell lineages in culture conditions. Some studies have also shown that neonatal TMSCs have immunomodulatory features and can differentiate into a cardiomyogenic lineage (29, 33). Moreover, neonatal TMSCs can express and *in vitro* secrete even more Sonic hedgehog (Shh), a proangiogenic and cardiac regenerative morphogen, than the bone-derived MSCs. Furthermore, in neonatal MSC organoid cultures, the expression of Shh ensures a cytoprotective effect for cardiomyocytes (33). TMSCs are negative for the hematopoietic surface antigens such as CD45, HLA-DR, HLA-ABC, CD34, CD38, CD40, CD40L, CD66, CD80, CD86, CD106 and positive for CD13, CD29, CD44, CD73, CD90, CD105, CD166 (4, 29, 37).

TMCs diversify at the early stage towards prethymic and intrathymic populations. The perithymic MCs form the thymic capsule, while the intrathymic populations differentiate into mFbs and pericytes (4, 118). However, how the generation of TMC diversity is regulated at the molecular level so far remains unknown (4). Neural crest-derived MCs in the adult thymus are presented by Fbs that are mainly located in the thymic capsule and medulla (4, 120). Thymic Fbs are important thymic stromal cells because of their large number and specific structure. They produce a collection of structural proteins such as collagens, as well as functional proteins that include FSP1, platelet-derived growth factor receptors α and β (PDGFRα and PDGFRβ), podoplanin/gp38, CD34, and epitopes for monoclonal antibodies known as MTS-15 and ERTR7 (4, 28, 30, 115, 118–121).

Capsular Fbs (capFbs) specifically express the surface protease dipeptidyl peptidase-4 (DPP4) or CD26, which is encoded by the differentially expressed gene *Dpp4* (4). This finding allowed the separation of the thymic fibroblast population on capFbs ($DPP4^+$ $gp38^+$) and mFbs ($DPP4^-$ $gp38^+$) (4, 122). Besides *Dpp4* expression, capFbs differ from mFbs by expression of *Pi16*, *Sema3c*, *Sema3d*, and *Aldh1a2* genes (4). Both capFbs and mFbs, are characterized by high expression of a set of fibroblast-associated genes, in particular, *Col1a1*, *Col3a1*, *Col6a1*, *Dcn*, *Lum*, *Mgp*, *Sparc* encoding the extracellular matrix proteins, *Serping1*, and *Serpinh1* encoding protease inhibitors, and *Htra1*, *Htra3*, *Mmp2*, *Mmp3*, *Mmp14*, *Mmp23* encoding extracellular proteases (4). Further, capFbs, in contrast to mFbs and other thymic stromal cells, have a higher level of Wnt family ligands and regulators (Wnt2, Wnt5a, Wnt5b, Wnt9a, Wnt10b, Wnt11, and Sfrp2 and Sfrp4) (4), suggesting that capFbs regulate cTEC development through the Wnt signaling.

mFbs are similar to fibroblastic reticular cells (FRCs) in the peripheral lymphoid organs, and these cells were previously described as thymic FRCs. However, they are a thymus-specific fibroblast subpopulation that is functionally distinct from FRCs of the secondary lymphoid organs (4). In adventitial layers surrounding the ECs and pericytes, the mFb subset of $CD34^+$ $podoplanin^+$ cells, better known as adventitial cells, has been identified (123, 124). mFbs predominantly express a set of genes that includes collagens (*Col6a5*, *Col6a6*), matrix metalloprotease-9 (*Mmp9*), metabolic enzymes (*Hmgcs2*, *Ltc4s*, and *Qprt*), and TGFb-binding proteins (*Ltbp1* and *Ltbp2*) (122). The lymphotoxin signal involving LTbR in mFbs regulates the adhesion molecules ICAM-1 and VCAM-1 (121, 124), suggesting a specific role of mFbs in the control of immune cell trafficking in the thymus (4). On the other hand, lymphotoxin, which in the thymus is produced by SP thymocytes, is required to develop mature mFbs and control the cellularity of $Aire^+$ mTECs (4, 80, 83). Moreover, lymphotoxin promotes the differentiation of $CCL21^+$ mTECs, Hassall's corpuscles, and thymic tuft cells (4, 53). These facts illustrate the thymic cell crosstalk between lymphoid and stromal-epithelial cells providing the medullary microenvironment that controls the negative selection of SP thymocytes (4, 125). This intrathymic interaction is regulated by RANKL, the main mediator of the intrathymic crosstalk (83). RANKL is a TNF superfamily ligand and is expressed predominantly by mTECs and SP thymocytes (4, 83). Through signaling mediated by IkB kinase (IKK), NIK, and TRAF6, it activates the transcription factor NF-kB and thus induces Aire expression in mTECs and their further differentiation (4, 126). SP thymocytes also produce CD40L, which in cooperation with RANKL, promotes the development of mTECs expressing Aire (83).

Thereby, the use of gene expression profiles at single-cell resolution in contrast to conventional flow cytometry has demonstrated a high heterogeneity among thymic stromal cells. Single-cell transcriptome analyses showed dynamic changes in the frequency of these cells across an extensive

range of developmental stages. However, the observed transcriptomic diversity of stromal subtypes is not fully supported by conventional flow cytometry due to a limited number of suitable cell surface markers. This limitation has yet hindered a comprehensive understanding functional role of nonepithelial stromal cell subtypes in the control of discrete stages of intrathymic T cell development. However, now there is no doubt that MCs and Fbs are the essential components of the thymic microenvironment, which is critical for its correct development and functioning.

THYMIC LYMPHOID STEM CELLS AND T CELL DEVELOPMENT

Early lymphoid precursor cells entering the thymus, the thymic lymphoid stem cells (TLSCs), are bone marrow migrants that in mice express low levels of CD4. In humans, they express CD34 and are negative for CD4, CD8, and TCRα/β (35, 37). These TLSCs can produce all known lymphoid lineages, including T cells, NK cells, B cells, and DCs (10, 34–37, 127, 128). In the bone marrow, the proliferation and fate HSCs are regulated by the stem cell factor (SCF) and its c-kit receptor in cooperation with Notch ligands and morphogenic factors, such as Wnt, Hedgehog, TGFβ, and BMP (34, 37, 127–130). They regulate the stem cell self-renewal and differentiation into multiple lineages (35, 127). The early TLSCs, which are bone marrow migrants, enter the thymus through the blood vessels in the CMJ area. They migrate consequentially to the thymic subcapsular zone (37), the thymic cortical, and medullary zones while differentiating first into immature DP ($CD4^+$ $CD8^+$) thymocytes. Finally, they become naive SP $CD4^+$ and $CD8^+$ T cells (**Figure 1**). Following this path, they undergo the positive and negative selection in the cortical and medullar regions of the thymus, respectively (2, 14, 37, 71). These intrathymic events are controlled by the direct interaction of thymocytes with the stromal-epithelial compartment. The chemokine, hormonal, and cytokine signals ensure the necessary conditions for the correct maturation, differentiation, and T cell trafficking through the thymus (4, 6, 8, 17, 34, 55, 56, 71).

In more detail, the development of thymocytes starts from early $CD25^-CD44^+$ TLPCs/TLSCs deriving from HSCs of the fetal liver or the adult bone marrow. The early stage of T cell maturation mainly occurs in the cortex of the thymus and is directed by contact with cTECs. A high level of chemokines, such as CCL25, CXCR4, CXCL12, Notch ligand DLL4, and cytokines IL-7 and SCF provided by these cTECs, is required for the development of these early thymocytes (2, 6, 15, 34, 37, 71). The CCL25 and CXCL12 chemokines ensure the growth and survival of TLPs in the cortex, the Notch ligand DLL4 enables the differentiation of TLPs into T cells (46–48, 131, 132), and IL-7 and c-kit ensure the proliferation of the immature T cells (47, 132, 133). Early TLPs go through the double negative 2 (DN2) stage when they express CD25 and CD44, proliferate, and downregulate CD44 to develop into the DN3 stage of $CD25^+CD44^-$ thymocytes. At this stage, they lose the B cell potential (15, 17, 128) and move through the cortex into the subcapsular area, and TCRβ undergoes rearrangement. DN3 cells further differentiate into DN4 $CD25^-CD44^-$ thymocytes. They actively proliferate and continue to develop into the DP $CD4^+CD8^+$ stage. DP thymocytes further rearrange the TCRα and finally express the mature TCRαβ complex. Thus, still immature T cells co-express CD4, CD8, and the TCRαβ complex in combination with CD3 (TCRαβ–CD3) (6, 15, 128).

In the outer cortex, cTECs activate the positive selection of the DP T cells with the help of MHC self-peptides and the TCR of maturing T cells (6, 15). This interaction of DP thymocytes with cTECs initiates the survival or cell death of DP thymocytes (6, 7, 15). Intrathymic location of $CD4^-CD8^-$ TLPs is regulated by chemokine CXCR4 and chemokine ligand CXCL12 interaction, while the maturation of these TLPs expressing pre-TCR requires the CXCR4–CXCL12 interaction together with Notch signaling to control β-selection (71, 131). In the later phase of thymocyte maturation, CXCL12 retains $CD4^+CD8^+$ thymocytes in the cortex to undergo correct maturational stages, including positive selection (69, 71, 131). cTECs can also support the positive selection of $CD8^+$ T cells mediated by MHC class I. However, the processing and presentation of peptides associated with molecules of MHC-I require the expression and degradation of thymic proteasomes in cTECs and the presentation of proteasomal catalytic subunit β5t (7, 52, 71, 106).

After positive selection, DP thymocytes develop into SP $CD4^+CD8^-$ or $CD4^-CD8^+$ T cells and they bind MHC II or MHC I, respectively (7, 15, 71, 128). These cells are transferred to the medulla, and this transfer is regulated by chemokine ligands CCL21 and CCL19 on mTECs (7, 15, 71). In the medulla, T lymphocytes with the self-reactive TCR undergo negative selection mediated by mTECs expressing a set of tissue-restricted antigens regulated by Aire and Fezf2 (6, 7, 14, 15, 64, 65, 71). In addition, thymic DCs also partake in the T cell selection through the expression of endogenous antigens or the presentation of antigens from other cell types (11, 15, 29, 55, 56, 122). In the medulla, the self-reactive T cells are removed by negative selection, and conventional regulatory T cells (Tregs) and $Foxp3^+$ Tregs that express diverse TCR repertoires are developed (15, 54, 123, 125). Finally, naïve $CD4^+$ and $CD8^+$ SP T cells and Tregs migrate from the medullar to the peripheral lymphoid tissue and circulation (15, 128).

In addition to the development of the conventional $TCR\alpha\beta^+$ T cells, the thymus also supports the progress of innate-like $TCR\gamma\delta^+$ T cells (γδ T cells) (54, 71). These cells do not require antigen-specific communications with the stromal microenvironment for development (71, 103). In mice, γδ T cells are generated at the transient DN2a-DN2b stages from the same DN1 early T cell progenitors (ETPs) (128), which appear as multipotent TLSCs (**Figure 1**). These γδ T cells are produced at some periods of ontogeny, and they are distributed to epithelial and mucosal tissues (15, 54, 103, 128). The development of γδ T cell populations was recently reviewed in detail by Parker and Ciofani (103). TLSCs can also contribute to the generation of the thymic B cells, DCs, NK cells, and MFs, at least in mice (10, 12, 128, 133), confirming their multipotent stem cell potential.

Human TLP development includes a $CD4^+$ SP stage, after which they diverge to the αβ or γδ lineages (15, 58, 59, 61). Human $CD4^+$ SP αβ thymocytes are found in the cortex, and they are turned to the DP form and then move to the medulla generating $CD4^+$ and $CD8^+$ SP T cells (2, 15, 103). Human negative and positive T cell selection also occurs in the cortex and medulla, probably similar to the selection in the mouse thymus (15, 58, 59). However, these events concerning the human thymus are yet under discussion. Another difference is that humans are born with an entire T cell repertoire, and T cell memory is formed during childhood (15, 58). The thymus suffers age-related involution throughout life, which is associated with an essential reduction in the proliferation and differentiation of early TLPs, altered T cell differentiation, and atrophy of the epithelial compartment (15). The malfunction or even impairment of thymus development is linked with several diseases like the DiGeorge Syndrome (DGS), Foxn1 deficiency, graft-versus-host disease (GVHD), HIV infection, or autoimmune diseases (1, 2, 9, 15).

THYMIC INNATE LYMPHOID CELLS

Innate lymphoid cells (ILCs) are tissue-resident cells. They comprise NK cells and lymphoid tissue inducer (LTi) cells triggered through receptors for pathogens or inflammatory cytokines but not through the BCR or TCR antigen-specific receptors (128, 134–136). They are usually located at the mucosal barrier sites, and they regulate homeostasis and tissue repair in non-barrier organs (128, 137, 138).

ILCs are very heterogeneous, and they have been grouped into subsets according to their surface marker expression, cytokine profiles, and transcription factors, similarly to the T cell classification, on ILC1/NK, ILC2, and ILC3/LTi (128, 139, 140). ILC1 are conventional NK cells and helper ILC1. They are identified by the production of interferon γ (IFN-γ) in response to IL-12, IL-15, and IL-18. ILC2 generates cytokines IL-5, IL-9, and IL-13 in response to stimulation by alarmins, IL-25, IL-33, and TSLP, and they depend on the expression of GATA3. Finally, ILC3 is characterized by the secretion of IL-17 and IL-22 in response to stimulation by IL-23 and IL-1β (128). IL-22 is critically important for thymus recovery after damage, and since ILC3 cells are highly resistant to damage, they play an essential role in thymus organogenesis and regeneration (144, 145). ILC3 cells are regulated by the transcription factors RORγt and RORα (127, 140–142). This population also contains an LTi family. LTi is generated during embryogenesis and facilitates the development of secondary lymphoid tissues (128, 143).

In general, the ILC1 population is essential for the clearance of intracellular pathogens, ILC2 for helminth infection and allergen-induced chronic airway inflammation, and ILC3 for gut immunity and for establishing gut tolerance and mucus secretion (128). Concerning the thymus, ILC3 is dominant in the embryo, and ILC2 in the postnatal thymus (128). A detailed analysis of ILC development in the thymus is presented in the review by Shin and McNagny (128), and it is shown in **Figure 1**.

THYMIC RADIORESISTANT AND RADIOSENSITIVE CELLS

As early as 1975, Kadish and Basch were the first to report that the thymus of adult mice contains cells resistant to radiation. These cells can temporarily restore the thymus cellularity after sublethal total body irradiation (38). This rare population of radioresistant thymic cells is $CD4^-CD8^-$ intrathymic TLPs (previously known as $L3T4^-Lyt2^-$), probably located in the subcapsular part of the thymic cortex (37–39). The significance and biological role of radioresistant intrathymic TLPs for the thymic function is yet unclear, and several research groups are tackling this intriguing research area (37–45, 47–71, 146–151).

The most important index of cell radioresistance is their stability to interphase death, which is measured by the dose irradiation that causes 63% of cell death (D_0). The radioresistance is an essential peculiarity of the resting cells. The radioresistance of lymphocytes is in the range of 1-10 Sievert (Sv), and it varies with the maturation stage and the cell subpopulation type. Concerning the thymus, a small population of intrathymic TLPs represents the most radioresistant lymphoid cells playing a special role in the post-radiation restoration of the thymus (37–45, 47–71, 146, 147). Studies of Shichkin's research group, which were recently revisited and updated (37), have shown that radioresistant intrathymic TLPs produce an autocrine thymocyte growth factor (THGF). The target cells for THGF are the radioresistant TLPs, which have D_0 of more than 50 Sv (37). With a radiation dose of more than 15 Sv, these TLPs persisted in an inactive or low activity state for a long time. However, exogenic THGF or its combination with IL-2 can activate and increase their proliferation. The gamma-irradiation at the dose of 12 Sv induces the secretion of THGF by the radioresistant cells, and this cytokine then supports the self-regulating proliferation of these cells in autocrine manner (37, 41, 146, 152, 153). They are probably early intrathymic TLPs, persisting at the DN1/DN2 stage as resting resident tissue-specific stem cells (**Figure 1**), which are the direct target-cells for TGHF. This assumption is supported by the data showing the existence of the radioresistant subpopulation of TLPs at the DN2 stage of thymocyte development that proliferate after irradiation in an IL-7-dependent manner and generate the conventional thymocytes. Moreover, their differentiation recapitulates normal thymic ontogeny (148). These data provide evidence concerning the specificity of THGF-dependent proliferation of radioresistant TLPs and successive change of THGF-sensitive stage to THGF/IL-2-sensitive stage (37).

Furthermore, there is evidence that THGF-dependent cells are self-renewing intrathymic $CD4^-CD8^-$ stem cells, activated by THGF and damage factors such as irradiation. THGF is probably a member of the SCF superfamily (37). Therefore, we are bringing together but not identifying THGF with IL-7, SCF, and GM-CSF. On the other hand, some authors have shown that the intrathymic progenitors are multipotent and may generate not only a T cell lineage but also NK cells (154), DCs (155), MFs, and B cells (156). These cells could, in turn, secrete IL-7, SCF, and other cytokines and thereby support T cell development during the reconstitution of the irradiated thymus.

A recently defined subpopulation of radioresistant TECs (149) may also contribute to the post-radiation restoration of the thymic function by producing these cytokines and providing signaling pathways essential for intercommunication with radioresistant TLPs. In particular, chemotherapy and radiotherapy, cytotoxic therapies cause apoptotic death of radiosensitive thymocytes and TECs (71). Following sublethal irradiation of mice, both cTECs and mTECs are reduced, indicating the radiosensitivity of most TECs (71, 149). However, after irradiation, some TECs can produce chemokines such as CCL19, CCL21, and CCL25 that are important for the recruitment of TLPs (71, 157). Furthermore, ECs are also radioresistant and can recruit TLPs (71). Therefore, together with radioresistant TLP and some TECs, ECs are essential in post-damage thymic regeneration.

Recent studies have shown that ILC3 and Th17 cells produce a cytokine (IL-22) that is critical for the thymic epithelial compartment recovery after high-dose chemotherapy or irradiation damage (144, 145, 151). IL-22 increased the number of TECs through the Stat3-dependent signaling pathway in the mTEC1 murine TEC line (158). Defects of IL-22 production delay thymus recovery in irradiated mice and act on the expression of genes associated with thymic function, such as *Foxn1*, *Aire*, and *Kgf*. In contrast, the use of IL-22 facilitates the repair of TECs, increases the number of T cells, increases the Aire level, and increases the proportion of natural regulatory T cells in the thymus (158), suggesting the critical role of the IL-22/Stat3/Mcl-1 pathway in the regeneration of TEC compartment after the irradiation damage. Following the total body irradiation or targeted irradiation of the thymus at the critical depletion of DP thymocytes, the intrathymic IL-22 level has been increased, suggesting a link of IL-22 with mechanisms of endogenous recovery (144, 151) and that is very similar to the effect of THGF (37). Production of IL-22 following damage is attributed to radioresistant thymic LTi/ILC3 cells, which were present in increased numbers following thymic insult, and RANKL molecule was implicated in thymus regeneration, which expression was upregulated by radioresistant LTi/ILC3 (71, 144).

In addition to the IL22 and THGF potential, a recent study highlighted the involvement of Bmp4 in thymus recovery following damage (112). Bmp4 is produced by multiple stromal cells within the thymus, including Fbs and ECs. However, following the total body irradiation, Bmp4 expression was upregulated only by ECs, resulting in increased cTECs, and an increase in Foxn1 levels and its target genes such as *Dll4*, *Kitl*, and *Cxcl12* (71, 112). Therefore, in this way, ECs involve Bmp4 to initiate thymus recovery. Since the number of ECs remains unchanged in the thymus after total body irradiation, ECs appear to be radioresistant thymic cells similarly to ILC3/LTi and resident THGF-sensitive TLPs. These new data provide a fresh look at the role of different radioresistant thymic cell populations in the thymus post-radiation regeneration and thymic function recovery.

INTHRATHYMIC CYTOKINE NETWORK

Thymocyte differentiation is regulated by direct contact with the stromal-epithelial microenvironment and responds to various cytokines produced by thymic stromal and lymphoid cells. IL-7, produced by TECs, is the key regulator of T cell maturation, differentiation, and survival in the early stages of their generation (37, 159). However, many other cytokines, usually presenting in the periphery, can also be found in the thymus. Since the thymus is a relatively closed organ for macromolecular migration into/from the organ, it is likely that the thymic cytokine network is adapted for the thymus itself and that cytokines do not leak to the periphery. However, for many intrathymic cytokines, their biological role is still unclear.

Various cytokines, such as IL-1, IL-3, IL-6, IL-7, IL-8, IL-12, IL-15, IL-25, as well as SCF, GM-CSF, G-CSF, M-CSF, TNFα, and TFRβ that are constitutively secreted by TECs, MCs, Fbs, and other stromal elements, have been identified in the thymus during the past decade. In addition, many cytokines, such as IL-1, IL-2, IL-3, IL-4, IL-5, IL-10, IL-17, IL-22, IL-23, THGF, IFN-γ, GM-CSF, G-CSF are the constitutive and/or inducible products of the thymic lymphoid populations, mainly DN TLPs, DP thymocytes, SP T cells, and thymic ILCs (6, 8, 16, 34, 35, 62, 108, 127, 132, 144, 146, 153, 159, 160).

TECs and T cells are two central populations of the thymic cells, which produce cytokines in the thymus. However, all thymic cells can secrete cytokines spontaneously or after stimulation. Among TECs, subcapsular and mTECs are more active cytokine producers than cTECs, and the cytokine profile of TECs is very close to the one of peripheral MFs and monocytes (2, 6, 7, 37, 71, 161). Although, when compared to the stromal thymic elements, thymocytes are relatively weak cytokine producers but being the most prominent population, their contribution to the intrathymic cytokine network is substantial. The cytokine-producing ability of thymocytes is gradually reduced during their maturation from the stage of DN $CD44^+CD3^-CD4^-CD8^-$ TLPs to the stage of immature DP $CD3^{lo}CD4^+CD8^+$ cortical thymocytes, and it is completely blocked in the latter DP stage. However, the capacity of thymocytes to produce cytokines and respond to their action is restored at the $CD4^+$ and $CD8^+$ SP stages of thymopoiesis following the completion of the selection process (37, 161, 162). The factors that activate and regulate the intrathymic cytokine secretion are still a matter of discussion, and they require further evaluation. Intercellular contacts, especially between TECs and thymocytes, play an important activation and modulating role in cytokine production in the thymus. In the mature thymocytes, the cytokines are produced in response to TCR-CD3 receptor complex binding (160, 162).

Inside the thymus, cytokines act as short distance factors, and their biological effects are determined by the expression of cytokine receptors on thymic cells. Some thymic cytokines can act as paracrine, and others appear as autocrine factors. IL-7 and SCF are examples of paracrine thymic cytokines that are produced by TECs and thymic MCs and induce the growth and differentiation of $CD4^-CD8^-$ TLPs (37, 159, 163). INFγ is another example of the paracrine cytokine, which is produced by mature SP thymocytes and participates in the control of T cell maturation and differentiation (6, 37, 161). IL-2 and IL-4, for which the producers and targets are thymocytes at the different

stages of maturing, can act both in a paracrine and autocrine manner. At the same time, THGF probably appears only as the autocrine factor for which the producers and targets are radioresistant intrathymic $CD4^-CD8^-$ TLPs (37, 41, 161). While SCF, IL-7, and THGF are essential to promote the proliferation and survival of $CD4^-CD8^-$ intrathymic TLPs, IL-2 and IL-4 are more specific for the final stages of T cell development (6, 37, 161). On the other hand, some $CD4^-CD8^-$ stages of intrathymic TLPs are also sensitive to IL-2 and IL-4, while IL-7, together with IL-12, IL-22, IL-23, and IFN-γ actively contribute to the negative selection and final stages of thymocyte differentiation (160).

SCF plays a key role in bone marrow hematopoiesis and lymphopoiesis (163); this growth factor is produced by thymic stromal cells, preferably by TECs and MCs, and SCF can directly stimulate the proliferation of $CD4^-CD8^-$ TLPs (71, 164, 165). Early TLPs exhibit high expression of the c-kit receptor for SCF. Therefore, the SCF/c-kit complex is essential during the early stages of thymopoiesis (35, 127, 128, 161), similar to IL-7 and THGF, suggesting that these cytokines belong to one functional group. Key signaling molecules of the intrathymic cellular network are summarized and presented in **Table 1**.

THYMUS RECONSTITUTION STRATEGIES

As early as 1961, Jacques Miller first reported the importance of the thymus for the development and function of the immune system function. Though many aspects are still a matter of intensive research, it is accepted that impaired thymus function can lead to various dramatic consequences, including the development of autoimmune diseases, increased susceptibility to infection, high risk of cancer, as well as a decreased immune response to vaccination (9, 166, 167). Complete thymectomy in neonates, especially if thymectomy was done at the age below 1 year, leads to the development of age-associated diseases, such as autoimmune and neurodegenerative diseases and atherosclerosis, and such patients have a persistent imbalance

TABLE 1 | Key signaling molecules of intrathymic cellular network.

Molecule	Cell expression	Functions in thymus	References
CD205	cTECs	Apoptopic cell clearance	(71)
β5t	cTECs, TEPCs	Thymic proteosome component, $CD8^+$ T cell selection	(71)
PRSS16	cTECs	Thymus specific serine protease, $CD4^+$ selection	(71)
DLL4	cTECs	Notch ligand, regulator of T cell commitment and β selection	(71)
CXCL12	cTECs	Chemokine ligand for CXCR4, regulation of β selection	(71)
CCL21	mTECs	Chemokine ligand for CCR7, regulator of cortex to medulla migration of SP thymocytes	(71)
CCL25	cTECs, mTECs	Chemokine ligand for CCR9, recruitment and positioning of TLPs, regulator of $CD4^+CD8^+$ thymocyte migration	(71)
LTβR	cTECs, mTECs	Ligand for lymphotoxin, regulator of mTEC and thymic endothelium development	(71)
Aire	mTECs	Tissue restricred antigen expression, tolerance	(71)
Fezf2	mTECs	Tissue restricred antigen expression, tolerance	(71)
RANK	mTECs, mTEPCs	mTEC development	(71)
Relb	mTECs	mTEPC development	(71)
IFNγ	Activated T cells, NK cells	T cell maturation and differentiation	(6)
SCF	cTECs	Maintenance of TLPs	(71)
THGF	Self-renewing TLPs	Activation and proliferation of self-renewing TLPs	(36, 152, 153)
IL-1	TECs, Macrophages	T cell activation and growth	(6)
IL-2	Activated T cells	T cell activation and development	(6)
IL-4	Activated T cells	T cell growth factor	(6)
IL-6	Macrophages, fibroblasts	T cell maturation and development	(6)
IL-7	cTECs and mTECs in adult thymus, TEPCs in embryonic thymus, stromal cells, DCs	Proliferation of TLPs	(6, 71)
IL-9	Activated T cells	T cell growth factor	(6)
IL-12	T cells	Maintenance of thymus integrity and function	(6)
IL-15	mTECs	Regulation of iNKT cells	(71)
IL-17	T cells	Activation of $CD4^+$ T cells, production of Treg17 cells	(6)
IL-21	Activated $CD4^+$ T cells	Differentiation of $CD4^+$ T cells, development of Treg17 cells	(6)
IL-22	Th17 cells, γδ T cells, NKT cells, ILCs	Proliferation and survival of TECs, Thymus regeneration	(6, 142, 143)
IL-25	Thymic tuft cells	Regulation of intrathymic ILCs and iNKT cells	(71)
TGFβ	Activated T cells	Inhibition of IL-1-, IL-2- and IL-7-dependent proliferation of thymocytes	(6)
TNFα	Macrophages	Promotion of T cell proliferation	(6)
TSLP	TECs, DCs	Promotion of Th2 cell differentiation of $CD4^+$ naïve T cells, activation of ILCs	(6)

DLL4, Delta like 4; LTβR, Lymphotoxin beta Receptor; Aire, Autoimmune Regulator; RANK, Receptor Activator of Nuclear Factor κB; ILCs, Innate Lymphoid Cells; iNKT, invariant Natural Killer T Cells; SP, Single Positive; DCs, Dendritic Cells; cTECs, cortical Thymic Epithelial Cells; mTECs; medullary Thymic Epithelial Cells; TEPCs, Thymic Epithelial Progenitor Cells; TLPs, Thymic Lymphocite Progenitors; Th, T helper; Treg, T regulator; IFNγ, Interferon gamma; SCF, Stem Cell Factor; THGF, Thymocyte Growth Factor; IL, Interleukin; TGFβ, Transforming Growth Factor beta; TNFα, Tumor Necrosis Factor alpha; TSLP, Thymic Stromal Lymphopoietin.

of naïve T cells in the periphery (9, 166, 167). During standard surgical procedures concerning congenital heart diseases, the thymus becomes biological waste. It can be used as a source of autologous tissue-specific stem cells for personalized treatment of thymectomized infants. With this, actual challenges are the optimization of thymectomy procedure in infants, collection and cryopreservation of thymic tissue, preparation of thymic stem cells, their clonal expansion, and development of robust protocols for autologous stem cell-based therapy (9, 37).

Current technologies for restoring the thymic function are focused mainly on using TEPCs/TESCs for remodeling functional thymic organoids *in vitro* or *in vivo* (15, 37, 71, 168, 169). The main obstacles to translating these technologies into medical practice are the small numbers of TESCs in the human thymus, difficulties of their isolation, purification, especially expansion *in vitro*, and formulation of the fully functional thymic organoids *ex vivo* (9, 37). The absence of effective methods for maintaining undifferentiated functional TESCs *in vitro* and the preferential growth of Fbs in such cultures, yet represent a significant challenge for the study and possible application of the cryopreserved TESCs (9, 37, 75, 76, 170).

Current approaches exploring how to reach a stable growth of TESCs *in vitro* apply the use of serum-free culture media, adding TESC-supporting compositions of growth factors, adding supplements that can inhibit the growth of other cell types, the use of nonadhesive materials to generate 3D TEC cultures (9, 15, 37, 71, 169). The use of small chemical compounds (SCC) blocking or enhancing signaling mediated by specific protein kinases and thus regulating the differentiation and clonal expansion of stem cells can be an additional practical component to reach this aim (9, 37, 171–173). Many studies have tested the target-specific SCC using human pluripotent ESCs, iPSCs, and HSCs (172). These studies validate the use of SCC for tissue engineering *in vitro* and for boosting the regenerative potential of stem cells *in vivo*. However, optimal SCC for TESCs has yet to be found and structurally optimized to achieve adequate efficiency and low toxicity (**Figure 3**).

Different molecules such as keratinocyte growth factor (KGF), Flt-3 Ligand (Flt3L), IL-7, IL-21, IL-22, RANKL, and growth hormones have been proposed as effective approaches to boost the endogenous thymic repair since these molecules are essential in thymopoiesis and for the maintenance of the epithelial compartment. In particular, the administration of IL-7, Flt3L, KGF, IL-21, and IL-22, have been used to support the thymus regeneration after high dose radio-chemotherapy injury (15, 144, 145, 151, 158). A clinical trial demonstrated that $CD4^+$ and $CD8^+$ T cells are increased by treating patients with IL-7, although an essential effect on thymic growth was not observed (15). A clinical trial with KGF to evaluate T cell recovery in HIV patients has not identified essential effects on thymic size or production of T cells in the thymus (15). In contrast, growth hormones have been influential in the reconstitution of the immune system and enhanced recovery of the thymus in HIV patients, demonstrating a higher thymic mass and numbers of circulating naïve T cells and $CD4^+$ T cells (15).

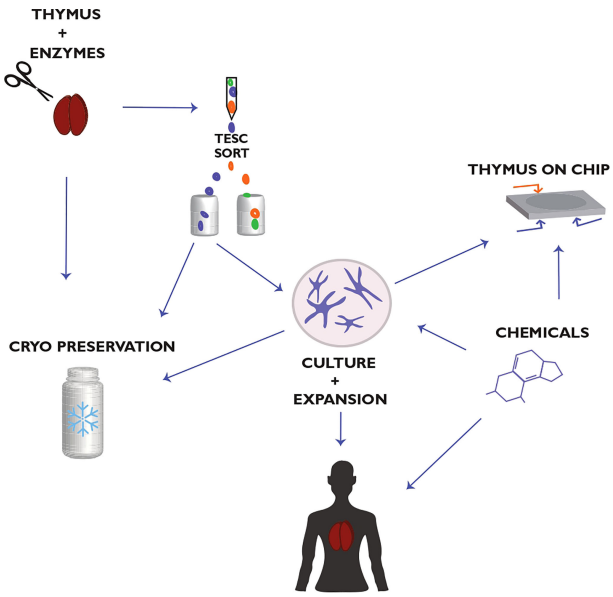


FIGURE 3 | Thymic epithelial stem cell (TESC)-based strategy for thymus regeneration with small chemical compounds (SCC). This strategy proposes the collection, preparation, and cryopreservation of primary thymic tissue and TESC-enriched cell samples. These thymic samples are used further to select TESC-specific SCC that can regulate the differentiation and proliferation of human TESCs and support their clonal expansion. The selected SCC are tested also for supporting thymic tissue growth *in vitro* as well as for reconstitution of thymic function in terms of differentiation, maturation, and tolerance of autologous T cells. The use of microfluidic chips in combination with human 3D thymic organ cultures (Thymus-on-Chip devices) to assess SCC specificity and toxicity is essential to accelerate drug development for thymus-compromised patients. Actual challenges are optimizing the thymectomy procedure in patients to preserve a thymic fragment for consequent postsurgical thymus regeneration and the quality life monitoring of thymectomized patients concerning their resistance to infections, allergies, autoimmune, oncological, and other diseases associated with the impaired thymic function. Modified from Shichkin and Antica, 2020 (9); the article is licensed under a Creative Commons Attribution 4.0 International License.

The first well-described pathway for endogenous thymus regeneration after the damage was centered on the production of IL-22 by resistance to injury ILCs (144, 151). Additionally to IL-22, ILCs also increase the production of RANKL that, in an autocrine manner, regulate IL-22 secretion by ILCs after thymic damage (144, 151). A second pathway is the IL-22-independent and connected with the production of BMP4 by ECs, which, similar to ILCs, are highly resistant to damage (112). The high radioresistance of these cells allows them to respond to activation by yet unknown signals and produce BMP4, which stimulates TECs to induce Foxn1 expression that controls DLL4 and Kit ligand transcription. These factors are critical for thymopoiesis and can control the thymic size (112, 151). The use of cytokines together with SCC that are essential for thymic regeneration *in vivo* may provide much more benefits for different groups of patients with thymic involution, including aging people representing the most significant population needed in this

medical treatment, than approaches using TESC-based technologies *in vitro* to generate the functional thymic organoids.

Several studies have evaluated the effectiveness of bone marrow-derived lymphoid progenitors, which were admixed with HSCs to accelerate and enhance immune rejuvenation. The limited supply of these lymphoid progenitors restricts this approach. However, with the development of new *in vitro* systems that use Notch-1 stimulation to generate T lineage-committed progenitors, 3D culture systems, and cell feeder-free culture conditions, this challenge can now be overcome (15, 45, 171–175).

In the last decade, essential efforts have been undertaken to generate *ex vivo* the functionally complete thymic microenvironment or thymic organoids that can be transplanted into patients. The advancement of these directions provides a promising framework for generating a *de novo* thymus from epithelial progenitors or pluripotent stem cells (15, 168, 169, 176). TEC-like cells, which are suitable for these modern technologies and can support T cell development, can be generated from Fbs with the help of targeted expression of *Foxn1* (174), demonstrating promising capabilities of these inducible TECs (iTECs).

Several studies have demonstrated the possibility of generating the functional thymus microenvironment from single mouse embryonal TESCs/TEPCs (19, 21, 175, 176) or iPSCs (174) injected under the kidney capsule in mice. These experiments illustrate the multipotency of the early TESC/TEPC and support ongoing efforts in the generation of the functional thymic organoids *in vitro* using epithelial progenitors and/or iPSCs. However, diversification of some thymic cell lineages essential for thymic function begins very early in the embryonal stage, and some of them have different embryonal precursors. Therefore, considering these factors, it appears problematic to develop a fully functional bioengineering thymus, which could provide the correct negative and positive selection of T cell repertoires only from epithelial precursors, and a combination at least with thymic mesenchymal and endothelial precursors may be needed. Moreover, the mouse thymus development and function are not entirely equivalent to the human thymus. Therefore, the knowledge received with mouse models may have a set of restrictions that should be considered when translating into medical practice. Considering these restrictions, combining different approaches instead of a single may better unlock the thymic regenerative potential and be more suitable for translating to the clinic. More detailed analyses of current thymic regenerative strategies are provided in the recent reviews (15, 177, 178).

CONCLUSION

Thymus function is based on the fine-tuning of specialized stromal, mesenchymal, epithelial, and endothelial cells and their products, which are essential for the continuous output of immunocompetent T lymphocytes. Although thymus involution occurs at puberty and its function decreases with aging, there is a great potential for restoring its function by either cell transplantation, regenerative therapy *in vivo*, or bioengineering strategies. Identifying cells and molecular factors that are important for differentiation, positive and negative selection, and generation of naïve T cells and translating of the experiments from the mouse models to humans is necessary for restoring a damaged thymus. The clinical application of the stem cells from adult tissues, that best resembles the *in vivo* conditions, will allow a better immune response in patients after iatrogenic thymus damage, patients born with thymus deficiency, or aged persons, increasing the efficiency of immunotherapy, including vaccination procedures.

AUTHOR CONTRIBUTIONS

VS reviewed the literature, wrote and redacted the manuscript, and designed and redacted the illustrations. MA redacted the manuscript and designed and redacted figures. All authors contributed to the article and approved the submitted version.

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A Proposed Link Between Acute Thymic Involution and Late Adverse Effects of Chemotherapy

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Epidemiologic data suggest that cancer survivors tend to develop a protuberant number of adverse late effects, including second primary malignancies (SPM), as a result of cytotoxic chemotherapy. Besides the genotoxic potential of these drugs that directly inflict mutational burden on genomic DNA, the precise mechanisms contributing to SPM development are poorly understood. Cancer is nowadays perceived as a complex process that goes beyond the concept of genetic disease and includes tumor cell interactions with complex stromal and immune cell microenvironments. The cancer immunoediting theory offers an explanation for the development of nascent neoplastic cells. Briefly, the theory suggests that newly emerging tumor cells are mostly eliminated by an effective tissue immunosurveillance, but certain tumor variants may occasionally escape innate and adaptive mechanisms of immunological destruction, entering an equilibrium phase, where immunologic tumor cell death "equals" new tumor cell birth. Subsequent microenvironmental pressures and accumulation of helpful mutations in certain variants may lead to escape from the equilibrium phase, and eventually cause an overt neoplasm. Cancer immunoediting functions as a dedicated sentinel under the auspice of a highly competent immune system. This perspective offers the fresh insight that chemotherapy-induced thymic involution, which is characterized by the extensive obliteration of the sensitive thymic epithelial cell (TEC) compartment, can cause long-term defects in thymopoiesis and in establishment of diverse T cell receptor repertoires and peripheral T cell pools of cancer survivors. Such delayed recovery of T cell adaptive immunity may result in prolonged hijacking of the cancer immunoediting mechanisms, and lead to development of persistent and mortal infections, inflammatory disorders, organ-specific autoimmunity lesions, and SPMs. Acknowledging that chemotherapy-induced thymic involution is a potential risk factor for the emergence of SPM demarcates new avenues for the rationalized development of pharmacologic interventions to promote thymic regeneration in patients receiving cytoreductive chemotherapies.

Keywords: chemotherapy, thymic involution, T cell, cancer immunoediting theory, immune surveillance, second primary malignacies

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INTRODUCTION

Despite the many advancements in the field of cancer therapeutics, including an array of targeted therapies and immunotherapies, chemotherapy still represents the frontier and standard-of-care therapeutic approach for the clinical management of the cancer patients (1). Today, a large number of cytoablative/cytostatic chemotherapies are available for clinical use in cancer patients, and are occasionally used alone, or more frequently under a combinatorial treatment strategy (2). These drugs are classified into five major classes (2), based on their mechanism of action: **(I)** Alkylating agents have the ability to covalently bind to and promote crosslinking of the two DNA strands *via* their alkyl group, thus leading to DNA strand break upon replication (i.e., during cell division) and triggering apoptosis (3). **(II)** Antimetabolites hinder the biosynthetic pathways of DNA/RNA, either because they inhibit enzymes that regulate DNA synthesis like DNA polymerase, or because they structurally resemble nucleobases/nucleosides lacking the proper chemical groups, thus preventing mitosis after their incorporation into the DNA (4). **(III)** Anti-microtubule agents interfere with microtubule dynamics, thus preventing key functions, such as the formation of the mitotic spindle during cell division and causing mitotic arrest (5). **(IV)** Topoisomerase inhibitors prevent the activity of topoisomerases, enzymes that physiologically introduce single- or double-strand breaks into the DNA to relieve strand tension and allow DNA to properly unwind during replication (6). **(V)** Cytotoxic antibiotics represent a large category of drugs with various modes of action, most notably prevention of cell division (7–9).

Beyond doubt, the survival rates of cancer patients have tremendously increased within the past decades due to more optimized and personalized use of chemotherapeutics, albeit with significant variations among different tumor types. Chemotherapy has even been successful in the radical treatment of certain tumor types, such as certain subtypes of testicular cancer and leukemias, although its therapeutic efficiency in most tumors of epithelial origin is rather limited, and at best suboptimal (10–15). An in-depth analysis of reasons behind the lack of its effectiveness is beyond the scope of this perspective. However, systemic toxicities rising from the lack of specificity in exclusively targeting neoplastic cells, drug resistance, and rapid drug metabolism/clearance of certain chemotherapeutics, signify only a few key reasons for their ineffectiveness against complete tumor eradication (2, 16, 17). More recent findings in preclinical mouse models of solid carcinomas suggest that chemotherapies may additionally promote neuroendocrine and stress responses, and elicit a proinflammatory cytokine surge, which together impede its short-term clinical benefits, by supporting a proangiogenic and prometastatic program in the tumor microenvironment, eventually leading to local and/or distant recurrence (18–21). Moreover, the long-term monitoring of cancer survivors (mostly pediatric cancer survivors) after years of receiving genotoxic treatments indicate a wide range of late adverse health effects, occurring mostly in, but not limited to, highly proliferating tissues, which include the hematopoietic, gastrointestinal, and reproductive systems. Such late adverse effects manifest as critical health issues in these patients, and include severe and long-term organ dysfunctions (including cardiotoxicity, neurotoxicity, nephrotoxicity, and hepatotoxicity, among others), infertility, cognitive impairment, and second primary malignancies (SPMs) (22–26). A thorough analysis on the occurrence and mechanisms behind all of the aforementioned adverse effects is beyond the scope of the current perspective. Here, we focus on the mechanistic origins of SPM, which represents one of the relatively understudied but most devastating late adverse effects in cancer survivors, as a paradigm for discussing the long-term consequences of chemotherapy on the immune system.

An SPM is defined as an unrelated primary cancer in a person who has experienced a different cancer sometime in their lifetime (22). By definition, SPM should be fundamentally distinguished from a secondary/metastatic cancer, especially if the latter occurs as a result of distant recurrence from a primary tumor, months or even years following treatment (27). The most prominent working model behind the development of such secondary cancers in the absence of a primary tumor relies on concrete, experimental evidence, collectively suggesting that cancer cell dissemination to distant metastatic sites, such as lungs, bone marrow, liver and brain, has occurred before the surgical excision or therapeutic management of the primary tumor (27). In this case, the long-term remission interval followed by relapse could be attributed to *cancer dormancy*, a stage of cancer progression, in which disseminated cancer cells either cease dividing (but survive in a quiescent state) or remain "locked" in a dynamic state, in which cancer cell proliferation balances cancer cell death (28, 29). Dormant cancers can remain clinically "silent" for months or even years, until the proper (micro)environmental conditions disrupt the dormancy program, and lead to a clinically overt tumor at the metastatic site (28, 29). On the contrary, SPM may rise on the same or a different organ and may either share a similar or different embryological origin with the first tumor; for example large B-cell lymphoma survivors are shown to be at high risk of developing colon, pancreas, breast (among other) tumors as late adverse SPMs (30). SPM is genetically distinct and independent from the first tumor that was experienced earlier in the patient's life, and typically harbors mutations as a result of genotoxicity from the chemotherapies used for the treatment of the first tumor (31–33).

Nowadays, revolutionary treatments and improvement in patient care have allowed oncologists to face a constantly increasing long-lived population of cancer survivors. As such, the late adverse health effects of cytotoxic cancer treatments have become a recent clinical issue, due to the better clinical outcomes and favorable prognostic potential. Hence, there exists an unmet clinical need to unravel risk factors for such late adverse, and especially fatal, as in the case of SPMs, health effects. A consequent unmet clinical need would thus be to establish new prognostic biomarkers to stratify cancer survivors that are at high risk of developing such late adverse effects, with an ultimate vision of adapting their therapies, strengthening follow-up, and identifying novel pharmacological targets for medical interventions. The mission of the basic cancer scientist against

this backdrop would thus be to provide a mechanistic insight on short- and long-term effects of cytotoxic cancer treatments on the immune system, as this appears to be the missing link for the development of devastating late adverse effects, such as SPMs. The current perspective offers a fresh working model, suggesting that acute thymic involution due to cytoreductive chemotherapy could significantly compromise the immune system of cancer survivors, thus leading to disturbed immune surveillance mechanisms and SPM development.

CHEMOTHERAPY-INDUCED SECOND PRIMARY MALIGNANCY – EPIDEMIOLOGY

Although prominent risk factors for the development of SPMs include predisposing genetic factors and the patient's lifestyle (as in the case of most primary cancers), it is important to mention that cytotoxic drugs that have been received for the clinical management of the first cancer and the patient's age at the onset of such treatments, represent two of the most well-established, independent risk factors of SPM development (34–37). In the United States, cancer survivors have a 14% higher risk of developing SPM when compared to the general population (22). Interestingly, the cumulative risk to develop an SPM within 30 years following diagnosis of a primary pediatric malignancy is ~6.8% (22). Along the same lines, effective control of early-onset malignancies through radiation therapy and multiagent chemotherapy, has on one hand achieved significant increase in the 5-year survival of pediatric cancer patients (up to 80%), but has detrimentally increased the relative risk of developing SPM at 30 years after the diagnosis of the first tumor, by approximately 6-fold (38). Hence, cancer survivors receiving cytoreductive chemotherapy for the treatment of their primary cancer are at high risk of developing an SPM, even years after the completion of therapy.

Commonly observed SPMs following pediatric cancer treatment with alkylating agents are of hematologic origin and include among others acute lymphoid leukemia (ALL), acute myeloid leukemia (AML), chronic myelogenous leukemia (CML), and myelodysplastic syndrome (MDS) (39). Depending on the dose and/or possible combination with doxorubicin, alkylating agents may increase the risk of developing leukemias as SPMs by at least 5-fold (22). Other chemotherapeutics, such as cyclophosphamide can increase the risk of developing bladder cancer as SPM (22). The combination of alkylating agents with radiation therapy can also result in the manifestation of solid carcinomas as SPMs, including breast (40), lung (41), stomach (42), pancreas (43), thyroid (44), and colorectal cancer (45), as well as bone or other sarcomas (46, 47). For more details, the readers are encouraged to consult excellent reviews and surveys for the most common pediatric first and second primary malignancies in childhood cancer survivors, along with a thorough analysis of the risk factors associated with those (22, 38, 48). Interestingly, there is vigorous epidemiologic evidence suggesting that pediatric cancer survivors carry significant risk of mortality due to SPMs that present as adverse late health effects (22, 49–52). This brief epidemiological synopsis of SPM incidence, risk factors, and prognosis in cancer survivors is intended to merely provide the readers with a fundamental clinical basis, to better conceptualize the causative link between chemotherapy and emergence of late adverse effects (e.g., SPM development).

CHEMOTHERAPY-INDUCED SECOND PRIMARY MALIGNANCY – MECHANISTIC ORIGINS

As described in the landmark review by Hanahan and Weinberg (2011), cancer is now accepted as a multifaceted disease, organized by the acquisition of certain biological capabilities, broadly known as the "hallmarks of cancer", and which can be summarized as the following: (i) Sustaining proliferative signaling, (ii) Evading growth suppressor mechanisms, (iii) Resisting cell death and apoptosis, (iv) Enabling replicative immortality, (v) Inducing angiogenesis, (vi) Activating invasion and metastasis, (vii) Reprogramming energy metabolism, and (viii) Evading immunological destruction (53). Underlying the acquisition of these acquired hallmark capabilities are two dimensions of tumor complexity. On one side is genome instability of transformed cells, which generates an essential genetic diversity (e.g., genomic mutations) that accelerates the acquisition of hallmark capabilities, while on the other side is a wide repertoire of recruited, seemingly normal cells that constitute the tumor microenvironment, and function as unwitting participants of cancer development and progression (53). Chemotherapy-based cancer treatments are highly genotoxic and are documented to increase the mutational burden of patients receiving them, thus providing an attractive rationale for the development of second independent malignancies as a late adverse effect. For instance, certain second primary leukemias developed by cancer survivors, including AML and MDS, present with deletion of 7q or monosomy 7 with normal chromosome 5, and deletions of 5q or monosomy 5, which are typical chromosomal aberrations due to prior exposure to alkylating agents (54). In another study, it was shown that topoisomerase II inhibitors, anthracyclines and mitoxantrone cause chromosomal translocations and chimeric rearrangements, leading to the manifestation of prolymphocytic leukemia as SPM (55–57). Topoisomerase II inhibitors have also been linked with translocations involving 11q23 or 21q22 in pediatric patients, leading to manifestation of AML within 1-5 years (58). Interestingly, genetic and epigenetic changes associated with cytotoxic treatments have also been reported for non-hematologic malignancies, such as pediatric ependymomas manifesting as SPMs, which depict hypermethylated phenotype leading to loss of tumor suppressor genes, such as CDKN2A, CDKN2B and p14ARF (59–61).

Although accumulation of such (epi)genetic defects due to chemotherapy treatment could partially explain early onset of SPMs, they cannot fully recapitulate the microenvironmental prerequisites that are essential for the development and

progression of clinically overt tumors. Interestingly, the "immune surveillance" theory, originally proposed by Burnet and Thomas more than half a century ago, suggested that the immune system functions as a sentry in identifying and eradicating newly-emerging neoplastic cells. An extensive refining of this theory based on experimental observations, culminated into the foundation of the more concrete "cancer immunoediting" theory, consisting of 3 biologically distinct phases, to describe the many aspects of immune-tumor cell interactions (62–67). In the first phase, Elimination, newly risen neoplastic cells are eliminated by a competent immune system, collectively described as the "immune surveillance". Intermittent tumor cells that manage to evade immunological destruction enter the second phase, Equilibrium, where immune-based elimination is balanced by the birth of new neoplastic cells. In the third phase, Escape, immunological "sculpting" allows tumors to progressively grow and lay the foundations for an immunosuppressive tumor microenvironment (62–67). As the primary site of T cell development and maturation, any intrinsic/extrinsic factors that negatively affect thymic integrity and functions could therefore affect the aforementioned immunoediting mechanisms, by shifting the balance toward the tumor-promoting end. Therefore, a critical question related to the origin of SPMs in cancer survivors is: "Could chemotherapy treatment have a long-lasting effect on the immune system, capable of hijacking the cancer immunoediting mechanism, thus facilitating SPM development in cancer survivors?"

To address this question, it is crucial to first recognize the key mediators of anticancer immunity. $CD8^+$ T lymphocytes and natural killer (NK) cells encompass the backbone of anticancer immune responses and cancer immunoediting (62–64, 67–71). T cell-mediated responses in particular, are mediated by cytotoxic $CD8^+$ T cells, which specifically recognize *via* their unique T cell receptor, one or more neoantigens on the cell surface of cancer cells (72–76). T cell-mediated anticancer immunity is supported by multiple stromal and immune cells, including cancer-associated endothelial cells and innate antigen-presenting cells (e.g., macrophages, dendritic cells), and leads to immunogenic cell death of tumor cells (77–79). To be able to recognize tumor cell neoantigens, a sufficient repertoire of T cell receptors and peripheral T cell pool with ability to monitor and elicit immunological attacks against neoplastic cells, must be generated in, and emerge from the thymus (80–82). As such, the thymus plays a critical role in the long-term establishment of anticancer immune surveillance and anticancer immunity (83). The thymus is a central lymphoid organ for T cell development, and signals derived from the thymic stromal epithelium are key determinants of thymocyte fate. The process of T cell development in the thymus is rather complex, and not the focus of the current perspective, but there are several checkpoints that determine efficient immune surveillance and anticancer immunity, such as: αβ-TCR gene rearrangement to acquire various specificities of neoantigen recognition, positive selection to achieve MHC restriction, and negative selection to establish central tolerance to self-antigens (84–87). Besides undergoing a natural decline termed age-related involution, the thymus is particularly sensitive to a variety of external stressors, as will be described in detail later, including cytoreductive chemotherapy, leading to its rapid involution and the consequent impairment of thymopoiesis (88–92).

Although thymic involution represents a logical mechanism for long-term immunosuppression and the failure of the immune system to control the emergence and survival of transformed cells, the link between thymus function and cancer development has been rather underrepresented in the "cancer immunology" literature. For instance, thymic involution could contribute to the long-term impairment of immune surveillance against tumor cells, enhanced ability of neoplastic cells to conceal their neoantigens and as such to evade immunological destruction, as well as the deployment of augmented immunosuppressive scaffolds in peripheral tissues (83). With regards to age-related thymic involution in particular, it has been documented that declined T cell-mediated immune surveillance is the outcome of reduced T cell repertoire diversity due to reduced thymic output, concurrent expansion of "immunosenescent" T cells expressing high levels of inhibitory checkpoint receptors (e.g., PD1), and a developmental shift towards immunosuppressive $CD4^+$ T regulatory (Treg) cells, capable of suppressing $CD8^+$ T cell functions in the periphery (83, 92–96). Therefore, the age-involuted thymus promotes the accumulation of multiple defects and the hijacking of the "cancer immunoediting machinery", which together promote the development of clinically overt cancers. A critical question in the context of SPM development is: "Would chemotherapy-induced involution present similar defects in immune surveillance and the cancer immunoediting process, as seen in the case of age-related thymic involution?"

Although there is sufficient evidence of short-term consequences of chemotherapy on the immune system, less is known about how chemotherapy or other extrinsic stressors could affect the cancer immunoediting process, and as a consequence, the emergence of SPMs in cancer survivors. With regards to short-term consequences of chemotherapy on the immune system, detailed investigations have unraveled conflicting data. It has been suggested that chemotherapy can exert desirable immunological effects, by boosting tumor cell immunogenicity and promoting immunologic cell death (ICD) of tumor cells, which is characterized by the mobilization of innate immune responses and tumor-specific adaptive immune responses (97–99). For example, doxorubicin and cyclophosphamide are capable of causing the translocation of calreticulin, an endoplasmic reticulum chaperone, to the tumor cell surface, thus offering a signal for phagocytosis by dendritic cells and as a consequence, tumor antigen uptake and presentation (100, 101). Chemotherapy is also capable of increasing expression of MHC-I molecules on the tumor cell surface, thus turning them into attractive targets for cytotoxic $CD8^+$ T cells, as well as of promoting the expression of NK stimulatory ligands, such as NKG2D, while suppressing NK inhibitory ligands (102–106). Finally, certain chemotherapeutics, including doxorubicin and cyclophosphamide, can enable type-I interferon signaling responses, and trigger macrophage recruitment, maturation, and NK cell proliferation (107, 108), thus establishing an immunostimulatory microenvironment. On

the other side, chemotherapy has been documented to induce a systemic cytokine surge, and the subsequent recruitment of bone marrow progenitors, including proangiogenic/prometastatic TIE2$^+$ monocytes, and myeloid-derived suppressor cells (MDSCs), which together promote a highly resilient and immunosuppressive tumor microenvironment (19, 21, 109–115). Moreover, certain chemotherapeutics, such as paclitaxel, can structurally mimic bacterial lipopolysaccharide, thus functioning as putative Toll-like receptor-4 (TLR4) agonists and leading to chronic inflammation, capable of hijacking the immune response against tumors (116–119). Besides the short-term effects, less has been unraveled on the immunological effects of chemotherapy over large periods of time.

However, indirect indications from epidemiologic data have hinted that cancer survivors may indeed suffer from suboptimal peripheral immune surveillance, due to receiving chemotherapeutics. For example, cancer survivors remain at elevated risk for developing infectious-related complications with a higher risk of persistent infections, and infection-related mortality, even years following chemotherapy (120–122), clearly suggesting that chemotherapy may exert long-term consequences to a patient's immune system. In certain hematological malignancies, it has been shown that the type and dose of chemotherapy treatment can determine the rate and magnitude of lymphocyte recovery following treatment, and as such, the re-establishment of proper immune surveillance (123–125). These observations do not only suggest that early lymphocyte recovery may be a favorable prognostic indicator in these patients, but also highlight the importance of developing therapeutic strategies to support faster lymphocyte recovery to avoid early or late adverse effects of chemotherapy-compromised immune surveillance (123–125).

Valuable insights in this regard have been provided by many groups studying long-term consequences of thymic involution in peripheral immune surveillance. In general, thymic epithelial cells (TECs) are necessary for T cell differentiation and maturation, by providing key growth factors, chemokines, cytokines, and strictly regulated selection processes within the thymic environments. The phenotypic heterogeneity of cortical (cTEC) and medullary (mTEC) thymic epithelial cells is critical for the precision and coordination of intrathymic pathways leading to the development of mature T cells (80, 126–132). Several common immunosuppressants used to prevent allograft rejection such as cyclosporine, corticosteroids such as dexamethasone, and cytoreductive chemotherapies used for cancer treatment such as cyclophosphamide, are all known to cause impaired thymopoiesis and even autoimmunity, primarily by targeting cTEC and mTEC populations (133–138). Acute thymic involution as a result of cytoreductive chemotherapy leads to delayed recovery of T cells, with imminent consequences in the peripheral T cell pool and immune surveillance. In a non-pediatric setting, it has been demonstrated that repopulation of certain subsets of CD4$^+$ T cells and B cells is delayed for almost a year following chemotherapy treatment in breast cancer patients (139). Although older studies have not looked into such extended periods of time, they have consistently reported that T cell recovery cannot be achieved between chemotherapy cycles, as opposed to the successful recovery of erythroid, myeloid and thrombocytic lineages (140, 141). In hematopoietic cell transplantation (HCT), cytoreductive chemotherapies are often used to prevent the transplant rejection, and as opposed to the fast recovery of non-lymphoid lineages post-chemotherapy, reconstitution of T cell adaptive immunity is profoundly delayed, often by a year or more (91, 142, 143). Post-chemotherapy T-cell deficiency in HCT recipients is not only associated with increased risk of infections and cancer relapse, but also with the development of SPMs, again due to failures in the cancer immunoediting mechanisms (141–147). Despite that all the non-T cell lineages are dependent on the bone marrow microenvironment for reconstitution following chemotherapy-mediated depletion, T lymphocytes are exclusively dependent on the thymus (148–150). The extensive delay in T cell reconstitution and the establishment of the peripheral T cell pool is therefore not attributed to impaired hematopoiesis, because the latter is restored soon after the termination of chemotherapy. Although the mentioned studies are quite indicative of the premise, the status of anticancer immune surveillance months or years following chemotherapy treatment has not been thoroughly assessed, and relevant animal models for such experimental testing are not, to our knowledge, standardized.

Besides impaired thymopoiesis leading to reduced peripheral T cell pool, chemotherapy-induced thymic involution may skew peripheral immune surveillance toward the development of precursor lesions for organ-specific autoimmune disease. As proof-of-concept, there is now clear epidemiologic evidence that post-chemotherapy rheumatism and other autoimmune syndromes may develop not only shortly, but even months or years, after completion of cytoablative treatments in (childhood) cancer survivors (151, 152). Mouse models of chemotherapy-induced thymic involution have determined that chemotherapy significantly obliterates the epithelial compartment of the thymus, most prominently the AIRE$^+$ MHC-II^{high} mTEC subset, whose endogenous repair is a rather time-demanding process (91, 126, 153–156). An elegant study by Fletcher and colleagues (2009) has previously demonstrated that AIRE$^+$ mTEC need approximately 7-10 days to be fully restored after treatment with immunosuppressive drugs or chemotherapeutics. Given that the restoration of AIRE$^+$ mTEC is significantly delayed compared to the other TEC subsets, the authors concluded that a 7–10-day period of impaired or suboptimal AIRE$^+$ mTEC function could be sufficient in allowing autoreactive T cells to escape the thymus and establish autoimmune lesions in the future (133). In general, the targeted deletion of the AIRE$^+$ mTEC subset, or the targeting of the optimal expansion of AIRE$^+$ mTEC *via* genetically engineered animals, both lead to organ-specific autoimmunity. For example, one study demonstrated that targeted deletion of the histone acetyltransferase KAT7 interferes with normal AIRE$^+$ mTEC development in the thymic environment and causes profound lymphocyte infiltration into a variety of peripheral organs, such as the lung, liver, salivary glands, stomach, and lacrimal glands (157). Although an increased release of

autoreactive T cells from the age-involuted thymus has been more strongly associated with chronic inflammation and autoimmunity (90, 158, 159), many investigators agree that the kinetically slow recovery of an acutely-involuted thymus can also provide a sufficient window to instigate the foundations of organ-specific autoimmunity.

What lessons can be learned by studying thymic involution, either age-induced or chemotherapy-induced, in the context of impaired thymopoiesis and autoimmunity? First, these studies collectively provide a proof-of-concept that damage/decline of various TEC components, including the most sensitive AIRE$^+$ mTEC subset, could potentially manifest as prolonged "disturbance" of tissue immune surveillance, characterized by profound deficiencies in T cell receptor repertoire, peripheral T cell pool, and the presence of autoreactive cytotoxic CD8$^+$ T cells. All these consequences can hinder the ability of the immune system to prevent nascent neoplastic cells *via* a competent immune surveillance, and to maintain control of tumor cell growth during the equilibrium phase of the cancer immunoediting process (154). Second, the thymus reaches its maximum relative size around birth and its maximum absolute size at puberty. As such, a significant impairment of thymopoiesis during this interval (i.e., the treatment of pediatric cancer patients with cytoreductive chemotherapy) would have a tremendous effect on the patient's immune system. Because chemotherapy-compromised cancer immunoediting mechanisms may persist for a long period of time (e.g., years) in pediatric cancer survivors, neoplastic cells can escape elimination and equilibrium phases much earlier, thus manifesting as early onset SPMs or other adverse health effects (22, 120). This newly proposed working model of establishing a causative link between chemotherapy-induced thymic involution and SPM development, with the defective cancer immunoediting mechanisms serving as an intermediary, is illustrated in **Figure 1**.

CHEMOTHERAPY-INDUCED THYMIC INVOLUTION – MECHANISTIC INSIGHTS AND REGENERATION STRATEGIES

The thymus is extremely sensitive to a wide array of external factors and stressors, including, but not limited to, acute/chronic infections, certain medications, glucocorticoids, cytoreductive chemotherapies, and even certain physiological states, such as pregnancy. Although these individual factors exert distinct effects on the thymic environment, they can all cause, in principle, extensive deterioration and/or complete elimination of the cTEC and mTEC compartments, leading to impaired thymopoiesis and escape of autoreactive T cells to the periphery (88, 91, 154, 155, 160, 161). In the case of cytoreductive treatments, the initial effect is dependent on the chemotherapy's mechanism of function, which is typically disruption of one or more steps associated with cell division, and as such the proliferating thymic epithelial cell pool is directly assaulted shortly after administration (88, 91, 161, 162). In general, chemotherapies that function by perturbing cell division will systemically suppress most of the actively proliferating niches, including the hematopoietic niche, which often leads to impaired multi-lineage hematopoiesis (163–165). Impaired lymphopoiesis leads to diminished mobilization of lymphocyte progenitors, and as such, it also leads to reduced homing of early thymic progenitors (ETPs) in the thymus environment (166, 167). Hence, the devastating effects on thymic architecture and function observed during chemotherapy are primarily related to its direct mechanism of action on proliferating niches in the mammalian body, and manifest as acute reduction of both thymocytes and TECs (**Figure 2A**).

Several investigations have interestingly revealed that when compared to cTEC subsets, AIRE$^+$ mTEC are more sensitive to stressor-mediated destruction, a feature that typically manifests as disproportional reconstitution of corticomedullary ratio with detrimental, long-term, organ-specific repercussions, such as development of autoimmunity and leukemic transformation (156, 162, 168, 169). Underlying this biased inefficiency of mTEC to repair from acute thymic involution may be indirect consequences of cytoablative treatments. Although, chemotherapies lead to severe reduction of thymocytes in the thymic environments as mentioned above, it is now well known that thymocytes and TECs participate in reciprocal signaling loops providing trophic and survival factors to one another (129, 131). For example, AIRE$^+$ mTEC are strongly dependent on RANK ligands (RANKL) provided by the single positive CD4$^+$ thymocytes and type 3 innate lymphoid cells (ILC3) for proliferation/differentiation and TEC regeneration (131, 170, 171). Therefore, chemotherapy-mediated disruption of lymphopoiesis will result in the elimination of lymphocyte homing and as such, the elimination of the TEC survival signals. In conclusion, besides the well-reported and direct mechanisms for chemotherapy-induced immunotoxicity, cytoablative treatments may also lead to prolonged "attritional" death of mTEC subsets due to the selective elimination of essential microenvironmental factors, such as RANKL (**Figure 2B**).

Naturally, the thymus has the endogenous capacity to regenerate from the loss of thymic epithelium (91, 172–174), although the time interval necessary for the completion of endogenous repair might be sufficient to cause critical failures in the aforementioned cancer immunoediting mechanisms, as already mentioned in the previous chapter. A recent, but active area of research, relies on the development of pharmacological interventions to facilitate thymic regeneration following chemotherapeutic or other cytotoxic insults. From the viewpoint of the current perspective, such strategies would be rather beneficial by boosting thymic functions and enhancing peripheral immune surveillance mechanisms in cancer survivors, to prevent early onset of SPMs and other late adverse effects of chemotherapy. In the following paragraphs, we briefly discuss the underlying principles of well-established regeneration strategies following acute thymic involution.

A significant number of regeneration strategies has focused on targeting cells, essential for thymic architecture and function, most notably cTEC and mTEC subsets (91), as thematically illustrated in **Figure 3A**. For instance, favorable outcomes have been reported from exposure to Fibroblast Growth Factor-7 (FGF7) (175, 176), Insulin-like Growth Factor-1 (IGF1) (177), Wingless-related

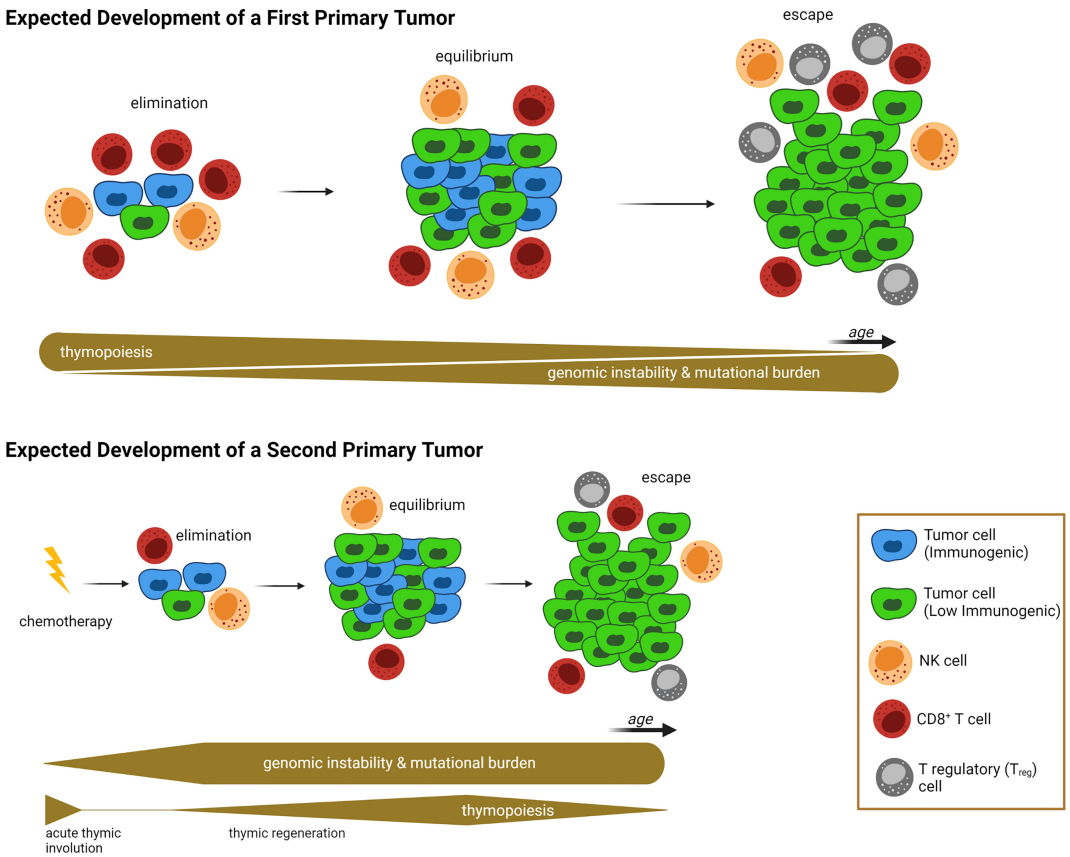

FIGURE 1 | Proposed Link Between Acute Thymic Involution and Development of Second Primary Malignancy. In the absence of exposure to prior treatments with cytoablative chemotherapies due to a first-primary tumor (upper half of illustration), the emergence of nascent transformed cells is subjected to a "competent" cancer immunoediting process. At the beginning, the competent immune system can eliminate neoplastic cells *via* an efficient immune surveillance machinery. Then tumor cell growth is balanced by immunogenic cell death, described as equilibrium phase. And finally, immunosculpting leads to the escape phase, during which anticancer immunity fails to control tumor growth and creates a clinically overt tumor. The succession of these three phases is a long-lasting process with two main contributing factors: First, genomic instability is increased over time, leading to accumulation of driver mutations and genetic diversity that allows immunoevasive and immunosuppressive mechanisms to evolve (e.g. development of tumor cell clones with absent or low immunogenicity). At the same time, age-related thymic involution causes a decreased T cell peripheral pool and T cell receptor repertoires, leading to failure of immune surveillance and equilibrium mechanisms. In contrast, following exposure to a first-primary tumor and associated treatment with cytoreductive chemotherapy (lower half of illustration), the failure of the immune surveillance and equilibrium mechanisms occurs at a much earlier timepoint, allowing for the onset of clinically overt second primary malignancies (SPMs) at a younger age, compared to first-primary tumors (compare timelines between upper and lower half of illustration). Contributing factors for the SPM are the genotoxic nature of cytotoxic chemotherapy (which grants genomic instability and mutational burden at a very early onset), and chemotherapy-induced acute thymic involution causing impaired thymopoiesis, T cell receptor repertoires, and peripheral T cell pools, thus weakening immune surveillance mechanisms during elimination and equilibrium phases. *Relative thickness of gray bars underneath the timelines in each condition indicates the strength of thymopoiesis (upper bar), and genomic instability (lower bar) over time (not drawn to scale). Illustration designed with Biorender.*

Integration site-4 (WNT4) (178), Bone Morphogenetic Protein-4 (BMP4) (174), and Interleukin-22 (IL22) (172). Most of these endogenous pathways orchestrate complex intrathymic circuitries, simultaneously involving multiple stromal epithelial, stromal non-epithelial (e.g., endothelial cells, mesenchymal cells), and immune cells (e.g., macrophages, dendritic cells, innate lymphoid cells), which cooperate to support the reconstitution of the appropriate thymic infrastructure for T cell development. For example, following radiation-induced thymic involution, a subset of dendritic cells secretes interleukin-23 (IL23) in the thymic environment, which stimulates innate lymphoid cells to subsequently secrete IL22, in turn promoting the survival and proliferation of radiation-affected TECs (172, 179). In another study, BMP4 was shown to be primarily secreted by the intrathymic endothelium and mesenchymal fibroblasts, and was significantly overexpressed following acute thymic involution to support the replenishment of $BMPR2^{high}$ cTEC populations, eventually facilitating thymic repair (174). A thorough analysis of all implicated studies in this category is beyond the scope of this perspective, but suffice is to claim that a concrete understanding of the paracrine/juxtacrine intrathymic milieu is paramount for the successful design of therapeutic modalities.

Because thymus physiology is under constant neuroendocrine control (180), a separate class of regeneration strategies has

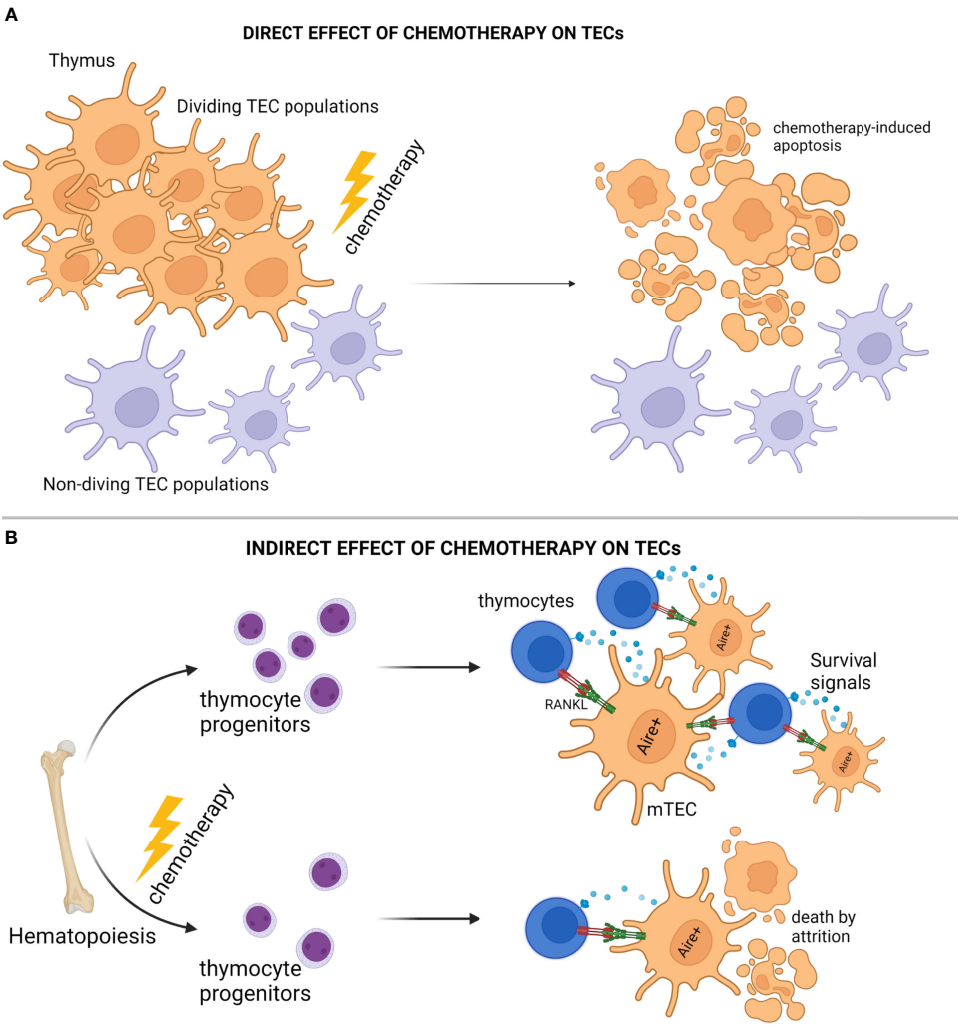

FIGURE 2 | Modes of Thymic Epithelial Cell Death After Chemotherapy Treatment. **(A)** Cytoreductive chemotherapy non-specifically and unconditionally targets proliferation niches in the entire organism, and as such, insults TEC subsets in the act of cell division. **(B)** Cytoreductive chemotherapy suppresses bone marrow hematopoiesis and subsequent early thymocyte progenitor homing in the thymic microenvironment, thus disrupting thymocyte-derived prosurvival signals essential for TEC homeostasis, and causing "attritional" cell death to sensitive TEC subsets (e.g., AIRE^{+} mTEC). *Illustration designed with Biorender*.

proposed the development of hormonal therapies to systemically control thymus growth (91), as thematically summarized in **Figure 3B**. Sex steroids have a negative impact on thymus function, and experimental models of chemical or surgical ablation of sex steroids have given positive results in thymic regeneration following acute thymic involution (153, 181–183). However, despite the beneficial effects of sex steroid inhibition in lymphoid potential and hematopoietic stem cell function, more studies need to be conducted in this direction, because animal models of castration often lead to increased release of autoreactive T cells (91, 155, 184). These findings raise the concern that regeneration strategies should carefully balance lymphocyte progenitor supply with the size of the thymic epithelial compartment to avoid detrimental consequences, such as autoimmunity.

Less explored thymic regeneration strategies include chemokine and cytokine therapy, to improve homing of bone marrow lymphocyte progenitors and expansion of thymic T cell precursors in the thymus (91), as exemplified in **Figure 3C**. The mechanistic principles behind the elicitation of such strategies rely on the fact that chemotherapy has a detrimental effect on bone marrow hematopoiesis, and the restoration of lymphopoiesis is rather restricted following the termination of the cytotoxic result (185, 186). A prominent example of such an approach includes pretreatment of bone marrow progenitors with CCL25 and CCL21 before autologous transplantation, to rescue their homing capacity in the thymus after exposure to the cytoreductive insult (187). Another strategy that circumvents hematopoietic cell transplantation involves the administration of IL7, a cytokine, endogenously secreted by cTEC subsets to

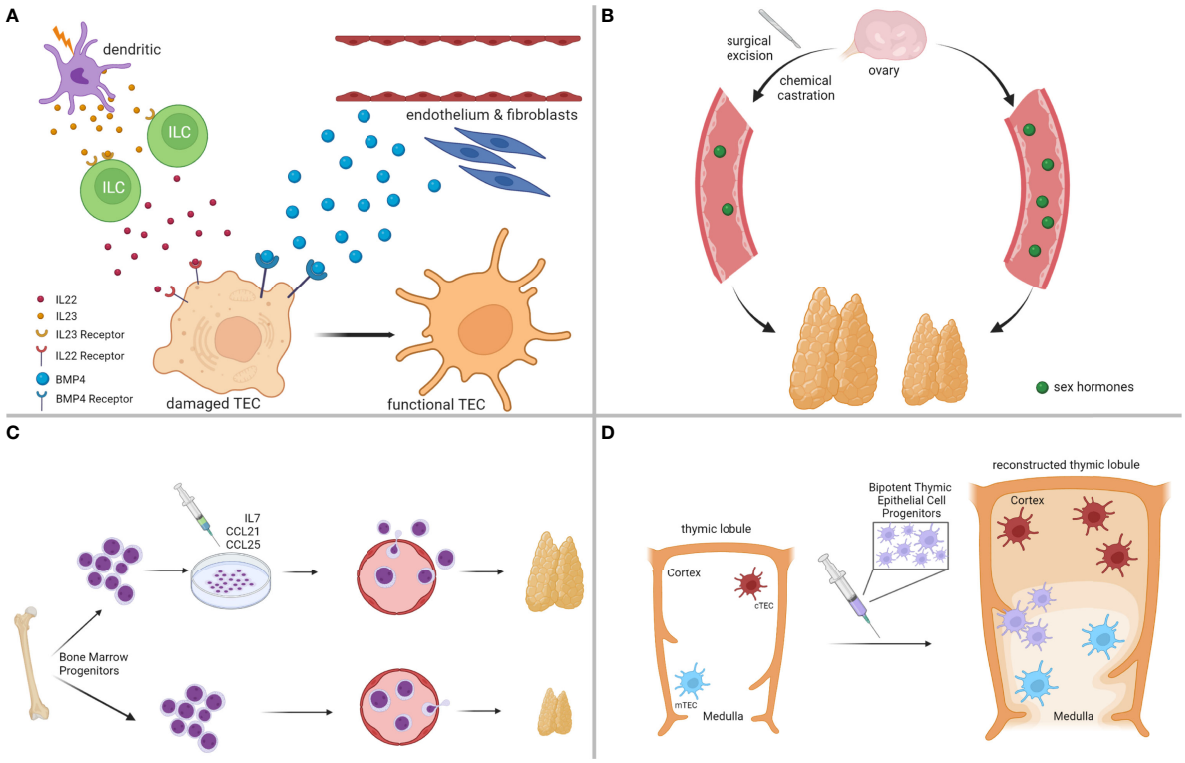

FIGURE 3 | Strategies for Enhancing Thymus Regeneration Following Chemotherapy. **(A)** Examples of thymus regeneration strategies targeting thymic stromal cell networks activated in endogenous thymic repair. **(B)** Examples of thymus regeneration strategies targeting negative feedback loops on thymus size/function from sex hormones. **(C)** Examples of thymus regeneration strategies involving the transplantation of (pre-conditioned) bone marrow-derived thymocyte progenitors. **(D)** Examples of thymus regeneration strategies that are not dependent on the endogenous thymus, such as transplantation of bipotent thymic epithelial cell progenitors to reconstitute thymus lobules and functions. *Illustration designed with Biorender*.

promote T cell proliferation and expansion, innate lymphoid cell development, and lymphoid tissue organization (188–190).

Other even less explored, but emerging strategies involve the development of artificial thymic niches to circumvent the reliance on the endogenous thymus upon cytoreductive insult (191), and the transplantation of bipotent TEC progenitor (TECP) cells to reconstruct the entire thymic environment (192), both of which show great promise (**Figure 3D**). Taken together, our goal in this section was not to provide an exhaustive discussion of all available regenerative strategies that are currently explored to boost thymic function following cytotoxic insults. Instead, we hoped to offer a brief overview of the most promising pharmacologic interventions that could help restore the cancer immunoediting mechanisms in cancer survivors receiving chemotherapy.

CRITICISMS OF THE PROPOSED MODEL AND FUTURE REPERCUSSIONS

The cancer immunoediting process functions as a devoted sentinel under the auspices of a highly competent immune system to put a tissue barrier on tumor development and progression. In this hypothesis and theory article, we explored the premise that cancer survivors who have received cytoreductive chemotherapy may present with multiple defects on the cancer immunoediting mechanisms, as a result of chemotherapy-induced thymic involution. These observations would further imply that the onset of late adverse effects of chemotherapy is not exclusively attributed to the genotoxic potential of these drugs, but also to their negative impact on thymic functions and T cell development. At this point, our proposed model is not intended to be a comprehensive and exhaustive analysis of all genomic and contextual intricacies governing the defects of the cancer immunoediting process that could lead to SPMs after chemotherapy. Rather, we have laid the groundwork for future expansions of the proposed model. For instance, we focused primarily on $CD8^+$ T cell-mediated anticancer immunity and immune surveillance to discuss the relevant defects on the cancer immunoediting mechanisms. However, there is now compelling evidence that both NK cells and NKT cells comprise a substantial component of the anticancer immune response, and cancer immune surveillance mechanisms (193–197), suggesting that the effects of chemotherapy on conventional intrathymic pathways for T cell development could be only one side of the coin. As such, it

would be important that future investigations focus on systematic immunology studies to address the impact of chemotherapy on the immune system.

The proposed model primarily focuses on the impact of chemotherapy on cancer immunoediting mechanisms, from the viewpoint of prolonged impaired thymopoiesis after chemotherapy. Our model, however, did not discuss the impact of chemotherapy on the quality of thymopoiesis upon chemotherapy treatment. A large body of evidence now suggests that age-related involution is related to immunosenescence, which is translated not only in defects on numbers of peripheral T cells during involution, but also in increased numbers of T regulatory cells and markers of T cell exhaustion in the periphery (83, 94, 198–204). In our opinion, "immunosenescence" has not been adequately addressed in the context of acute thymic involution, but regardless, it should be taken into account during the experimental design of future thymus regeneration strategies.

Our proposed model has not made clear distinctions between types or schemes of chemotherapy and specific defects on cancer immunoediting mechanisms and development of SPMs. In part, this is due to the fact that not many such studies currently exist. However, it would be an oversimplification to claim that all chemotherapies exert similar effects or have the same capacity to inflict SPMs, given that, for example, there are well-known specific mutations tied to specific drug classes (22). In addition, chemotherapies may potentially affect thymic environments in a heterogeneous manner. As mentioned, paclitaxel has been shown to function as a lipopolysaccharide mimetic, thus promoting an acute proinflammatory milieu by functioning directly as a TLR4 agonist, besides the traditional mechanism of microtubule stabilization (116–119). Many groups have compared neoadjuvant versus adjuvant chemotherapy settings, either on the local tumor microenvironment or the systemic tumor "macroenvironment", and also reported fundamental epidemiological differences in their response (19, 115, 205–212). Because the choice of cytoreductive treatments could have a unique effect on thymopoiesis, such considerations should be carefully taken into account as the scientific community moves forward in the field, to properly enrich and revisit our currently proposed model.

Our proposed model focuses on pediatric cancer patient survivors, to propose a causative link between acute thymic involution and defective cancer immunoediting mechanisms leading to SPMs. Nevertheless, SPM development also occurs in non-pediatric patients, and similar mechanisms could also be relevant in these populations (41, 213–221). The pediatric cancer survivor paradigm was easier to discuss in our model, first because there are long-term follow-up epidemiological data that can be used as a better proof-of-concept (222–224), and second, because thymic functions are relatively stronger in childhood, as compared to other ages (201). However, due to scientific advancements, oncologists are nowadays faced with an increasing population of cancer survivors at all ages, and as such, we anticipate that studies on acute thymic involution will eventually become relevant for older cancer survivors.

To conclude, acknowledging that chemotherapy-induced thymic involution is a risk factor for the emergence of SPMs opens a new avenue for the rationalized development of pharmacologic interventions to promote thymic regeneration in patients receiving cytoreductive chemotherapies. Here, we articulated that this research field is promising and exciting, and we further anticipate that it will be at the frontier of personalized medicine in the next decade.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

GK conceptualized the working hypothesis/model. ML designed all illustrations; ML, DA, and GK wrote, drafted, and edited the manuscript. All authors contributed to the article and approved the submitted version.

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Thymus Reconstitution in Young and Aged Mice Is Facilitated by *In Vitro*-Generated Progenitor T Cells

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The prolonged lag in T cell recovery seen in older patients undergoing hematopoietic stem cell transplant (HSCT), after chemo-/radiotherapy, can lead to immune dysfunction. As a result, recovering patients may experience a relapse in malignancies and opportunistic infections, leading to high mortality rates. The delay in T cell recovery is partly due to thymic involution, a natural collapse in the size and function of the thymus, as individuals age, and partly due to the damage sustained by the thymic stromal cells through exposure to chemo-/radiotherapy. There is a clear need for new strategies to accelerate intrathymic T cell reconstitution when treating aged patients to counter the effects of involution and cancer therapy regimens. Adoptive transfer of human progenitor T (proT) cells has been shown to accelerate T cell regeneration in radiation-treated young mice and to restore thymic architecture in immunodeficient mice. Here, we demonstrate that the adoptive transfer of *in vitro*-generated proT cells in aged mice (18-24 months) accelerated thymic reconstitution after treatment with chemotherapy and gamma irradiation compared to HSCT alone. We noted that aged mice appeared to have a more limited expansion of CD4-CD8- thymocytes and slower temporal kinetics in the development of donor proT cells into mature T cells, when compared to younger mice, despite following the same chemo/radiation regimen. This suggests a greater resilience of the young thymus compared to the aged thymus. Nevertheless, newly generated T cells from proT cell engrafted aged and young mice were readily present in the periphery accelerating the reappearance of new naïve T cells. Accelerated T cell recovery was also observed in both aged and young mice receiving both proT cells and HSCT. The strategy of transferring proT cells can potentially be used as an effective cellular therapy in aged patients to improve immune recovery and reduce the risk of opportunistic infections post-HSCT.

Keywords: hematopoietic stem cell (HSC) transplantation, progenitor T cells, T cell development, thymus, homing

INTRODUCTION

T cells play a key role in the adaptive immunity to protect individuals from infections and malignancies. Most of the events during T cell development occur within the thymus, which is a primary immune organ that normally lacks self-renewing T cell progenitors (1). Therefore, the thymus relies on the semicontinuous supply of thymus-seeding progenitors (TSPs) from the bone marrow (BM) (2). TSPs receive strong Notch signals in the thymus (3), which guides them through a series of regulated developmental steps, including $CD4^-$ $CD8^-$ double negative (DN), $CD4^+$ $CD8^+$ double positive (DP), and $CD4^+$ or $CD8^+$ single positive (SP) stages (4). TSPs commit to the T-lineage during intrathymic differentiation and become mature T cells when receiving signals from the specialized thymic microenvironment (5).

Interventions that disrupt the generation of T cells can lead to immunodeficiencies, such as irradiation/chemotherapy required for the treatment of some cancers (6, 7). Clinically, patients with hematological malignancies require myeloablative chemotherapy and/or radiotherapy to eliminate leukemic cells followed by hematopoietic and stem cell transplant (HSCT) from compatible donors to reconstitute the depleted BM niche. Although most blood borne cells recover relatively quickly, T cells have a prolonged recovery period, leading to higher risks of opportunistic infections or relapse (8). This is likely due by the disrupted process of lymphopoiesis as a result of irradiation and chemotherapy (*e.g.*, cyclophosphamide), particularly due to damage to thymic epithelial cells (TECs) (9, 10).

The delay in T cell recovery, which can last for over a year, is exacerbated in elderly patients (11), largely as a result of thymic involution, the natural age-related atrophy of the T-lymphopoietic organ. This has been modeled by showing that TECs in aged mice showed a higher rate of apoptosis and lower proliferative capacity (12), which was concomitant with a decline in the expression of the TEC-specific master transcription factor, Forkhead box N1 (FOXN1) (13, 14). In addition, aged thymus has a deficiency in the enzyme catalase, leading to an accumulation of damaging reactive oxygen species in TECs (15–17). The aged thymus also has a disorganized thymic structure, with a thinning of cortical regions, and more adipocytes and fibroblasts, providing a suboptimal microenvironment for the development and survival of proT cells (18, 19). This is further exacerbated by the absence of thymocyte/TEC crosstalk following HSCT, which may include RANK stimulation, required for proper thymic maintenance (20–22), potentially adding to the decline of the aged thymus compared to young (23).

A potential strategy to circumvent the paucity in T cell regeneration seen with HSCT is to adoptively transfer *in vitro*-generated progenitor T (proT) cells along with HSCT to facilitate thymic engraftment and accelerate T cell reconstitution (23–25). We define mouse proT cells as $CD25^+$ DN cells (DN2 and DN3 stages) that can home to thymus, while possessing limited potential for non-T lineages outcomes. ProT cells have been shown to effectively reconstitute a host thymus and differentiate into all T cell subsets. Since donor proT cells undergo positive- and negative-selections in the host thymus, newly generated T cells are then restricted to host self-MHC, and tolerized to self-antigens, free from the risk of graft-vs-host-disease (26). Additionally, we have shown that through lymphocyte/TEC crosstalk, proT cells improve the thymic architecture of immunodeficient mice and enhance subsequent recruitment of bone marrow-derived progenitors (27). However, the kinetics of thymus recovery after combined therapy of proT cell and HSCT in aged mice remained unknown.

Here, we show that co-administration of *in vitro*-generated proT cells and HSCT can accelerate thymic reconstitution in aged and young mice after chemotherapy and radiotherapy, as compared to HSCT alone. Furthermore, we demonstrate that aged mice receiving proT cells have accelerated T cell recovery in the periphery, as compared to mice given HSCT alone. Notably, proT cells showed a similar ability to home to the thymuses of aged and young mice. However, aged recipient hosts showed a more limited expansion of donor thymocytes and slower kinetics of T cell development as compared to the young mice. Our preclinical results confirm that co-transferring proT cells with HSCT can potentially be used as an effective cellular therapy to enhance the immune recovery and lower the risk of opportunistic infections in aged patients post-HSCT.

METHODS

Mice

C57BL/6 (B6 CD45.2) and congenic B6.SJL-Ptprca Pepcb/BoyJ (B6 CD45.1) mice were purchased from The Jackson Laboratory (stock numbers 000664 and 002014, respectively). Young (8 to 12 weeks), and in-house aged (18-20 months) cohorts of mice were used. Green fluorescent protein (GFP^+) hematopoietic cells were generated by breeding ROSA26-rtTA transgenic mice (3) to Vav-iCre transgenic mice (4) to establish VaviCre-ROSA26rtTA mice on the B6 CD45.2 background. In hematopoietic cells, GFP was expressed upon Cre-dependent removal of a loxP-stop-loxP cassette within the ROSA26 locus. DsRed (B6.Cg-Tg(CAG-DsRed*MST)1Nagy/J) transgenic mice were purchased from The Jackson Laboratory (stock number 006051). All mice were maintained and bred at Sunnybrook Health Sciences Centre, and all animal procedures were approved by the Sunnybrook Health Sciences Centre Animal Care Committee.

Progenitor T Cell Co-Cultures With OP9-DL4-7FS Cells

Lineage-negative (Lin^-) $Sca\text{-}1^+$ Kit^+ (LSK)/OP9-DL4 cell co-cultures were implemented, as previously described (5) with several modifications. Briefly, we cultured mouse-BM-derived LSK cells with the newly generated OP9-DL4-7FS cell line, transduced to express the Notch ligand *Dll4* as well as human cytokines IL-7, FLT3-L, and SCF, as described (28). BM cells were collected from wild type B6 mice by dissecting and crushing the leg bones using sterile utensils in Hanks' Balanced Salt Solution (HBSS). BM cells were then filtered through 40 μm filter to get a single cells suspension. $CD117^+$ (Kit^+) cells were enriched using anti-CD117-MicroBeads and LS column

(Miltenyi) according to manufacturer's instructions. Subsequently, the CD117-enriched population was labelled with FITC-conjugated antibodies against lineage (Lin) markers [anti-B220 (RA3–6B2), anti-CD19 (1D3), anti-CD11b (M1/70), anti-Gr-1 (8C5), anti-NK1.1 (PK136), anti-CD3 (2C11), anti-CD8α (53.6–7), anti-CD4 (GK1.5)], as well as with anti-CD117-APC (2B8) and anti-Sca1-PE (D7) (all from BioLegend). LSK cells were sorted using cell sorter FACSAria Fusion (BD Biosciences). In experiments using LSK cells from DsRed$^+$ donor mice, the same procedure was performed with Sca-1 coupled to APC-Cy7 fluorophore (BioLegend).

In each 15 cm culture dish of OP9-DL4-7FS cells at ~90% confluency, 50,000 to 70,000 LSK cells were seeded and maintained in α-Minimum Essential Medium Eagle (α-MEM) supplemented with 5% FBS and 1% Penicillin/Streptomycin (Gibco) in the presence of 1 ng/ml IL-7 (Miltenyi Biotec) and 5 ng/ml Flt-3L (Miltenyi Biotec). Old culture media was ½ replaced with fresh media with no additional cytokines on days 5 and 8 after the start of co-cultures. On day 10 after seeding the LSK cells, the co-cultures were harvested and filtered through 40 μm cell strainers (Thermo-Fisher). The single cell suspension was labelled with anti-CD25-APC (PC61, Bio-Legend) and subsequently incubated in anti-APC-MicroBeads (Miltenyi Biotec), and enriched for CD25$^+$ cells using LS column (Miltenyi Biotec) according to manufacturer's instructions. The flow through (CD25$^-$ cells) was also collected for injection in some experiments.

Adoptive Transfer of Progenitor T Cells

B6 CD45.2 or CD45.1 congenic hosts were IP-injected with 150 μg/kg of Cyclophosphamide (Procytox (CTX), Baxter Corp.) 5 days and 3 days prior to exposure to 1.05 Gy total body irradiation using a Cs137 source gamma irradiator. 4-6 hours post-irradiation, all mice were intravenously injected with 1×10^6 B6 GFP$^+$ BM-extracted cells from VaviCre-ROSA26rtTA mice that were T cells depleted (T-depleted Bone Marrow, TDBM). T cells in the BM VaviCre-ROSA26rtTA mice were depleted by anti-CD3-MicroBeads (Miltenyi). The "proT+TDBM" experimental group also received 5×10^5, 1×10^6, 2×10^6, or 4×10^6 CD25-enriched proT cells derived from B6 CD45.2 or CD45.1 mice, at 99% purity. Cells were resuspended in 200 μL of serum-free αMEM in preparation for injections. In the CD25$^+$ vs. CD25$^-$ experiment, each experimental group was intravenously injected with 5×10^5 CD25$^+$ or CD25$^-$ cells.

Flow Cytometric Analysis

Single cell suspensions of dissected thymus, spleen, and BM were prepared by mashing followed by filtering through 40 μL cell strainers in HBSS supplemented with 1% bovine serum albumin and 2 mM EDTA. Single-cell suspensions were labelled with fluorescently-conjugated antibodies purchased from BioLegend as follows: CD45.1(A20)-Percp/Cy5.5, CD45.2(104)-APC/Cy7, CD4 (GK1.5)-Alexafluor 700, CD8(53.6–7)-PE/Cy7, CD44(IM7)-PE, CD25-APC, CD3(17A2)-PE/Cy7, CD11b(M1/70)-APC, CD19 (1D3)-PE, CD45(30F-11)-APC/Cy7. Flow cytometry was performed on LSR II (BD Biosciences). Dead cells were excluded by 4′,6-diamidino-2-phenylindole (DAPI) uptake. Data were analyzed using FlowJo Version 10.8.1 software (TreeStar).

Statistical Analysis

Statistically significance between different adoptive transfer dosages of proT cells and between aged and young mice in their thymuses and spleens were analyzed using one-way or two-way ANOVA and were performed using Prism software. All data are represented as mean ± SEM in error bars, with asterisks representing statistical significance (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001).

RESULTS

Available Thymic Niches Are Similar in Young and Aged Mice

To determine whether the thymuses of aged and young mice have an intrinsically different capacity to recruit proT cells and/or a different number of available thymic niches under steady state conditions, we adoptively transferred increasing numbers of *in vitro*-generated proT cells into non-irradiated mice in each age group (**Figure 1**). To this end, sorted mouse bone marrow (BM) Lineage$^-$ Sca-1$^+$ Kit$^+$ (LSK) cells were cocultured with OP9-DL4-7FS cells (28) for 10 days to generate CD25$^+$ proT cells at the

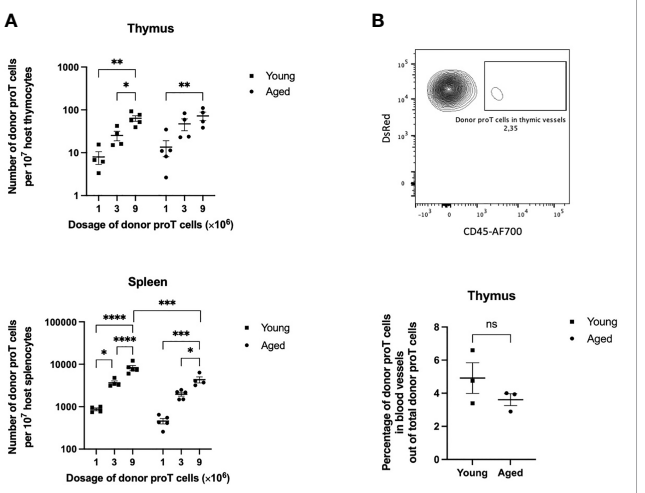

FIGURE 1 | Thymus engraftment by *in vitro*-generated progenitor T cells in unmanipulated young and old mice. **(A)** CD25$^+$ DsRed$^+$ donor proT cells per 10^7 host thymocytes (top) and splenocytes (bottom) isolated from host thymuses and spleens of non-irradiated aged and young mice. Each group of mice (n=4-5) were injected with 1, 3, or 9 x10^6 proT cells. Error bars depict SEM; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 analyzed by two-way ANOVA. **(B)** Representative flow cytometric analysis of detected DsRed$^+$ proT cells from the thymus of a host mouse (top). Aggregated percentages of DsRed$^+$ proT cells in blood vessels out of total DsRed$^+$ proT cells from host thymuses injected with either 1, 3, or 9 x10^6 proT cells (bottom). Hosts were intravenously injected with CD45-AF700 antibodies 3 minutes prior to sacrificing. Error bars depict SEM; difference between the aged and young mice groups was not significantly (ns) different as analyzed by two tailed unpaired student's *t*-test.

DN2/DN3 stage of T cell development (**Supplementary Figure 1A**). Cocultures were subjected to magnetic-assisted cell sorting (MACS) to enrich for $CD25^+$ and the $CD25^-$ subsets.

$CD25^+$ proT cells (1, 3, or 9 x10^6 $CD25^+$ cells), *in vitro*-generated from LSK cells from DsRed mice, were injected intravenously (i.v.) into host mice, and the presence of donor cells within the thymus was assessed at 40 h after injection. To rule out proT cells outside the thymic parenchyma, a labelled anti-CD45 antibody was injected i.v. just prior to sacrificing the host mice. Flow cytometric analysis of thymuses from young mice revealed the presence of donor $DsRed^+$ cells when injected with 1 x10^6 proT cells, and the numbers of donor cells appeared to increase linearly when 3 and 9 x10^6 donor proT cells were adoptively transferred (**Figure 1A**). However, the thymus of aged mice showed saturated niches with increasing numbers of injected proT cells. Of note, the frequency of proT cells present outside the thymic parenchyma, or perivascular space, was on average ≤5% in both aged and young mice (**Figure 1B**). Surprisingly, the thymus of aged mice showed a similar capacity in recruiting donor proT cells, with the thymus of young and aged mice failing to show a significantly different number of receptive niches. Similar to the thymus, host spleens showed a dose-dependent appearance of donor $DsRed^+$ cells. However, the number of donor cells present in spleens was over 40-fold higher than what was detected in the thymus. This suggested that at steady state the thymus has a highly restricted entry and/or limited number of niches for proT cells.

Thymus Engraftment by *In Vitro* Generated ProT Cells

The use of non-irradiated host mice established that the thymus of both young and aged mice showed a similar but low number of receptive niches. We then assessed whether the use of clinically relevant conditioning regimens, including chemo/radiotherapy, would impact the effectiveness of proT cell engraftment in aged and young mice. To this end, young host mice were treated with cyclophosphamide (CTX, 150 μg/kg) 5 and 3 days prior to lethal irradiation (1.05 Gy) (**Figure 2A**). To verify that *in vitro*-generated proT cells and no other cocultured-derived cells would home to the thymus, as described before (24, 25) and shown above, we compared the homing and engraftment ability of LSK/OP9-DL47FS coculture-generated $CD25^+$ DN proT cells to that of the remaining $CD25^-$ DN cells (**Supplementary Figure 1A**). In addition to the culture-derived cells, young mice (8-12 wks old) were co-injected with 1 x10^6 GFP^+ T cell-depleted BM (TDBM) cells. The thymuses of host mice were analyzed by flow cytometry 8 days (D8) after injection, and we noted a significantly greater number of donor $CD25^+$ proT cells homing to and developing within the thymus, constituting ~50% of total thymocytes, including $CD4^+$ $CD8^+$ DP cells, in contrast to $CD25^-$ DN cells, which failed to engraft (**Figure 2B**). In this regard, mice injected with $CD25^-$ DN cells showed only host-derived cells in the thymus (**Figure 2B**). Remarkably, on D8, some of the $CD25^+$ proT-derived thymocytes still exhibited CD25 expression, suggesting long-lasting self-renewal of proT cells within the thymus (**Figure 2B**, left panel). Nevertheless, mice injected with $CD25^-$ DN cells showed a minor fraction (~0.5%) of donor cells within the thymus that progressed towards the DP stage of differentiation, which we attributed to the fact that the MACS-enriched $CD25^-$ DN population contained about 8% $CD25^+$ proT cells (**Supplementary Figure 1A**, middle panel). In contrast to the poor thymic engraftment, $CD25^-$ DN cells were readily detected in the spleen of host mice, comprising the 28% of splenocytes, with the vast majority expressing CD11b (**Figure 2C** and **Supplementary Figure 1B**). As expected, the other major contributor to $CD11b^+$ myeloid cells in the spleen are derived from the GFP^+ TDBM donor graft. Remarkably, the contribution of culture-derived donor cells to the BM was minimal for both $CD25^+$ proT and $CD25^-$ DN cells, while as expected a strong contribution by GFP^+ TDBM donor cells was observed (**Figure 2D**). Taken together, our findings further validate the use of *in vitro*-generated $CD25^+$ proT cells as an effective thymus seeding cell in the context of HSCT.

Thymus Engraftment by Increasing Numbers of ProT Cells in Aged Mice

To determine the number of proT cells required to saturate thymic engraftment in aged mice, in the context of combined chemo/radiation conditioning and HSCT, we adoptively transferred 0, 0.5, 1, 2 or 4 x10^6 congenic *in vitro*-generated proT cells along with 1 x10^6 GFP^+ TDBM cells into aged mice, as illustrated in **Figure 2A**. On D14, we used flow cytometry to determine the contribution of host, GFP^+ TDBM and proT cells to thymus cellularity (**Figure 3**). All aged mice receiving proT cells showed the presence of donor-derived DP cells by D14. Mice injected with only TDBM (0 proT) did not show the presence of DPs, either host or TDBM derived, rather contained host CD4 or CD8 SP T cells and very few, if any, TDBM derived cells, which were nearly all at the DN stage. There was a positive correlation between engraftment efficiency and the number of proT cells injected (**Figure 3B**), with the provision of 4 x10^6 cells resulting in nearly 100% of thymic cellularity corresponding to proT-derived cells. This suggested that all the possible niches were likely occupied by the injection of 4 x10^6 proT cells, competing out host and GFP^+ TDBM donor cells to exclusively participate in early thymic engraftment.

ProT Cells Accelerate Thymus Engraftment in Both Young and Aged Mice

Having established the number of proT cells required to fully engraft the thymus in aged mice, we then compared proT cell engraftment and differentiation in aged vs. young mice. We analyzed host mice, young and aged, in 2-week intervals following the administration of 4 x10^6 proT cells and 1 x10^6 GFP^+ TDBM cells (**Figure 4A**). On D14, proT-derived cells constitute ~95% of thymocytes present in young mice and ~87% in aged mice (**Figure 4B**), with most cells (~90%) having reached the CD4/CD8 DP stage of differentiation in both young and aged thymuses. Of note, a major difference between young and aged host mice was the number of thymocytes after engraftment, with thymus of young mice having ~5-fold higher total cellularity

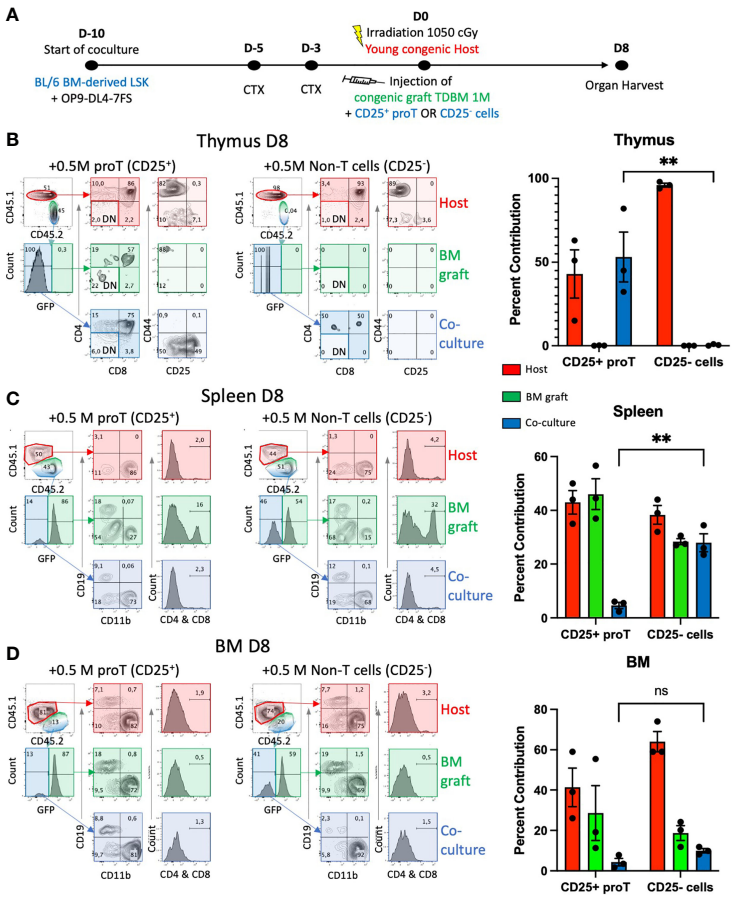

FIGURE 2 | Thymus engraftment by *in vitro*-generated proT cells. **(A)** Experimental schematic for generating proT cells in the HSC/OP9DL47FS system. BM-derived LSK cells from CD45.2 mice were obtained and co-cultured with OP9-DL4-7FS cells 10 days prior to injection. Young (8-12 wks) B6 CD45.1 mice were intraperitoneally injected with cyclophosphamide (CTX) 5 days and 3 days prior to injection and lethally irradiated at 1.05 Gy on the day of injection. All mice were intravenously injected with 1 x10^6 hematopoietic cells extracted from the BM of CD45.2 GFP$^+$ mice that were T cell-depleted (TDBM cells) and 5 x10^5 CD25$^+$ or CD25$^-$ culture-derived cells. On D8 post injection, graft and host contribution to the host **(B)** thymus, **(C)** spleen, **(D)** BM, were analyzed by flow cytometry. In **(B)**, CD44 vs. CD25 panels were gated on CD4- CD8- DN cells (lighter shade for DN-gated panels). For each organ, the contribution of host (red), GFP$^+$ BM graft (green) or coculture derived CD25$^+$ or CD25$^-$ cells (blue) were calculated and graphed. Significant difference between coculture-derived cells was noted (**p<0.01 analyzed by two-way ANOVA, error bars depict SEM), ns, not significant.

than aged mice at D14 (**Figure 4C**). In contrast, mice receiving only TDBM cells showed a delayed thymic engraftment. Importantly, at D14, TDBM mice had ~4-fold fewer thymocytes when compared to their age-matched mice that had also received proT cells, confirming that proT cells significantly enhance thymic reconstitution in both young and aged mice.

In vitro generated proT cells may represent a finite source of thymic seeding cells, after entering the thymus and differentiating into later stages of T cell development. However, whether proT cells could undergo limited self-renewal after thymic entry was addressed by establishing whether proT-derived short lived DP cells, as well as SP cells, could be seen at later time points. We noted an increase in the proportion of proT-derived SPs in both young and aged thymus by day 28 (**Figure 4A**). However, only the thymuses of young mice revealed the presence of a large percentage of proT-derived DP cells at D28. Of note, even as late as D42, proT-derived DP cells were still detected in the thymus of young mice, albeit coming from a much-reduced frequency of proT-derived cells.

By day 28, the next wave of TSPs derived from the GFP$^+$ TDBM cells becomes apparent. We observed a significant decline in the percentage of total thymocytes that were derived from proT cells in young mice, from 96% by D14 to an average of 36% by D28 and only 7% by D42 (**Figure 4B**). This was concomitant with a shift towards an increase in GFP$^+$ donor-derived cells within the thymus of young mice (**Figure 4B**). Additionally, total thymus cellularity in young mice, reached ~84 x10^6 cells by D42, which is similar to unmanipulated aged-matched mice (**Figure 4C**). Remarkably, we noted a dip in thymocyte cellularity at D28 in young mice receiving proT cells (**Figure 4C**), which though statistically not significant, it appears to correspond to the transition from proT-derived cells being the major contributor of thymic cellularity to GFP$^+$

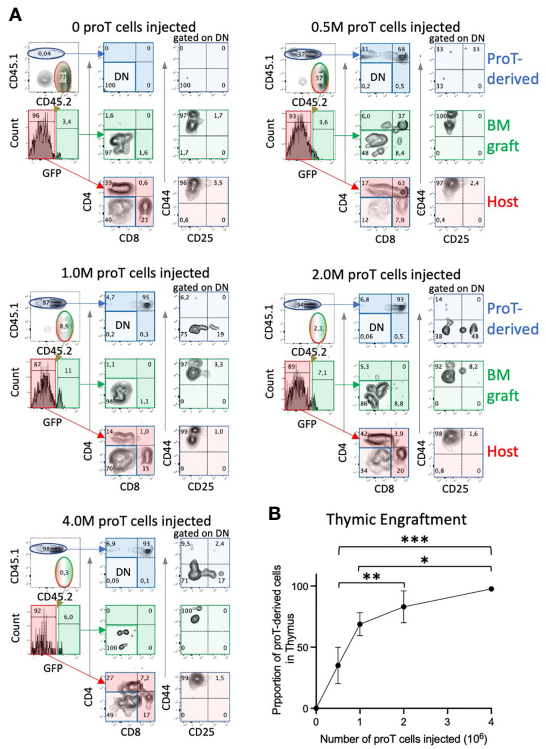

FIGURE 3 | Thymic engraftment of aged mice is dependent on proT cell dosage. Conditioned aged B6 CD45.2 mice were i.v. injected with 1 x 10^6 TDBM from CD45.2 GFP$^+$ mice and an increasing number of proT cells, *in vitro*-generated from CD45.1 mice, ranging from 0.5, 1, 2 and 4 x10^6. Thymuses were harvested on D14 and assessed for the presence of T cell markers using flow cytometry. Representative plots are shown in **(A)**. CD44 vs. CD25 panels were gated on CD4$^-$ CD8$^-$ DN cells. Percentage of thymic cells derived from CD45.1$^+$ proT cells are shown in graph in **(B)** (n=4; error bars depict SEM; *p<0.05, **p<0.01, ***p<0.001. analyzed by two-way ANOVA). ns, not significant.

donor-derived cells taking over, with over 90% derived from GFP$^+$ donor cells (**Figure 4B**).

In aged mice, the frequency of proT-derived thymocytes also significantly declined in percent contribution over time, as these cells are replaced by donor GFP$^+$ cells, but also by host cells as well; this latter occurrence was not as readily observed in young mice (**Figure 4**). On D28, both proT- and TDBM-injected aged mice host cells contributed to about 50% of the thymus cellularity, in contrast to less than 5% host contribution in young mice (**Figure 4B**). A similar trend was observed on D42, with the thymuses of aged mice showing a large fraction of host-derived cells irrespective of whether they received proT or TDBM GFP$^+$ donor cells.

Accelerated Peripheral T Cell Reconstitution in Aged ProT-Treated Mice

We next examined whether the rapid thymic engraftment observed in proT-injected mice had an effect on the appearance of peripheral T cells in the spleen. Mice receiving chemo/radio conditioning treatment showed altered immune cell subset distribution in their spleens. While there is variation amongst mouse strains, as well as effects of ageing on the cellular composition of spleen in control unmanipulated mice (29, 30), we observed on average 62% B cells, 25% T cells, 7% myeloid cells and 6% other cells in C57BL/6 spleen. In contrast, on D14 following conditioning, myeloid cells made up the majority of the splenocytes in young or aged mice, whether given proT cells or GFP$^+$ TDBM cells (**Figure 5A**). By D28, the proportion of B cells began to recover and approached normal levels, which were reached by D42, and by this time point the proportion of myeloid, B and T cells had returned to control levels.

Focusing on T cells, we noted that the frequency of T cells remain low at D14, both in young and aged mice (**Figure 5B**). By D28, a clear recovery in the percentage of T cells was observed in young mice that were given either proT or TDBM cells, with an increase in T cells to ~14% and ~10%, respectively. Aged mice given proT cells showed an equal recovery, with an increase in T cells to ~8%; whereas, aged mice given TDBM cells failed to recover the proportion of T cells, and remained at less than 2% (**Figure 5B**). This suggests that aged mice given TDBM remain T cell deficient for a significantly longer period when compared to proT-injected aged mice. Nevertheless, by D42 we observed the recovery of T cells in TDBM-injected aged mice.

In terms of the contribution of host, GFP$^+$ TDBM or proT-derived cells to splenic T cell population, we found that for proT-injected mice, whether young or aged, the majority of T cells present on D28 were derived from proT cells (**Figure 5C**). This trend continued up to D42, even as the frequency of GFP$^+$ TDBM- and host-derived T cells increased, but remained a minority. For young and aged mice given TDBM, there was a trend towards an increased contribution of GFP$^+$ donor cells over host cells at all time points. Taken together, these findings support the notion that aged mice given proT cells exhibit an earlier recovery of their T cell compartment.

DISCUSSION

One of the clinical shortcomings of HSCT is the extended period of time needed for the T cell compartment to reemerge, leading to increased susceptibility to infections and relapse. This is exacerbated in elderly patients (≥60 years old) that comprise the majority of individuals undergoing HSCT for leukemia (6, 31, 32). Adoptive transfer of proT cells into patients has been proposed as a viable future option to combat the prolonged paucity of T cells, since preclinical studies showed that proT cells, generated in culture from HSPCs, can engraft the thymus and accelerate recovery of functional T cells (24, 25). In this study, we addressed whether proT cells can function in a similar manner when given to aged mice, as a preclinical model for elderly patients receiving HSCT. To this end, we also included the administration of cyclophosphamide, a chemotherapy reagent, to better simulate conditioning regimens. Additionally, we expand the pool of proT cells to include all CD3$^-$CD4$^-$CD8$^-$ (DN) CD25$^+$ cells, based on the observations that both DN2 and DN3 can engraft the thymus (24).

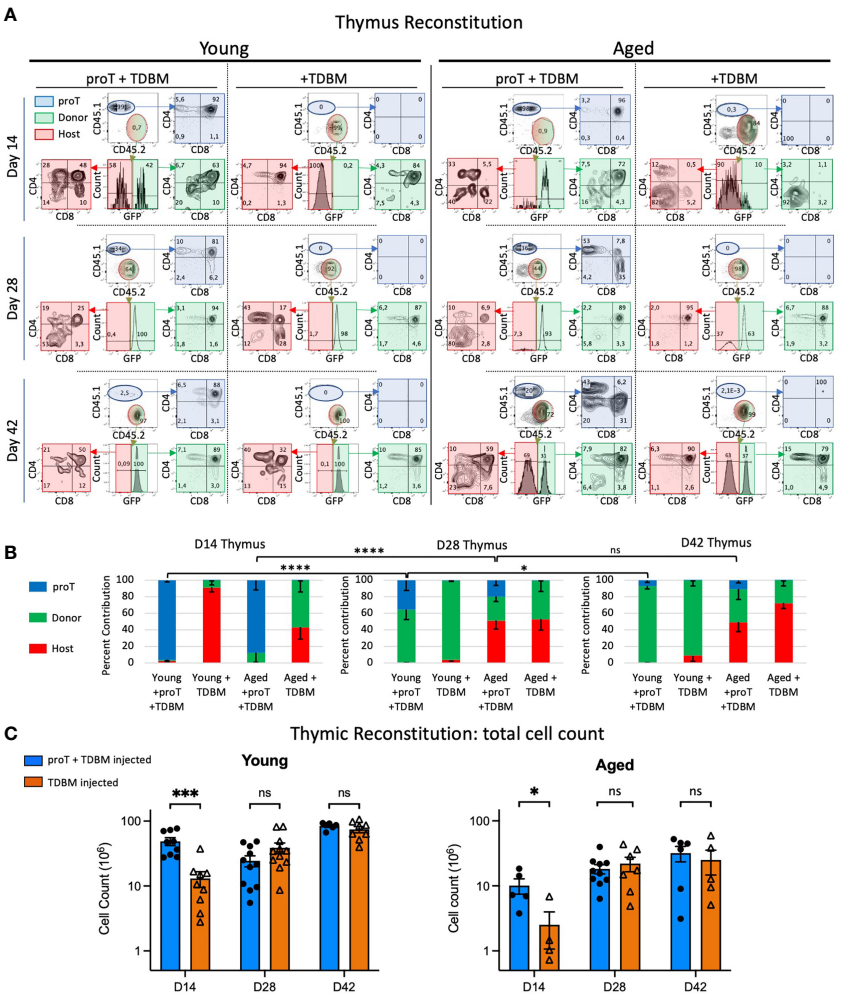

FIGURE 4 | Thymic engraftment of young and aged mice. Young (8-12wks) and aged (18-20 months) mice were treated with CTX followed by lethal irradiation in preparation for i.v. injection of either 4 x 10^6 proT cells derived from LSK/OP9-DL4 cocultures plus 1 x10^6 TDBM or 1 x10^6 TDBM alone as indicated. Thymus and other organs were harvested on days 14 (D14), D28 and D42. Thymocytes were labelled with appropriate lineage markers and analyzed by flow cytometry represented by plots shown in **(A)**. The thymocyte population was contributed by cells derived from the host (red), GFP$^+$ BM graft (green) and proT coculture (blue). The percent contribution of the source, whether host (CD45.2$^+$, GFP$^-$), BM graft-derived (CD45.2$^+$, GFP$^+$) or proT-derived (CD45.1$^+$) is shown in **(B)** for the three timepoints. Total cell number of thymocytes is depicted in **(C)** with young in the left panel and the aged in the right panel. For all samples at all time points, n≥4 up to 12. Error bars indicated SEM as indicated in *Methods*; significance was measured with two-way ANOVA. *p<0.05, ***p<0.001, ****p<0.0001.

One of the issues with performing irradiation as the sole approach in mouse models, is that it does not reflect the reality of most clinical modalities, where multiple regimens of chemo- and radio- treatments may be combined. There is also the issue of early auto-reconstitution of host thymocytes by the endogenous radioresistant DN2 subset, which reduces the availability of niches for thymic seeding cells (23). In our model and clinically, as radioresistant DN cells expand and differentiate to repopulate the thymus affected by chemotherapy, these cells are exposed to irradiation, leading to a reduction in the pool of radioresistant DN cells, and potentially less competition for incoming adoptively transferred proT cells. This notion is reflected in the reduction in the requirement of the number of proT cells, from 6-10 x10^6 (23, 25, 33) to 4 x10^6, to reach saturation of thymic niches, and with a concomitant increase in the average proportion of proT-derived thymocytes to ~90% within two weeks after adoptive transfer.

Consistent with previous results (12), engraftment of proT cells into the thymus of aged mice appeared to be very similar in efficiency as that seen in young mice, when comparing the number of incoming proT cells within 40 hours after adoptive transfer in absence of conditioning. We extended our analysis to young and aged mice receiving chemo/radio-conditioning and noted by day 14, the total cellularity of the thymus in young mice was 5-fold greater than that obtained in aged mice. This difference in cellularity is likely due to thymic involution, the natural age-related atrophy of the thymus, limiting the niches available for expansion (34). Our results confirmed the

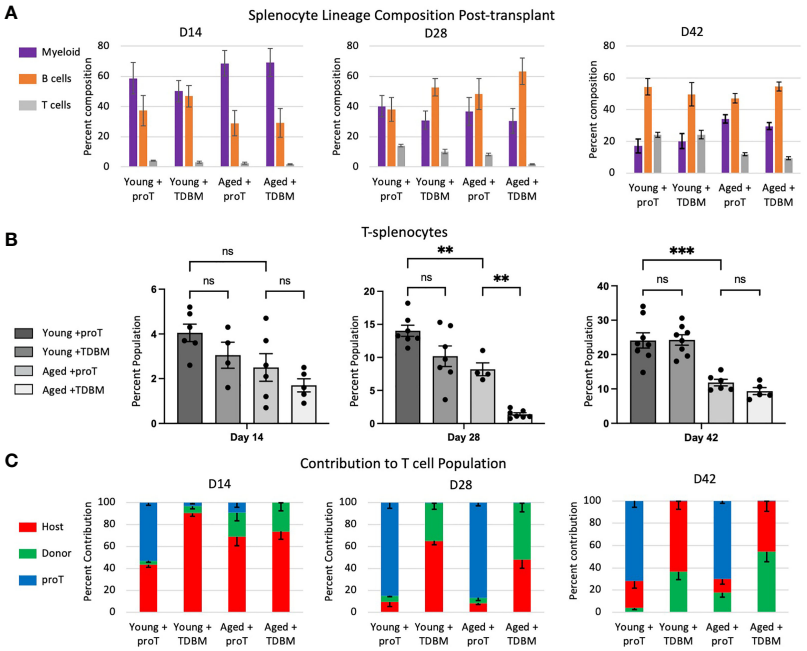

FIGURE 5 | Cellular composition of the spleen post proT adoptive transfer. Mice were treated as indicated in **Figure 4**. Spleens dissected from these mice were processed into single cells and their lineage composition of myeloid ($CD11b^+$), B cell ($CD19^+$) and T cells (gated first on $CD11b^-$ and $CD19^-$ population and then on $CD3^+$ or $CD4^+$ and $CD8^+$) was determined through flow cytometry **(A)**. In **(B)**, only the T cell percentage is shown for direct comparison. **(C)** We determined which origin T splenocytes are derived by flow cytometry and expression of $CD45.2^+$ GFP^- (Host, red), $CD45.2^+$ GFP^+ (Donor, green) and $CD45.1^+$ (proT, blue). Error bars indicated SEM. Statistical significance was determined using one-way ANOVA, $^{**}p < 0.01$, $^{***}p < 0.001$.

likelihood of limiting or poor-quality niches within the thymus of aged mice, affecting the proliferation of donor-derived thymocytes (12, 35).

In particular, we noted that, in the thymus of young mice, short-lived immature DP cells were still being generated from proT cell grafts even after one month since their adoptive transfer. Strikingly, this capacity of proT cells to give rise to DPs for such an extended period after transplant was not seen in the thymus of aged mice. These findings point to a major difference between the thymic microenvironment of young and aged mice, such that proT-derived cells were able to give rise to short-lived DPs for a much longer period of time within the young thymus than when seeding the thymus of aged mice, suggesting that their ability to undergo self-renewal was severely limited within the older thymic microenvironment. Understanding what these deficiencies are within the aged thymic niche will provide important insights as to how to improve T-lymphopoiesis in the elderly.

Despite the known age-associated decrease in thymic output (36, 37), we show here that the thymus of aged mice can export newly generated T cells to the periphery, as detected in the spleen by D28. Of importance, with respect to our modeling, we replicated the lag in the reemergence of peripheral T cells in aged mice receiving TDBM only, when compared to mice given both proT cells and TDBM cells, which showed an earlier appearance of donor-derived T cells in the periphery. We postulate that the aged mice with delayed T cell recovery would be more susceptible to infections, similar to what is seen in elderly patients receiving HSCT. While peripheral T cell reconstitution is accelerated by the provision of proT cells, it is not clear from our results whether earlier thymic engraftment by proT cells facilitated the subsequent wave of TSPs from the GFP^+ TDBM donor graft, as we had postulated earlier (27). As such, for both the young and aged thymus, the initial delay in T cell cellularity seen at D14 when given only TDBM cells, is replaced by an equivalent or greater cellularity by D28 and beyond. This suggests that a delay in thymic crosstalk does not appear to significantly alter the recruitment capacity of the recovering thymus, despite the damage incurred by TECs due to the conditioning regimen.

Apart from lower cellularity, the thymuses of aged mice are quite distinct in their composition from their young counterparts, in that they showed a much larger contribution of host-derived cells. This may be due to the incomplete replacement of host-derived cells by GFP^+ TDBM donor cells within the BM of aged mice, unlike their young counterparts. The ability of host cells to compete against the GFP^+ graft and repopulate the BM conflicts with current literature showing many instances of aging cells being more susceptible to radiation exposure (38). One explanation could be that host cells in aged mice are being protected from the effects of radiation simply due to their higher body weight, as aged mice typically weighed twice as much as their young counterpart. While we set the CTX dose according to weight, a similar

increase in radiation dose according to weight may have led to irreparable cellular damage. Nevertheless, mouse weight and size, as well as other metabolic differences, may add to the complexity in comparing the effects of conditioning in aged and young mice.

Future clinical applications of our findings will rely on the recent replacement of the xenogenic OP9-DL cells with a serum-free, cell-free system of plate-bound Dll4 or DL4-μbeads methods for the generation of human proT cells (39–41), which have increased the potential therapeutic use of proT cells. In addition, our findings strongly suggest that proTs could provide an immune boost to the elderly, the population that comprises the majority of patients undergoing HSCT. The next hurdles remaining before the therapeutic use of proT cells appear surmountable, though wide-ranging. Focusing on the preclinical side, it is paramount to demonstrate that proT-derived mature T cells can confer immunity against diseases in aged mice. Further, there is the need to address the standard practices of care in hospitals during HSCT and use them as a guide for our preclinical modelling. This includes details such as administration of both chemo- and radiation treatments, which we have done here, but also including anti-thymoglobulin (ATG) following HSCT (42) or the use of allogeneic grafts instead of congenic HSPCs. ATG treatment, for example, is given to curtail host vs graft rejection and other complications of HSCT, a practice that would likely counter proT cell therapy, as these cells would also be targeted by ATG. Thus, it requires further consideration before embarking on clinical trials.

In short, here we have improved upon the conditioning regimen and discovered that providing proT cells allows for the effective reconstitution of the aged mouse thymus with accelerated T cell regeneration. The favorable consequences afforded by rapid thymic reconstitution includes the appearance of mature T cells in secondary immune organs, providing a potential advantageous immune boost to aged recipients.

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 Health Sciences Centre Animal Care Committee.

AUTHOR CONTRIBUTIONS

MM, YRL and CL performed all the experiments. MM and YRL designed and analyzed the data, and wrote the manuscript. JCZ-P. conceived the project, analyzed the data, and wrote and edited 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.2022.926773/full#supplementary-material

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