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Revisiting the thymus: The origin of T cells

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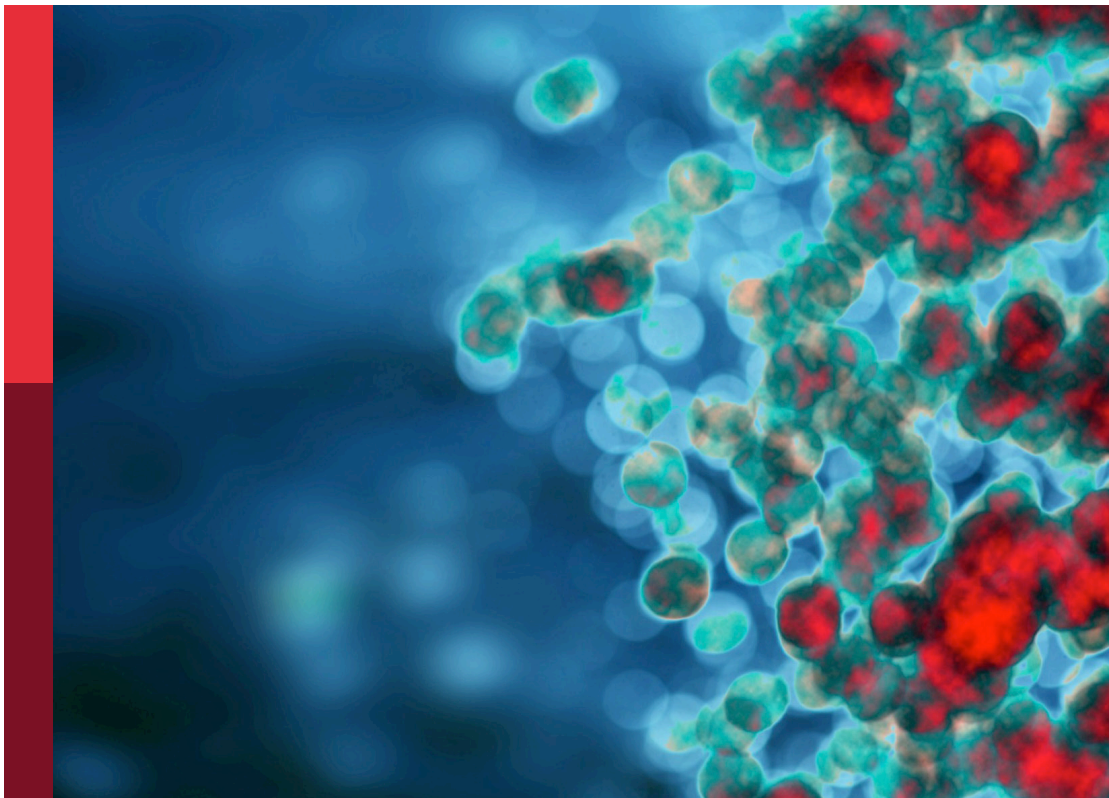

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Revisiting the thymus: The origin of T cells

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Editorial: Revisiting the thymus: the origin of T cells

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T cell development, thymus, thymocyte, T cell differentiation, T cell signaling

Editorial on the Research Topic
Revisiting the thymus: the origin of T cells

T cells are crucial components of adaptive immunity, playing an indispensable role in protecting against external pathogenic agents and maintaining immunological homeostasis. T cell development in the thymus involves a series of highly structured procedures, including lineage commitment, T cell receptor (TCR) rearrangement, positive and negative selection, and other checkpoints (1). Disruption in this process can lead to T cell dysfunction, potentially leading to severe immunodeficiency or autoimmune diseases. Numerous studies have demonstrated that T cell development is a complex process, regulated by multiple factors and intricate signaling pathway networks (1, 2). Despite significant advancements in this field, unraveling the underlying mechanisms for T cell development remains a formidable challenge. Therefore, the purpose of this Research Topic was to collect evidence for a deeper understanding of underlying mechanisms involved in thymocyte development as this will not only expand our knowledge of cell fate determination through gene regulatory networks but also could pave the way for potential immunotherapeutic strategies.

The thymus provides a microenvironment to support T cell development, including stromal cells, primarily thymic epithelial cells (TECs), and other immune cells. Although TECs have been extensively studied for their role in T cell selection, the contribution of other immune cells such as macrophages and dendritic cells (DCs), to T cell development remains to be elucidated. Wang et al. provided a systemic review of the heterogeneity of DC and macrophage subpopulations in the thymus and their potential roles in T cell selection and maintenance of thymic homeostasis. Original research by Hou and Yuki showed that CD11c, a surface marker of DCs, is important for maintaining T cell survival as *Itgax* (encodes CD11c) deficiency leads to increased apoptosis of DP, CD4SP, and CD8DP T cells. These findings suggest that thymic DCs may contribute to supporting T cell survival through CD11c.

T cell development is regulated by multiple levels, including gene mutation, transcriptional regulation, epigenetic modification, and post-translational modifications. Gene mutations can affect T cell development, especially some key mutations that can lead to severe combined immunodeficiency (SCID). Sertori et al. explored a causal etiology in a

patient with T-B+NK+ immunodeficiency and found the MED14^{V763A} variant that may be responsible for the disease. However, this deficiency can be observed in neither male siblings of the proband nor mouse models carrying the same variant. Therefore, one possibility is that the MED14^{V763A} variant may contribute to the disease, but the degree and direction of this effect may be modulated by specific modifier genes that interact with MED14 to enhance or suppress its expression or function. Another possibility is that the disease may result from another unknown noncoding mutation. Transcription factors play a critical role in T cell development by regulating the expression of target genes involved in cell differentiation, proliferation, survival, and maturation. Bao et al. provided a comprehensive review of key transcription factors and epigenetic regulatory factors in each stage of T cell development, including both classic transcription factors such as T-cell-specific transcription factor (TCF-1) and Runx transcription factor family, and some newly reported transcription factors such as Zinc finger protein Yin Yang 1 (YY1). However, the roles of newly discovered transcription factors are less clear. Ji et al. demonstrated the importance of serine/arginine-rich splicing factor 1 (SRSF1) in T cell development by regulating the expression of the key transcription factor Runx3. Specifically, they show that the selective knockout of SRSF1 in the DP stage (SRSF1$^{fl/fl}$CD4^{cre}) results in decreased CD8SP cell numbers and maturation defects, which can be partially rescued by the overexpression of Runx3. However, the exact mechanism by which SPSF1 regulates the expression of Runx3 requires further investigation. In addition, as an important form of post-translational modification, protein ubiquitination regulates various biological processes, including T cell development. Zhong et al. reviewed the molecular mechanisms and cellular pathways that regulate thymocyte ubiquitination and focused on the roles of E3 ligases and deubiquitinating enzymes (DUBs).

Previous studies primarily focus on transcriptional regulation and post-translational modification. However, recent research highlights the importance of cell metabolism and metabolic reprogramming during T cell development in adapting to specific functional requirements. Zhang et al. provided an overview of the metabolic changes that occur during T cell development and summarized key metabolic regulators and regulation mechanisms. Furthermore, mitochondrial oxidative phosphorylation (OXPHOS) is central to cell metabolism and regulates various critical cellular processes, including proliferation, apoptosis, and differentiation by supplying energy and metabolites. The original research from Limper et al. reported that maintaining a high-fidelity replication of mtDNA is crucial for T cell development. They found that accumulated mtDNA mutations can impede proliferation during the DN stage and decrease mitochondrial density. This may be attributed to the dependence on mitochondria to provide energy and substrates during the highly proliferative DN3 stage and mitochondrial function is impaired by accumulated mtDNA mutations.

The interaction between major histocompatibility complex (MHC) and TCR is a critical link in T cell development. TCR signaling strength contributes to the determination of the fate of thymocytes. The E protein is one of the key transcription factors involved in T cell development and is negatively regulated by the Id protein, but the specific regulatory mechanism remains unclear. Anderson proposed the "Clutch" model to describe this process and discussed how Id3 participates in the development of T cells by regulating the activity of the E protein. They proposed that Id3 changes the accessibility of E protein target genes and guides T cells into different developmental pathways. During T cell development, the activity of Id3 is mainly regulated by the strength of the TCR signal. For example, pre-TCR transmits weaker TCR signals, which reduces the activity of Id3 and promotes the development of αβT cells. In contrast, γδ-TCR transmits stronger TCR signals and guides thymocytes to develop into γδT cells. Besides, CD4/CD8 lineage fate determination is also regulated by TCR signals. Stronger TCR signaling favors CD4 T cell development, whereas weaker TCR signaling favors CD8 T cell development.

It is widely accepted that MHC restriction is the key to T cell development, leading to the selection of functional and self-tolerant T cells. However, recent studies have shown that MHC restriction may not always be necessary for αβT cell development. Van Laethem et al. revealed the mechanism of MHC-independent αβTCR selection and the potential non-MHC ligands that may be involved in this process. DP thymocytes require TCR signals to maintain their survival after TCR rearrangement, but the initiation of αβTCR signaling relies on Lck tyrosine kinase, which binds to the coreceptors CD4 and CD8. Lck is typically sequestered by the coreceptors and TCRs can recruit Lck and initiate downstream signaling only when they bind to MHC-antigen peptide complexes corresponding to the coreceptors. However, without coreceptors sequestered, TCRs can recognize non-MHC ligands, such as CD155, CD102, and CD48, and complete signal transduction through free Lck to guide the development and maturation of MHC-independent αβT cells. However, it is worth noting that free Lck is not capable of transmitting high-affinity TCR signals effectively; therefore, it may not effectively clear self-reactive T cells.

In addition to the factors discussed earlier, sex steroids are found to play a role in T cell development as well. However, the direct effects of sex steroids on thymocytes and underlying mechanisms remain unclear. Taves and Ashwell concluded on the expression of sex steroid receptors on thymic cells and TECs and the mechanisms by which sex steroids regulate T cell development. In this review, they emphasized the suggestive finding of sex steroid production within the thymus itself.

The thymus not only contributes to central immune tolerance but also plays a critical role in establishing immune tolerance by generating immunosuppressive T cell subsets that migrate to the periphery. Regulatory T cells (Tregs) are a critical subset of these T cells that play a crucial role in maintaining immune homeostasis by suppressing effector T cells. While Tregs can differentiate from CD4 + T cells in the periphery, most Tregs are generated directly in the thymus, but their development is not completely understood. While TCR/CD28 co-stimulation and cytokine IL-2 are thought to be involved, a recent review by Tang et al. discussed the role of cytokines in thymic Treg (tTreg) development. The development

of TCRαβ+CD8αα+ and TCRγδ+CD8αα+ intestinal intraepithelial lymphocytes (IELs), which play a role in the intestinal immune barrier and immune regulation, also originates in the thymus. Gui et al. concluded that the pivotal molecules and their functions are involved in the development process of these two specific IEL subsets.

In summary, these findings underscore the intricate and dynamic nature of T cell development, emphasizing the importance of a comprehensive understanding of the mechanisms governing this process.

Author contributions

XL wrote the first draft of the present Editorial article. YG, YD and BZ revised it and provided their valuable and precious comments and suggestions. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

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ORIGINAL RESEARCH
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SRSF1 Deficiency Impairs the Late Thymocyte Maturation and the CD8 Single-Positive Lineage Fate Decision

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The underlying mechanisms of thymocyte development and lineage determination remain incompletely understood, and the emerging evidences demonstrated that RNA binding proteins (RBPs) are deeply involved in governing T cell fate in thymus. Serine/arginine-rich splicing factor 1 (SRSF1), as a classical splicing factor, is a pivotal RBP for gene expression in various biological processes. Our recent study demonstrated that SRSF1 plays essential roles in the development of late thymocytes by modulating the T cell regulatory gene networks post-transcriptionally, which are critical in response to type I interferon signaling for supporting thymocyte maturation. Here, we report SRSF1 also contributes to the determination of the $CD8^+$ T cell fate. By specific ablation of SRSF1 in $CD4^+CD8^+$ double positive (DP) thymocytes, we found that SRSF1 deficiency impaired the maturation of late thymocytes and diminished the output of both $CD4^+$ and $CD8^+$ single positive T cells. Interestingly, the ratio of mature $CD4^+$ to $CD8^+$ cells was notably altered and more severe defects were exhibited in $CD8^+$ lineage than those in $CD4^+$ lineage, reflecting the specific function of SRSF1 in $CD8^+$ T cell fate decision. Mechanistically, SRSF1-deficient cells downregulate their expression of *Runx3*, which is a crucial transcriptional regulator in sustaining $CD8^+$ single positive (SP) thymocyte development and lineage choice. Moreover, forced expression of Runx3 partially rectified the defects in SRSF1-deficient $CD8^+$ thymocyte maturation. Thus, our data uncovered the previous unknown role of SRSF1 in establishment of $CD8^+$ cell identity.

Keywords: SRSF1, thymocyte, lineage choice, CD8+ T cell, Runx3, development

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INTRODUCTION

T cell development occurs in the thymus and consists of several ordered processes, such as T cell lineage commitment, T cell receptor (TCR) rearrangements, expression of diverse TCR repertoire, positive and negative selection, and the terminal maturation for acquisition of their functions as helper, cytotoxic or regulatory T cells (1–4). A lymphoid precursor developing into a mature αβT cell undergoes three major sequential phases defined by the CD4 and CD8 expression, including $CD4^-CD8^-$ double negative (DN), $CD4^+CD8^+$ double positive (DP), and either $CD4^+CD8^-$ or $CD4^-CD8^+$ single positive (SP) stages (2, 5). The dynamic expression of cell surface markers which are related to functional alterations is essential to delineate the stages of thymocyte development (6). For instance, the thymocytes are stratified into distinct developmental stages defined by the expression of TCRβ (or CD3e) and the activation marker CD69, representing preselection ($TCR\beta^{lo}CD69^{lo}$), initial stage of selection ($TCR\beta^{int}CD69^{lo}$), undergoing selection ($TCR\beta^{int}CD69^{hi}$), post selected immature ($TCR\beta^{hi}CD69^{hi}$), and post selected mature ($TCR\beta^{hi}CD69^{lo}$) thymocytes, respectively (7–9). In addition, SP thymocytes are also a heterogeneous population which gradually proceed to downregulate heat-stable antigen (HSA, CD24) and upregulate Qa2 before entry into the periphery T cell pool (6, 10). Hence, the post selected $TCR\beta^{hi}$ thymocytes can be further compartmentalized by the dynamic expression level of CD69, CD24, CD4 and CD8 on their cell surface, reflecting the heterogeneity and defining the developmental stages of late thymocytes (11, 12).

DP thymocytes first express the mature αβTCR complex which allows the engagement by intrathymic peptide major histocompatibility complex (MHC) ligands and interact with stromal cells that are localized in the cortex for positive and negative selection (13). After positive selection, DP cells expressing MHC class I- or MHC class II-TCRs selectively differentiate into either conventional $CD4^+$ helper or $CD8^+$ cytotoxic T cells, which is a critical developmental event known as the CD4/CD8 lineage choice. Based on the theory of the kinetic signaling model, most of positively selected DP thymocytes must pass through an intermediate $CD4^+CD8^{lo}$ stage and both duration and intensity of TCR signaling exert essential impact on cell fate decision (14). To comprehend the underlying intracellular mechanisms involved in the CD4/CD8 lineage commitment, a few transcription factors have been identified, such as Thpok, Runx3, Mazr, Myb, Bcl11b, Gata3, Tox, Tcf1/Lef1, and Tle factors (11, 12, 15–21). Among them, Thpok and Runx3 are critical for specification of $CD4^+$ helper or $CD8^+$ cytotoxic cells, respectively, and play central roles in controlling CD4/CD8 lineage choice (22). To date, a complete understanding of the process awaits elucidation of the precise mechanisms involved in the extensive regulatory network.

The RNA-binding protein serine/arginine splicing factor 1 (SRSF1, also named ASF/SF2) belongs to the highly conserved SR protein family which functions as a key regulator in most cell types *via* mediating mRNA metabolism, such as constitutive and alternative splicing, RNA polymerase II transcription, nuclear export of mature mRNA and translation, and genomic stability (23–27). Our recent studies have demonstrated that SRSF1 not only plays a critical role in the late stage development of conventional T cells by controlling the expression of *Il27ra* and *Irf7* transcripts (28), but also serves as an important post-transcriptional regulator in promoting the development and functional differentiation of iNKT cell *via* balancing the abundances of two transcriptional isoforms of Myb (29). These findings suggest that SRSF1 is profoundly involved in the development and function of both conventional and unconventional T cells.

In this study, we employed $Srsf1^{fl/fl}$*Cd4*-Cre mice to investigate the potential role of SRSF1 in determination of CD4/CD8 lineage fate by specific ablation of SRSF1 in DP thymocytes. The ratio of mature $CD4^+$ to $CD8^+$ cells was notably altered and more severe defects were exhibited in $CD8^+$ lineage, albeit the maturation of both $CD4^+$ and $CD8^+$ SP T cell was impaired in SRSF1-deficient mice, suggesting the specific function of SRSF1 in $CD8^+$ T cell fate decision. Moreover, SRSF1-deficient cells exhibit the reduced abundance of *Runx3* and forced expression of Runx3 partially rectifies the defects in $CD8^+$ lineage proportion.

RESULTS

Conditional Ablation of SRSF1 at DP Stage Impairs the Maturation of Late Thymocytes

Our recent study has shown that SRSF1 regulates the terminal maturation of thymocytes by post-transcriptionally regulating the abundances of *Il27ra* and *Irf7* functional transcripts *via* alternative splicing (28). By reviewing the phenotype of thymocytes from $Srsf1^{fl/fl}Lck^{Cre/+}$ mice, we found that the numbers of CD8 single-positive (SP) cells are more severe reduction than those of $CD4^+$ SP cells, resulting in the substantially altered ratio of $CD4^+$ to $CD8^+$ cells (**Figures S1A–C**). In addition, we performed gene set enrichment analysis (GSEA) by using our published RNA-seq data (GSE141349). The results indicated that $CD8^+$ cell-specific genes were enriched in wild-type DP cells relative to SRSF1-deficient DP cells, suggesting that the differentiation capacity of DP cell toward $CD8^+$ SP was more significantly reduced in absence of SRSF1, although both $CD4^+$ and $CD8^+$ SP thymocyte-related genes exhibited the enrichment in wild-type DP cells (**Figure S1D**). To address the potential role of SRSF1 involved in the lineage choice of CD4-versus-CD8 thymocytes, we established the genetic mouse model with conditional inactivation of SRSF1 in DP stage by crossing $Srsf1^{fl/fl}$ mice with *Cd4*-Cre mice (30), which is widely applied for the lineage determination analysis of late thymocytes (**Figure S2A**). The deletion efficiency of SRSF1 was further confirmed in district subsets along with the sequential developmental phases, indicating the effective deletion of *Srsf1* was achieved in DP and CD4/CD8 SP thymocytes from $Srsf1^{fl/fl}$*Cd4*-Cre mice compared with those in their littermate control mice (henceforth called Control) (**Figure S2B**).

We next analyzed the phenotype of these conditional knock out mice. Compared with their controls, $Srsf1^{fl/fl}$*Cd4*-Cre mice exhibited comparable size and cellularity of thymus and spleen, but diminished cell number in lymph nodes (**Figures 1A, B**).

The frequency of both $CD4^+$ and $CD8^+$ thymocytes from SRSF1-deficient mice was significantly decreased (**Figures 1C, D**), whereas the percentage of DP thymocytes was correspondingly increased, reflecting a blockade of DP thymocyte development. The cell numbers of $CD8^+$ thymocytes in SRSF1-deficient mice were significantly reduced, but no statistical difference in absolute numbers of DP and $CD4^+$ thymocytes was observed. The ratio of $CD4^+$ cells to $CD8^+$ cells was notably altered (**Figure 1E**), implying more severe impacts on $CD8^+$ lineage development caused by conditional *Srsf1* deletion in DP thymocytes. To determine the specific developmental stage of thymocytes that was impaired in $Srsf1^{fl/fl}Cd4$-Cre mice, we carved up thymocytes at five distinct developmental phases defined by the expression of TCRβ and the activation marker CD69 as previous described (8, 9, 31) (**Figure 1F**). There was no significant difference observed from populations 1 to 3 between $Srsf1^{fl/fl}Cd4$-Cre mice and their controls, implying the DP thymocytes at pre-selection and the initial stage of positive selection were not affected in absence of SRSF1 (**Figures 1F, G**). In contrast, $Srsf1^{fl/fl}Cd4$-Cre mice had significantly fewer cells in populations 4 to 5 which include post-selected DP, immature SP, and mature SP thymocytes, respectively. These results indicate that ablation of SRSF1 at DP thymocytes mainly impairs the T cell development beyond the post-selection phase.

SRSF1 Deficiency Alters the Ratio of $CD4^+$ to $CD8^+$ Cells in $TCR\beta^{hi}$ Thymocytes

We next focused on the post-selection $TCR\beta^{hi}$ thymocytes with an additional maturation marker CD24 staining combined with the activation marker CD69 of thymocytes as previously described (28). The frequency and cell numbers of $TCR\beta^{hi}CD69^-CD24^-$ mature subset were decreased from $Srsf1^{fl/fl}Cd4$-Cre mice compared with those from Controls (**Figures 2A, B**). The frequency of $TCR\beta^{hi}CD69^+CD24^+$ immature T cell exhibited a relative increase, but the numbers were slightly diminished (**Figures 2A, B**). By further analysis of the expression of CD4 and CD8 in $TCR\beta^{hi}CD69^+CD24^+$ immature subsets, we found that the frequency and numbers

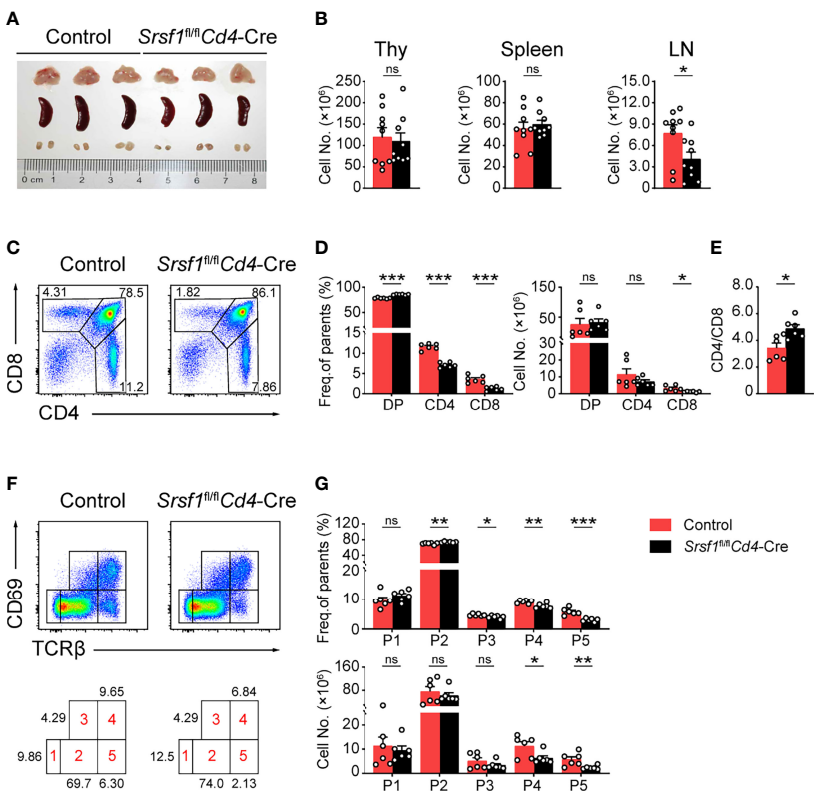

FIGURE 1 | $Srsf1^{fl/fl}Cd4$-Cre mice exhibits defects in the maturation of late thymocytes. **(A)** Images of Thymus (Thy), spleen, and lymph nodes (LNs) from Control and $Srsf1^{fl/fl}Cd4$-Cre mice were shown (n = 3 per group). **(B)** Total cell numbers of Thy, spleen, and LNs from Control and $Srsf1^{fl/fl}Cd4$-Cre mice were shown (n = 9). **(C–E)** Flow cytometry analysis of $CD4^+$, $CD8^+$, and $CD4^+CD8^+$ double positive (DP) thymocytes. Representative pseudocolor plots show the indicated populations in Control and $Srsf1^{fl/fl}Cd4$-Cre mice in **(C)**, and the frequency and numbers of indicated populations were shown in **(D)**, accordingly. The ratio of frequency between $CD4^+$, and $CD8^+$ thymocytes was calculated and shown in **(E)** (n = 6). **(F, G)** Flow cytometry analysis of the sequentially developmental stages. **(F)** Representative pseudocolor plots show five subsets, including population 1 (P1: $TCR\beta^{lo}CD69^{lo}$), population 2 (P2: $TCR\beta^{int}CD69^{lo}$), population 3 (P3: $TCR\beta^{int}CD69^{hi}$), population 4 (P4: $TCR\beta^{hi}CD69^{hi}$), and population 5 (P5: $TCR\beta^{hi}CD69^{lo}$) in Control and $Srsf1^{fl/fl}Cd4$-Cre mice. The frequency and numbers of indicated subsets were shown in **(G)** (n = 6). Data were collected from at least three independent experiments. The error bars are means ± standard deviation (SD). Statistical significance was determined by one-tailed Student's *t*-test. ns, not statistically significant; $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$.

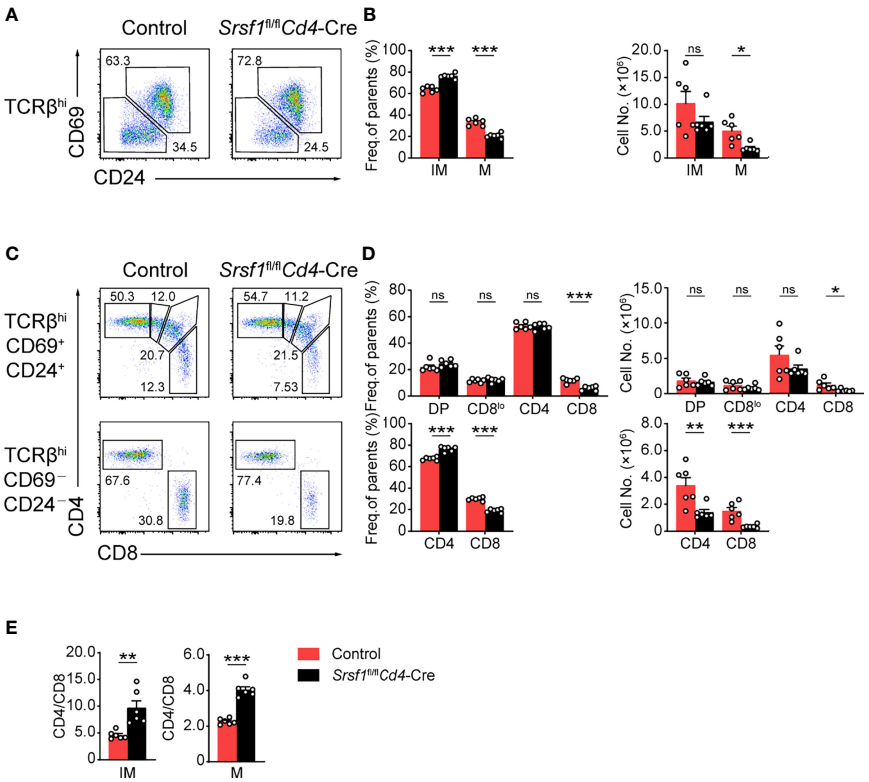

FIGURE 2 | Ablation of SRSF1 severely hinders the maturation of CD8 single-positive thymocytes. **(A–D)** Characterization of the post-selection thymocytes. **(A)** TCRβ^{hi} thymocytes (populations 4 and 5 in **Figure 1F**) were further fractionated into CD69^{+}CD24^{+} immature (IM) and CD69^{-}CD24^{-} mature (M) subsets. The immature subsets were subdivided into CD4^{+}, CD4^{+}CD8^{lo} (CD8^{lo}), DP, and CD8^{+} sub-populations (clockwise from top left in the top row), and the mature subsets were further subdivided into CD4^{+} and CD8^{+} populations (bottom row) **(C)**. The frequency and numbers of indicated subsets were shown in **(B, D)**, respectively. **(E)** The ratio of CD4^{+} to CD8^{+} thymocytes was calculated and shown (n = 6). Data were collected from at least three independent experiments. The error bars are means ± SD. Statistical significance was determined by one-tailed Student's *t*-test. ns, not statistically significant; *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$.

of DP, CD4^{+}CD8^{lo} intermediate cells, and CD4^{+} SP subsets were not significantly alerted, but the frequency and numbers of CD8^{+} SP were remarkably decreased in *Srsf1*$^{fl/fl}$*Cd4*-Cre mice (**Figures 2C, D**). In SRSF1-deficient TCRβ^{hi}CD69^{-}CD24^{-} mature population, the numbers of CD4^{+} and CD8^{+} SP were dramatically diminished, though the frequency of CD4^{+} SP cells was increased whereas the frequency of CD8^{+} SP cells was reduced (**Figures 2C, D**). Moreover, the ratio of CD4^{+} to CD8^{+} SP cells was notably increased in both TCRβ^{hi}CD69^{+}CD24^{+} immature and TCRβ^{hi}CD69^{-}CD24^{-} mature thymocytes from *Srsf1*$^{fl/fl}$*Cd4*-Cre mice (**Figure 2E**). Collectively, these data indicated that SRSF1 deficiency impaired the terminal maturation of both CD4^{+} and CD8^{+} SP cells, and led to the aberrant ratio of CD4^{+} to CD8^{+} SP cells.

SRSF1 Deficiency Disturbs the Proportion of CD4^{+} to CD8^{+} Cells in Periphery T Cell Pool

We next checked whether the peripheral T cell pool was affected in *Srsf1*$^{fl/fl}$*Cd4*-Cre mice. The mature CD4^{+} and CD8^{+} T cell populations in spleens, LNs and PBCs were remarkably diminished in *Srsf1*$^{fl/fl}$*Cd4*-Cre mice (**Figures 3A, B**). By further analysis of the proportion of CD4^{+} to CD8^{+} cells in peripheral tissues, we found the frequency of CD4^{+} T cells was increased in SRSF1-deficient TCRβ^{+} cells, and the ratio of CD4/CD8 in peripheral tissues was increased, accordingly (**Figures 3C–E**). These results suggested the critical requirement of SRSF1 in maintaining the numbers of mature T cells, especially CD8^{+} cells in periphery T cell pool.

SRSF1 Regulates the Maturation of Late Thymocytes in a Cell-Intrinsic Manner

To determine whether the developmental defects in *Srsf1*$^{fl/fl}$*Cd4*-Cre were T cell autonomous, we generated bone marrow chimeric mice as described in **Figure 4A**. We found thymocytes derived from *Srsf1*$^{fl/fl}$*Cd4*-Cre mice had a phenotype identical to that of thymocytes in primary SRSF1-deficient mice as described above (**Figures 4B–H**). The severe defects were detected in population 4 and 5 of thymocytes derived from *Srsf1*$^{fl/fl}$*Cd4*-Cre mice (**Figures 4B, C**), and the frequency of TCRβ^{hi}CD69^{-}CD24^{-} mature population was substantially reduced (**Figures 4D, E**). In chimeric mice transplanted with *Srsf1*$^{fl/fl}$*Cd4*-Cre donor cells, the frequency of donor-derived CD8^{+} SP cells was remarkably reduced in both

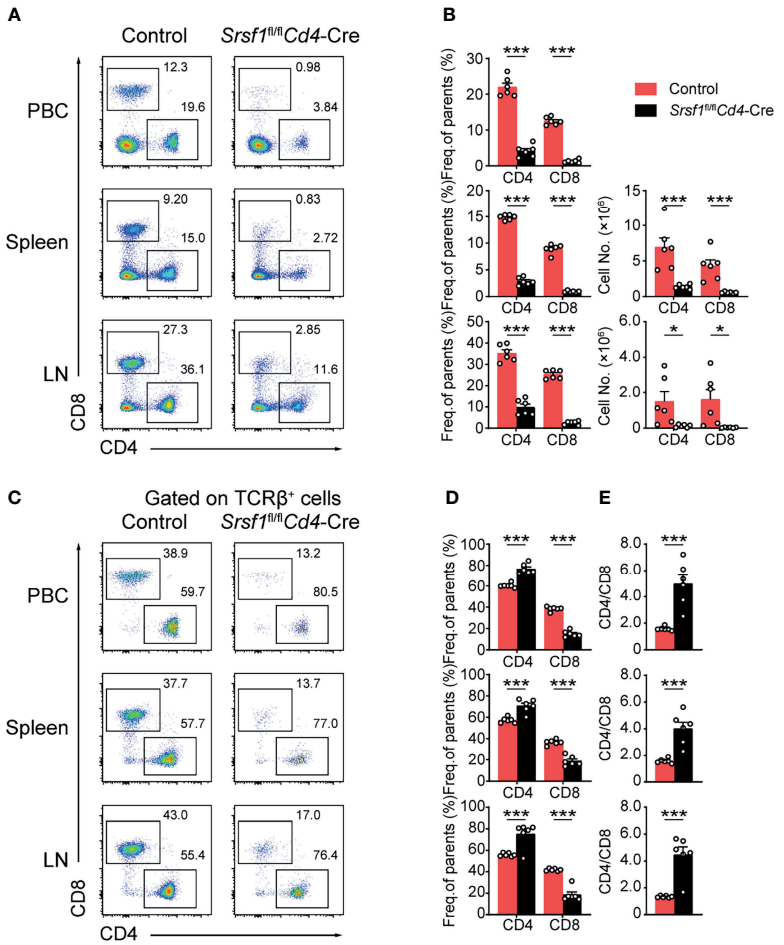

FIGURE 3 | The proportion of peripheral CD4$^+$ to CD8$^+$ T cells was disturbed in *Srsf1*$^{fl/fl}$*Cd4*-Cre mice. **(A, B)** Flow cytometry analysis of T cells in peripheral tissues. **(A)** Representative pseudocolor plots show CD4$^+$ and CD8$^+$ T cells in PBC, spleen, and LNs from Control and *Srsf1*$^{fl/fl}$*Cd4*-Cre mice. The frequency and numbers of indicated subsets in spleen, and LNs were shown in **(B)**, accordingly (n = 6). **(C–E)** Analysis of the ratio of frequency between peripheral CD4$^+$ T cells and CD8$^+$ T cells. **(C)** Representative pseudocolor plots show CD4$^+$ and CD8$^+$ T cells from TCRβ$^+$ populations in PBCs, spleen, and LNs. The frequency and numbers of indicated subsets were shown in **(D)** (n = 6), and the ratio of frequency between CD4$^+$ T cells to CD8$^+$ T cells was calculated and shown in **(E)**, respectively. Data were collected from at least three independent experiments. The error bars are means ± SD. Statistical significance was determined by one-tailed Student's t-test. $^*P < 0.05$ and $^{***}P < 0.001$.

TCRβ^{hi}CD69$^+$CD24$^+$ immature and TCRβ^{hi}CD69$^-$CD24$^-$ mature thymocytes, and the ratio of CD4$^+$ to CD8$^+$ SP cells was notably increased, accordingly (**Figures 4F–H**). These data thus demonstrated the impacts on maturation of late thymocytes and CD8 lineage fate were T cell intrinsic.

SRSF1 Contributes to the Lineage Determination of CD4-Versus-CD8 Thymocytes

To further evaluate how SRSF1 contributes to CD8$^+$ lineage choice, we crossed *Srsf1*$^{fl/fl}$*Cd4*-Cre mice with MHC class II-deficient (*H2ab1*$^{-/-}$) mice, which lack mature CD4$^+$ SP thymocytes (**Figure 5A**). We found the frequency of CD8$^+$ SP cells in both immature and mature thymocytes from *H2ab1*$^{-/-}$ *Srsf1*$^{fl/fl}$*Cd4*-Cre mice was substantially lower compared with those in their control mice (**Figure 5B**). The frequency of CD4$^+$ SP cells in mature thymocytes from *H2ab1*$^{-/-}$*Srsf1*$^{fl/fl}$*Cd4*-Cre mice was significantly higher than those from their control mice (**Figure 5B**). The number of both mature and immature CD8$^+$ SP cells was dramatically lower in *H2ab1*$^{-/-}$*Srsf1*$^{fl/fl}$*Cd4*-Cre mice, accordingly (**Figure 5C**). In contrast, the number of immature CD4$^+$ SP cells was comparable from *H2ab1*$^{-/-}$*Srsf1*$^{fl/fl}$*Cd4*-Cre and Control mice, whereas the number of mature CD4$^+$ SP cells was diminished in *H2ab1*$^{-/-}$*Srsf1*$^{fl/fl}$*Cd4*-Cre due to SRSF1 deficiency (**Figure 5C**). These data collectively indicated that SRSF1 deficiency impaired the CD8 lineage identity. We next detected the expression of genes involved in lineage selection in immature TCRβ$^+$ DP, CD4$^+$CD8^{lo}, and mature CD8$^+$ SP thymocytes, including *Runx3*, *Thpok (Zbtb7b)*, *Tle3*, *Bcl11b*, *Tcf7*, *Tox*, *Gata3*, *IL7Rα* and *Mazr*. The abundance of CD8 master regulator *Runx3* was substantially reduced in all three stages, and the significant elevation of *Tox* and *Mazr* was observed in

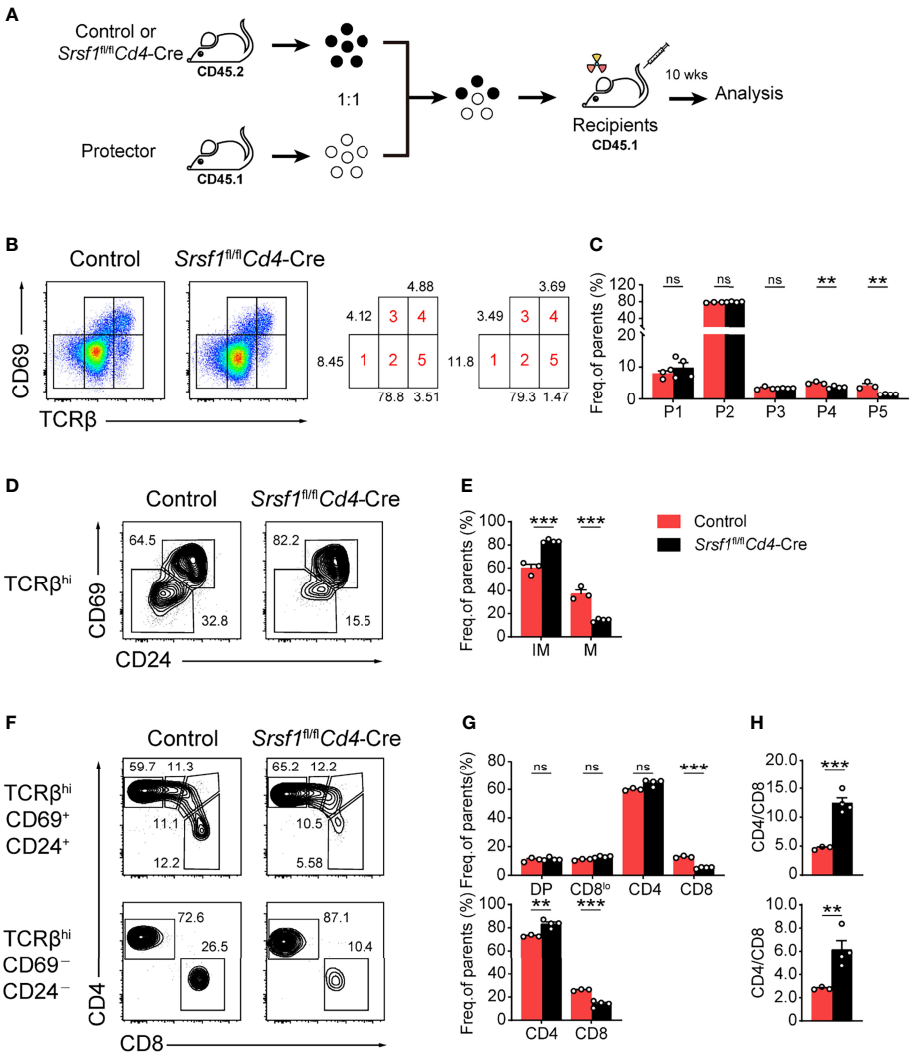

FIGURE 4 | SRSF1 intrinsically regulates the maturation of late thymocytes. **(A)** The scheme of bone marrow chimeric mice generation. A 1:1 mixture of bone marrow cells from Control or *Srsf1*$^{fl/fl}$*Cd4*-Cre mice (CD45.2$^+$) together with protector bone marrow cells from B6.SJL wild-type (CD45.1$^+$) was transplanted into lethally irradiated B6.SJL recipients (CD45.1$^+$). The thymocyte development of recipients was analyzed 10 weeks post transplantation. **(B, C)** Flow cytometry analysis of the sequentially developmental stages of donor-derived thymocytes. **(B)** Representative pseudocolor plots show five subsets, including population 1 (P1: TCRβ^{lo}CD69^{lo}), population 2 (P2: TCRβ^{int}CD69^{lo}), population 3 (P3: TCRβ^{int}CD69^{hi}), population 4 (P4: TCRβ^{hi}CD69^{hi}), and population 5 (P5: TCRβ^{hi}CD69^{lo}) in donor-derived thymocytes from Control and *Srsf1*$^{fl/fl}$*Cd4*-Cre mice, respectively. The frequency of indicated subsets was shown in **(C)** (n ≥ 3). **(D–H)** Analysis of the post-selection TCRβ^{hi} thymocytes from donor-derived mice. **(D)**TCRβ^{hi} thymocytes [populations 4 and 5 in **(B)**] were further fractionated into CD69$^+$CD24$^+$ immature and CD69$^-$CD24$^-$ mature subsets. **(F)** The immature subsets were subdivided into CD4$^+$, CD4$^+$CD8^{lo} (CD8^{lo}), DP, and CD8$^+$ sub-populations (clockwise from left in the top row), and the mature subsets were further subdivided into CD4$^+$ and CD8$^+$ populations (bottom row). The frequency of indicated subsets was shown in **(E, G)**, accordingly. **(H)** The ratio of CD4$^+$ and CD8$^+$ thymocytes was calculated and shown (n ≥ 3). Data are representative from at least two independent experiments. The error bars are means ± SD. Statistical significance was determined by one-tailed Student's *t*-test. ns, not statistically significant; $^{**}P < 0.01$ and $^{***}P < 0.001$.

DP stage but no changes in CD4$^+$CD8^{lo} and mature CD8$^+$ SP thymocytes in SRSF1-deficient cells (**Figure 5D**). Although the expression of *Tle3, Bcl11b*, and *IL7Rα* was dramatically decreased in CD8$^+$ SP thymocytes, most of detected lineage commitment-related genes were not altered in the essential transient stages (DP and CD4$^+$CD8^{lo}), such as *Thpok, Tcf7, Tle3, Bcl11b*, and *Gata3* (**Figure 5D**). These results imply that SRSF1 may contribute to the CD8 lineage fate by primarily controlling *Runx3* expression.

Overexpression of Runx3 Partially Rectify the Ratio of CD4$^+$ to CD8$^+$ Cells in *Srsf1*$^{fl/fl}$*Cd4*-Cre Mice

We next attempted to explore whether enforced expression of Runx3 could rectify the defects in the CD8 lineage fate caused by SRSF1 deficiency. To achieve this goal, the retrogenic mouse models were established and analyzed as described in the flowchart (**Figure 6A**). We confirmed the transduced efficiency

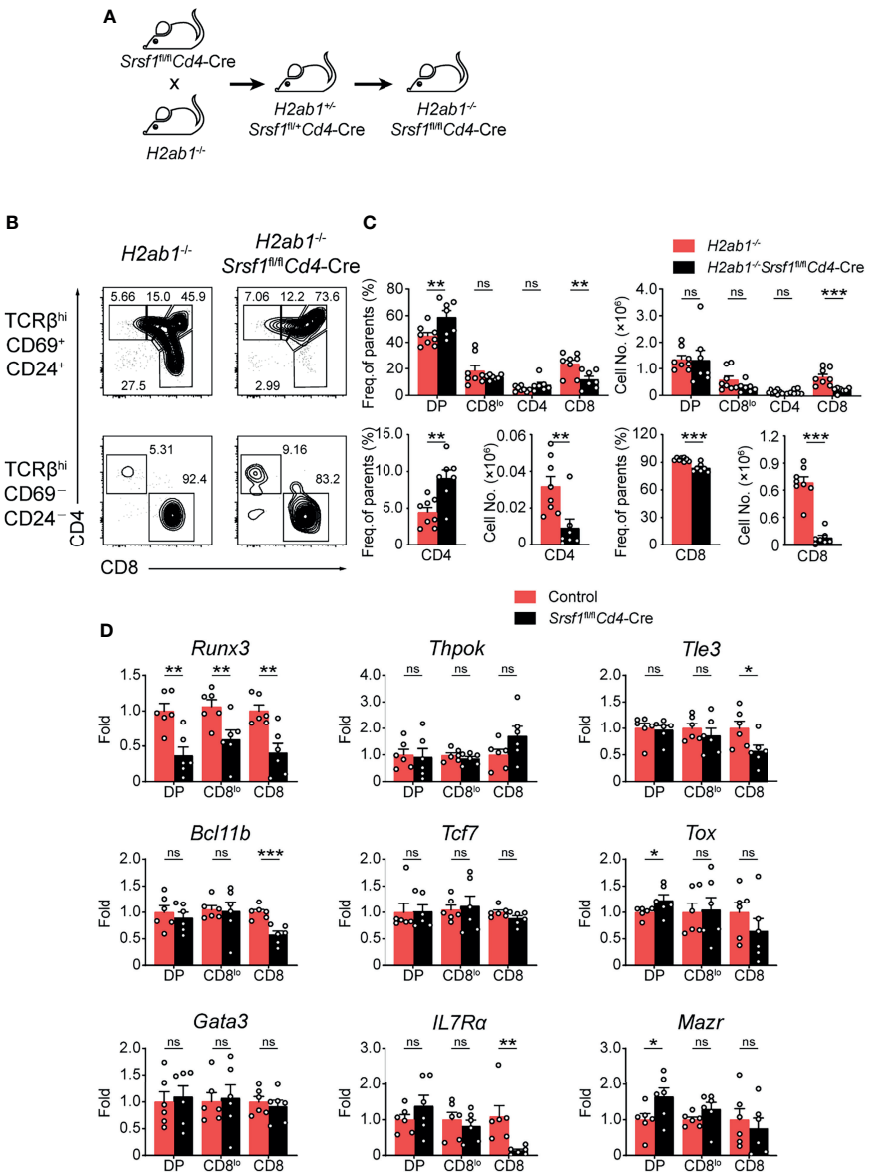

FIGURE 5 | SRSF1 is involved in the lineage selection of CD4-versus-CD8 T cells. **(A)** The scheme shows the generation of *H2ab1*$^{-/-}$*Srsf1*$^{fl/fl}$*Cd4*-Cre mice. **(B, C)** Analysis of the post-selection TCRβ^{hi} thymocytes from *H2ab1*$^{-/-}$*Srsf1*$^{fl/fl}$*Cd4*-Cre mice. The immature (TCRβ^{hi}CD69^{+}CD24^{+}) subsets were subdivided into CD4^{+}, CD4^{+}CD8^{lo}, DP, and CD8^{+} sub-populations (clockwise from left in top row), and the mature (TCRβ^{hi}CD69^{-}CD24^{-}) subsets were further subdivided into CD4^{+} and CD8^{+} populations (bottom row). The frequency and numbers of indicated subsets were shown in **(C)**, respectively (n ≥ 6). **(D)** Analyzing the expression of *Runx3*, *Thpok* (*Zbtb7b*), *Tle3, Bcl11b, Tcf7, Tox, Gata3, IL7Rα* and *Mazr* in immature TCRβ^{+} DP, CD4^{+}CD8^{lo}, and mature CD8^{+} SP thymocytes from Control or *Srsf1*$^{fl/fl}$*Cd4*-Cre mice. The relative expression of *Srsf1* transcript in indicated T cell subsets (after normalization to *Gapdh*) in Control cells was set as 1, and its relative expression in cells from *Srsf1*$^{fl/fl}$*Cd4*-Cre mice was normalized, accordingly. Data were collected from at least two independent experiments. The error bars are means ± SD. Statistical significance was determined by one-tailed Student's *t*-test. ns, not statistically significant; *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$.

of BM LSK cells was more than 50% before transplantation (**Figure S3**) to ensure the successful construction of chimeric mice. By analyzing donor-derived TCRβ^{hi} post-selection thymocytes, we found that the reduction of mature (TCRβ^{hi}CD69^{-}CD24^{-}) thymocytes was substantially restored by forced expression of SRSF1, but not by forced expression of Runx3 compared with those derived from Control-*MigR1* or *Srsf1*$^{fl/fl}$*Cd4*-Cre-*MigR1* donors (**Figures 6B, C**). Meanwhile, the ectopic expression of SRSF1 also rectified the ratio of CD4^{+} to CD8^{+} SP cells in both TCRβ^{hi}CD69^{+}CD24^{+} immature and TCRβ^{hi}CD69^{-}CD24^{-} mature thymocytes (**Figures 6D–F**). However, overexpression of Runx3 could largely restore the ratio of CD4^{+} to CD8^{+} SP cells in TCRβ^{hi}CD69^{-}CD24^{-} mature stage while no rescue was observed in the

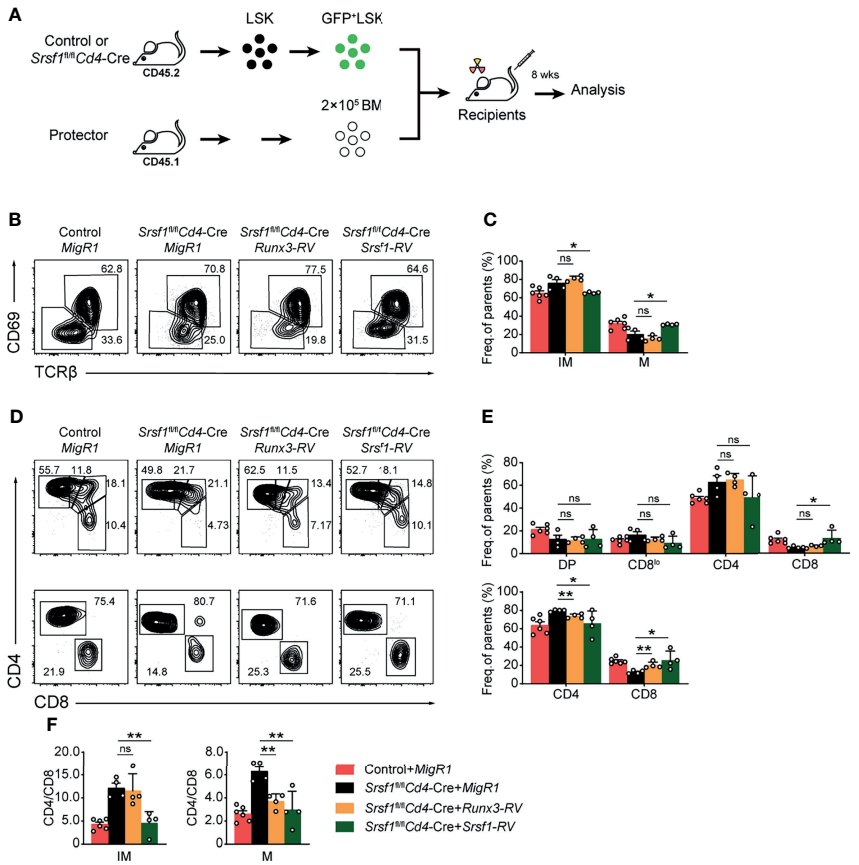

FIGURE 6 | Overexpression of Runx3 partially rescues the defects of CD8$^+$ T proportion in *Srsf1*$^{fl/fl}$*Cd4*-Cre mice. **(A)** The flow chart shows the experimental design of bone marrow chimeric mice by using *Runx3*- or *Srsf1*-contained retroviral transduction. **(B)** Analysis the post-selection thymocytes from chimeric mice. TCRβ^{hi} thymocytes were further fractionated into CD69$^+$CD24$^+$ immature and CD69$^-$CD24$^-$ mature subsets. **(C)** The frequency of indicated subsets in **(B)** was shown, accordingly (n ≥ 4). **(D)** The immature subsets were subdivided into CD4$^+$, CD4$^+$CD8^{lo}, DP, and CD8$^+$ sub-populations (clockwise from top left in the top row), and the mature subsets were further subdivided into CD4$^+$ and CD8$^+$ populations (bottom row). **(E)** The frequency of indicated subsets in **(D)** was shown, respectively. **(F)** The ratio of CD4$^+$ to CD8$^+$ cells from immature and mature subsets of TCRβ^{hi} thymocytes was calculated and shown, accordingly (n ≥ 4). Data were collected from at least two independent experiments. The error bars are means ± SD. Statistical significance was determined by one-tailed Student's *t*-test. ns, not statistically significant; **P* < 0.05 and ***P* < 0.01.

TCRβ^{hi}CD69$^+$CD24$^+$ immature stage (**Figures 6D–F**). These data collectively revealed that Runx3 serves as a regulator downstream SRSF1 for CD8 lineage fate decision, but other regulators and more complicated mechanisms may involve in the SRSF1-dependent regulatory network of late thymocyte maturation and lineage fate decision.

DISCUSSION

The lineage commitment of T cell to either CD8$^+$ or CD4$^+$ lineage before egress from thymus has been a fundamental research interest in the field of immunology, but the precise mechanism remains incompletely understood. Increasing evidences demonstrate that RBPs are indispensable for the development and function of immune cells by modulating gene expression through mRNA destabilization or stabilization, or by controlling translation (32–34), which provide a new direction to decode the complicated regulatory network in T cell fate decision. As a prototypical splicing factor, SRSF1 is well characterized for its roles in the maintenance of genomic stability, cell viability and cell-cycle progression (23, 35, 36), over the past twenty years, SRSF1 has been extensively investigated owing to its critical involvement in multiple cancers and autoimmune diseases (37–41). However, the roles of SRSF1 in T cell development and function have not been exposited until we recently found that it serves as a key posttranscriptional regulator in sustaining both the conventional T cell development and iNKT cell differentiation (28, 29).

As a follow-up study of the work by Qi et al. (28), we here report that conditionally targeting SRSF1 in DP thymocytes impairs the post selected T cell development and CD8$^+$ T cell fate decision. Although previous study established the importance of SRSF1 in late thymocyte development and

terminal maturation by using *Srsf1*$^{fl/fl}$*Lck*$^{Cre/+}$ mice (28), the altered ratio of CD4$^+$ to CD8$^+$ cells has not been specifically addressed. To avoid the impacts caused by SRSF1 deletion at early stage, we employed *Srsf1*$^{fl/fl}$*Cd4*-Cre mice to investigate the stage-specific role of SRSF1 in lineage choice in current study. We found the phenotypic defects were weaker in late stage of thymocyte development and maturation from *Srsf1*$^{fl/fl}$*Cd4*-Cre mice than those from *Srsf1*$^{fl/fl}$*Lck*$^{Cre/+}$ mice. Consistent with previous results from *Srsf1*$^{fl/fl}$*Lck*$^{Cre/+}$ mice, the peripheral T cells were substantially decreased from *Srsf1*$^{fl/fl}$*Cd4*-Cre mice, and most of the existing mature T cells were escapees in secondary lymphatic organ, which was caused by increased apoptosis and the shortened lifespan of SRSF1-deficient cells (28). Despite the substantial reduction of CD8$^+$ SP cells was exhibited in both TCRβ^{hi}CD69$^+$CD24$^+$ immature and TCRβ^{hi}CD69$^-$CD24$^-$ mature thymocytes, the CD4$^+$ SP cells were only notably reduced in TCRβ^{hi}CD69$^-$CD24$^-$ mature stage, suggesting SRSF1 deficiency has more severe effects in CD8$^+$ lineage differentiation.

To inspect whether SRSF1 contributes to the lineage choice of post selected DP thymocytes, we crossed the *Cd4*-Cre-mediated SRSF1 deletion mouse strain with the MHC-II-deficient *H2ab1*$^{-/-}$ mice. As expected, post selected mature thymocytes from control mice were largely restricted to the CD8$^+$ T cell lineage because of the defective MHC-II expression. In contrast, mature thymocytes from *H2ab1*$^{-/-}$*Srsf1*$^{fl/fl}$*Cd4*-Cre mice contained fewer CD8$^+$ SP cells but more CD4$^+$ SP cells, indicating MHC-I–selected thymocytes are redirected from CD8$^+$ to CD4$^+$ T cell lineage in the absence of SRSF1. For potential targets involved in lineage choice and CD8 cell identity downstream SRSF1, we measured the well-established lineage commitment-related genes in three sequential developmental stages DP, CD4$^+$CD8^{lo}, and mature CD8$^+$ T cells. In SRSF1-deficient cells, we found significant reduced expression of *Runx3* in three sequential developmental stages, and elevated expression of *Tox* and *Mazr* in only DP, but not CD4$^+$CD8^{lo} stage, which is an essential transient population from DP thymocytes to either CD4$^+$ or CD8$^+$ SP cells (14). In addition, the expression of *Tle3, Bcl11b*, and *IL7Rα* was only reduced in CD8$^+$ SP cells, which may miss the critical time point for lineage selection but affect the CD8 cell terminal maturation and survival. The dysregulation of lineage commitment-related genes leads to the aberrant differentiation of CD8$^+$ SP thymocytes and jointly contributes to the abnormal ratio of CD4 to CD8 cells in *Srsf1*$^{fl/fl}$*Cd4*-Cre mice, and Runx3 plays a central role downstream of SRSF1, particularly. However, overexpression of Runx3 could rectify the ratio of CD4$^+$ to CD8$^+$ SP cells in TCRβ^{hi}CD69$^-$CD24$^-$ mature stage, but not completely rescue the defects in SRSF1-deficient mice, suggesting the complex mechanisms involved in the defective identity of CD8$^+$ T cell in absence of SRSF1. Therefore, further understanding of how SRSF1 controls the expression of Runx3 as well as CD8 cell fate decision is required in future study.

In summary, our data revealed that SRSF1 exerts its developmental stage-specific effects in late thymocytes and contributes to CD8$^+$ T cell lineage fate decision and identity. This study represents an important step to further decipher the physiological functions of SR proteins, providing new insights of RBPs in regulating T cell development and lineage commitment.

MATERIALS AND METHODS

Animals

All mice used in this study were between 7 and 10 weeks of age on a fully C57BL/6J background. *Srsf1*$^{fl/fl}$ mice were kindly provided by Dr Xiang-Dong Fu (University of California, San Diego). *Cd4*-Cre and *H2ab1*$^{-/-}$ mice from Jackson Laboratories were maintained in the animal facility of China Agricultural University. Mice were housed in specific pathogen-free conditions under controlled temperature (22 ± 1°C) and exposed to a constant 12-hour light/dark cycle. All institutional and national guidelines for the care and use of laboratory animals were followed and all animal protocols used in this study were approved by the Institutional Animal Care and Use Committee at China Agricultural University.

Flow Cytometry

Single cell suspensions obtained from thymus (Thy), spleen, lymph node (LN), and peripheral blood cells (PBCs) were stained with fluorochrome-conjugated antibody as described previously (42). The fluorochrome-conjugated antibodies listed below: CD4 (RM4-5), CD8a (53-6.7), CD24 (M1/69), CD69 (H1.2F3), TCRβ (H57-597), B220 (RA3-6B2), CD11b (M1/70), CD11c (N418), CD45.1 (A20), CD45.2 (104), CD49b (DX5), Gr.1 (RB6-8C5), TER119 (TER-119), TCRγδ (GL-3), ScaI (D7), cKit (2B8) and 7AAD (00-6993-50) were purchased from eBiosciences. The fluorochrome-conjugated streptavidin (554063) was purchased from BD Biosciences. Samples were acquired on a LSRFortessa or FACSVerse (BD Biosciences) and analyzed with FlowJo software v10.4.0 (Tree Star, Inc.). For cell sorting, cells were surface-stained with indicated fluorochrome-conjugated antibodies and subjected to sorting on a FACSAria II (BD Biosciences).

Gene Expression Analysis

The gene expression was measured by qPCR as previously described (43). Briefly, total RNA was extracted from sorted cells using RNasey Mini Kit (Cat. # 74106, Qiagen) according to manufacturer's instructions. FastQuant RT Kit (Cat. # KR106-02, Tiangen) was used to synthesize cDNA. Quantitative RT-PCR (qPCR) was performed with SYBR Green Master Mix (Cat. # FP205-02, Tiangen) using CFX96 Connect™ Real-Time System (Bio-Rad). The primers were shown in **Supplementary Table 1**. Fold differences in expression levels were calculated according to the $2^{-\Delta\Delta CT}$ method and the relative expression of indicated genes was normalized to *Gapdh*.

BM Chimeras

The BM chimeric mice were generated as previously described (44). Briefly, the lethally irradiated B6.SJL (CD45.1$^+$) mice were transferred intravenously with a 1:1 mixture of 1 × 10^6 BM cells

from *Srsf1*$^{fl/fl}$*Cd4*-Cre (CD45.2$^+$) or control mice together with BM cells from congenic B6.SJL (CD45.1$^+$) mice. After 10 weeks reconstitution, recipients were sacrificed and analyzed.

Retroviral Transduction

The retrogenic chimera mouse models were generated by a modified protocol as previously described (28, 45). Briefly, retroviral packaging was carried out by transfection of HEK293T cells with *Runx3* cDNA bearing retroviral vector or empty pMigR1 vector along with pCLeco using Lipofectamine 2000 (Cat. # 11668019, Invitrogen), and the retrovirus-containing medium was collected at 24- and 48-hours post-transfection. After being filtered by 0.45 μm filters, the retrovirus-containing medium was loaded and centrifuged onto RetroNectin-coated [10 μg/mL (Cat. # T100A, TaKaRa)] non-tissue culture 24 well plates (Cat. # 351147, Falcon). BM cells from Control and *Srsf1*$^{fl/fl}$*Cd4*-Cre mice were depleted of lineage positive cells and cultured for 24 hours in IMDM medium in the presence of thrombopoietin (20 ng/mL), stem cell factor (50 ng/mL), 15% FBS, 2-mercaptoethanol (50 μm), streptomycin and penicillin (100 μg/mL) in retrovirus contained RetroNectin plate as described above. Then, cells were infected with fresh retrovirus-containing medium in the presence of 8 μg/mL Polybrene (Cat. # H9268, Sigma-Aldrich) by centrifuging at 1,000 rcf for 90 min at 32°C. Subsequently, the cells were cultured for 2 hours at 37°C 5% CO_2 incubator and resuspended in IMDM medium supplemented with components and cytokines as above. On the next day, the cells were spino-infected again. The infected cells were collected and analyzed by flow cytometry 24 hours later, and then these cells containing 5,000 GFP$^+$ lineage$^-$Scal$^+$cKit^{hi} (LSK) cells were transplanted into lethally irradiated (7.5 Gray) recipients (CD45.1$^+$). The recipients were sacrificed to analyze at 8 weeks after transplantation.

Gene Set Enrichment Assay

GSEA (v4.0.2) was used to analyze RNA-Seq data (GSE141349) from the GEO database, and the gene sets used in the article were obtained from MSigDB.

Statistical Analysis

Statistical analysis was carried out through using GraphPad Prism software (version 8.0). Statistical significance was determined by one-tailed Student's *t*-test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The animal study was reviewed and approved by China Agricultural University Laboratory Animal Welfare and Animal Experiment Ethics Review Committee.

AUTHOR CONTRIBUTIONS

SYY designed the project and supervised the overall experiments. CJ, LB, and SZY performed the major experiments. CJ and MY analyzed the overall data and generated figures. MY analyzed the high throughput data. ZQ, FW, GY, JiL, XC, ZW, JuL, WG, MF, and FC assisted the overall experiments. SYY, YK, and CJ wrote the manuscript with the revision from all authors. 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.2022.838719/full#supplementary-material

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Role of Cytokines in Thymic Regulatory T Cell Generation: Overview and Updates

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$CD4^+CD25^+Foxp3^+$ Regulatory (Treg) T cells are mainly generated within the thymus. However, the mechanism of thymic Treg cell (tTreg cell) generation remains to be fully revealed. Although the functions of TCR/CD28 co-stimulation have been widely accepted, the functions of cytokines in the generation of tTreg cells remain highly controversial. In this review, we summarize the existing studies on cytokine regulation of tTreg cell generation. By integrating the key findings of cytokines in tTreg cell generation, we have concluded that four members of γc family cytokines (IL-2, IL-4, IL-7 and IL-15), transforming growth factor β (TGF-β), and three members of TNF superfamily cytokines (GITRL, OX40L and TNF-α) play vitally important roles in regulating tTreg cell generation. We also point out all disputed points and highlight critical scientific questions that need to be addressed in the future.

Keywords: tTreg cells, IL-2, IL-15, TGF-β, γc family cytokines, TNF superfamily, TNFRSF

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INTRODUCTION

$CD4^+$ Regulatory T (Treg) cells that express IL-2 receptor α-chain (CD25) and the transcription factor forkhead box P3 (Foxp3) are the major cell population that maintains immune tolerance (1–6). Since these cells were identified in 1995 (2), Treg cells have been demonstrated to play extremely important roles in maintaining tolerance to auto-antigens (7, 8) and commensal microbiota (9, 10), controlling maternal-fetal immune interactions (11, 12), and suppressing overactive immune responses during infection (13, 14). On the other hand, Treg cell-mediated immune suppression can also promote tumor immune escape (15, 16). Therefore, targeting Treg cells could be a promising strategy to treat autoimmune disorders, maternal-fetal conflict, infections, and malignant tumors.

A majority of Treg cells are generated in the thymus (thymic Treg cells, tTreg cells), however some Treg cells can also be generated in periphery (pTreg cells) (17). Although it has been well documented that tTreg cells are generated during $CD4^+$ thymocyte development, the clear mechanisms of tTreg cell development is still not completely understood. Since T-cell receptor (TCR) stimulation from self-antigens and CD28 co-stimulation during thymocyte development are indispensable for tTreg cell generation (18–20), the mainstream view once believed that high-affinity TCR signal is the main driving force for inducing Treg cell differentiation (21–23). However, later studies demonstrated that tTreg cells could be generated from developing $CD4^+$ thymocytes

expressing TCRs with a broad range of self-reactivity (24, 25), showing that the self-reactivity of the TCR signal is not the deciding factor for tTreg cell generation.

In contrast, a two-step model of tTreg cell generation is gaining acceptance (26–29). The first step is driven by self-antigen induced TCR stimulation and CD28 co-stimulation, which leads to differentiation of $CD4^+$ $CD8^-$ $Foxp3^-$ $CD25^+$ tTreg cell precursors ($CD25^+$ $Foxp3^-$ tTreg precursors) and $CD4^+$ $CD8^-$ $Foxp3^+$ $CD25^-$ Treg cell precursors ($Foxp3^+$ $CD25^-$ tTreg precursors) from developing $CD4^+$ $CD8^-$ thymocytes. The second step relies on IL-2, which leads to the generation of $CD25^+Foxp3^+$ mature tTreg cells from $CD25^+$ $Foxp3^-$ tTreg precursors and $Foxp3^+$ $CD25^-$ tTreg precursors. This model proposes that both precursor populations are induced by TCR/CD28 co-stimulation, and both precursor populations rely on IL-2 to differentiate into mature tTreg cells. However, one recent study indicated that $CD25^+$ $Foxp3^-$ tTreg precursors and $Foxp3^+$ $CD25^-$ tTreg precursors are generated through two distinct developmental programs (30), suggesting that besides TCR/CD28 co-stimulation, some other key factors must be involved during development of these two tTreg precursor populations. All this evidence shows that this model still needs further refinements.

Besides TCR/CD28 co-stimulation, the most probable factors that mediate the distinct developmental programs of tTreg cell are different cytokines. Other than IL-2 and IL-15, three members of the tumor necrosis factor (TNF) superfamily cytokines (GITRL, OX40L and TNF-α) were demonstrated to promote tTreg generation (31). Moreover, TGF-β has also been shown to be important for tTreg cell generation (25, 32). In this review, we summarize the existing studies showing the important functions of cytokines in tTreg cell generation. We conclude that IL-2, IL-7, IL-15, IL-4, TGF-β, GITRL, OX40L, and TNF-α all play important roles in regulating tTreg cell generation, although regulation mechanisms of these cytokines have yet to be confirmed.

FOUR γC FAMILY CYTOKINES (IL-2, IL-7, IL-15 AND IL-4)

Function of IL-2, IL-7, IL-15 and IL-4 in tTreg Cell Generation

Before Treg cells were well identified, it was determined that mice deficient in IL-2 (33–35), IL-2 receptor α chain (IL-2Rα, also called CD25) (36) or IL-2 receptor β chain (IL-2Rβ, also called CD122) (37) would develop severe autoimmunity. It was a surprising finding since IL-2 was found to be a critical T cell growth factor (38–40). Since Treg cells have been identified, CD25 was proven to be a surface marker of Treg cells (2), and then it was determined that Treg cell-deficient scurfy mice develop severe autoimmunity as well (3–5, 41). These findings suggested that IL-2 might play a vital role in Treg cell generation.

However, the function of IL-2 in tTreg cell generation is still contentious. Some studies are against the idea that IL-2 is key for tTreg cell generation, because a significant number of $CD4^+$ $CD8^-$ $CD25^-$ $FOXP3^+$ thymocytes were still present in IL-2 knockout ($Il2^{-/-}$) mice, and these cells could still suppress inflammation in adaptive transfer mice model (42–44), although $CD25^-$ $FOXP3^+$ thymocytes were defined as tTreg precursors in the two-step model (29). Moreover, a recent study found that IL-2 could modulate the tTreg cell epigenetic landscape by targeting genome wide chromatin accessibility (45). These studies showed that IL-2 is dispensable for tTreg cell development, but important for mature tTreg cell survival, tTreg cell stabilization, and tTreg cell suppression function. Consistent with this idea, it was determined that Foxp3 is a proapoptotic protein and these $Foxp3^+$ $CD25^-$ tTreg precursors completed for the limited IL-2 to support their survival (28). In contrast, some studies found that although mice deficient in IL-2 or IL-2Rα had a certain number of Foxp3+ cells, their tTreg cells were not mature, and mice deficient in IL-2Rβ were shown to have a significant decrease in Treg numbers (44, 46), suggesting IL-2 should be important for tTreg cell development. Consistent with this idea, in the two-step model of tTreg cell development, it was found that $CD25^+$ $FOXP3^-$ tTreg precursors needed IL-2 to convert to mature tTreg cells (26, 27).

IL-2 receptor γ chain (IL-2Rγ), also known as the common cytokine receptor γ chain (γc) or CD132, is a common component of the receptors for IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21 (γc family cytokines) (47, 48). Therefore, besides IL-2, functions of other γc family cytokines in tTreg cell generation have also attracted a lot of attention. Importantly, mice deficient in IL-2Rβ resulted in a large reduction in the number of tTreg cells, whereas mice deficient in IL-2 or IL-2Rα still have high Foxp3 expression (42, 44, 46). IL-2Rβ is the receptor for both IL-2 and IL-15, so the function of IL-15 in tTreg cell generation was determined. Indeed, IL-2 and IL-15 double knockout ($Il2^{-/-}$x$Il15^{-/-}$) mice have a significant decrease in Treg numbers compared with $Il2^{-/-}$ mice (44), showing that IL-2 and IL-15 are important for tTreg cell generation. Moreover, mice deficient in IL-2Rγ were shown to be devoid of tTreg cells and have no expression of Foxp3 (42, 49), suggesting other γc family cytokines might also be important for tTreg cell generation. After in-depth research and verification, IL-7 was proven to be important for tTreg cell generation (50, 51). Moreover, IL-2Rβ and IL-7 receptor subunit α (IL-7Rα, also known as CD127) double knockout ($Il2rb^{-/-}$x$Il7ra^{-/-}$) mice were also devoid of tTreg cells, just like mice deficient in IL-2Rγ (50). Further studies proved that IL-2, IL-7, and IL-15 induces STAT5 phosphorylation and this process is indispensable for tTreg cell generation (49, 50), as STAT5 phosphorylation is critical for tTreg cell development by regulating Foxp3 expression (52–55). Taken together, three γc family cytokines, IL-2, IL-7, and IL-15 are essential for Treg cell generation (**Figure 1**). However, it remains to be confirmed whether these cytokines mainly induce tTreg cell development, promote tTreg cell survival, and/or maintain tTreg cell stabilization.

In the beginning, another γc family cytokine IL-4 was thought to be not important for tTreg cell generation as mice deficient in IL-4 receptor α (IL-4Rα) had absolutely normal tTreg cell generation (50). Moreover, IL-4 was actually shown to suppress Treg cell generation and induce T helper-9 cells (Th9

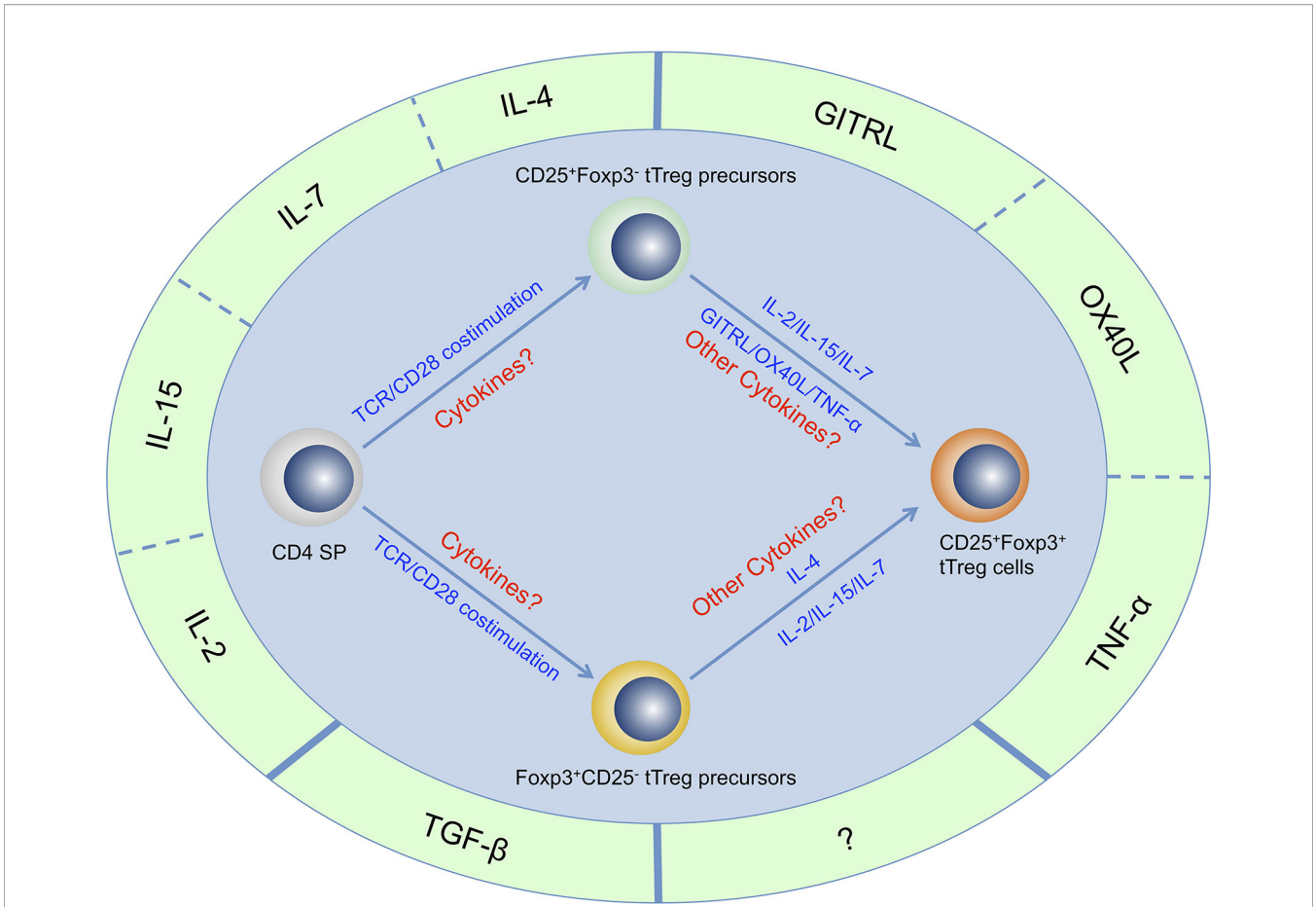

FIGURE 1 | Cytokines that are important for tTreg cell generation. Four γc family cytokines (IL-2, IL-4, IL-7, and IL-15), Three TNF superfamily cytokines (GITRL, OX40L, and TNF-α) and TGF-β have been determined to be important for tTreg cell generation. There may be other cytokines that are important for tTreg cell generation but have not yet been identified. It has been proven that $CD4^+CD8^-Foxp3^-CD25^+$ thymocytes and $CD4^+CD8^-Foxp3^+CD25^-$ thymocytes are two populations of tTreg cell precursors that generated through two distinct developmental programs, but the regulatory network of these cytokines in the development of these two precursor populations and mature tTreg cell has not been fully revealed.

cells) in periphery and *in vitro* (56–58). However, The same research team corrected the views (59), as they found that IL-4 could promote tTreg cell generation from $Foxp3^+$ $CD25^-$ tTreg precursors, although IL-4 could not support tTreg cell generation from $CD25^+Foxp3^-$ tTreg precursors (30). This evidence shows that IL-4 plays a role in tTreg cell development from $Foxp3^+CD25^-$ tTreg precursors.

Source of IL-2, IL-7, IL-15 and IL-4 in the Thymus

Determining the cellular sources of IL-2, IL-7, and IL-15 within the thymus are important in revealing the generation of tTreg cells, and it is also important for autoimmunity treatment through the manipulation of tTreg cells. It has been shown that tTreg cells could not produce IL-2 to support tTreg cell development and survival because Foxp3 represses expression of IL-2 (3, 60). More than that, in IL-2 wild type ($Il2^{+/+}$) and $Il2^{-/-}$ bone marrow chimera mice, tTreg cell generation was totally rectified in $Il2^{-/-}$ thymocytes and these bone marrow chimera mice did not develop autoimmunity (20). Therefore, tTreg cell generation mainly relied on IL-2 produced by non-Treg cells.

Although dendritic cells (DCs) and B cells were shown to be able to produce IL-2, mice that have selectively deleted IL-2 in DCs and B cells had been shown to have normal tTreg cell development and homeostasis (61, 62), showing DCs and B cells are not the major cellular sources of IL-2 in the thymus. In contrast, tTreg cell development was largely impaired in $Il2^{f/f}$ CD4-Cre mice, suggesting T cells are the key cellular source of IL-2 in the thymus (62). Moreover, a recent study determined that cells that secrete IL-2 are predominantly mature $CD4^+$ $CD8^-$ (CD4SP) thymocytes in the thymus; it has further been identified that IL-2 is mainly produced by self-reactive CD4SP thymocytes through single-cell RNA sequencing analysis (63). This evidence shows that self-reactive CD4SP thymocytes are the major cellular sources of IL-2 in the thymus.

Unlike IL-2, the major cellular sources of IL-7 and IL-15 are not T cells. It was determined that both cortical thymic epithelial cells (TECs) and medullary TECs express high levels of IL-7, and

IL-7 expression in cortical TECs is even higher than in medullary TECs (64). However, medullary TECs that highly expressed MHC class II were the major cellular source of IL-15 (65). Interestingly, it is well documented that tTreg cells are mainly generated in the medulla (66–69), suggesting it might be why IL-7 is not as important as IL-2 and IL-15 during tTreg cell generation in thymus. So far, the major cellular source of IL-4 in the thymus has not been determined (30).

TGF-β

Function of TGF-β in tTreg Cell Generation

Although it has been determined that TGF-β is the key inducer of Foxp3 in periphery and *in vitro* (70, 71), the function of TGF-β in tTreg cell generation is still in dispute. During early research, TGF-β was thought to be dispensable for tTreg cell development, because TGF-β1 deficient (8-10 days old) mice (*Tgfb1*$^{-/-}$) had normal frequency of tTreg cell in thymus (72), and T cell-specific TGF-β receptor II-deficient mice (*Tgfbr2*$^{f/f}$ x CD4-Cre) did not change the frequency of tTreg cell in thymus (12-14 days old mice) either (73, 74). In contrast, it was shown that TGF-β is critical for tTreg cell stabilization and regulatory function (72–74). Although the same research team repudiated their earlier study and thought TGF-β was not important for tTreg cell function and stabilization (75, 76), a recent study determined that TGF-β is critical for tTreg cell function in specific tissue environments, but not important for tTreg cell stabilization (77).

Surprisingly, TGF-β was identified to be important for tTreg cell development by studying tTreg cell generation in 3-5 days old neonatal mice (32, 78). It was shown that deletion of TGF-β receptor I (*Tgfbr1*$^{f/f}$ x Lck-Cre) in T cells blocks tTreg cell development largely in 3-5 days old neonatal mice, then tTreg cell frequency was recovered and became even higher in thymus of 3-4 weeks old *Tgfbr1*$^{f/f}$ x Lck-Cre mice than that in WT mice (32). It was then shown that tTreg cell frequency was increased in thymus due to increased tTreg cell proliferation in *Tgfbr1*$^{f/f}$ x Lck-Cre mice, as thymocytes lacking TGF-β receptor I produced more IL-2 and tTreg cells lacking TGF-β receptor I proliferated much faster in response to IL-2 (32). More importantly, further deletion of IL-2 in *Tgfbr1*$^{f/f}$ x Lck-Cre mice (*Tgfbr1*$^{f/f}$ x Lck-Cre x *Il2*$^{-/-}$) blocked tTreg cell development and expansion totally, as 3-4 weeks old *Tgfbr1*$^{f/f}$ x Lck-Cre x *Il2*$^{-/-}$ mice were devoid of tTreg cells as well (32).

The other group also reported a lack of tTreg cells in the thymus of 3-5 days old neonatal *Tgfbr2*$^{f/f}$ x CD4-Cre mice, but they proposed that this was due to increased tTreg cell apoptosis caused by the deletion of TGF-β signaling (78). Since TGF-β promotes thymocyte cell survival (79), a Treg cell-specific TGF-β receptor I-deficient mice (*Tgfbr1*$^{f/f}$ x Foxp3-Cre) was generated to determine whether the main function of TGF-β is to promote tTreg cell survival in the thymus (25). Surprisingly, it was found that tTreg cell frequency and number in *Tgfbr1*$^{f/f}$ x Foxp3-Cre mice did not decrease at all (25), and the aged *Tgfbr1*$^{f/f}$ x Foxp3-Cre mice had even more tTreg cells (77), showing the main function of TGF-β in tTreg cell generation is not to support tTreg cell survival. Existing mechanism studies have found that Smad3 could bind at the conserved noncoding sequence 1 (CNS1) of Foxp3 enhancer and induce Foxp3 expression (80, 81), but it was argued that Smad3 binding to the foxp3 enhancer was dispensable for tTreg cell development (82). Taken together, these findings show that TGF-β is critical to tTreg cell development, although the exact mechanisms need to be further identified (**Figure 1**).

Source of TGF-β in the Thymus

Thymocyte apoptosis has been identified to increase by day 2 after birth (83), TGF-β level was found to increase significantly in the thymus by day 3 after birth (25), and tTreg cells were shown to appear in large numbers in the thymus by day 3 after birth (84). This evidence suggests that tTreg cell generation, thymocyte apoptosis, and TGF-β production are highly correlated. Indeed, one study showed that the intrathymic concentration of TGF-β is highly dependent on thymocyte apoptosis (25). However, the major cellular source of TGF-β in the thymus has not been uncovered. Based on the existing studies, TGF-β is likely to be released from two possible cellular sources. The first possible source is apoptotic T cells that release TGF-β directly (85), and the second possible source is phagocytes that release TGF-β after these cells phagocytize apoptotic cells (86, 87).

It is worth mentioning that TGF-β is secreted into the extracellular matrix in an inactive latent form (latent TGF-β) and needs to be activated to produce bioactive TGF-β (88, 89). By now, it has not been determined how TGF-β is activated in the thymus. One possible mechanism for the activation of TGF-β in the thymus is through apoptotic cell-released ROS, as apoptotic thymocytes could release a high level of ROS (85), and ROS has been shown to induce TGF-β activation and promote Treg cell generation in periphery (90–92).

THREE TNF SUPERFAMILY CYTOKINES (GITRL, OX40L AND TNF-α)

Function of GITRL, OX40L, and TNF-α in tTreg Cell Generation

The tumor necrosis factor (TNF) superfamily is a protein superfamily originally produced as type-II transmembrane proteins, but these proteins can function as cytokines once they are cleaved off the cell membrane by metalloproteinases (93). The receptors of the TNF superfamily are tumor necrosis factor receptor superfamily (TNFRSF) (94). It has been determined that CD25^{+} Foxp3^{-} tTreg precursors and mature tTreg cells express high levels of TNFRSF members called Glucocorticoid-induced tumor necrosis factor receptor-related protein (GITR, also known as CD357 or TNFRSF18), OX40 (also known as CD134 or TNFRSF4) and TNFR2 (also known as CD120b or TNFRSF1B) (26, 31, 95). Moreover, it was found that a TNF superfamily member, TNF-α, a ligand of TNFR2, could promote Treg cell expansion *in vivo* (96–98). These findings suggest that the TNF superfamily might be important for tTreg cell generation.

Three TNF superfamily members, GITRL, OX40L, and TNF-α have been identified to promote tTreg cell generation (31, 99). One study reported that deficiency in TNFR2 reduced tTreg cell generation significantly (99). Another study showed that deficiency in all three of the TNFRSF members GITR, OX40, and TNFR2, or neutralization of TNF superfamily members GITRL, OX40L, and TNF-α together, markedly inhibited the generation of tTreg cells (31) (**Figure 1**). It was shown that GITRL, OX40L, and TNF-α could convert $CD25^+$ $Foxp3^-$ tTreg precursors into mature Foxp3+ Treg cells at very low dose of IL-2 (31), showing these three TNF superfamily members promote tTreg cell mature from $CD25^+$ $Foxp3^-$ tTreg precursors. However, it is still not clear whether TNF superfamily members and IL-2 complement each other, or TNF superfamily members just function as compensatory signals of IL-2 signal.

Source of GITRL, OX40L and TNF-α in the Thymus

Although the major cellular sources of GITRL, OX40L, and TNF-α have not been well defined, it was identified that medullary TECs expressed GITRL, OX40L, and TNF-α, while conventional dendritic cells (cDCs) and plasmacytoid dendritic cells (pDCs) expressed only GITRL and TNF-α (31). Further studies are needed to determine which kind of APCs are the major cellular source of GITRL, OX40L, and TNF-α. Moreover, whether membrane-bound or soluble GITRL, OX40L, and TNF-α play a more important role in tTreg cell generation has not yet been determined either.

CONCLUSIONS AND FUTURE PERSPECTIVE

By summarizing the existing studies of cytokines in tTreg cell generation, we conclude that four members of γc family cytokines (IL-2, IL-4, IL-7 and IL-15), transforming growth factor β (TGF-β), and three members of TNF superfamily cytokines (GITRL, OX40L, and TNF-α) play vitally important roles in regulating tTreg cell generation, although regulation mechanisms of these cytokines have yet to be confirmed. Functions of these cytokines in tTreg cell generation are still divisive. For example, opinions are still divided on the functions of TGF-β and IL-2, whether they are important for tTreg cell development, survival, and/or proliferation are still controversial.

On the other hand, when and how cytokines interact with each other and mediate tTreg cell generation in the thymus remains to be fully revealed. Also, when and how these cytokines take effect during tTreg cell development is still unclear. Therefore, future studies should focus on why developing tTreg cells are divided into two populations of tTreg precursors. Since $CD25^+$ $Foxp3^-$ tTreg precursors and $Foxp3^+$ $CD25^-$ tTreg precursors are generated through two distinct developmental programs (30), it is very likely that cytokines play key roles in inducing these two precursor populations besides TCR/CD28 co-stimulation. So far, it has been proven that IL-4 can support tTreg cell generation from $Foxp3^+$ $CD25^-$ tTreg precursors (30), and TNF superfamily cytokines (GITRL, OX40L and TNF-α) can support tTreg cell generation from $CD25^+$ $Foxp3^-$ tTreg precursors (31). These findings can partially explain the differences of $CD25^+$ $Foxp3^-$ tTreg precursors and $Foxp3^+$ $CD25^-$ tTreg precursors. However, the regulatory network of these cytokines during the development of tTreg precursors and mature tTreg cell has not yet been fully revealed. It is beyond all doubt that answering these basic questions is extremely important for fully disclosing the generation of tTreg cells.

AUTHOR CONTRIBUTIONS

MT drafted the manuscript. FJ, FN, FZ, and ZY reviewed and edited the manuscript. DZ supervised the work and wrote the manuscript. All authors contributed to the article and approved it for publication.

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Transcriptional Regulation of Early T-Lymphocyte Development in Thymus

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T-lymphocytes play crucial roles for maintaining immune homeostasis by fighting against various pathogenic microorganisms and establishing self-antigen tolerance. They will go through several stages and checkpoints in the thymus from progenitors to mature T cells, from $CD4^-CD8^-$ double negative (DN) cells to $CD4^+CD8^+$ double positive (DP) cells, finally become $CD4^+$ or $CD8^+$ single positive (SP) cells. The mature SP cells then emigrate out of the thymus and further differentiate into distinct subsets under different environment signals to perform specific functions. Each step is regulated by various transcriptional regulators downstream of T cell receptors (TCRs) that have been extensively studied both *in vivo* and *vitro via* multiple mouse models and advanced techniques, such as single cell RNA sequencing (scRNA-seq) and Chromatin Immunoprecipitation sequencing (ChIP-seq). This review will summarize the transcriptional regulators participating in the early stage of T cell development reported in the past decade, trying to figure out cascade networks in each process and provide possible research directions in the future.

Keywords: transcriptional regulators, T-lymphocytes, double negative (DN) cells, double positive (DP) cells, single positive (SP) cells, T cell receptor (TCR)

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INTRODUCTION

T cells widely participate in the innate and adaptive immune responses throughout the lifetime. T cell development is tightly regulated by numerous factors including transcriptional and epigenetic regulators. The proper development and differentiation of thymocytes is the foundation for the function of the immune system.

There is no doubt that the thymus is the fundamental place of thymocytes development that is highly organized, where thymocytes go through several stages and checkpoints before maturation and under-control of a network of multiple players (1, 2). Thymocyte development is driven by TCR activation and can be disrupted by defects in signaling components involved in the TCR signaling pathways (3, 4).

The early thymic precursor (ETPs) that come from bone marrow will go through different thymocyte developmental stages including $CD4^-CD8^-$ double negative (DN), $CD4^+CD8^+$ double positive (DP) and $CD4^+CD8^-$ or $CD4^-CD8^+$ single positive (SP). Then, mature SP cells will migrate to the periphery. Particularly, the DN population can be divided into four stages according to the expression of CD25 and CD44, starting from DN1 ($CD44^+CD25^-$), followed by DN2 ($CD44^+CD25^+$),

DN3 ($CD44^-CD25^+$) and DN4 ($CD44^-CD25^-$) (5). In addition, the DN1 cells are known as ETPs. There are several check points during T cell development. β-selection is the first major checkpoint occurs at the DN3 stage. At this stage, a properly rearranged TCRβ chain will be produced that mediated by recombinant activating genes 1 and 2 (RAG1 and RAG2). Cells with successful β-selection downregulate the expression of CD25 and become DN4 cells, which then progress to the DP cells through the immature CD8 single positive (ISP) stage. In contrast, unsuccessful β-selection of DN3 cells will undergo apoptosis.

At the DP stage, TCRα gene rearrangements initiate and mature αβ-TCR will be produced. Subsequently, thymocytes must pass through both positive and negative selections to become mature T cells. Thymocytes with functional TCRs interact with the major histocompatibility complex (MHC) on cortical epithelial cells (cTECs) presenting foreign antigens will survive (6). Thus, positive selection is vital for MHC restrictions. During negative selection, thymocytes respond to self-antigens presented by mTECs (medullary epithelial cells) will be eliminated. Finally, the selected thymocytes differentiate into mature SP cells, emigrate out of thymus to periphery, and then differentiate into distinct functional subsets such as regulatory T cells (Treg), helper T cells (Th) and cytotoxic T cells. Less than 5% DP thymocytes will survive during all the checkpoints.

Each developmental step requires the participants of transcriptional regulators that have been elucidated through advanced genomic techniques to identify the binding sites (7–9). The transcription factors bind to regulatory elements of target genes, such as promoters, enhancers or silencers, to regulate the gene expression. In this review, we will briefly summarize the critical transcriptional factors and related epigenetic regulators during the T-lymphocyte development reported in the past decade and provide a comprehensive understanding of the thymocytes regulation.

DN STAGES

Notch signaling is one of the most important pathways to initiate the transcriptional program of the progenitor cells (10). Firstly, Notch signaling induces T cell-specific transcription factor TCF-1 (T cell factor 1, encoded by *Tcf7* gene) expression at the ETP stage. Then lead to the activation of two major target genes, Gata3 and Bcl11b (B-cell lymphoma/leukemia 11B) (11). Three waves of chromatin remodeling were observed at the ETP, DN2b and SP stage respectively. TCF-1 is enriched at recognition sites and regulatory regions that become accessible during the ETP and DN2b wave and persist until maturation in both humans and mice (12, 13). TCF-1 deficiency at distinct phases redirects bifurcation among divergent cell fates and subdivide the DN cells to different clusters *via* scRNA-seq. In addition, TCF-1 directly binds and mediates chromatin accessibility contributing to tumorigenesis (14). Moreover, TCF-1 is also found to directly interact with actin-nucleating factor WASp by ChIP-seq to promote T cell development (15). Most recently, Notch1 target genes HES1 and HES4 have been reported to be upregulated in a Notch-dependent manner promoting early T-cell development (16). Collectively, these studies emphasize the essential role of TCF-1 and Notch signaling in regulating T cell development.

Gata3 and Bcl11b are the major targets of TCF-1. Gata3 is a member of the Gata transcription factor family, plays multiple roles in the transcriptional network of thymocyte development. Gata3 deficiency will affect T-cell survival, growth, commitment and progression into mature cells. Gata3 has been proved to be additionally required at the earliest stage of thymopoiesis for the development of ETP population and DN2 to DN4 stages, since the mRNA levels of Gata3 are gradually increased between the ETP and DN3 stages and slightly diminish again in DN4 cells (7, 17). In mouse DN4 cells, Gata3 is bound by F-box protein Fbw7 and augmented in Fbw7-deficient thymocytes (18), while it is negatively regulated by E-box binding protein HEB *via* Notch1 (19). Furthermore, Gata3 positively regulates Bcl11b at the transition stage of T cell commitment. Over 10 years ago, the important roles of Bcl11b in the differentiation and survival of DN cells have been revealed (20–22). It is stimulated not only by Notch signaling but also by MAP kinase-and Gsk3-dependent signaling. The kinetic modifications of Bcl11b in DN cells are somewhat different from the patterns observed in DP cells, suggesting the essential function of Bcl11b in DN to DP transition (23). In addition, the expression of Bcl11b is impaired in CD147 deficient mice which results in failed T cell identity determination (24). More interestingly, the intraepithelial lymphocytes are decreased when Bcl11b is deficient (25). Cooperating with Bcl protein, transcription factor NFATc1 also plays a critical role in DN thymocytes survival and differentiation (26). It is activated by IL-7-Jak3 signals during the DN1 to DN3 stages (27, 28).

The function of each RUNT-related transcription factors (Runx) family member is still poorly understood based on current studies. Nevertheless, it is well known that Runx family members, including Runx1, Runx2 and Runx3 are another crucial transcription factors facilitating early T cell development. The activity of Runx1 has been highlighted in the relationship with other key transcription factors such as Bcl11b and Pu.1, which regulate the dynamic changes of transcriptional signatures before and after T cell commitment respectively. In addition, enforced expression of Runx2 affects β-selection resulting in an expansion of DN cells (29). The intronic silencer *(S4)* of *Cd4* gene cooperates with RUNX which is involved in T-helper inducing POZ-Kruppel factor (ThPOK) pathway (30). Herein, Runx family members are involved in various stages such as β-selection of double-negative thymocytes (22). The hypomorphic mutation of Runx component core-binding factor β (Cbfβ) results in a consecutive differentiation block within the DN population, as evidenced by a decrease of ETP followed by an inefficient ETP-to-DN2 transition as well as DN2-to-DN3 transition (22, 31).

DN-TO-DP TRANSITION

T cells that have formed a functional pre-TCR complex, consisting of CD3, TCRβ, and pre-TCRα, can develop into DP cells. As a consequence, pre-TCR signaling is required for thymocyte development from DN to DP cells, following by dozens of transcriptional responses to pre-TCR signaling (32, 33).

Moreover, pre-TCR signaling leads to increased expression of the transcriptional repressor Bcl6 which is required for differentiation to DP cells (34). Another member of the Bcl family is the antiapoptotic molecule Bcl2, whose down regulation induces enhanced apoptosis during the transition from the DN3 to the DN4 stage and contribute to DN4 cell number reduction. While the proliferation of ISP thymocytes is compensated, the number of ISP cells is normal eventually (35, 36). The successful assembly of pre-TCR promotes rapid self-renewal of DN3b cells and sequentially differentiate into cycling DN4, CD8 ISP and early DP (eDP) blast cells, then stop proliferating to become quiescent late DP (lDP) cells (37). TCR has multiple gene segments as alpha, beta, gamma and delta (*Tcra, Tcrb, Tcrg* and *Tcrd*). Murine *Tcra* and *Tcrd* are organized into a single genetic locus (*Tcra/Tcrd* locus) that undergoes V(D)J recombination in DN thymocytes to assemble the *Tcrd* gene and in DP thymocytes to assemble *Tcra* gene, to generate diverse TCR repertoires (38, 39). In addition, the formation of a functional VDJ join signals is required for robust proliferation of DN thymocytes and their differentiation into DP cells, whereas *Tcrb* recombination is suppressed by allelic exclusion (40).

Subsequently, pre-TCR complexes activate Notch1, whose activation is essential for generating the huge pool of DP thymocytes as physiological Notch1 signals are highest expressed in DN3 cells and decreased in DP cells. Thus, Notch1 signaling is crucial and transiently upregulated in DN-to-DP transition. There are two types of Notch1 related transcriptional regulators which are activators and repressors. Notch1 can be activated by Delta-like Notch ligands such as DL4, which is critically regulated by Lunatic Fringe (Lfng) (41, 42). Another activator is Zmiz1, which is a stage-specific cofactor of Notch1. Withdrawal of Zmiz1 at the later pre-T cell stage impairs the DN-to-DP transition by inhibiting proliferation. Furthermore, the Zmiz1-deficient DN-to-DP defect can be rescued by enforced activation of Notch1 or its target gene (43). However, DN4 and DP cells will be oncogenic when Notch1 is activated inappropriately (44). The repressors of Notch1 are vital for homeostasis. It is confirmed that Notch1 signaling can be attenuated by Bcl6 (34), NKAP (45) and Early growth response 2 (Egr-2) (46) in ISP thymocytes. Forced expression of these repressors may result in a severe reduction of DP cells in the thymus. Furthermore, downstream transcriptional factors of Notch1 also influence DN-to-DP transition. Induced TCF-1 form complex with β-catenin that will lead to transcriptional activation of cell-fate specific target genes in the transition and DP thymocytes survival *via* canonical Wnt pathway. On the contrary, TCF-1 interacts with co-repressors such as Groucho/Transducin-like enhancer (GRG/TLE) and turns off-target gene expression in the absence of Wnt signals. In the absence of TCF-1, ISP thymocyte development is blocked which contributes to DP thymocytes reduction (47).

As we mentioned in the previous section, Runx1 binds to the *CD4* silencer and represses transcription factors in immature DN thymocytes followed by CD8 expression to promote DN-to-DP transition, then down regulate in DP stage (48). The growth rate of DN4 cells and the transition of DN4 to the DP stage are impaired by overexpressed Runx1, resulting in the substantial reduction of DP thymocytes (49). Coincidentally, a sequence-specific transcription factor Ets1 specifically associates with Runx1 in DN and TCF-1 in DP cells by binding distal nucleosome-occupied and depleted regions respectively (50). Another critical transcription factors family is Ikaros which transiently increased from DN to DP developmental stage (51). Nevertheless, Ikaros maybe not a conventional activator or repressor according to defined sets of genes (52). As a tumor suppressor, Ikaros directly repress most Notch target genes through genome-wide analyses, such as ChIP-seq (53). Furthermore, a newly reported transcriptional regulator, Zinc finger protein Yin Yang 1 (YY1), is functional in DN thymocytes survival and apoptosis by suppressing the expression of p53, which can eliminate thymocytes that fail to pass β-selection. Early ablation of YY1 caused severely impaired development to DP cells due to increased apoptosis of DN thymocytes that prevented the expansion of post-β-selection thymocytes (54). Nevertheless, the comprehensive mechanism of YY1 in thymocyte development remains unclear though it is essential for iNKT cell development by ChIP-seq analysis (55).

DP-TO-SP TRANSITION

DP Survival

Appropriate TCR signaling is crucial for the survival of DP thymocytes and determines positive or negative selection (56). Without proper selective signaling, DP cells will be eliminated by apoptosis within 3~4 days during this pre-selection period.

RORγt is one of the most important survival transcription factors in pre-selective DP cells that activates the gene encoding the antiapoptotic protein Bcl-x_L. It is well-known that the γc cytokine receptor subunit provides critical signals for T cell survival and differentiation. Recently, it is found that RORγt is abundant in immature DP thymocytes and act through Bcl-x_L to reduce the surface expression of γc. More importantly, Ligons et al. demonstrate that loss of RORγt in mouse DP thymocytes is associated with increased γc surface abundance and this phenomenon can be restored by forced expression of Bcl-x_L in RORγt-deficient thymocytes (57). Moreover, RORγt can be upregulated by TCF-1. Both TCF-1 and RORγt-knockout DP thymocytes undergo similarly accelerated apoptosis, while only in the presence of RORγt, the activation of TCF-1 by stabilized β-catenin can enhance DP thymocyte survival. Specifically, RORγt overexpression could rescue TCF-1 deficient DP thymocytes from apoptosis but overexpressed TCF-1 in RORγt$^{-/-}$ DP thymocytes doesn't show any rescue, which indicate that RORγt acts downstream of TCF-1. In addition, TCF-1 directly interacts with the promoter region of RORγt and induces its activity (58, 59). According to the most recent studies, TCF-1 may cooperate with transcription factors Zeb family member Zeb1 to participate in the cell cycle and TCR signaling by transcriptomic analysis (60).

Both Bim (Bcl2l11) and Nur77 are TCR-induced proteins with pro-apoptotic function. Bim is important for clonal deletion whereas Nur77 is often dispensable but able to influence late DP thymocytes apoptosis (61, 62).

Interestingly, nuclear speckle-related protein 70 (NSrp70) is selectively expressed on developing thymocytes and highest at DP stage. NSrp70 could regulate cell cycle and survival of thymocytes by governing the alternative processing of various RNA splicing factors, such as oncogenic serine/arginine-rich splicing factor SRSF1 (63). This finding may provide a new angle to dig up larger scale of transcription network in DP survival.

Positive Selection

The DP thymocytes will undergo positive selection in the cortex of the thymus by recognizing antigen-MHC complex presented by cTECs and transducing intra-thymic TCR signals, then become $CD4^+$ or $CD8^+$ expressing SP cells. Calcium flux signaling is required for positive selection of T cells. Our results demonstrate a newly discovered adaptor named Tespa1 (Thymocyte-expressed, positive selection-associated 1) is essential for positive selection by modulating the interaction with a Ca^{2+} release channel - inositol 1,4,5-trisphosphate receptor (IP3R) which express on ER membranes (64–66).

The positive selection is also induced by forkhead box (Fox) family. In a way, the pioneer transcription factors Foxa1 and Foxa2 (forkhead box protein A) regulate alternative RNA splicing during thymocyte positive selection. Another Fox protein Foxo1 may induce the selection and maturation of DP thymocytes that can be accelerated in the deficiency of transcription repressor Gfi1(Growth factor independent 1). Thus, the Gfi1-Foxo1 axis shapes the proper generation of SP cells (67, 68). Additionally, Egr-2 also regulates the survival of stage-specific thymocytes and enhanced the maturation of DP cells into SP cells in thymus (46, 69).

Lastly, the achievement of positive selection is inseparable from epigenetic regulation which cross-work with transcriptional signals. HDAC7 (Histone deacetylase 7) has been reported exporting from the cell nucleus during positive selection in mouse thymocytes and modifying genes to mediate the coupling between TCR engagement and downstream events that determine cell survival including MAPK activity (70).

Negative Selection

Negative selection is critical to delete highly self-reactive thymocytes to prevent autoimmunity. The thymocytes who pass the negative selection will become mature T cells with low self-reactivity and export to periphery immune organs.

The proceed of negative selection depends on functional mTECs, whose development is powerfully promoted by transcription factors Foxn1 (forkhead box family N1) and Aire (autoimmune regulator), which control the differentiation and maturation repectively (71). Conditional Foxn1 knockout results in defective negative selection contribute to less clonal deletion of autoreactive thymocytes (72), which possibly attribute to abnormal mTECs. Therefore, the Foxn1-TEC axis has been considered to repair negative selection and rejuvenation of thymic involution which is critical for counteracting inflammaging (73). Foxn1 is also the downstream target of Wnts which are a large family to secret glycoproteins and participate in cell fate determination, migration, proliferation, polarity and death in TECs. Existing evidences show Wnt4 and Wnt5b regulate Foxn1 expression in TECs through TCF-4 and LEF-1 by both autocrine and paracrine manners (74).

On the other hand, the function of mTECs is highly dependent on their characteristic features such as ectopic expression of tissue-restricted antigens (TRAs) and their master regulator Aire, whose expression is restricted to a mature subset of mTECs. Aire induces tissue-specific antigens to ensure negative selection by directly binding the promoter of the target gene within the medulla (75, 76). The transcriptional function of Aire in the process of mTECs adhesion is reconfirmed by CRISPR/Cas9 technology (77). Subsequently, it is shown that Aire targets 5'-URR (5'-untranslated regulatory region) of immune checkpoint HLA-G lead to increased intracellular HLA-G protein expression in TECs (78). Surprisingly, Aire can bind to sequence-independent epigenetic tags, such as unmethylated histone 3, and be recruited to a locus. After demethylation and Aire binding, Aire either directly enhances transcription or recruits other transcriptional activators (75).

In addition to the promoters, the transcriptional repressors of negative selection are indispensable. NCoR1 is a nuclear receptor co-repressor to connect repressive chromatin-modifying enzymes to gene-specific transcription factors. NCoR1 restrains negative selection by repressing pro-apoptotic factor Bim expression, which is expressed elevated in the absence of NCoR1. NCoR1-null thymocytes show excessive negative selection and reduced mature SP thymocytes (79–81). NCoR1 interacts with a predominant member of the HDAC family named HDAC3 which is a major and specific molecular switch that is crucial for mTECs differentiation and highly specific to histone deacetylases (82). Capicua (CIC) (83) and Sphingomyelin microdomains (SM) (84) also work as repressive factors together to ensure the proper negative selection and prevent autoimmunity.

DISCUSSION

We conclude the map of T-lymphocyte development in the thymus and related transcriptional regulators that have been reported in the past decade (**Figure 1**), hoping to give some clues or inspiration to the future research. These selected regulators may have redundant or opposite functions in the thymocyte's maintenance, proliferation, differentiation and maturation. Indeed, our understanding of the early stage of T lymphocytes development is still limited yet, the modulators we reviewed here are still poorly understood. Surprisingly, in recent years, more and more advanced techniques including various sequencing are invented or improved in order to elucidate the function of transcription factors involved in T cell development. However, the regulatory network among them and the precise mechanism still need further investigation both *in vivo* and *vitro* using ingenious animal models and molecular biological approaches.

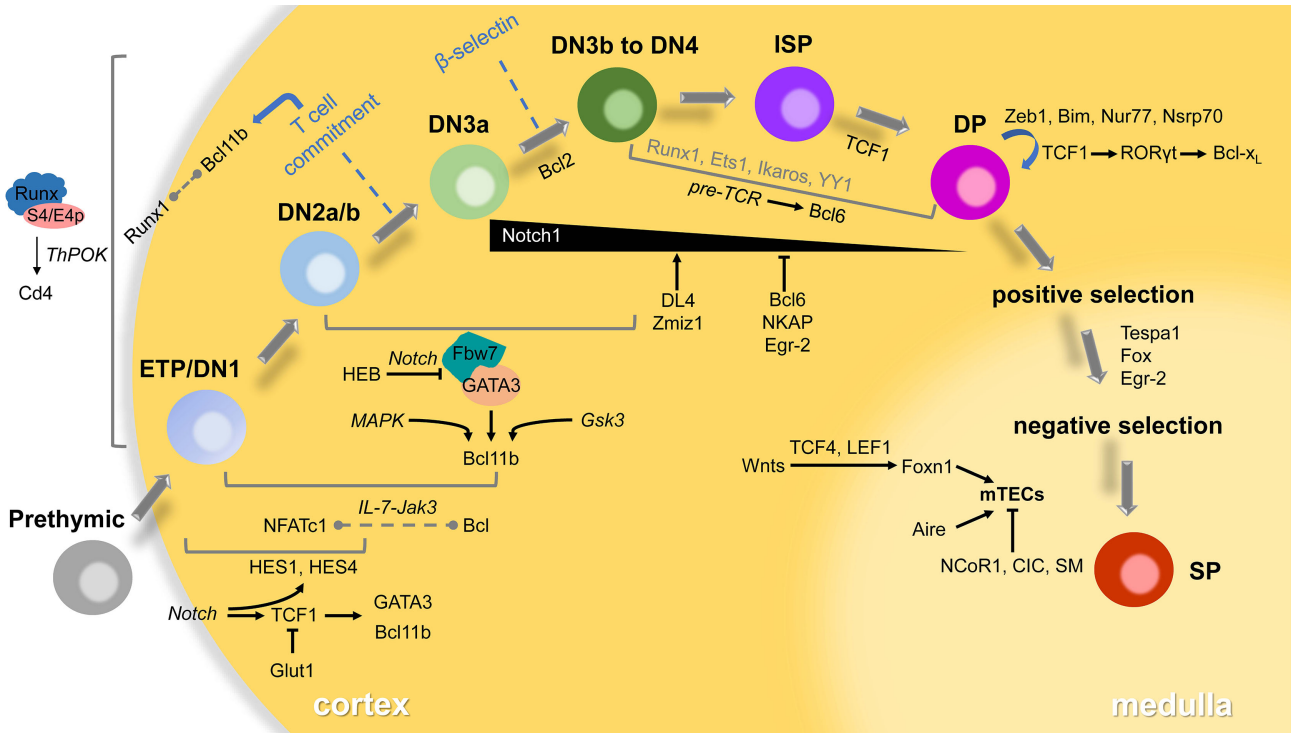

FIGURE 1 | Transcriptional regulators in thymocyte development. T-lymphocytes go through several distinct stages according to expressed TCRs or surface markers in the thymus. The developing cells will pass checkpoints (β-selection, positive selection and negative selection) to become mature SP thymocytes, then emigrate to the periphery to differentiate to functional subsets. Most of the transcriptional regulators play roles in specific stages *via* small networks involving signaling in italics. Some interaction between factors remains unclear that displayed in the dotted line. The participants of a few transcriptional factors in grey are also poorly understood.

AUTHOR CONTRIBUTIONS

XB and YQ prepared the initial draft. LL and MZ revised and finalized the manuscript. All authors contributed to the article and approved the submitted version.

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MINI REVIEW
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Thymic Microenvironment: Interactions Between Innate Immune Cells and Developing Thymocytes

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The thymus is a crucial organ for the development of T cells. T cell progenitors first migrate from the bone marrow into the thymus. During the journey to become a mature T cell, progenitors require interactions with many different cell types within the thymic microenvironment, such as stromal cells, which include epithelial, mesenchymal and other non-T-lineage immune cells. There are two crucial decision steps that are required for generating mature T cells: positive and negative selection. Each of these two processes needs to be performed efficiently to produce functional MHC-restricted T cells, while simultaneously restricting the production of auto-reactive T cells. In each step, there are various cell types that are required for the process to be carried out suitably, such as scavengers to clean up apoptotic thymocytes that fail positive or negative selection, and antigen presenting cells to display self-antigens during positive and negative selection. In this review, we will focus on thymic non-T-lineage immune cells, particularly dendritic cells and macrophages, and the role they play in positive and negative selection. We will also examine recent advances in the understanding of their participation in thymus homeostasis and T cell development. This review will provide a perspective on how the thymic microenvironment contributes to thymocyte differentiation and T cell maturation.

Keywords: thymus, macrophage, dendritic cell, T cell development, positive selection, negative selection, thymus repair

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INTRODUCTION

The thymus is an essential organ for T cell development (1). It is home to many cell types, such as stromal and immune cells, which not only aid in T cell development, but are also integral to thymus homeostasis (2–4). During T cell development, bone marrow-derived early thymic progenitors (ETPs) first seed the thymus where they receive Notch signals from cortical thymic epithelial cells (cTECs) and are signaled to enter the T-lineage differentiation pathway (5). These early progenitor T cells are double negative (DN) for CD4 and CD8 expression and their T cell receptor (TCR) genes have not yet undergone V(D)J rearrangement (6). At this stage, DN cells rearrange their γ, δ and β TCR gene loci, and following successful TCRβ gene assembly gain CD4 and CD8 expressions, a checkpoint termed β-selection, and advance to the CD4 and CD8 double positive (DP) stage. Cells that properly rearrange their γδ TCRs mature into the γδ-T cell lineage (7). However, the majority of

cells become DP cells, and following rearrangement of their TCRα gene loci are subjected to positive selection, which is conducted by cTECs presenting peptide self-antigens on their major histocompatibility complex (MHC) class I and MHC class II molecules to DP cells (8).

Proper TCR-MHC interactions predicate whether DP cells are allowed to differentiate to the next stage of αβ-T cell development. Conversely, DPs with non-functional TCR-MHC interactions undergo death by neglect, which occurs for over 95% of DPs (9, 10). Following positive selection, DPs migrate to the thymus medullary region and undergo negative selection against strong TCR-MHC interactions. This process, which helps to ensure self-tolerance, is conducted by medullary thymic epithelial cells (mTECs), which under the regulation of autoimmune regulator (AIRE) express a vast array of self-antigens, and with the help from other thymic antigen presenting cells (APC), such as dendritic cells (DCs) (**Figure 1**) (11–13). The purpose of this process is to eliminate potential self-reactive T cells, which could lead to autoimmune diseases if released into the periphery. In total, it is estimated that only 3-5% of developing thymocytes become mature CD4 or CD8 single positive (SP) T cells and exit the thymus (14).

The two-step selection process is repeated every day in the thymus and is only diminished during thymus aging or due to external injuries, such as irradiation and inflammatory stress (12, 15, 16). One necessary aspect of the selection process, which is critical to ensure that randomly generated TCRs are both able to properly interact with self-MHC and not lead to autoimmunity, is the need to eliminate a vast number of potentially useless or harmful cells on a continuous basis. Due to the daily massive cell death during T cell selection, thymic homeostasis needs to be strictly maintained by other cell types. Thymic macrophages are immune cells that are crucial for clearing apoptotic thymocytes in the thymus. Remarkably, thymic macrophages only make up 0.1% of all cells in the thymus (17). This suggests that they are highly efficient in efferocytosis since there are over 50 million DPs generated in a mouse thymus every day, a majority of which are likely destined for cell death and need to be cleared by thymic macrophages (13). These cells have also been shown to play a role in maintaining thymus homeostasis and thymus repair after injuries (18). As for the negative selection process, thymic DCs are also present in the medulla and have been shown to play a pivotal role in T cell selection alongside mTECs to curtail the generation self-reactive T cells and promote central tolerance (19). In this review, we will focus

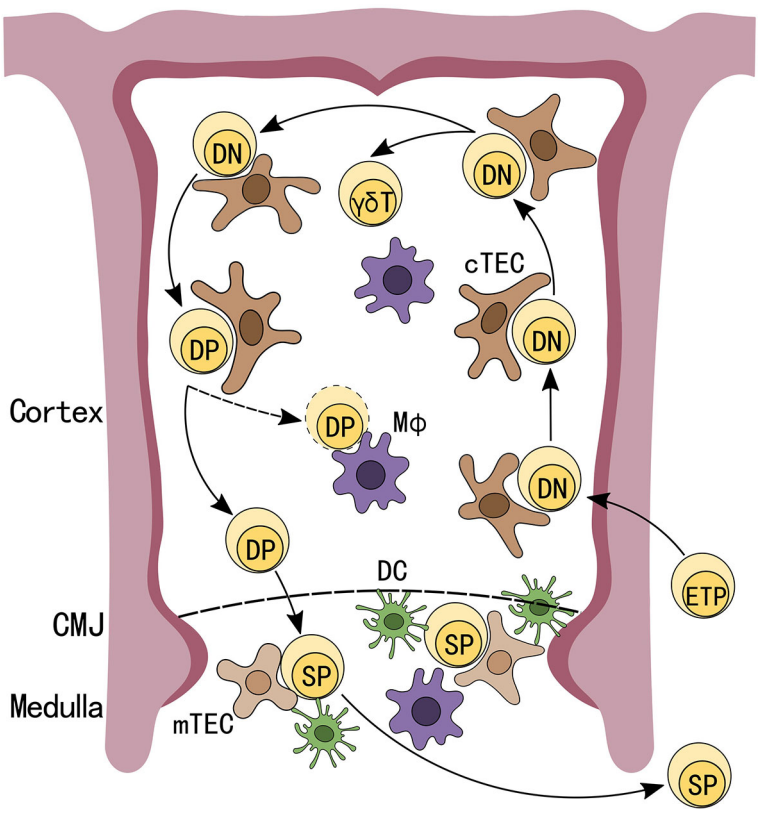

FIGURE 1 | Schematic depiction of T cell development in the thymus. Early thymic progenitors (ETP), arriving from the bone marrow, seed the thymus and receive Notch signals from thymic epithelial cells (TECs) to differentiate into $CD4^{-}CD8^{-}$ double negative (DN) T-lineage cells. DN cells that have undergone successful V(D)J rearrangement at TCRβ gene loci differentiate into $CD4^{+}CD8^{+}$ double positive (DP) cells. After completing TCRα rearrangements and successfully undergoing positive selection, DPs migrate to the thymus medullary region and are subjected to negative selections while DPs that fail positive selection will be programmed for apoptosis. Cells that successfully passed these checkpoints will exit the thymus as CD4 or CD8 single positive (SP) T cells.

on these two important cell types in the thymus, DCs and macrophages, by examining their developmental origin, localization, function, and recent advances on their role in T cell selection and thymus repair post injury.

THYMIC DENDRITIC CELLS

DCs in the thymus make up 0.5% of thymus cellularity and are mainly composed of three different groups: plasmacytoid DCs (pDCs), $CD8^+SIRP\alpha^-$ ($CD8^+$ DCs), and $CD8^-SIRP\alpha^+$ ($SIRP\alpha^+$ DCs) (20). $SIRP\alpha^+$ DCs and pDCs are migratory DCs that developed in the bone marrow and migrate from the periphery to the thymus, while a small fraction of $CD8^+$ DCs originate intrathymically from a common T/DC progenitor, majority of $CD8^+$ DCs develop outside the thymus (21–26). Typically, mature $SIRP\alpha^+$ DCs are located in the cortico-medullary perivascular space, $CD8^+$ DCs are located within the medulla, and pDCs are located at the cortical-medullary junction (CMJ) (27–29). A recent paper published by Sarah Teichman's group using single-cell (sc) RNA-sequencing (seq) of human thymus cells, allowed them to identify a new subtype of DCs, which they named as activated DCs (aDCs), due to their high expression in costimulatory molecules (30). These aDCs could be further clustered into aDC1, aDC2, and aDC3 subsets, where aDC1 and aDC2 expressed similar gene profile as $CD8^+$ DCs and $SIRP\alpha^+$ DCs, respectively. While the aDC3 cluster expressed lower levels of co-stimulatory molecules compared with other aDCs, suggesting that these are post-activated aDCs. The distinct gene expression profiles from the different aDCs subsets suggests they are derived from different DCs population. This new aDC subtype is located at the center of the medulla, and uniquely expresses *LAMP3* and *CCR7*, which are not found in other DC subtypes in the thymus. Their data also showed that aDCs can recruit naïve and regulatory T cells (Treg) into the thymus medullary through CCR7:CCL19 and CCR4: CCL17/CCL22 interactions, respectively. Interestingly, some aDCs also expressed AIRE, which validated other group's previous findings (31, 32). It has been proposed that AIRE can regulate intercellular transfers of self-antigen from mTECs to thymic DCs to promote thymic tolerance (32, 33). Combined with their high costimulatory molecule expression and their interaction with developing T cells, these aDCs may play a role in T cell negative selection, however, functional analyses are needed to further determine the exact role that aDCs may play in T cell selection. Furthermore, whether these aDCs share a common developmental origin as $CD8^+$ DCs and $SIRP\alpha^+$ DCs, or whether aDCs merely represent an activate stage of conventional DCs in the thymus requires further elucidation.

THYMIC DENDRITIC CELLS ON T CELL SELECTION

Thymic DCs are known to express high levels of class I and II MHC molecules (34). It has been well established that thymic DCs play a role in central tolerance and clonal deletion during T cell development (35). Particularly, $SIRP\alpha^+$ DCs have been shown to transport antigens through blood and induce Treg development in mice (36). Further validating this point, Dominik Filipp's group recently found a novel $CD14^+SIRP\alpha^+$ monocyte DCs (moDCs) subset in the thymus that was important for the generation of Tregs (37). While moDCs expressed some genes overlapping with $SIRP\alpha^+$ DCs, they also expressed high levels of monocyte associated genes (*Mafb*, *Apoe*, and *Csf2ra*), which are absent in the $SIRP\alpha^+$ DC subset, indicating that moDCs are likely a distinct population. Their findings suggested that the TLR9/MyD88 pathway induced mTECs to express chemokines that promoted the recruitment of moDCs to the thymus. These moDCs could also acquire antigens from mTECs. However, whether these or other DCs are able to transfer self-antigens expressed by medullary fibroblast, which were recently shown to express TRAs that contribute to central tolerance was not addressed (38, 39). Of note, $MyD88^{\Delta TEC}$ mice that conditionally lacked MyD88 in mTECs, there was a decrease in moDCs populations in the thymus, leading the impaired generation of Tregs, and those Tregs that were generated displayed reduced suppressive capacity. The same group also found specific DCs subsets in the thymus have a preference in antigen transfer from different TEC subsets (40). Notably, moDCs were most efficient in antigen transfer compared with all other thymic DC subsets, and moDCs were able to acquire antigens from multiple mTECs. However, the mechanism of how these cells acquire self-antigens for T cell negative selection remained unclear.

Attempting to answer the above questions, Charles J Kroger et al. illustrated how thymic DCs can acquire MHC molecules from TECs through intercellular transfer (41). By coculturing thymic DCs from NOD mice and TECs from BALB/c mice that express $H2\text{-}D^d$ (an MHC class I antigen) and IE^d (an MHC class II antigen), the authors found thymic DCs, compared with splenic DCs, had a higher efficiency in acquiring $H2\text{-}D^d$ and IE^d. The capacity for MHC molecules uptake by thymus $CD8^+$ DCs and $SIRP\alpha^+$ DCs were similar. However, this intercellular transfer ability was only found between thymic DCs and TECs, and not with other APCs, such as B cells, when cocultured with thymic DCs. Using qRT-PCR, the authors identified that this intercellular antigen transfer process was correlated with the unique expression of the epithelial marker EpCAM only in DCs found in the thymus. Thymic DCs were previously thought to acquire EpCAM protein from TECs, while this paper showed that both thymic $CD8^+$ DCs and $SIRP\alpha^+$ DCs can express EpCAM, while $SIRP\alpha^+$ DCs expressed the highest level of EpCAM compared with all other DC subtypes in the thymus (42). This intercellular transfer ability in DCs is organ specific and is regulated differently between the different subsets of DCs in the thymus. This was shown when the authors blocked PI3K signaling and the transfer of MHC antigens to $CD8^+$ DCs was reduced, while transfer to $SIRP\alpha^+$ DCs was not impacted. This work provided new insights on how thymic DCs can specifically acquire antigens from neighboring TECs in the thymus, and the mechanism for antigen transfer in thymus DCs subtypes are regulated by different pathways. Further studies can be

done to determine the exact mechanism that regulates intercellular antigen transfer between TECs and SIRPα$^+$ DCs in the thymus since these DCs are known to play a role in the generation of Tregs.

Because a majority of thymic DCs are periphery-derived that migrate to the thymus, they also have the capacity to carry antigens from the periphery to the thymus for T cell selection (35). However, the specific molecules that each thymic DCs subtype carries remains unclear. A recent paper from Ulrich von Adrian's group found a new population of DCs that expresses CX_3CR1 in both human and mice, which they named transendothelial DCs (TE-DCs) (43). Using multi-photon intravital microscopy, they found that these TE-DCs are located between the microvessels and the thymus where they can transport blood born proteins into the thymus and then use it for T cell selection (**Figure 2**). They also reported that these TE-DCs are a heterogeneous population of DCs, a majority of which are composed of SIRPα$^+$ DCs, followed by pDCs. Only a small fraction of TE-DCs was identified as CD8$^+$ DCs. This finding was supported by previous research that looked at the origin of thymic DCs and showed that SIRPα$^+$ DCs and pDCs were migratory DCs from the periphery, while CD8$^+$ DCs can be intrathymically derived. This new antigen transport system by CX_3CR1 TE-DCs depends on its ligand CX_3CL1, which is expressed by thymus endothelial cells. Recent work by Gretchen Diehl's group also showed CX_3CR1^+ DCs can capture microbial antigens, present these antigens to developing T cells, and induce microbial-specific T cell expansion (44). Altogether, these findings introduced a new model for T cell selection by thymic DCs where a specialized subset of CX_3CR1^+ DCs, located at microvessels, are actively taking up blood born antigens and transporting them into the thymus for T cell selection. However, whether these CX_3CR1^+ DCs have distinct developmental origin and what signals are responsible for the polarization of CX_3CR1 DCs are still unclear.

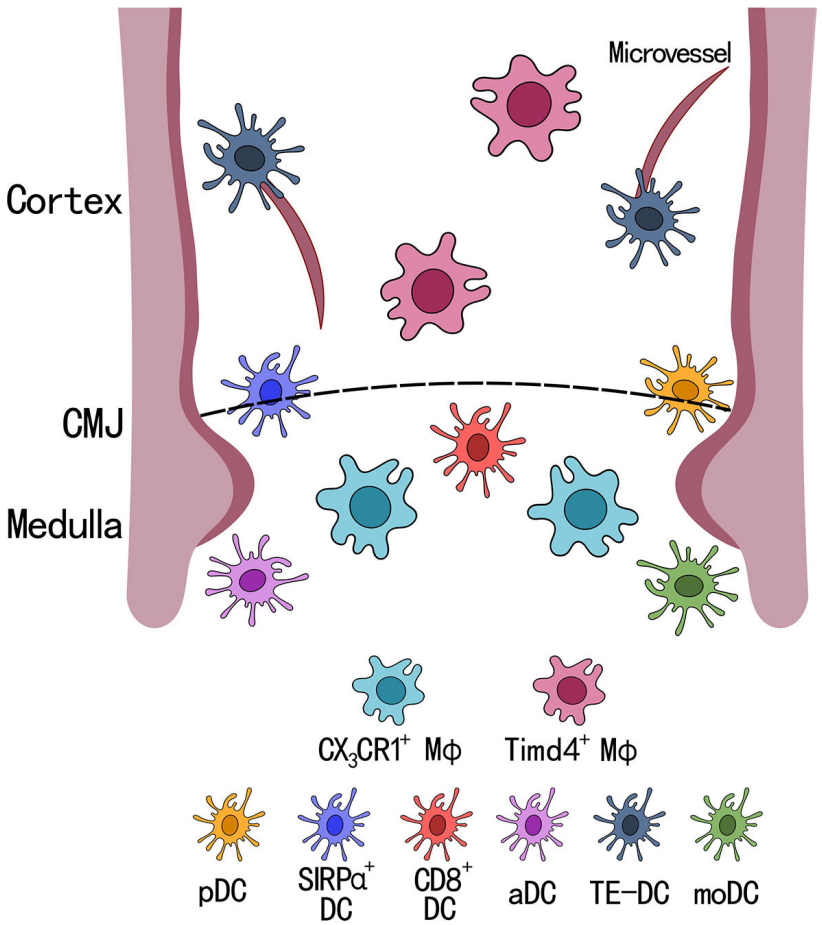

FIGURE 2 | Localization of dendritic cell and macrophage subsets in the thymus. There are 6 subsets of dendritic cells (DCs) and 2 subsets of macrophage (MΦ) in the thymus. SIRPα$^+$ DCs and pDCs are located closely to the cortical-medullary junction (CMJ), CD8$^+$ DCs, activated DCs (aDCs), and CD14$^+$SIRPα$^+$ moDCs (moDCs) are located within the medullary region, and transendothelial DCs (TE-DCs) are located between the microvessels in the thymus. Timd4$^+$ macrophages are located within the cortex and uniquely express *Spic* and *Vcam1*, while CX_3CR1^+ macrophages are located at the CMJ expressing *Runx3* and antigen presenting genes, such as *H2-Q7*.

THYMIC DENDRITIC CELLS IN POST INFECTION

It has been shown that the generation of mature T cells from the thymus is attenuated during and post infections (45, 46). Since a majority of thymic DCs come from the periphery, whether migratory DCs play a role in thymus damage post infection was unclear. A recent publication by Haojie Wu et al. showed that mature DCs from the circulation can enter the thymus and induce thymus involution through the Notch signaling pathway (47). Upon activation by antigens such as lipopolysaccharide and ovalbumin, DCs have been shown to enhance Jagged1 expression (48, 49). Their work showed that these activated DCs expressing Jagged1 can bind to Notch3-expressing mTECs and this interaction through the Notch signaling pathway induces apoptosis in mTECs. This in turn led to the disruption in SP cell generation in the thymus. However, this finding needs to be validated in disease models, such as post viral infections. Nonetheless, this work provided a new perspective on thymic atrophy upon infection by activated DCs, suggesting that DCs in the thymus may play a deleterious role during an infection, which as previously thought that this may be critical to prevent the thymus from inducing self-tolerance against virally encoded antigens. It would also be interesting to test whether blocking DCs infiltration into the thymus post infection could prevent thymic atrophy.

THYMIC MACROPHAGES

During T cell development, cells that do not pass positive or negative selection undergo apoptosis (50). It is estimated that over 95% of cells undergo apoptosis in the thymus every day (50, 51). However, when isolating cells from the thymus of healthy adult mice, one typically finds that nearly all the thymocytes are live cells, suggesting that apoptotic cells within the thymus are actively and effectively cleared (52, 53). The clearing of apoptotic cells is done by intrathymic macrophages (9, 30, 50, 54–56). For many years, macrophages in the thymus have not been well characterized nor understood, due to technical limitations in analyzing these cells and performing functional studies. There are only a few well known macrophage markers that have been found to be expressed on thymic macrophages (ED1 and ED2 in rats, CD68, F4/80 and CD11b in mice) making it difficult to study the origin of these thymic macrophages and identify their heterogeneity in the thymus (57–60). With the advent of scRNA-seq technology, characterizing small cell populations, and performing ontogeny analysis on thymic macrophages have become possible.

A recent publication by Tyng-An Zhou et al. identified two macrophage subsets (Timd4$^+$ and CX$_3$CR1$^+$) in the thymus of adult mice using scRNA-seq (**Figure 2**). Both populations of thymic macrophages were found to developed during embryonic life, and the authors found Timd4$^+$ thymic macrophages were derived from CX$_3$CR1$^+$ cells during embryogenesis. The two different subsets of thymic macrophages showed distinct gene expression profiles, where Timd4$^+$ thymic macrophages expressed high levels of *SpiC*, *MafB*, and *Vcam1*, which showed high similarity with the transcriptomic landscape of spleen red pulp macrophages (61, 62). While CX$_3$CR1$^+$ thymic macrophages had high expression of *Runx3* (which is important for cytotoxic CD8$^+$ T cell development), and genes involved in antigen presentation (*B2m*, *H2-M2, H2-K1, and H2-Q7*) (63–66). These two tissue resident macrophage subsets found in the thymus agreed with recent findings by Slava Epelman's group, in which they showed Timd4$^+$ and CX$_3$CR1$^+$ tissue resident macrophages were found across many organs (heart, liver, lung, kidney, and brain) in mice (67).

The distinct gene profile for these two subsets of thymic macrophages suggested they may have different functions within the thymus. Using immunofluorescence to examine thymic histological sections, Zhou et al. found that Timd4$^+$ macrophages are found mainly in the cortex, while CX$_3$CR1$^+$ macrophages are localized in the CMJ. In combination with their transcriptomic profile, this suggests that Timd4$^+$ thymic macrophages are the main cells performing efferocytosis of apoptotic thymocytes. Their findings were also supported by Catherine C. Hedrick's group who demonstrated that Timd4$^+$F4/80$^+$ thymic macrophages have the highest phagocytic efficiency compared with other macrophage subsets, and that the depletion of these macrophages accelerated thymic involution, suggesting an important role in thymic homeostasis (68).

Conversely, CX$_3$CR1$^+$ thymic macrophages may play a role in T cell negative selection. This is supported by their location at the CMJ, which is where negative selection initiates, as positively selected thymocytes migrate into the medulla. Combined with their gene expression profile and migratory ability, these thymic macrophages may have the potential to carry self-antigen through blood vessels and present them to developing T cells for negative selection and tolerance induction. However, further studies need to be performed to validate their potential functions *in vivo* (69).

THYMIC MACROPHAGE IN T CELL SELECTION

As the findings from Zhou et al. suggest, thymic macrophages may play a role in T cell selection by their antigen presenting ability. Other groups have shown Timd4$^+$ cells in the thymus can also present MHC-I peptides and induce negative selection of CD8$^+$ T cells (70, 71). However, as these authors mentioned, Timd4 can also be expressed on thymic DCs, thus it is difficult to distinguish whether Timd4$^+$ thymic macrophage are the true players for culling self-reactive CD8$^+$ T cells and whether they play a defining role in presenting antigens to developing T cells during negative selection. These data contrast the scRNA-seq results presented by Zhou's group, where CX$_3$CR1$^+$ thymic macrophages by their location and gene expression profile were suggested to have a higher probability in presenting self-antigens for negative selection.

Vijay K. Kuchroo's group generated a Timd4$^{-/-}$ mice, and found that Timd4-deficient mice had hyperactive T and B cells, as well as displaying an impairment in efferocytosis by peritoneal macrophages (70). However, the absolute cell number of thymocytes in Timd4 deficient mice did not differ from control wild-type mice, which contrasts other group's findings, where the

depletion of thymic macrophage led to an acceleration of thymic involution, and hence decreasing the size of the thymus (68, 71). This could be attributed by the compensation from other phagocytes in the thymus of Timd4$^{-/-}$ mice to maintain thymus homeostasis. This was evidenced in other organs where depletion of a specific subset of tissue resident macrophages led to empty niches in the organ where infiltrating monocytes or other tissue resident macrophages quickly occupied these niches and performed functions similar to the original tissue resident macrophage (72–74). Thus, whether thymic macrophages play a role in T cell selection remains to be elucidated.

THYMIC MACROPHAGE DURING THYMUS INJURY

In addition to efferocytosis, phagocytosis and antigen presentation, tissue resident macrophages have been shown to play a crucial role in tissue repair across many organs (75–77). After tissue injury, tissue resident macrophages can secrete cytokines (IL-10 and TGFβ), growth factors (FGF, TGFα, and PDGF), and exosomes to promote cell differentiation and suppress inflammation (78). Depletion of tissue resident macrophages in the heart and liver were shown to impair organ healing (67, 76, 79–81). However, whether thymic macrophages can play a similar role in thymus repair is still unclear.

One clinically relevant source of injury to the thymus is irradiation, a process that some cancer patients are subjected to as part of their treatment (82, 83). The rate of recovery is crucial as the thymus is integral for generating T cells that form an immune response. Several groups have sought new approaches to improve thymic recovery post irradiation treatment (84–86). A recent publication by Gen Yamada's group used a *MafB*/green fluorescent protein knock-in (*MafB*$^{+/GFP}$) mouse to demonstrated that MafB expressing cells in the thymus play a crucial role in thymus repair after irradiation. When comparing thymus recovery post irradiation between *MafB*$^{+/+}$ and *MafB*$^{+/GFP}$, the authors found that there was a decrease in immature TECs (Krt5^{+}FoxN1^{+}) generated in the *MafB*$^{+/GFP}$ thymus. The organization of the medulla was also found to be abnormal post-irradiation injury, where mTECs in the *MafB*$^{+/GFP}$ thymus formed only one prominent medullary compartment, while *MafB*$^{+/+}$ maintained multiple medullary compartments after recovery. Since *MafB* is a common marker used to identify macrophage populations, it stands to reason that a majority of the cells expressing *MafB* in the thymus are likely macrophages (17, 18, 87). This new finding showed that thymic macrophages may play a role in thymus repair, potentially by engulfing apoptotic cells and controlling inflammation in the thymus. These results also suggested that post thymic injury, macrophages are important for the repair of the thymus architecture and supporting the regeneration of thymic endothelial cells. However, exactly which of the two thymic macrophage populations is playing a role in thymus repair after injury remains unclear. Further studies are needed to assess the role of the two thymic macrophage subsets, Timd4^{+} and CX$_3$CR1^{+}, in clinically relevant injury models.

CONCLUSION

The thymus is a sophisticated organ that is important for generating T cells, which play a critical role in immune function. As a result, severe consequences can arise if thymic homeostasis is not properly regulated. This therefore demands the need to have a thorough understanding of the thymus environment that induces and support T cell development. Although the T cell selection process by TECs has been well studied, whether thymic DCs and macrophages are important players in T cell development, selection and thymus homeostasis remain to be further elucidated. With scRNA-seq technology, several groups have been able to identify new populations of DCs in the thymus (aDCs, TE-DCs, and CX$_3$CR1^{+} DCs), each of which appears to serve distinct functions. Macrophage heterogeneity in the thymus was also elucidated using this technology, and we can now appreciate that there are two macrophage populations in the thymus, Timd4^{+} and CX$_3$CR1^{+}. However, there are still many questions remaining, such as which thymic macrophage subset plays a role in thymus repair? Do thymic macrophages play a role in the negative selection of T cells, if so, which subset? By addressing these questions, we can pave the way to promoting new clinical therapies for the repairing of the thymus post injuries.

AUTHOR CONTRIBUTIONS

HW wrote the manuscript. JCZ-P wrote and edited the manuscript. All authors contributed to the article and approved the submitted version.

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MHC-independent αβT cells: Lessons learned about thymic selection and MHC-restriction

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Understanding the generation of an MHC-restricted T cell repertoire is the cornerstone of modern T cell immunology. The unique ability of αβT cells to only recognize peptide antigens presented by MHC molecules but not conformational antigens is referred to as MHC restriction. How MHC restriction is imposed on a very large T cell receptor (TCR) repertoire is still heavily debated. We recently proposed the selection model, which posits that newly re-arranged TCRs can structurally recognize a wide variety of antigens, ranging from peptides presented by MHC molecules to native proteins like cell surface markers. However, on a molecular level, the sequestration of the essential tyrosine kinase Lck by the coreceptors CD4 and CD8 allows only MHC-restricted TCRs to signal. In the absence of Lck sequestration, MHC-independent TCRs can signal and instruct the generation of mature αβT cells that can recognize native protein ligands. The selection model thus explains how only MHC-restricted TCRs can signal and survive thymic selection. In this review, we will discuss the genetic evidence that led to our selection model. We will summarize the selection mechanism and structural properties of MHC-independent TCRs and further discuss the various non-MHC ligands we have identified.

KEYWORDS

thymic selection, MHC restriction, T cell receptor, tyrosine kinases, Lck, coreceptors, T cell repertoire

Introduction

Adaptive immunity depends on the ability of T lymphocytes to recognize foreign antigens. The last three decades have brought tremendous insight into the antigen recognition properties of αβT cells. Experiments performed by Zinkernagel and Doherty

more than forty years ago documented the ability of αβT cells from lymphocytic choriomeningitis virus (LCMV)-infected mice to kill *in vitro* LCMV-infected target cells only if the T cells and the target cells shared at least one H-2 antigen (1–3). Their observations and interpretation led them to their 1996 Nobel Prize award for discovering major histocompatibility complex (MHC) restricted antigen recognition, now a well-established T cell immunology hallmark (4, 5). The simultaneous recognition of antigenic peptides with self-MHC molecules highlights a unique receptor-ligand interaction that is unparalleled in biology. A fragile balance in this unusual interplay is required to control T cell immunity, providing effective protection from infection while avoiding T cell mediated autoimmunity.

Both T and B lymphocytes use the same gene reco0mbination machinery to create their antigen receptor diversity, but those receptors recognize their ligands in fundamentally different ways (6). Antibodies generated by B cells recognize a wide array of three-dimensional epitopes on native antigenic proteins or glycolipids (7). Somatic recombination of the TCR loci generates tremendous diversity, but αβTCRs focus only on foreign and self-peptides presented by self MHC molecules (8). Thymocytes rearrange genomic regions on both TCRα and TCRβ loci, generating in the process diverse *de novo* segments called complementarity determining regions 3 (CDR3) that are responsible for peptide recognition (9, 10), with diversity being further enhanced by random addition and deletion of nucleotides. The other two regions, CDR1 and CDR2, are germline-encoded and carry limited diversity which is encoded in the variable domains of both α and β TCR chains (11).

On the other side of the equation, MHC proteins present peptides to T cells to discriminate between self and non-self. Immune evasion by pathogens is rendered more difficult by two major characteristics of the MHC loci. First, the MHC is polygenic and contains several different MHC-I and MHC-II genes so that each individual possesses a set of MHC molecules with different ranges of peptide-binding specificities. Second, the MHC genes show the greatest degree of polymorphism in the human genome (12). Multiple variants of the same gene exist within the population as a whole and therefore the extent of peptides presented to T cells is virtually unlimited. This heterogeneity of MHC alleles at the individual and population levels provides the immune system a robust mechanism to counteract pathogens evading MHC presentation and T cell responses.

During T cell development in the thymus, positive and negative selection allow immature thymocytes to be screened for ligand specificity. To survive selection and undergo differentiation, thymocytes must express TCRs that engage intra-thymic ligands and successfully generate intracellular signals. This process is crucial for thymic selection, as the vast majority of T cell precursors bear "useless" TCRs that are incapable of producing signals and therefore undergo death by neglect.

A few years ago, we proposed the selection model to describe the molecular basis of MHC restriction (13). In the selection model, nothing intrinsic to the TCR structure imposes MHC restriction on the randomly generated αβT cell repertoire (**Figure 1**). Like antibodies generated by the same recombination machinery, the pre-selection αβTCR repertoire can recognize a wide variety of antigens, including MHC and non-MHC ligands but only MHC-restricted αβTCRs can signal in the thymus. The TCR itself does not possess intrinsic signaling capabilities but requires the co-engagement of coreceptors to initiate signaling. TCR ligation leads to the tyrosine phosphorylation within immunoreceptor tyrosine-based activation motifs (ITAMs) on all TCR-associated CD3 chains (14). This phosphorylation is carried out by the tyrosine kinases of the Src family of kinases, i.e. Lck and Fyn. Subsequently, another tyrosine kinase, ZAP-70, is recruited to the TCR/CD3 complex, where it binds the phosphorylated ITAMs and can now be phosphorylated and activated by Lck. The adaptor proteins LAT and SLP-76 are then phosphorylated by active ZAP-70 and recruit mediators to propagate downstream signaling pathways. Additionally, signaling initiation is strictly dependent on coreceptor binding to its specific MHC.

Thus, the fact that coreceptors only recognize MHC ligands invokes the hypothesis that MHC restriction is directly imposed by the TCR signaling requirements for thymic selection (**Figure 1**).

After recalling fundamental aspects of early TCR signaling, we will review experimental evidence in favor of the selection model of MHC restriction.

LCK and TCR signaling

Lck is a member of the Src family of protein tyrosine kinases first identified in the 1980s and plays a crucial role in initiating the TCR signaling cascade (15, 16). Lck is critically important during T cell development and T cell activation. Germline Lck-deficient mice or immune-deficient patients with Lck mutations show profound T cell developmental defects (17, 18). The function of Lck and its conformational state are regulated by several tyrosine kinases and phosphatases acting on its phosphorylation status (19, 20). The phosphorylation of the activating tyrosine (Y394) in the catalytic domain results in an open conformation of Lck and therefore induces its kinase activity, whereas the phosphorylation of tyrosine (Y505) at the C-terminal domain is thought to induce a closed conformation and therefore inhibits Lck activity (21). Collectively, the activity of Lck is tightly regulated by a great number of biochemical modifications, conformational changes and signaling circuits. These complicated regulatory mechanisms highlight the importance of Lck in the initiation of the proximal signaling events downstream of the TCR and consequently, T cell

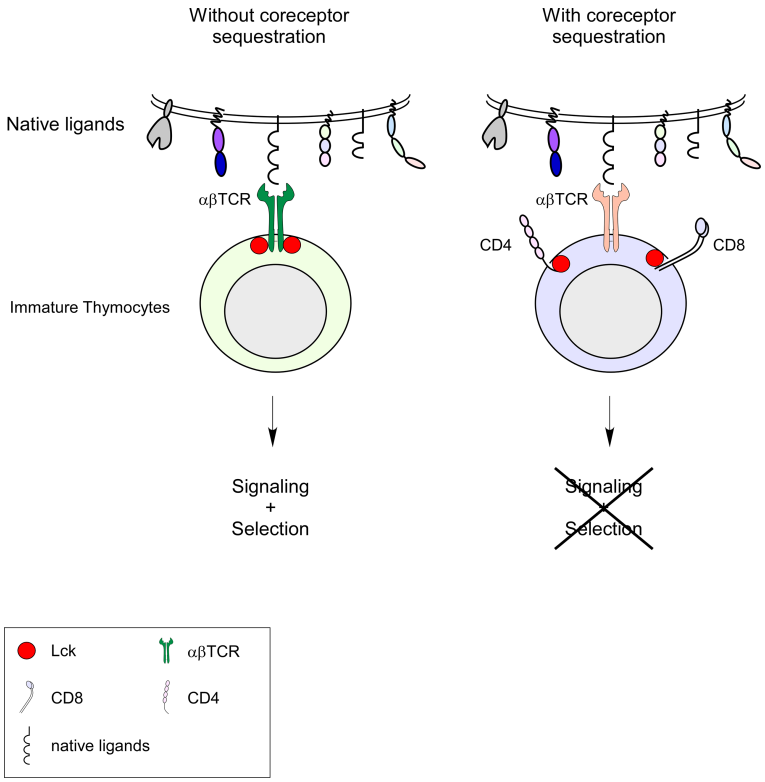

FIGURE 1
Selection model and MHC-independent αβT cell selection. In immature DP thymocytes, the protein kinase Lck is sequestered by the CD4 and CD8 coreceptors. In the presence of coreceptors (right side of panel), MHC-independent αβTCRs cannot receive any Lck-mediated signaling and therefore cannot be selected. In the absence of coreceptors (left side of the panel), MHC-independent αβTCRs have access to Lck (coreceptor-free and TCR-associated) and can be signaled and selected. Lck sequestration by CD4 and CD8 coreceptors ensures that only MHC-specific αβTCRs can be signaled and selected.

responses. It is therefore not surprising that the absence of Lck in both humans and murine models results in significant defects in immune functions whereas deregulation of Lck activity is often associated with cellular transformation. All these observations further emphasize the crucial role played by this kinase.

LCK and coreceptors

Lck binds to the coreceptors CD4 and CD8 *via* a cytoplasmic "zinc clasp" formed by the double cysteine motif found in the coreceptor tails and the cysteines in Lck's SH4 domain (22). The association of Lck with coreceptors is essential for coreceptor function as transgenic T cells or T cell hybridomas with truncated coreceptor tails, lacking the Lck binding domain, have diminished responses *in vitro* (23–31). How much Lck is physically bound to coreceptors is still debated and likely depends on the type of T cell studied (immature *vs.* mature, for example). Early studies using co-immunoprecipitation assays showed a significant fraction of cytoplasmic Lck bound to coreceptors (32, 33). However, more recent experiments showed much lower Lck to coreceptor occupancy, notably between 6 and 37% for CD4-Lck interactions in CD4+ single-positive (SP) cells (34, 35). Even lower occupancy values were found for CD8 SP cells and double-positive (DP) thymocytes. Targeting of Lck to membranes (plasma, vesicles, Golgi or ER membranes) is mediated by myristylation and palmitoylation modifications and preventing these modifications drastically impairs membrane targeting and TCR signaling (36). Consequently, an unknown amount of Lck is associated with plasma membranes versus internal membranes that do not contain coreceptors and these would appear in anti-coreceptor immunoprecipitates as "coreceptor-free' Lck. As a result, immunoprecipitation experiments invariably under-estimate the true fraction of coreceptor-associated Lck in plasma membranes. Even so, the majority of Lck in immature double

positive thymocytes is coreceptor-bound and that genetic knockdown of one the coreceptors leads to a dramatic increase in Lck associated with the remaining coreceptor (37). More precise biochemical or imaging techniques will be needed to settle the substantial discrepancies in Lck-coreceptor occupancy.

Selection model

The selection model proposes that the delivery of Lck by the coreceptors during thymic development is the critical factor in imposing MHC restriction. Coreceptors play two fundamental roles; first, their specificity for invariant regions on MHC molecules allows tethering of the TCR to MHC, and second, their association with Lck allows the delivery of this kinase to the TCR-pMHC complexes to initiate signal transduction. Because all available Lck is bound to coreceptors in immature thymocytes, TCRs can only be signaled if they engage the same pMHC complexes as the coreceptors (CD4 for pMHCII-TCR complexes and CD8 for pMHCI-TCR complexes). TCRs that are specific for non-MHC ligands would not be signaled because Lck would not be recruited. Our model emphasizes that the sequestration of Lck away from the TCRs by the coreceptors ensures that only MHC-restricted TCRs can signal and be selected in the thymus (**Figure 1**).

In vivo evidence of the selection model

To test the selection model, we generated several genetically manipulated mice. By disabling coreceptor-mediated Lck sequestration through germline deletion of both CD4 and CD8 coreceptors or by transgenic expression of a mutant Lck that cannot bind to coreceptors, we tested the hypothesis that non-MHC specific TCRs could signal in the thymus by "free" Lck and be positively selected to generate mature MHC-independent αβT cells. We called such mice Quad-KO mice since they are deficient for both CD4 and CD8 coreceptors and also lack MHC class I and II expression (38). Thymocytes in these mice were strongly signaled *in vivo* as shown by very high-level surface CD5 expression. Importantly we confirmed that MHC-independent signaling *in vivo* required the expression of αβTCR and Lck proteins as thymocytes deficient in TCRα, RAG2, pTα (unpublished data) and Lck showed reduced or absent CD5 upregulation (39). Furthermore, by forcing or preventing Lck sequestration through transgenic expression of wildtype or tailless CD4 proteins that encode either full-length CD4 or CD4 lacking the cytosolic tail, we confirmed that Lck sequestration significantly impairs TCR signaling in the absence of MHC (39). Importantly, deleting both coreceptors allowed the generation of mature αβT cells that were non-MHC specific (38). Notably, these TCRs had antibody-like properties in that they recognized conformational antigens with high affinity and in the absence of any antigen processing (40).

We have characterized in detail various MHC-independent αβTCRs isolated from Quad-KO mice. In our original studies, two of the Quad-KO TCRs recognized CD155, the mouse homolog of the poliovirus receptor, in its unprocessed form, independently of MHC and with affinities close to 200nM (40). These affinities are approximately 10- to 100-fold higher than conventional micromolar affinities of MHC-restricted TCRs (41, 42). As one of the TCRs we isolated used the same Vβ8 gene segments that contain germline-encoded residues and have been shown to contact MHC in crystal structures (43), we tested if the same residues were involved in non-MHC specific signaling and *in vivo* selection (39, 40). We found that the same germline-encoded CDR2 residues were also required for the thymic selection of the CD155-specific MHC-independent αβTCRs (39). These residues within the antigen-binding pocket are likely involved in contacting any protein, including, in this case, CD155. This result argues strongly against the model that these evolutionary conserved germline CDR residues enforce MHC binding (9, 44).

Selection of MHC-independent αβTCRS

Our *in vivo* experiments showed that thymic signaling by CD155-specific αβTCRs occurred in the absence of any MHC and coreceptors, demonstrating the presence of αβTCRs that do not require MHC for their selection. Surprisingly, both CD155-specific TCRs absolutely required the presence of intra-thymic CD155 to signal thymic positive selection (39). These observations sharply contrast with conventional MHC-restricted αβTCRs, which require very low affinity ligand engagements for positive selection and for which very few selecting ligands have been identified (45–48). Our studies were the first to show a loss of function for a positively selecting ligand for any given TCRs that induce positive selection (39). Interestingly, using a series of mixed bone-marrow chimaeras, we demonstrated that the selection of mature CD155-specific αβT cells was achieved by all thymic elements (radio-resistant and radio-sensitive cells) and correlated with the amount of CD155 expressed (39). Ligands expressed on lymphoid elements in the thymus have been shown to select innate-like T cells, cells that can be characterized by the expression of the transcription factors PLZF for NKT cells or Sox13 for γδ-lineage T cells (49–51). None of our CD155-selected T cells expressed either PLZF or Sox13, confirming that CD155-specific peripheral T cells were neither innate-like NKT cells nor γδ-lineage T cells (39). As a matter of fact, thymocyte differentiation and lineage specification occurred normally in Quad-KO mice, as evidenced by CD4 reporter or TCR transgenic mice in which CD4 and CD8 αβT cells

expressed the appropriate helper- and cytotoxic-lineage genes (38, 39). In our Quad-KO TCR transgenic mice, transgenic TCR expression occurs early at the DN stage and could have led to aberrant γδT cell differentiation. However, neither transgenic thymocytes nor peripheral Quad-KO αβT cells expressed specific γδT-lineage genes. Moreover, premature expression of a wildtype CD4 transgene, enabling CD4-mediated Lck sequestration at the DN stage, dramatically impaired positive selection (39). In summary, MHC-independent αβTCRs require *in vivo* expression of their cognate ligand for thymic selection, and they can be selected *in vivo* in the absence of coreceptors and MHC. This contrasts sharply with conventional MHC-restricted TCRs for which no defined *in vivo* ligands have been described to date and that MHC-restricted TCRs require coreceptor and MHC molecules for their selection.

Diversity of MHC-independent αβTCRS

It was surprising that our first described MHC-independent αβTCRs were all specific for the same adhesion molecule CD155 and that both engaged CD155 with such high affinity. We therefore decided to test if CD155 was the only ligand for MHC-independent TCRs and if high affinity ligand engagement were a general feature of MHC-independent TCRs (52). Our first observation showed that Quad-KO mice that also lacked CD155 had the same number of peripheral MHC-independent αβT cells as did CD155-sufficient Quad-KO control mice, demonstrating that, *in vivo*, CD155 was not the sole thymic selecting ligand. We isolated and fully characterized additional Quad-KO TCRs that displayed high-affinity recognition of cell surface antigens CD155, CD102, and CD48. These native self-proteins normally function as low-affinity cell adhesion molecules. Like CD155 recognition, these newly isolated Quad-KO αβTCRs bind to and can be signaled by native unprocessed CD102 and CD48 in the absence of MHC (52). We used T-cell specific transgenic expression for one of those TCRs (specific to mouse CD102) and showed that this TCR signaled *in vivo* selection in the absence of coreceptors and MHC. Importantly, like the previously described CD155-specific TCRs, thymic positive selection required the expression of the native self-ligand CD102 (52). It was surprising to find that all the ligands identified for our MHC-independent TCRs were involved in cell adhesion. One reason could be that adhesion proteins are generally highly expressed on thymic cells (thymocytes and epithelial cells), increasing the likelihood of productive selecting signals (53, 54). Moreover, we have previously observed that some molecules including adhesion molecules like CD155 are downregulated during T-hybridoma fusions (unpublished data). Downregulation of these molecules impairs the fratricide of T-hybridomas expressing TCRs with those ligand specificities and allows their recovery during T-hybridoma fusions.

Other naturally occurring MHC-independent TCRs have been described over the years. These αβTCRs were also obtained from mature T cells but showed lower affinities (55–59). Because of their low affinity, it has been argued that their non-MHC ligands might not be their primary specificities (9). However, these TCRs were obtained from mature αβT cells that had undergone MHC-specific thymic selection and may cross-react incidentally with MHC-independent ligands. In Quad-KO mice, MHC-independent TCRs were signaled and selected by self-ligands with much higher affinity than those observed by conventional MHC-restricted TCRs.

The presence of high-affinity self-reactive αβTCRs in Quad-KO mice raises the possibility that signaling with free Lck prevents efficient clonal deletion. However, the reactivity of Quad-KO T cells selected in the presence or absence of the anti-apoptotic transgenic Bcl-2 (Bcl-2^{Tg}) that is known to rescue deletion was identical to self and allogenic spleen stimulator cells. Irrespective of the transgenic Bcl-2^{Tg} expression, Quad-KO αβT cells were self-reactive as they proliferated in the presence of syngeneic (own Quad-KO) stimulator cells as well as against third party C57BL/6 and B10.A or BALB/c allogeneic splenic stimulator cells (52). We think that signaling by free Lck in the absence of coreceptor sequestration is inefficient in transducing high-affinity TCR signals to efficiently delete autoreactive thymocytes and prevent their emergence in the peripheral organs.

Repertoire analysis of quad-KO T mice

Positive selection in the absence of MHC requires high-affinity TCR-ligand engagement, which could strongly affect the self-reactivity and diversity of the mature αβTCR repertoire. To test this hypothesis and learn what molecular constraints distinguish MHC-independent and MHC-restricted repertoire selection, TCR repertoire sequences in pre-selection thymocytes, mature MHC-restricted αβT cells, and MHC-independent αβT cells from Quad-KO mice were compared (60). Interestingly, we found that molecular constraints are imposed on hypervariable CDR3 segments during thymic selection of conventional MHC-selected repertoires. The length and amino acid composition of CDR3 segments were the primary parameters distinguishing both MHC-restricted and MHC-independent TCR repertoires (60). CDR3 lengths are known to vary greatly among αβTCRs, γδTCRs cells and immunoglobulins (61). Indeed, whereas CDR3s of both IgH and TCRδ are more variable in size and are longer than those in IgL and TCRγ chains, TCRα and TCRβ have almost identical CDR3 length, which is usually shorter than that of γδTCR and immunoglobulins. Interestingly, these differences are in accordance with their profoundly different recognition properties and requirements, both γδTCR and immunoglobulins functioning independently of MHC-and are, therefore, not constrained by the

size of the MHC peptide binding groove. The conserved structure of peptide-MHC complexes limits the length of CDR3 on TCRs to favor shorter CDR3s, usually 8-13 amino-acids. TCRs with longer CDR3 structurally impair the contacts of their CDR1 and CDR2 with MHC. The position of the CDR3α and CDR3β at the center of the TCR-MHC contact interface requires the movement of the exterior CDR1 and CDR2 regions to accommodate longer CDR3s. In addition to peripheral MHC-independent TCRs, preselection thymocytes from normal MHC-expressing mice also contained TCRs with longer CDR3s, suggesting that MHC-dependent selection prevents the selection of TCRs with long CDR3s (60). Longer CDR3 could either signal MHC-specific clonal deletion or might fail to produce any MHC-specific signal and induce death by neglect. In fact, preventing clonal deletion in the thymus by introducing a Bcl-2 transgene did not result in the appearance of TCRs with longer CDR3 segments in the periphery MHC positive animals. Therefore, longer CDR3s does impair MHC binding and TCR signaling of MHC-specific positive selection in the thymus.

The usage of specific amino acids in CDR3s also puts some constraints on MHC-specific TCRs. For example, positively charged amino-acids (such as Lysine, Histidine or Arginine) were disfavored in CDR3 FGβ loops during MHC-restricted selection (60). The mature TCR repertoire is also controlled by clonal deletion, thereby eliminating TCRs with an excessive affinity for self-peptide/MHC ligands. Interestingly, we observed that clonal deletion during MHC-specific selection eliminated TCRs containing cysteines in their FG-loops (60). In fact, cysteines were present in 1-3% of TCRs in pre-selection and MHC-independent repertoires. Cysteines were, however, absent from mature MHC-restricted repertoires but were present in mice expressing the Bcl-2 transgene that prevents clonal deletion. Cysteines present in MHC-specific FG-loops would be crosslinked by MHC-presented peptides and induce clonal deletion (60). Interestingly, surface ligands recognized by MHC-independent TCRs do not contain free cysteines but rather have disulfide-linked cysteines (**Figure 2**). Such surface ligands would, therefore, not interact with the FG-loop cysteines from MHC-independent TCRs. Cysteines have a unique and critical role in protein function, structure, and stability. For extracellular and secreted proteins such as immunoglobulins, disulfide bonds formed between cysteine residues regulate protein scaffolding that allows proteins to maintain their three-dimensional structure (62, 63). Non-canonical cysteines can be found, although rarely, in immunoglobulins, even in the variable regions and are thought to participate in the generation of repertoire diversity (64) (**Figure 2**). Interestingly, Daley and colleagues found increased CDR3 cysteine usage in CD8αα intraepithelial T cells and their thymic precursors compared to regulatory T cells and conventional T cells (65). Thus, the presence of cysteine in the FG-loops serves as a TCR-intrinsic motif that could mark immature pre-selection thymocytes or some T cells with specific selection, such as the intraepithelial T cell or T cells selected by MHC-independent selection.

The comparison of TCR sequences from MHC-restricted and MHC-independent (both pre-selection cells and peripheral Quad-KO αβT cells) T cell populations allowed us to propose the important structural requirements of CDR3 for MHC-restricted and MHC-independent selection. The selection of MHC-independent TCRs seems to be largely unrestrained compared to the much more restrained selection of MHC-restricted TCRs. MHC restriction favors shorter than 13 amino-acids CDR3, prevents cysteine inclusion, and limits positively charged and hydrophobic amino acids in the CDR3β regions. The presence of conserved positively charged residues near CDR3β contact sites on both MHCI and MHCII molecules likely interferes with positively charged amino acids in the TCRβ sequences, inducing an electrostatic repulsion and preventing productive TCR-MHC interactions (60). Intriguingly, rare TCRs with longer CDR3α and multiple positively charged residues in CDR3β have been observed to bind MHC in a reversed orientation (66, 67). This reversed polarity could potentially be explained by the inability of the highly positively charged CDR3 FGβ loops and positive charges on MHC to form the canonical binding mode.

We also analyzed if thymic selection affected the usage of germline-encoded V- and J-genes and, their pairings. Interestingly, we observed similar frequencies of Vα-, Vβ-, Jα- and Jβ-genes between pre-selection, MHC-restricted and MHC-independent repertoires and animals of the same strain exhibited the highest similarities. The VJ pairing also showed similar frequencies among all groups (60). We concluded that neither V- and J-gene usages nor their pairing is significantly affected by thymic selection.

To assess the size of the TCR repertoire from Quad-KO mice, deep RNA sequencing of individual TCRα and TCRβ chains in Quad-KO mice was performed and compared with those from MHC-restricted wild-type strains. Importantly, wildtype mice had much greater repertoire overlap compared to Quad-KO mice. Common sequences were also shared among MHC-restricted strains but not among individual Quad-KO mice (60). We concluded that MHC-restricted repertoires show significantly higher sequence conservation than MHC-independent repertoires. The lack of shared sequences in the Quad-KO mice resembles that of antibody repertoires. Sequence diversities of the TCRα and TCRβ chains from MHC-restricted and MHC-independent TCRs were also analyzed. In fact, TCRs selected in the absence of MHC had dramatically lower (10- to 50-fold less) diversity compared to those from TCRs selected in the presence of MHC.

Overall, our repertoire analysis has shown that MHC-restriction severely constraints the length and composition of the hyper-variable CDR3 segments. In addition, positive

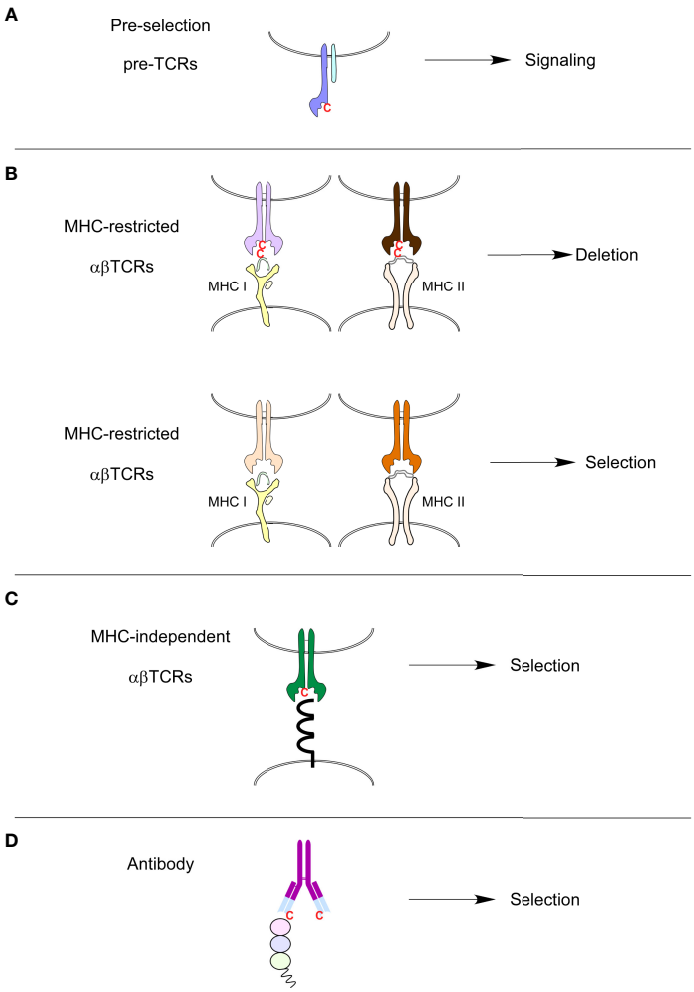

FIGURE 2
Presence or absence of cysteines in immune receptors. **(A)** Cysteines can be found in the FG loops of preselection TCRs. **(B)** MHC-restricted TCRs that contain cysteines in their FG loops are clonally deleted because cysteines will form disulfide-bonds with free cysteines in MHC-bound peptides present in the thymus. **(C)** MHC-independent ligands are extra-cellular proteins that very rarely possess free cysteines that could potentially link cysteines present in FG loops of MHC-independent TCRs. MHC-independent TCRs, just like antibodies **(D)**, do contain cysteines in their variable antigen-binding sites.

selection by high affinity TCR-ligand interactions, such as those observed for MHC-independent TCRs, has dramatic effects on TCR repertoire diversity. Therefore, the presence of coreceptors during thymic selection permits the selection of a great variety of diverse TCRs with low affinity to self-peptide/MHC complexes.

Structural analysis of MHC-independent αβTCRS

To gain further insight into the biophysical properties of MHC-independent ligand interactions, we generated the first crystal structures of two MHC-independent αβTCRs and described their conformational epitopes on their ligand (CD155). Both TCRs (A11 and B12A) showed very high binding affinity to their CD155 ligand (230-280nM), values much higher than those typically observed for typical TCR-MHC binding (40, 68). A V-domain single chain of one TCR was sufficient to bind to CD155 with a 400nM binding affinity, a value only slightly lower than that of the two V-C domains. The B12A V-domain alone is, therefore, sufficient to recognize CD155. The structures of both αβTCR A11 and B12A αβTCR heterodimers were nearly identical and exhibited canonical structures when superimposed on the structure of an MHC-

restricted TCR (68). Domain swapping experiment with murine and human CD155 revealed that both A11 and B12A TCRs required both D1 and D2 domains on CD155. This finding was confirmed using negative staining electron microscopy images of B12A TCR and murine CD155 and modeled in **Figure 3**. These images suggested that the CDR3s regions of B12A docked onto the D1 domain of CD155. Additionally, mutational experiments showed that A11 and B12A TCRs recognize two closely related but distinct epitopes on the D1 domain of CD155, a domain involved in binding to the poliovirus in humans (68). Interestingly, a third CD155-specific TCR (TCR 25) showed a different recognition motif (52), with domain swapping experiments for this TCR revealing that TCR-25 recognizes a novel epitope formed by all three external CD155 domains.

Structural experiments have provided evidence that some evolutionarily conserved residues (Y48 and E54) in the CDR2 region of Vβ8 TCR were necessary to engage MHC and impose MHC specificity on thymic selection (9, 43, 69). If these residues were conserved to engage MHC molecules, we would predict that such conserved CDR2 germline-encoded residues would not promote TCR selection by non-MHC ligands. However, we showed that the selection of an MHC-independent TCR containing Vβ8 also required the presence of the same conserved residues (39). These residues may have evolved for reasons unrelated to MHC binding but could possibly be involved in maintaining the integrity of the TCR combining site. It was also recently shown that an MHC-restricted TCR repertoire was still generated without the conserved germline-encoded CDR1 and CDR2 sequences (70). Dyson and colleagues replaced the TCRβ germline CDR1 and CDR2 regions with TCRγ chain CDRs (70). The resulting γβTCR hybrids paired with endogenous TCRα chains, provided efficient recognition of MHC and did not alter positive selection or CD4/CD8 lineage commitment. Receptors on γδT cells do not recognize MHC class I and II as natural ligands and, therefore, their germline encoded CDRs have not coevolved with MHC molecules. They concluded that T cell selection is not dependent on germline TCR structures and that the TCR can embrace antibody like strategies to engage MHC-peptide complexes. These observations were further confirmed by replacing the TCRβ germline CDRs with immunoglobulin (Ig) heavy and light chain germline CDRs, the resulting hybrid TCRs also led to the thymic selection of both CD4 and CD8 αβT cell repertoires (70). A novel population of naturally occurring T cells expressing a hybrid Vγ-Cβ TCR together with a TCRα has also been described (71). It suggests that the entire Vβ domain can be dispensable for MHC recognition. In summary, biophysical experiments have shown that, unlike conventional TCRs that only recognize peptide fragments complexed to MHC molecules, MHC-independent TCRs recognize a broad spectrum of conformational antigens. The combination of high-affinity binding and a variety of conformational antigens are typical characteristics of antibody recognition.

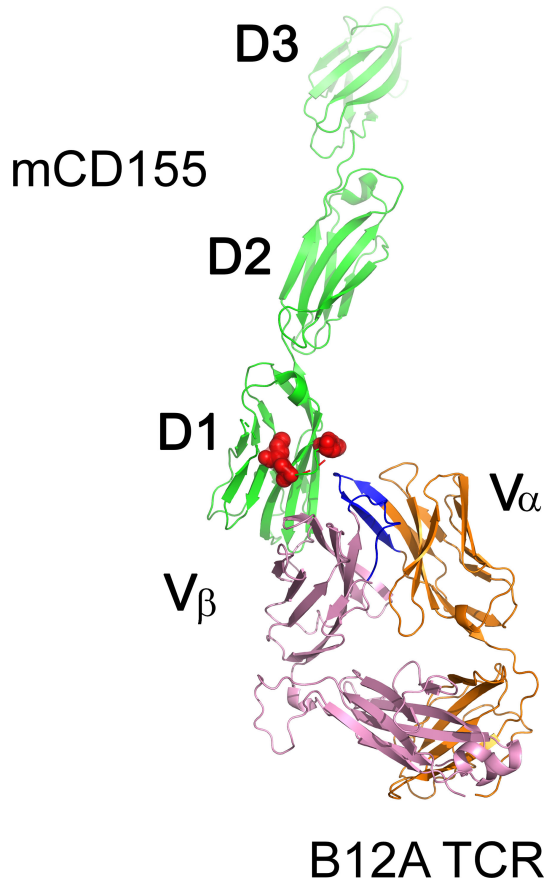

FIGURE 3
Surface representation of the MHC-independent B12A TCR with murine CD155. TCR B12A α β chains, and mCD155 are displayed as cartoons in orange, pink and green, respectively. CDR3 loops of TCR B12A are highlighted in blue and its binding epitopes on mCD155, revealed by mutagenesis studies, are highlighted as red surface.

Timing of coreceptor expression and LCK expression

Unconventional T cells such as mucosal-associated invariant T (MAIT) cells, natural killer T cells (NKT) and γδT cells are stimulated by lipid or metabolite antigens presented by monomorphic MHC-like molecules such as CD1 and MR1. Structural analyses have shown that the main characteristics of conventional TCR/MHC binding, namely the TCR conserved docking polarity of the TCR in which the TCR is placed over the peptide and simultaneously binds both MHC the peptide cargo, is also seen in unconventional TCR recognition of non-classical MHC molecules. Recognition of CD1 molecules, however, is

inconsistent as some TCRs bind only to CD1 and not the lipid antigen. Interestingly, γδT cells, known to interact with ligands independently of MHC, have also been shown to interact with CD1d with a conserved polarity and docking angle.

As mentioned above, γδTCRs are mostly MHC-independent and are selected in the thymus before the DP stage (72–74). This early selection before the DP stage allows them access to free Lck (**Figure 4**). In normal conditions, all TCRs that are signaled and selected in the thymus before Lck sequestration by the coreceptors, such as γδTCRs, are MHC-independent. Early CD4 transgenic expression at the DN stage dramatically impairs the generation of γδT cells (39). We therefore think that the timing of endogenous γδ and αβTCR expression is precisely adjusted. This timing has evolved to permit different TCR complexes to selectively access either coreceptor-free or coreceptor-associated Lck so that ligand recognition by γδTCRs would be MHC-independent and ligand recognition by αβTCR would be MHC-restricted (**Figure 4**). Therefore, the appearance of coreceptors and the subsequent sequestration of Lck at the DP stage prevents positive selection signaling by MHC-independent ligands.

Conclusions

A lot of information has been gathered since the first description of MHC restriction by Zinkernagel and Doherty more than 40 years ago (3). Recent experimental evidence supports both the germline-encoded and selection models, and both likely play a role in shaping an MHC-restricted TCR repertoire. The preselection repertoire may contain some proportion of MHC-biased TCRs but the requirement of Lck-coreceptor associations only permits and enhances the selection of a diverse but MHC-centric T cell repertoire. We think CD4 and CD8 play a central role in dictating the MHC specificity of the T cell repertoire and may have driven the co-evolution of αβTCRs with MHC. In other words, CD4 and CD8 coreceptors bestow the evolutionary pressure to skew germline TCR sequences toward MHC recognition. A better understanding of the biology of MHC-independent T cells will offer alternative therapeutic strategies, for example in immunotherapy. In conclusion, we think that MHC restriction of αβT cells is the consequence of thymic selection that imposes MHC-specificity

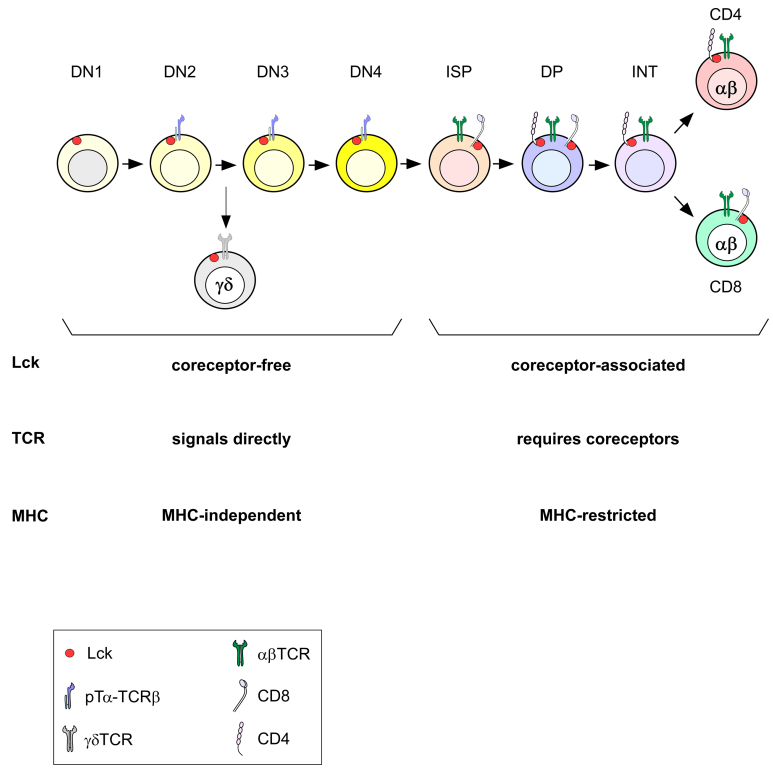

FIGURE 4
Timing of Lck expression during T cell development. During DN stages, Lck is necessarily coreceptor-independent (free). When coreceptors start being expressed at the DP stage, Lck becomes coreceptor-bound. In the absence of coreceptors at the DN stage, Lck can signal TCRs directly and signals independently of MHC (pre-TCR signaling at the DN to DP transition and during γδTCR selection). Once coreceptors sequester Lck, TCR signaling requires coreceptor engagement and is MHC-restricted.

by precisely timed expression of both CD4 and CD8 coreceptors on thymocytes.

Author contributions

FL prepared the initial draft. AB, MC, JL, PS and AS revised and finalized the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

Publisher's note

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Metabolic regulation of T cell development

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T cell development in the thymus is tightly controlled by complex regulatory mechanisms at multiple checkpoints. Currently, many studies have focused on the transcriptional and posttranslational control of the intrathymic journey of T-cell precursors. However, over the last few years, compelling evidence has highlighted cell metabolism as a critical regulator in this process. Different thymocyte subsets are directed by distinct metabolic pathways and signaling networks to match the specific functional requirements of the stage. Here, we epitomize these metabolic alterations during the development of a T cell and review several recent works that provide insights into equilibrating metabolic quiescence and activation programs. Ultimately, understanding the interplay between cellular metabolism and T cell developmental programs may offer an opportunity to selectively regulate T cell subset functions and to provide potential novel therapeutic approaches to modulate autoimmunity.

KEYWORDS

T cell development, thymocytes, T cell metabolism, thymus, thymocyte metabolism

Introduction

T cell development is tightly regulated by multiple checkpoints and proliferative events before the emergence of naive T cells from the thymus (1–3). Early thymic progenitors (ETPs), also known as double-negative 1 (DN1) cells, differentiate into DN2 thymocytes in the thymic parenchyma, gaining T-lineage commitment (4, 5). A paramount event in T cell development called β-selection occurs during the DN3 stage, and thymocytes that successfully assemble the pre-T cell receptor (TCR) hasten to the DN4 phase and initiate rapid cell cycling governed by complex regulatory metabolism (6, 7). Additionally, γδ and αβ T cell lineages diverge at the DN3 stage (8, 9). Thymocytes return to a quiescence state in double-positive (DP) stage, undergoing

positive/negative selection and DP to single-positive (SP) transition before becoming mature CD4+ or CD8+ T cells (3, 10, 11).

Cellular metabolism integrates multiple pathways and large networks of chemical reactions, and plays a critical role in regulating almost all cellular processes (12). Different thymocyte subsets have distinct metabolic patterns tailored to meet the bioenergetic demands required at each stage (13–15). In summary, catabolic metabolism of amino acids and glucose promotes the energy and biosynthesis that quiescent thymocytes require, whereas the transition from resting cells into highly activated phenotypes requires substantial metabolic reprogramming comprising aerobic glycolysis (Warburg effect), glutaminolysis, and mitochondrial biogenesis, which expedites oxidative phosphorylation (OXPHOS) and one-carbon metabolism (10, 15–17). These metabolic alterations constitute complex signaling mechanisms that connect external signals with transcriptional events and fate verdicts (17, 18). Here, we summarize recent findings on the metabolic control of T cell development, and highlight the roles of cell-intrinsic and cell-extrinsic metabolic factors involved in these processes.

Thymocytes are relatively quiescent before β-selection

ETPs settling in the thymus are quiescent before the first run of proliferation (11). Cytokines, such as Interleukin-7 (IL-7), Kit, and C-X-C motif chemokine receptor 4 (CXCR4), as well as Notch and Wnt signaling, account for the proliferation of thymocytes before β-selection (6, 19–29). During the four DN stages, Notch and IL-7 signaling drive the maturation of thymocytes and regulate cellular metabolism by interacting with the phosphatidylinositol-3-kinase/protein kinase B/mammalian target of rapamycin (PI3K/Akt/mTOR) signaling pathway (18, 21, 30–38). Before accelerating the multiplication at the DN3b stage, thymocytes are quiescent and primarily rely on OXPHOS to maintain bioenergetic homeostasis. AMP-activated protein kinase (AMPK) is a major regulator of metabolism that senses bioenergetic undulation and maintains energy homeostasis in cells (39–41). AMPK works in concert with liver kinase B1 (LKB1) to suppress biosynthesis and energy production, such as glycolysis and lipid synthesis, to restrain the anabolic growth of thymocytes (41–43). Loss of LKB1 leads to thymocyte developmental block and reduction of peripheral T cells (44, 45). Fatty acid-binding protein 3 (FABP3) regulates cellular lipid metabolism by binding to polyunsaturated fatty acids (PUFAs), such as ω-6 PUFAs. Recently, it has been shown that loss of FABP3 redirects DN2 thymocytes to pathogenic Vγ4+ γδ T cells (46). Mitochondrial metabolism also plays a role in thymocyte development. For example, the Janus mitochondrial protein apoptosis-inducing factor (AIF) has been shown to affect thymocyte transitions from DN1 to DN4 by modulating mitochondrial metabolism (47).

β-selected thymocytes exhibit robust cell proliferation and metabolic reprogramming

Transition from the DN3 to the immature single-positive (ISP) phase, thymocytes undergo V(D)J rearrangement and β-selection. This exclusive selection event then gives rise to robust cell growth and proliferation in which cells have to thoroughly alter their metabolism to meet the increased energy demand. Energy generation by cycling thymocytes after β-selection is largely dependent on aerobic glycolytic metabolism for prioritizing efficient and rapid biosynthesis of intracellular constituents, including nucleic acids and lipids (3, 11). The metabolic switch is mainly triggered by pre-TCR signaling (6, 48–50). Signaling of pre-TCR, Notch, and IL-7 converges to activate PI3K signaling, which stimulates the transition to anabolic metabolism, especially glycolysis. Glucose transporter type 1 (Glut1), an important glucose transporter, is induced at these phases, and its expression level is dependent on the activation of PI3K-Akt signaling (51–54).

The PI3K-phosphoinositol-dependent protein kinase-1 (PDK1)-Akt axis plays a critical role in thymocyte maturation (55, 56). In addition to their well-described role in protein synthesis *via* mTOR signaling, PI3K dominates aerobic glycolysis and glucose metabolism in a variety of biological processes (57, 58). PDK1 regulates the expression of key amino acid and iron transporters and controls the switch of glucose metabolism from aerobic oxidation to glycolysis in the thymus (55, 59). Loss of PDK1 impairs nutrient receptor expression and hence renders metabolically deficient to meet the energy demands from the DN to DP stage transition (55, 60). Furthermore, Akt signaling is a major stimulus of anabolism (21, 61, 62). Deletion of Akt alters thymocyte subsets with a development blocked after DN3 stage (56, 63). Similar to AKT, PIM kinases are also linchpins that regulate the expansion of thymocytes undergoing β-selection (64–66). Of note, the lipid kinase inositol-trisphosphate 3-kinase B (Itpkb) affects β-selection by restricting metabolic activation in DN3 thymocytes. The deficiency of Itpkb leads to accelerated development from DN3 cells to DP cells by activating Akt-mTOR signaling and breaking the balance between Notch and pre-TCR signaling (67).

As a master regulator of cell growth and metabolism, mTOR regulates multiple metabolic pathways, such as glutaminolysis, glycolysis, mitochondrial biogenesis, and protein synthesis (68, 69). It has been shown that mTOR signaling regulates thymocyte proliferation, anabolism, and development *via*

integrated signals from TCRs, costimulatory molecules, cytokines, and nutrients (70–72). mTOR forms two structurally and functionally distinct complexes, mTOR complex 1 (mTORC1) and mTORC2 (71). Specifically, mTORC1 could integrate TCR and Notch signaling and induce the expression of transcription factors such as cellular myelocytomatosis oncogene (c-Myc) and Sterol-regulatory element binding proteins (SREBPs) for lipid synthesis and ROS production (71, 73). It has been reported that mTORC1 is involved in the reciprocal development of two fundamentally distinct T cell lineages, αβ and γδ T thymocytes (70). Loss of Raptor-mediated mTORC1 activity impairs the development of αβ T cells but promotes γδ T cell generation (70, 74). In addition, hypoxia-inducible factor 1-α (HIF1α) induced by mTORC1 signaling could increase glycolysis metabolism and the pentose phosphate pathway by controlling the production of glycolytic cascade members such as hexokinase 2 (HK2) and PDK1 (75–78). On the other hand, mTORC2 regulates glycolysis by activating Akt (61, 63, 79). Deletion of the mTORC2 component Rictor leads to thymocyte developmental blocks at the ISP phase, resulting in a reduction of DP cells (80, 81). Thymocyte specific ablation of Sin1, an important component of mTORC2, leads to developmental block at the DN3 to DN4 transition due to impaired proliferation and reduced expression of the glycolytic enzyme pyruvate kinase M2 (PKM2) through mTORC2-peroxisome proliferator-activated receptor γ (PPAR-γ)-PKM2 axes (82, 83). Taken together, the distinct regulation of thymocyte metabolism between mTORC1 and mTORC2 warrants further biological validation.

Myc expression is transcriptionally induced in thymocytes to facilitate the developmental progression from the DN to the DP stage (84–87). As Myc downstream target genes, the thioredoxin-1 (Trx1) system is a biosensor of glucose and energy metabolism that maintains cellular redox balance. Recent data from an animal study have shown that deletion of thioredoxin reductase-1 (Txnrd1), which is critical for the last step of nucleotide biosynthesis, precludes the expansion of DN cells (88). c-Myc gene expression is also regulated by bromodomain protein 4 (BRD4), which is a transcriptional and epigenetic regulator with functions throughout the cell cycle for proliferative regulation (89–93). It has been shown that BRD4 governs the development and differentiation of ISP thymocytes by modulating metabolic pathways and cell cycle progression (94). Deletion of BRD4 in ISP cells leads to a block in the transition to the DP phase, as well as inhibition of glycolysis (94). The mitochondrial protein Optic atrophy 1 (Opa1) was demonstrated to regulate OXPHOS and was required for thymocyte development during β-selection at the DN3 stage (37). The absence of Opa1 damages cellular respiration and induces apoptotic cell death. Mothe-Satney and collaborators found that overexpression of the transcription factor peroxisome proliferator-activated receptor β (PPARβ) increases fatty acid oxidation instead of glucose oxidation, hence restricting the expansion of DN4 cells (95). Furthermore, Zhao et al. demonstrated that PPARβ regulates the expression of the key genes and enzymes in glycolysis, oxidative phosphorylation, and lipogenesis in β-selected thymocytes. PPARβ^{mut} mice exhibited a reduction in thymocyte cell number starting at the DN4 stage (96). Recent laboratory work demonstrated that depletion of mitochondrial pyruvate carrier (MPC) 1, an MPC transporter subunit responsible for moving pyruvate into mitochondria, led to impaired β-selection and decreased αβT cells due to upregulated glycolysis and reduced OXPHOS (97). MicroRNAs and other noncoding RNAs have also been documented to be involved in T cell development as regulators of anabolism and catabolism (98–103). For example, as an important regulator of PI3K, miR-181 has been shown to regulate T cell development, including conventional and unconventional T cells (104–108). The transition from the DN to DP stage was severely impaired in miR-181-deficient mice (109). Single-cell RNA data showed that key genes of the glycolytic, pentose phosphate, and nucleotide biosynthetic pathways were deregulated in miR-181a1b1-deficient DP cells (104). Furthermore, miR-146a, a suppressor of nuclear factor-kappa B (NF-κB) signaling, participates in the regulation of thymocyte positive selection and amino acid metabolism (110).

Metabolic programming has been proposed to govern distinct immune cell lineages and functions, whereas γδ T cell metabolism is still poorly understood (70, 111). γδ T cells express mature γδTCR complex and undergo extensive proliferation comparable to αβ T cells (112–114). Unlike conventional T cells that exit the thymus as naïve T cells and further differentiate in peripheral organs upon activation, a large portion of γδ T cells commits to producing either IL-17 or IFN-γ during the development in the thymus, called the IL-17-producing γδ T cell (γδT17) and the IFN-γ-producing γδ T cell (γδT1), respectively (115–117). The two have intrinsically different metabolic requirements. Notably, the TCRγδ signaling appears to be more favorable for the metabolic transition of thymic γδ progenitors to γδT1 cells that are highly glycolytic. In contrast, γδT17 mainly engages OXPHOS (118). The metabolic dichotomy established in the thymus has a significant impact on the expansion and function of γδT1/17 cells, which could be used in tumor and autoimmune disease therapies (118–123). Yang et al. demonstrated that both γδT1 and γδT17 cells require mTORC1 for proliferation and survival, whereas mTORC2 is only essential for γδT17 cells. In Raptor KO mice, γδT17 differentiation was impaired due to suppressed glycolysis. In contrast, mTORC2 potentiated γδT17 induction by reducing mitochondrial ROS production (122). Moreover, nitric oxide synthase 2 (NOS2) deficient mice exhibited substantially reduced glycolysis and proliferation of γδ T cells (124). Glutaminase 1-mediated glutaminolysis was aberrantly activated and promoted γδT17 differentiation, thereby resulting in the development and immune imbalance of psoriasis (125). A recent study revealed a key role of c-Maf in regulating the function of γδT17 effectors through IDH2-mediated metabolic reprogramming (126). In conclusion, thymocyte development is orchestrated by these key metabolic regulators in the thymus.

Thymocytes return to a resting state from the small DP stage to CD4 or CD8 SP stage

After massive proliferation, DP thymocytes return to a resting state and initiate rearrangement of the Tcra gene for positive and negative selection. When quiescence occurs along a continuum as thymocytes differentiate from DP cells into SP cells, glucose metabolism must drastically decrease and revert to mitochondrial oxidative metabolism for maximal ATP generation (10, 127). Key metabolic regulators, such as c-Myc and HIF1α, need to be downregulated to safeguard the transition from the DP blast to the resting small DP stage (77, 84, 128). Moreover, the rise in metabolic activity in SP cells after the DP late stage could be due to functional TCR signaling that promotes the restoration of sensitivity to cytokines that fine-tune cellular metabolism (129, 130).

Although apoptosis of those DP cells that fail to be selected is crucial for thymocyte maturation program, it is also essential for pre-selection thymocytes to maintain a relatively low metabolic state to survive long enough waiting to be tested for their responses to self-peptide/MHC. Retinoid-related orphan nuclear receptor γt (RORγt) belongs to the nuclear hormone receptor superfamily of transcription factors and serves as a signaling node to connect lipid metabolism, inflammation, and immune cell responses (131). It has been demonstrated that RORγt expression reduces the abundance of cytokine receptors of the common chain (γc) and suppresses cellular metabolism and mitochondrial biogenesis in preselection DP thymocytes. DP thymocytes lacking RORγt exhibit features identical to persistent T cell expansion (132). O-linked β-N-acetylglucosamine protein O-GlcNAcylation shows a concomitant dynamic regulation with consecutive phases of T cell development and is controlled by Notch, c-Myc, and the T cell antigen receptors (133). O-GlcNAc transferase (OGT) acts as a signaling hub to integrate thymocyte responses to developmental stimuli through modifying changes in glucose and glutamine supply (134). An interesting finding has been shown that $Ogt^{-/-}CD4^{cre/+}$ mice had normal numbers of DP thymocytes but failed to differentiate into mature CD4+ or CD8 + SP thymocytes (134, 135). Pyruvate dehydrogenase (PDH) is required for thymocytes to regulate metabolic processes such as the tricarboxylic acid (TCA) cycle, redox homeostasis, glutathione levels, and pyruvate accumulation (136–138). Thymocytes can oxidize more glucose in the TCA cycle through PDH, and loss of PDH decreases the number of DP cells (139). Heme (iron-protoporphyrin IX) is an essential cofactor and signaling molecule involved in a vast array of biological processes, including cellular metabolism (140). Philip et al. reported a surprising finding that feline leukemia virus subgroup C receptor (FLVCR), the major facilitator superfamily (MFS) metabolite transporter and the heme exporter, was required for thymocyte development beyond the DP stage by supporting heme metabolism (141, 142). With FLVCR deletion during the DN stage, mice had a complete block in αβ T cell development at the DN-DP transition, whereas loss of FLVCR at the DP stage affects peripheral T cell proliferation and apoptosis (143).

THEMIS, a T cell specific protein that is highly expressed in DP cells, directly regulates the catalytic activity of SHP-1 by promoting ROS-mediated oxidation of the SHP-1 active site cysteine to facilitate thymic positive selection (144). N-linked glycosylation (NLG) also has an important impact on thymocyte selection. On the one hand, NLG negatively regulates the activity of high-affinity TCR, allowing thymocytes with these receptors to survive during negative selection. On the other hand, NLG increases expression of the CD4/CD8 co-receptors, allowing thymocytes with low-affinity TCR to survive during positive selection (145, 146).

Thymocyte egress is a crucial determinant factor of T cell homeostasis and adaptive immunity (147). Recently, protein geranylgeranyltransferase type I catalytic β-subunit (Pggt1b) has been shown to be involved in thymocyte egress by maintaining mevalonate metabolism–fueled posttranslational modification. Du et al. demonstrated that the expression of Pggt1b was upregulated in SP cells in comparison with DP cells, and deletion of Pggt1b impaired thymocyte egress, resulting in severe peripheral T cell lymphopenia but the accumulation of mature SP thymocytes in the thymus (148). Ultimately, CD4+ or CD8+ single-positive cells exit the thymus and circulate to peripheral organs, such as the spleen and lymph node.

The development of invariant natural killer T (iNKT) cells is more sensitive to changes in mitochondrial electron transport chain function than conventional αβT cells (149). There have been several informative reviews recently, so we won't go over those details here (150–152).

Conclusion and perspective

In the past few years, extensive studies have largely focused on the metabolic regulation of T cell differentiation and responses. However, our knowledge of how cellular metabolism modulates thymocyte maturation in response to developmental signaling pathways and microenvironmental cues remains limited. Emerging studies using gene knockout mouse models have demonstrated that key metabolic regulators and enzymes are involved in different stages of thymocyte maturation by modulating the metabolic pathways and signaling networks to match the specific functional requirements of the stage (Figures 1 and 2). Recent progress in single-cell metabolomics, CRISPR/Cas9, and spatially resolved metabolomics will continue to add valuable findings to this field (10, 153). Future work on the molecular mechanisms of cell context-dependent regulation of these metabolic processes will not only enhance our understanding of the interplay between cellular metabolism and T cell developmental programs

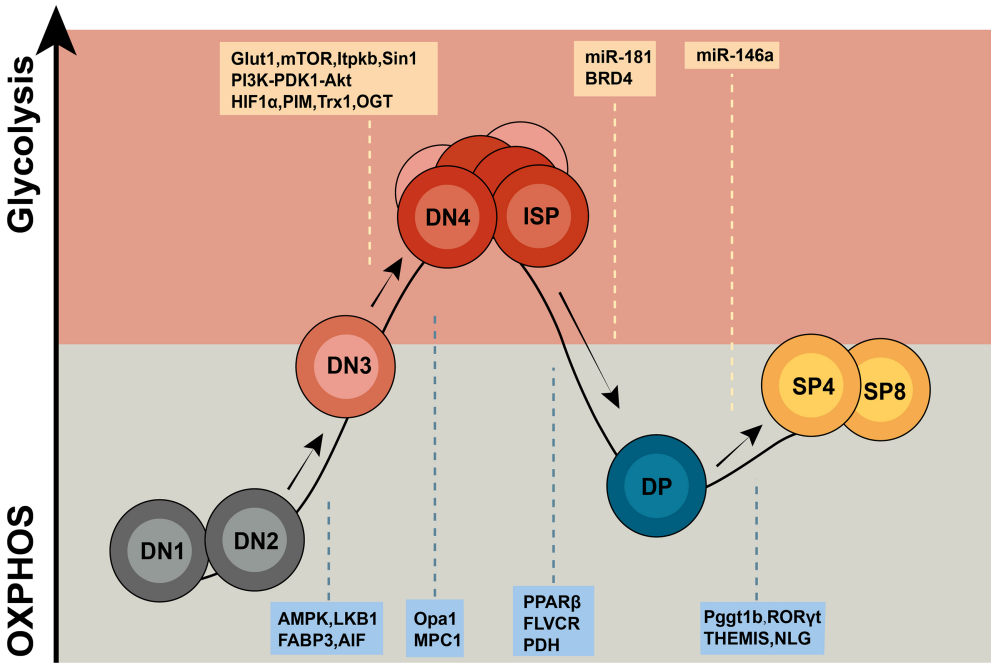

FIGURE 1
Overview of metabolic regulators in T cell development. Thymocytes display distinct metabolic profiles depending upon their states of development. DN1 and DN2 T cells are metabolically quiescent and adopt a basal level of nutritional intake, relying on OXPHOS as the primary approach of ATP production. Upon proliferation, T cells from the DN3b stage to early DP stage shift to a state of metabolic activation characterized by incremental nutrient uptake and elevated glycolysis. Then, T cells return to a resting state from the small DP stage to CD4 +/CD8+ SP stage. The letters in the yellow box represent glycolysis regulators, and the letters in the blue box represent OXPHOS regulators.

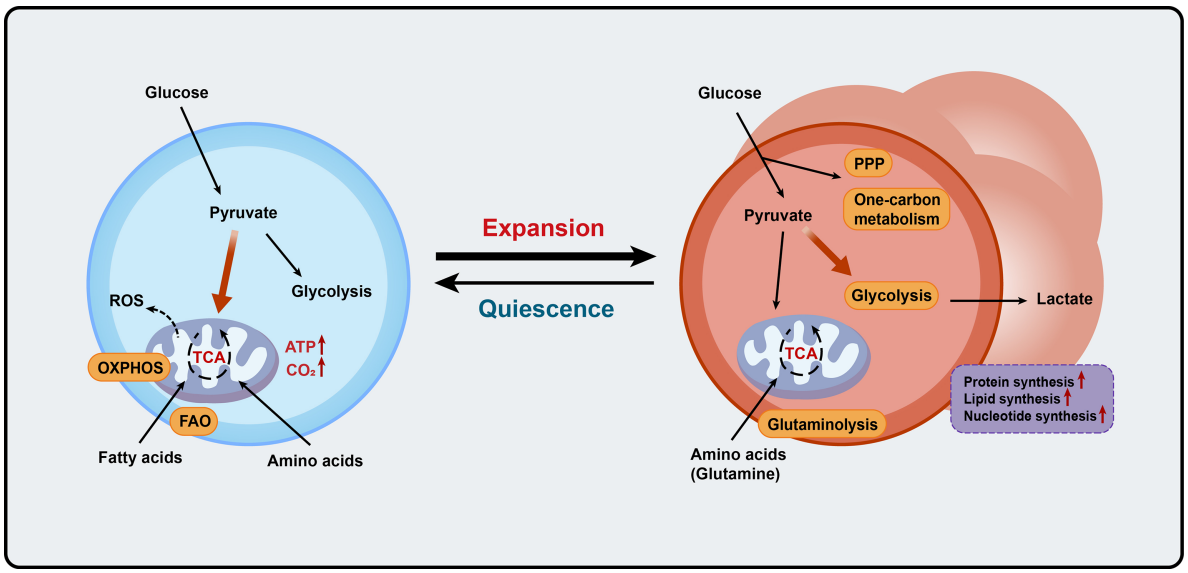

FIGURE 2
Metabolic programs match expansion demands of thymocytes. Blue cells on the left represent the quiescent thymocytes, and red cells on the right represent proliferative thymocytes. OXPHOS, oxidative phosphorylation; FAO, fatty acid oxidation; ROS, reactive oxygen species; ATP, adenosine triphosphate; PPP, pentose phosphate pathway; TCA, the tricarboxylic acid.

but also provide potential novel therapeutic strategies to modulate immune responses.

Author contributions

JH and BZ: conceptualization and guidance. MZ wrote and edited the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

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Investigation of the causal etiology in a patient with T-B+NK+ immunodeficiency

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Newborn screening for severe combined immunodeficiency (SCID) has not only accelerated diagnosis and improved treatment for affected infants, but also led to identification of novel genes required for human T cell development. A male proband had SCID newborn screening showing very low T cell receptor excision circles (TRECs), a biomarker for thymic output of nascent T cells. He had persistent profound T lymphopenia, but normal numbers of B and natural killer (NK) cells. Despite an allogeneic hematopoietic stem cell transplant from his brother, he failed to develop normal T cells. Targeted resequencing excluded known SCID genes; however, whole exome sequencing (WES) of the proband and parents revealed a maternally inherited X-linked missense mutation in *MED14* (*MED14*^{V763A}), a component of the mediator complex. Morpholino (MO)-mediated loss of MED14 function attenuated T cell development in zebrafish. Moreover, this arrest was rescued by ectopic expression of cDNA encoding the wild type human *MED14* ortholog, but not by *MED14*^{V763A}, suggesting that the variant impaired MED14 function. Modeling of the equivalent mutation in mouse (*Med14*^{V769A}) did not disrupt T cell development at baseline. However, repopulation of peripheral T cells upon competitive bone marrow transplantation was compromised, consistent with the incomplete T cell reconstitution experienced by the proband upon transplantation with bone marrow from his healthy male sibling, who was found to have the same *MED14*^{V763A} variant. Suspecting that the variable phenotypic expression between the siblings was influenced by further

mutation(s), we sought to identify genetic variants present only in the affected proband. Indeed, WES revealed a mutation in the L1 cell adhesion molecule *(L1CAM^{Q498H})*; however, introducing that mutation *in vivo* in mice did not disrupt T cell development. Consequently, immunodeficiency in the proband may depend upon additional, unidentified gene variants.

KEYWORDS

immunodeficiency, newborn screening, zebrafish, thymus, MED14, T cell lymphopenia, severe combined immunodeficiency (SCID)

Introduction

Primary immune deficiencies are rare, with severe combined immunodeficiency (SCID) occurring approximately 1/66,000 live births in the United States (1). SCID is defined as the absence of T lymphocytes and absent or nonfunctional B lymphocytes (2). Historically, SCID was diagnosed when patients manifested life-threatening infections in the first few months of life (3); however, in 2005, a newborn screening approach was developed that enabled reliable identification of patients with SCID prior to the onset of infections (4). The newborn screening assay measures the presence of T cell receptor excision circles (TRECs) in dried blood spots from peripheral blood. TRECs are a biproduct of T cell receptor (TCR) gene rearrangement and constitute a biomarker for normal T cell development in the thymus. TREC-based newborn screening, now adopted throughout the United States and several countries, afforded two significant benefits. First, earlier diagnosis of SCID enables the initiation of treatment prior to the onset of infection, thereby markedly increasing treatment efficacy (5, 6). Second, TREC screening has facilitated efforts to establish the molecular etiology of T cell lymphopenic conditions, leading to identification of a number of novel regulators of T cell development (7–10). Specifically, identifying the causative mutation in a T lymphopenic patient entails the targeted resequencing of known immunodeficiency genes to determine if disease results from a mutation in a known gene. Upon exclusion of known causes, whole exome sequencing (WES) is performed on the patient and parents to identify candidate variants, which then must be functionally studied to identify the causal variant. The zebrafish model is useful to evaluate human candidate variants, having high conservation of genes and processes controlling hematopoiesis and immune cell development (11) and ease of genetic manipulation through direct injection of embryos (12).

Here we describe a male proband identified by newborn screening as having low TRECs and reduced T lymphocytes. After exclusion of known causes of immunodeficiency, WES revealed a missense mutation in a component of the Mediator Complex, *MED14*, which is inherited in an X-linked manner. The multiprotein mediator complex is required for gene transcription by RNA polymerase II, and has been shown to influence epigenetic regulation, transcriptional elongation, termination, mRNA processing, noncoding RNA activation, and super-enhancer formation, making it a critical regulator of development and lineage determination (13, 14). MED14 functions as a backbone of the complex, and loss of MED14 is lethal (15, 16). Functional screening of the patient MED14 variant suggested that it may have contributed to the patient's disease.

Materials and methods

Human subjects and genomic analysis

Immunodeficiency was identified in the male proband by routine newborn SCID screening of a blood filter card (17). Research activities were performed with parental informed consent under protocols approved by the institutional review boards (IRBs) at the University of California, San Francisco and National Heart, Lung, and Blood Institute (NHLBI), National Institutes of Health (NIH), Bethesda, MD. Research-based WES was performed on cells from the patient and parents with bioinformatic analysis and variant calling as described (7). Additional WES was performed using genomic DNA (gDNA) from EBV lines derived from the patient and his parents and PBMC from his healthy brother. Briefly, gDNA (3 µg) was fragmented by sonication to generate 100-500 bp fragments. The DNA fragments were then end-repaired, 3' dA overhangs were added, and adaptors were ligated per the manufacturer's instructions. After removing free adaptors using Agencourt AMPure XP beads, the DNA fragments were amplified by 6 cycles of PCR. The exons and UTRs were enriched using Exon V4 plus UTR (SureSelect^{XT} Target Enrichment System for Illumina, Agilent Technologies); the enriched DNA was additionally amplified by 12 cycles of PCR, and 250-400 bp fragments were purified by 2% E-gel (ThermoFisher) and Gel

Purification Kit (Zymo Research) and sequenced on a HiSeq platform (Illumina). Sequencing reads were mapped to hg19 using Bowtie2 and BWA using default parameters. Aligned reads were piled up by Samtools Mpileup using the following parameters: (-A -B -Q 30 -q 20 -d 10000 -L 1000 -h 50 -o 10 -e 17 -m 3). The output was converted to VCF file using Bcftools (view -vcg) and was converted to Annovar format for annotation using Convert2annovar.pl. All common variants (AF>0.0001) in the ExAC database were filtered. Remaining variants were annotated using Annovar, and non-exonic variants were removed. Nonsynonymous variants were categorized as X-linked, de-novo, or autosomal recessive (AR). X-linked variants hemizygous in the proband and heterozygous or absent in the mother were examined, as were autosomal de-novo variants absent in either parent and AR variants that were heterozygous in each parent, heterozygous or absent in the unaffected sibling, but homozygous or compound heterozygous in the proband.

Animals

Tuebingen long fin zebrafish were maintained at 28.5°C under standard aquaculture conditions. Animal housing and handling were all performed in accordance with the approved protocols from the Fox Chase Cancer Center Institutional Animal Care and Use Committee (IACUC). Likewise, mouse experiments were performed under the auspices of IACUC-approved animal protocols, and all mouse strains were housed in accredited facilities at either Fox Chase Cancer Center or NIH. All experiments using mice at NHLBI were performed using protocols approved by the NHLBI Animal Care and Use Committee and followed NIH guidelines for use of animals in intramural research.

Ortholog analysis

Genomic sequences were obtained by searching the NCBI and ENSEMBL databases. Multiple alignments of human, mouse and zebrafish MED14 and SMARCAL1 amino acid sequences were obtained using Clustal X. Clustal X was also used for a multiple species alignment of human, mouse, rat, bovine, frog, zebrafish, worm and fly sequences.

Structural modeling

To assess the extent to which the V763A MED14 patient variant alters MED14 structure, we performed structural modeling by surveying all PDB structures that contain MED14, including the following PDB codes: 7EMF, 7ENA, 7ENC, 7ENJ (18), 7LBM (19), and 7NVR (representative of 9 other companion structures from the same paper) (20). The PDB structure code 7ENA (MED14 is author chain n) was chosen as the representative structure. The topology and overall conformation of amino acids 637-884 of the protein were conserved for all the structures examined, making it suitable for a computational analysis of the missense change V763A. In the MED14 fragment containing amino acids 637-884, the Valine at position 763 was substituted with Alanine using a backbone dependent rotamer library employed in the UCSF Chimera 1.15 software package (21, 22). Both the wildtype and V763A versions of this MED14 fragment were subjected to two different protein relaxation methods using the Rosetta molecular modeling suite to optimize side chain packing and to obtain an energy score that would reflect the stability of the V763A variant compared to wildtype MED14 (23). Either a coordinate-constrained method of relaxation or a full atom relax were employed alone or in combination, to allow for backbone movement in addition to side chain packing steps (24, 25). The stability of the V763A variant compared to wildtype was assessed by the All-Atom Rosetta energy scoring function of the conserved fragment from amino acids 637 to 884 (26). The same modeling analysis was also performed on the murine equivalent (V769A) to the human V763A MED14 variant. As there were two chain breaks in the coordinates of the mouse MED14 in the PDB entry 6W1S (chain I), we submitted the equivalent fragment of mouse MED14 (residues 643 to 890) to the ColabFold advanced version python notebook (27).

Zebrafish experiments

The zebrafish orthologs of candidate patient variants, MED14 (*med14*; NM_212765.2) and SMARCAL1 (*smarcal1*; NM_001127466.1), were identified by homology and synteny as described (28). We designed and obtained antisense morpholino (MO) oligonucleotides to block the pre-mRNA splicing of zebrafish *med14* and *smarcal1* from Gene Tools (Table 1). MO dose was established by injecting titrated quantities of MO into one-cell zebrafish embryos, following which MO efficacy was assessed by reverse-transcriptase (RT)–PCR as described using the indicated primers (Table 1) (12, 29). The effect of MO knockdown on T cell development was assessed by whole mount *in situ* hybridization (WISH) as described (30), using the following probes: *lck, ikaros, tcrd* and *foxn1* (28, 31). The stained embryos were photographed using a Nikon SMZ1500 stereomicroscope equipped with DS-Fi1 digital camera and Nikon Ar imaging software. Image J software was used to measure integrated staining density of zebrafish thymi. Experiments to assess the capacity of wild type and patient variant MED14 (V763A) to rescue the arrest of T cell development caused by MO depletion of endogenous *med14* comprised heat-inducible re-expression as described (7). Wild type and patient variant (V763A) human *MED14* constructs

TABLE 1 Oligonucleotides used in this study.

Zebrafish *med14* MO	ACTGGGAGATAAATCACATACCGCA
Zebrafish *smarcal1 MO*	GCTGAGTCTGTAAAGATGAGCATAA
Zebrafish *med14* RT-PCR-Fwd	GATGAAATCGCTTCCGCTG
Zebrafish *med14* RT-PCR-Rev	TTGACTCGTCCATTGGCCAC
Zebrafish *smarcal1* RT-PCR-Fwd	TTGTGTCAGTAAGCGCCTGT
Zebrafish *smarcal1* RT-PCR-Rev	CATCCCTTCCAGAGGTTTGA
Zebrafish *actb2* RT-PCR-Fwd	TGGCATCACACCTTCTAC
Zebrafish *actb2* RT-PCR-Rev	AGACCATCACCAGAGTCC
Mouse *Med14* V769A sgRNA	UUGAAAUGUUUCUUAATGAC
Mouse V769A mutant sgRNA binding site HDR donor oligo1	ACCATCCCGACATGTTTACCTGACGTATG AAAATTTGTTGTCTGAACCTGTTGGTGGC AGAAAAGTAGCTGAGATGTTCTTGAACGA TTGGAGTAGCATTGCCCGTTTATACGAGTG TGTGTTGGAATTTGCACGTTCTCTACCAGgta CACTTGGGTGGCTGAATTAG
Mouse V769A genotyping	GAGAAAGAGAGACTATACACTGCGG
Mouse V769A genotyping	TGTTCTGGTCATTGGCAGCCTGG
Human MED14 PCR-Rev	AAAGGAGATTATCTCCACACGTAC
Human MED14 PCR-Fwd	GTATAACTGAGGAAACCCAAAAGG
Human L1CAM PCR-Rev	TCTGAGTTGCATCTGAGGGTAA
Human L1CAM PCR-Fwd	TTCAGTGGTGAGTGTCTCGTC
Mouse *L1cam* Q497H sgRNA	GCCAATGGAACGCTGAGCATCAGAGACCTC CAGGCCAA
Mouse *L1cam* Q497H Donor Oligo	TGACACTGGACGCTATTTCTGCCAGGCCGCA AACGATCACAACAATGTGACCATTTTGGCTA ACCTACAGGTTAAAGGTTAGATGATGAGCAC ACATGACTG
Mouse *L1cam* Q497H PCR-Rev	ATCTCCACGCCAAGTGATGCT
Mouse *L1cam* Q497H PCR-Fwd	AGTGGTGAGTGCCCATC

were produced using Vector Builder, sequenced, and subcloned into pSGH2. Ectopic expression of wild-type and mutant human MED14 was achieved by injection of the heat-inducible pSGH2 vector into one-cell–stage embryos (32), following which re-expression was induced by elevating the temperature to 37°C for 1 hour at 30 hours post fertilization (hpf). GFP^+ embryos in which re-expression of *MED14* was induced were selected at 5 days post fertilization (dpf) for analysis by WISH using an lck probe. Image J software was used to measure integrated staining density of zebrafish thymi.

Construction of knockin mice

Med14^{V769A} knockin mice were generated by the Fox Chase Transgenic Mouse Facility using CRISPR-induced cutting and HDR repair (33). *Med14*^{V769A} mice were created using a single guide RNA (sgRNA) close to the mutation site (all oligonucleotides are listed in Table 1) and a 150 bp oligonucleotide donor encoding the T to C change plus 5 silent mutations in the sgRNA binding site to prevent cutting of the altered allele. The knockin mutation created a new Alu I restriction enzyme site which was used for screening for the allele. The *L1cam*^{Q497H} mouse line was also generated using the CRISPR/Cas9 method. Briefly, an sgRNA (Table 1) designed to cut near the *L1cam* mutation site was purchased from Synthego (Menlo Park, CA). Cas9 mRNA was purchased from TriLink BioTechnologies (San Diego, CA). Single strand donor oligonucleotide for *L1cam* (Table 1) was used to introduce point mutations (IDT, Coralville, IA). Besides the desired nucleotide changes to convert the Q to H, four silent nucleotide substitutions that prevented Cas9 from continuously cutting the DNA after donor knock-in were also included in the donor oligonucleotides. For making the *L1cam* knockin mouse line, the sgRNA (20 ng/µl) and its corresponding donor oligonucleotides (100 ng/µl) were co-microinjected with Cas9 mRNA (50 ng/µl) into the cytoplasm of zygotes from C57BL/6 mice (Charles River Laboratory) and the resulting embryos were implanted into the oviducts of pseudo-pregnant surrogate mothers. Offspring born to the foster mothers were genotyped by PCR and Sanger DNA sequencing and founders with the desired nucleotide changes were identified. Founder mice were backcrossed to C57BL/6J (JAX 000664) background for 4-6 generations before using for experiments.

Flow cytometry

Single-cell suspensions from thymus and spleen were stained, as indicated, with optimal amounts of the following fluorochrome-conjugated antibodies: anti-CD3ϵ (145-2C11), anti-CD4 (GK1.5), anti-CD8 (53-6.7), anti-CD24 (M1/69), anti-CD25 (PC61), anti-CD44 (IM7), anti-CD62L (MEL-14), anti-CD69 (H1.2F3), anti-CD73 (TY/11.8), anti-CD90.2 (30-H12) anti-B220 (RA3-6B2), anti-NK1.1 (PK136), anti-CD122 (TM-β1), anti-TCRδ (GL3), anti-TCRβ (H57-597), anti-IgM (RMM-1) and CFSE Cell Division Tracker Kit 423801. The antibodies were purchased from BD Biosciences, eBioscience, BioLegend, or Tonbo Biosciences. Dead cells were excluded from analyses using propidium iodide (PI). Data were acquired on an LSRII flow cytometer (BD Pharmigen) or FACS Canto II flow cytometer (BD Pharmingen) and analyzed with Flowjo 9.96 software (Treestar, Inc.). CSFE dilution was employed to monitor proliferation of splenic T cells from WT or *L1cam*^{Q497H} mutant mice according to manufacturers specifications (CellTracer CFSE Cell Proliferation Kit, Invitrogen, Carlsbad, CA) following stimulation with 2 µg/ml plate-bound anti-CD3 and 1 µg/ml soluble anti-CD28.

Graphing and statistics

Graphical analysis was conducted using GraphPad prism V9 and statistical significance was calculated using one-way ANOVA and student t tests. Significance values are indicated.

Results

Identification of a proband with T lymphopenia

The male proband was born at term to nonconsanguineous, healthy parents who had no known family history of immunodeficiency, as described (patient #5) (17). Newborn SCID screening was positive with a TREC level of 3, and confirmatory testing showed T-cell lymphopenia with 1,021 T cells/μl, essentially absent naïve T cells, and normal B and NK cell numbers (Table 2). The patient had a non-dysmorphic appearance, had no syndromic features, and negative testing included FISH for 22q11.2 microdeletion, ADA/PNP metabolites, and sequencing a panel of previously reported SCID genes. Imaging analysis at age 6 months, 15 months, and 4 years detected tissue in the anatomic location of the thymus, but it was diminished in size and displayed fatty infiltration. T cell proliferation to mitogen was normal, but was severely impaired following TCR cross-linking. Testing for maternal engraftment was negative. Thus, the immune phenotype was leaky/atypical SCID per Primary Immune Deficiency Treatment Consortium (PIDTC) criteria (35). The patient was closely monitored in protective isolation, maternal breast milk was discontinued, and IVIG and *Pneumocystis jirovecci* prophylaxis were given. Low-level CMV was detected by PCR in the patient's blood at 10 months of age. The patient was asymptomatic, but was subsequently treated with valganciclovir for persistent low-level CMV viremia. At 14 months of age, EBV was detected by PCR in the patient's blood, again without signs or symptoms for EBV infection. Because of the leaky/atypical SCID phenotype and persistent CMV and EBV, an allogeneic conditioned hematopoietic stem cell transplant (HSCT) was performed at 15 months of age using the patient's healthy HLA-matched older brother as the donor. The brother was in good health, had passed the newborn SCID screen, and normal extended immune phenotyping, normal quantitative immunoglobulins and robust vaccine titers. The conditioned HSCT was uncomplicated, with both T and myeloid donor engraftment (81% and >95% respectively at Day +60) and undetectable levels of EBV and CMV at Day +60 after HSCT. The patient's T cell functional studies normalized; however, his T lymphopenia did not improve over time (Table 2). The patient remains alive and well off all immune supportive therapies.

TABLE 2 Patient Immune Characteristics.

AGE	Days post-HSCT	Abs. CD3 num./μL (Ref. range)	Abs. CD4 T cell num./μL (Ref. range)	Abs. CD8 T cell num./μL (Ref. range)	Abs. B cell (CD19+) num./μL(Ref. range)	Abs. NK cell num. (CD3-CD16+/CD56+)/μL(Ref. range)	%CD3+CD4+CD45RA+(Ref. range)	TREC level*
1 week	n/a	1021 (2500-5500)	756 (1600-4000)	265 (560-1700)	1777 (300-2000)	869 (170-1100)	2 (64-95)	3
1 month	n/a	702 (2500-5500)	488 (1600-4000)	214 (560-1700)	1922 (300-2000)	397 (170-1100)	2 (64-95)	0
3 months	n/a	834 (2500-5500)	516 (1600-4000)	278 (560-1700)	2184 (300-2000)	953 (170-1100)	3 (64-95)	n.d.
6 months	n/a	497 (1900-5900)	276 (1400-4300)	221 (500-1700)	1586 (610-2600)	390 (160-950)	6 (64-93)	0
12 months	n/a	708 (2100-6200)	248 (1300-3400)	425 (620-2000)	1876 (720-2600)	920 (180-920)	6 (63-91)	0
21 months	6 months	662 (2100-6200)	95 (1300-3400)	93 (620-2000)	761 (720-2600)	683 (180-920)	0 (63-91)	n.d.
27 months	1 year	529 (1400-3700)	88 (700-2200)	382 (490-1300)	764 (390-1400)	176 (130-720)	1 (53-86)	n.d.
39 months	2 years	347 (1400-3700)	95 (700-2200)	210 (490-1300)	599 (390-1400)	95 (130-720)	3 (53-86)	0
6 years	5 years	684 (1200-2600)	151 (650-1500)	456 (370-1100)	n.d	n.d.	10 (46-77)	0
10 years	9 years	750 (1200-2600)	392 (650-1500)	303 (370-1100)	n.d.	n.d.	6 (46-77)	98 (≥5270)

TREC level assayed by two methods: through newborn screening laboratory with blood filter card and liquid blood sample through CLIA laboratory,
n/a, not applicable.
n.d., not done.
Reference ranges (34).

Identification of candidate variants by exome sequencing

To determine the genetic cause for the patient's disease, WES was performed on genomic DNA from the proband and his parents as described (7). The variants prioritized by our initial analysis were an X-linked V763A variant in *MED14* and a homozygous autosomal R114H variant in *SMARCAL1* (Figure 1A and Supplementary Figure 1A). SMARCAL1/HARP is an annealing helicase that functions in the repair and restart of damaged DNA replication forks and has been linked to AR Schimke immuno-osseous dysplasia (SIOD), which can cause T-cell immunodeficiency, but is also accompanied by short stature and other phenotypes (36). The affected arginine residue (R114) in SMARCAL1 was not conserved between human, mouse, and zebrafish (Supplementary Figure 1A) and its location in the SMARCAL1 protein was distinct from that of reported pathogenic mutations that cause SIOD (37, 38).

Functional testing of candidate variants

To test in zebrafish the role of the *smarcal1* gene in supporting T cell development, expression of *smarcal1* was knocked down using MO that disrupted pre-mRNA splicing, following which the impact on T cell development was evaluated by WISH using an *lck* probe to identify T cells (Supplementary Figure 1B). Importantly, despite effective induction of *smarcal1* mis-splicing, the development of T cells at 5 dpf was not impaired, suggesting that *smarcal1* was not essential for T cell development in zebrafish and that the SMARCAL1 R114H variant was unlikely to be responsible for the T lymphopenia observed in the proband.

The other highly ranked variant was in MED14, an integral component of the mediator complex that links the head and neck of the complex (39). While mediator complex component MED23 has been implicated in T cell activation, mediator complex function has not been explored in T cell development (40). The MED14 V763 residue that was mutated in the proband was conserved from human to zebrafish (Supplementary Figure 2A). To explore the extent to which the V763A variant might damage MED14 function, we performed structural modeling using Rosetta to optimize side chain packing and obtain an energy score reflective of the stability of the V763A variant compared to wildtype MED14 (23, 25). The results showed that the V763A substitution resulted in a decrease in hydrophobic contacts between two key helices at the 'elbow' of MED14 between repetitive modules (RM) 5 and 6 (Figure 1B). V763

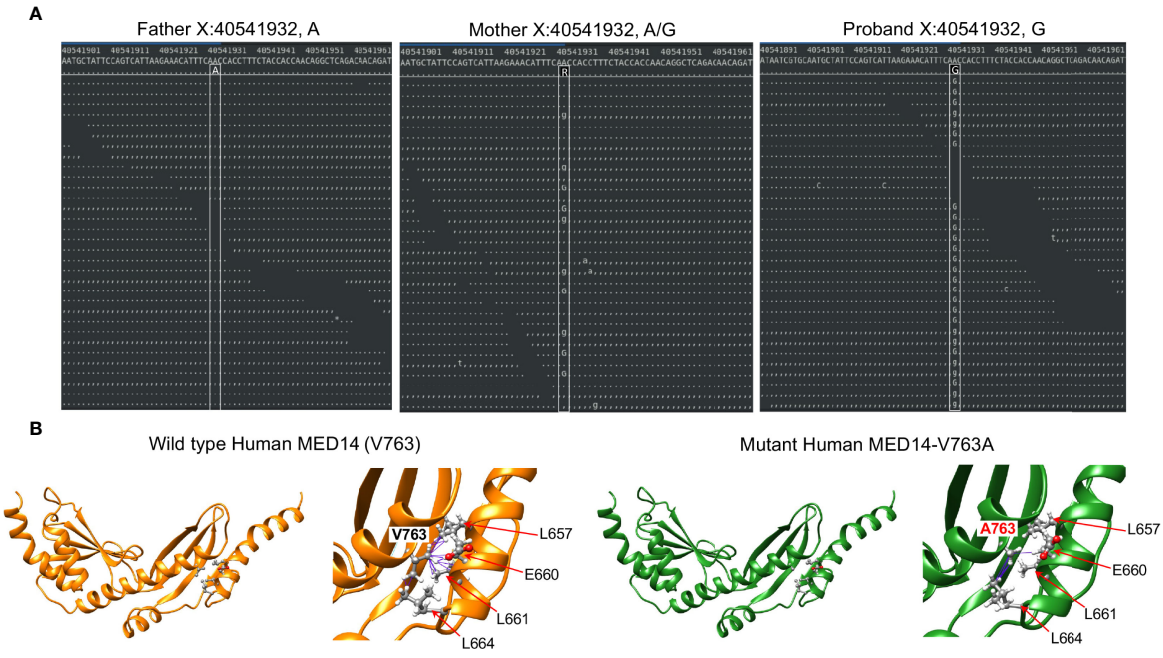

FIGURE 1
Identification of the *MED14* missense mutation by next generation sequencing. **(A)** Screen shots of NGS sequencing runs of the proband and parents are depicted. The A>G mutation is indicated by upper and lower case G. Dots or commas indicate wild type sequence. **(B)** Molecular models of the wild type and variant MED14 proteins. Two views of wild type (orange) and V763A mutant (green) human MED14 are depicted. The right half of each panel shows a zoomed in view of aa 763 with nearby residues on the opposing helix that are capable of making contacts with the A or V763. The left panel shows wild type MED14 V763 from known PDB structure 7ENA chain n, residues 637 to 884. The right panel shows the human V763A MED14 variant. Hydrophobic contacts are shown with purple lines.

made 16 contacts to 4 different residues (L657, E660, L661, L664), while the A763 variant made only 5 contacts to 2 residues (E660, L664), decreasing the stability of the A763 variant by 7.9 Rosetta Energy Units (REU) (26), far more profoundly than V to A substitutions in 11 other model proteins, which averaged reductions of 2.4 kcal/mole (41). It was not clear whether the reduction in hydrophobic contacts observed in the V763A variant was sufficient to cause major changes in MED14 conformation, but it was likely to cause local structure perturbation that could affect protein turnover and/or alter conformational dynamics.

To investigate the role of Med14 protein in supporting T-cell development *in vivo*, we performed MO knockdown of *med14* in zebrafish. The role of zebrafish *med14* in T cell development had not previously been evaluated because the zebrafish *logelei* mutant (in *med14)* arrests embryo development at 2 dpf (15). Knockdown of *med14* using splice-site blocking MO at the indicated dose disrupted the splicing of *med14* pre-mRNA, without generally disrupting zebrafish development or altering morphology; however, WISH using an *lck* probe to identify T cells in the thymus at 5 dpf revealed that *med14* knockdown markedly impaired T cell development (Figure 2A and Supplementary Figure 2B). The decrease in Lck^+ T cells indicated that Med14 plays a critical role in supporting T cell development in zebrafish. To determine whether the specific V763A patient variant (Figure 1A and Supplementary Figure 2A) would impair MED14 function, as predicted by structural modeling (Figure 1B), we performed rescue experiments. After knocking down endogenous *med14* expression using splice blocking MO, wild type or patient variant human *MED14* were re-expressed using heat-mediated induction (7). Expression of the human wildtype MED14 protein rescued the arrest in T cell development caused by knockdown of endogenous *med14*, indicating conservation of function between zebrafish and human MED14 (Figure 2B and Supplementary Figure 2C). Importantly, however, re-expression of the patient $MED14^{V763A}$ variant failed to rescue the loss of endogenous *med14* (Figure 2B), indicating that the $MED14^{V763A}$ variant significantly damaged MED14 function. These findings suggested that the $MED14^{V763A}$ variant might have caused the proband's immunodeficiency.

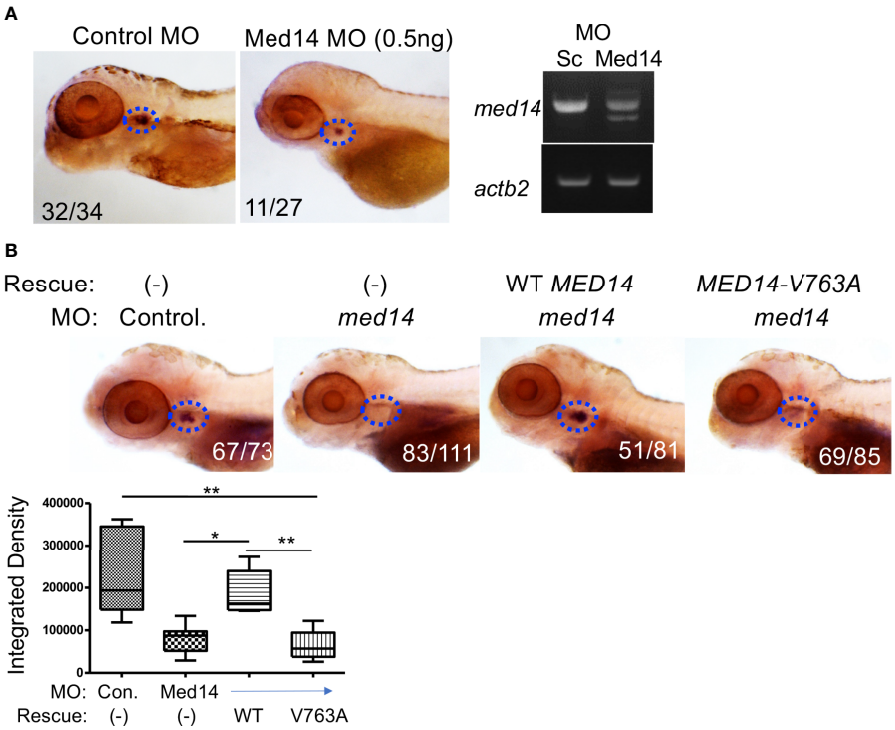

FIGURE 2
Role of Med14 in Zebrafish T cell Development. **(A)** The effect of MO knockdown of *med14* on T cell development at 5 dpf was assessed by WISH using an *lck* probe to identify T cells. The numbers on the images reflect the fraction of the embryos with the depicted staining pattern. Thymus staining is outlined by blue dashed ovals. The panel on the right confirms MO induced mis-splicing of *med14* mRNA by RT-PCR at 1 dpf with β-actin (*actb2)* as a loading control. **(B)** The ability of the wild type and human MED14 variant to rescue loss of endogenous zebrafish *med14* was assessed by heat-inducible re-expression of wild type or variant MED14. The effect on T cell development was assessed as above by WISH using an *lck* probe. The integrated density of WISH staining was measured by ImageJ software and depicted graphically as box plots. Significantly altered groups are indicated. Data are representative of 3 experiments. * $p < 0.05$, ** $p < 0.01$.

Basis for impaired T cell development upon Med14 loss

To determine how Med14 loss blocked zebrafish T cell development, we examined whether other precursor and cell populations were impacted. Because *lck* is expressed in all T cell precursors, the reduction in the *lck* WISH signal (Figure 3) indicated that overall thymic cellularity was reduced in zebrafish in the absence of Med14. To determine if the impairment of T cell development was restricted to the αβ T cell lineage or affected both αβ and γδ T lineage cells, we performed WISH with a probe for *tcrd*, which marks γδ T lineage cells (Figure 3). The reduction of both *lck*- and *tcrd*-marked T lineage precursors indicated that Med14 loss impaired the development of both the αβ and γδ T cell lineages (Figure 3). WISH employing a probe for *ikaros* to mark thymic seeding cells revealed that the loss of Med14 reduced thymic seeding (**Figure 3**). Finally, WISH using *foxn1*, which marks thymic stroma, revealed reduced staining, suggesting that the thymic structure itself might also have been disrupted (Figure 3). Importantly, similar results were obtained when the zebrafish *med14* knockdown was replaced with the $MED14^{V763A}$ variant, indicating that the patient variant was unable to rescue the attenuation of thymic seeding or perturbation of thymic stroma (Supplementary Figure 3). Taken together, these observations indicated that loss of Med14 function interfered with development by attenuating thymic seeding and might involve impaired thymic organogenesis.

Effect of the $MED14^{V763A}$ mutation on T cell development in mice

Because the zebrafish model did not allow one to ascertain the precise stage of developmental arrest upon Med14 loss or whether it was cell-autonomous, we next developed a mouse model in which these questions could be addressed by generating V769A knock-in mice ($Med14^{V769A}$) (Supplementary Figure 4). These mice were viable and fertile and were analyzed after being outcrossed to C57BL/6 mice for at least 4 generations. To determine if the $Med14^{V769A}$ variant knock-in mice exhibited a defect in T cell development, we performed flow cytometry on thymic and splenic explants (Figure 4). Surprisingly, analysis of the thymus revealed no reduction in cellularity in the $Med14^{V769A}$ knockin mice or in the proportion of thymic subsets (Figure 4A). Moreover, there was no change in splenic cellularity or the distribution of B, T, or NK cells in the spleen, including in memory T cell subsets defined by CD44 and CD62L (Figure 4B). However, competitive transplantation analysis revealed that hematopoietic stem and progenitor cells (HSPC) from $Med14^{V769A}$ mice exhibited a mild impairment of differentiation beyond the β-selection checkpoint as evidenced by an accumulation of $CD4^{-}CD8^{-}CD44^{-}CD25^{+}$ (DN3) thymocytes, reduced DN3b (CD25+CD98+) and DN4 (CD44-CD25-), and strongly reduced T cell repopulation of the periphery (Supplementary Figure 5). The reduction of peripheral T cells was not associated with decreases in thymic emigrating

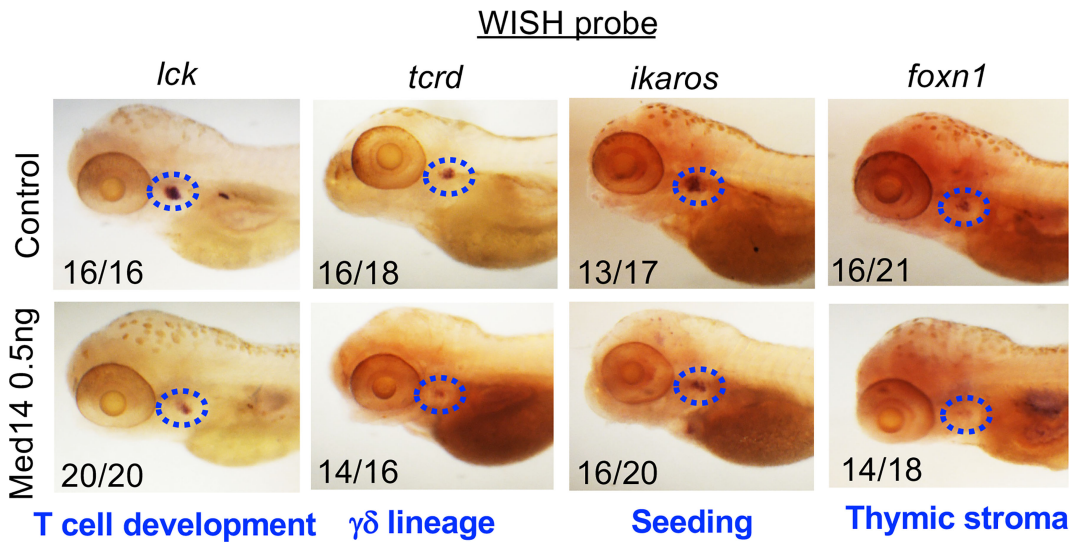

FIGURE 3
Role of MED14 in Development of Thymic Subpopulations in Zebrafish. The effect of *med14* knockdown on cell subpopulations was evaluated at 5 dpf by performing WISH on TLF zebrafish embryos with the indicated probes: *lck* marks most developing thymocytes, *ikaros* marks thymic seeding progenitors, *tcrd* marks γδ lineage progenitors, and *foxn1* marks thymic epithelial cells. Blue ovals mark the thymus and frequencies of embryos with the exhibited staining pattern are indicated at the lower left of each image. Data are representative of 3 experiments.

cells (CD69-CD62L+S1PR1+) or reduced proliferation in the periphery (Supplementary Figure 5).

The absence of a baseline defect in T cell development in the *Med14*^{V769A} mice prompted us to investigate whether the V to A substitutions impacted the structure of mouse and human MED14 protein differently. Consequently, we replicated the structural modeling analysis that we had performed on the human V763A variant (Figure 1B) using ColabFold (27). The resulting AlphaFold 2 model had a complete chain structure that was highly similar to the conformation of the PDB 6W1S mouse MED14, with a root-mean-square deviation (RMSD) of 160 alpha carbons of 0.983 Å, and an overall RMSD of 228 protein structure pairs of 1.531 Å (Supplementary Figure 6). The human and mouse sequences were highly conserved in this region of MED14, with 98% identity. Only 4 positions in the aligned fragment (residues 643 to 890) differed. Nevertheless, the same coordinate-constrained Rosetta relaxation protocol (25, 26) demonstrated that the murine V769A variant exhibited only a minor reduction in predicted stability of 1.2 REUs relative to the wildtype protein (Supplementary Figure 6). The difference relative to human MED14 V763 was that murine MED14 V769 made 17 hydrophobic contacts (relative to 7 in the human MED14 V763) with 4 different residues (L663, E666, L667, L670) on the adjacent helix (Supplementary Figure 6). This increase in contacts apparently rendered mouse MED14 resistant to the A substitution, consistent with a possible difference in mouse versus human MED14 proteins harboring the V763A change.

Sequence analysis of the unaffected sibling

The inability of the murine *Med14*^{V769A} variant to attenuate baseline T cell development diminished the likelihood that this

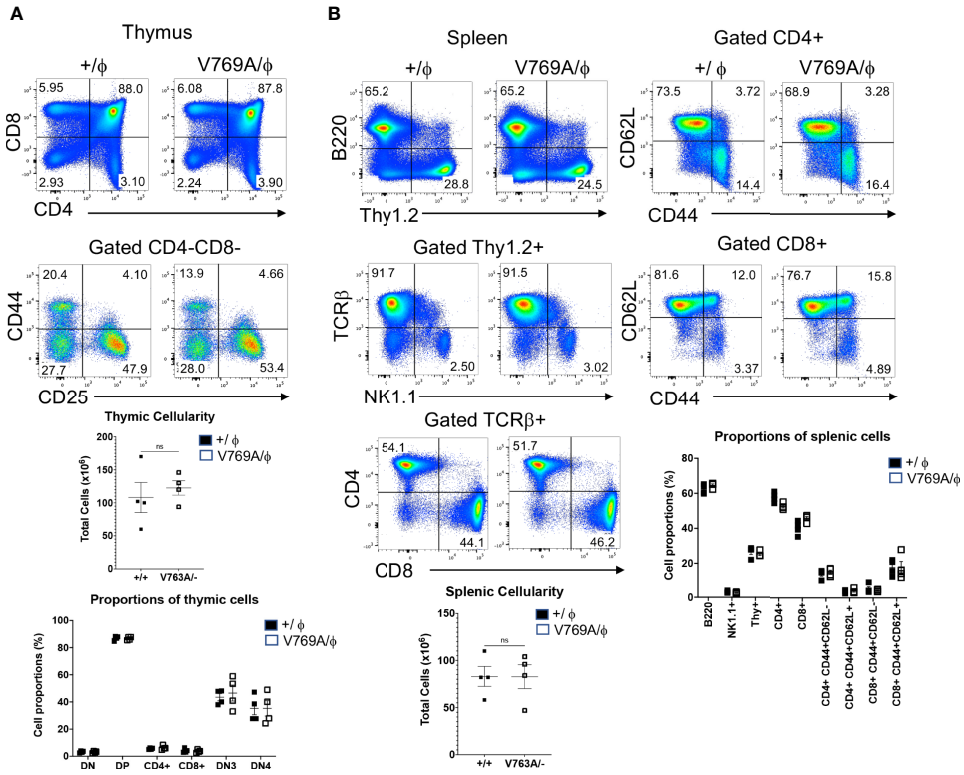

FIGURE 4
Phenotypic analysis of lymphoid development in *Med14* mutant mice. **(A)** Histograms are displayed of flow cytometry analysis of thymic cell suspensions from wildtype (+/ϕ) and hemizygous *Med14* mutant (V769A/ϕ) mice. The following antibodies were used: CD4, CD8, CD44, and CD25. Scatter plots of total thymic cellularity and the frequencies of the indicated populations are depicted. The following populations are graphed: DN, CD4-CD8-; DP, CD4+CD8+; CD4+; CD8+; DN3, CD4-CD8-CD44-CD25+; DN4, CD4-CD8-CD44-CD25-. Proportion of DN3 and DN4 subpopulations among DN thymocytes is depicted graphically **(B)** Histograms are displayed that illustrate flow cytometric analysis of the lymphoid content of spleens from +/ϕ and V769A/ϕ mice. The following antibodies were used: B220, Thy1.2, NK1.1, CD4, CD8, TCRβ, CD44, and CD62L. Scatter plots of total splenic cellularity and the frequencies of the indicated populations are depicted. Each symbol represents an individual mouse. The proportions of CD4 and CD8 T cells among Thy1+ cells and the proportions of memory subsets among CD4+ and CD8+ subsets are depicted graphically. Data are representative of 3 experiments performed. No statistically significant differences were found in any of the indicated populations.

variant alone could fully account for the immunodeficiency in the patient. To seek other potentially disease-causing candidate gene(s), WES was repeated using the patient, his parents, and his healthy brother, the HSCT donor. Unexpectedly, the brother shared the same *Med14*^{V763A} variant as the patient (Figure 5A), indicating that this genotype alone could not explain the disease. This raised the possibility that there was variable penetrance of a phenotype due variable expressivity, in which identical mutations may be associated with a spectrum of disease severity due to the contributions of secondary mutations that differ between patients (42–44). Another possibility was that a gene other than *MED14* could be responsible for the disease (see Discussion). Additional variants were indeed identified in 6 genes (Table 3), including an X-linked variant in *L1CAM* (p.Q498H) present in the patient but not in his healthy brother (Figure 5B). L1CAM is a transmembrane glycoprotein belonging to the immunoglobulin superfamily of cell adhesion molecules (45). It contains six immunoglobulin (Ig) and five fibronectin III-like domains at the extracellular surface, a single-pass transmembrane domain, and a short cytoplasmic domain (46). The Q498H variant was located in the fifth Ig domain. Mutation or deletion of L1CAM has been associated with an X-linked recessive neurological disorder (47), with at least 248 variants/mutations having been identified (48), but the roles of the variants, including the one in the proband, have not been studied in the immune system. To further examine whether an orthologous murine *L1CAM*^{Q497H} variant (corresponding to *L1CAM*^{Q498H} in human) would result in T cell deficiency, we generated *L1cam*^{Q497H} knock-in mice (Supplementary Figures 7A, B). However, no defects were observed, with normal development of CD4$^+$CD8$^+$ (DP), CD4$^+$ and CD8$^+$ (SP), CD4$^-$ CD8$^-$ (DN), DN1 (CD25$^-$CD44$^+$), DN2 (CD25$^+$CD44$^+$), DN3, and DN4 (CD25$^-$CD44$^-$) cells in thymus (Figures 6A, B); normal overall frequencies and numbers of T, B (B220$^+$IgM$^+$) and NK

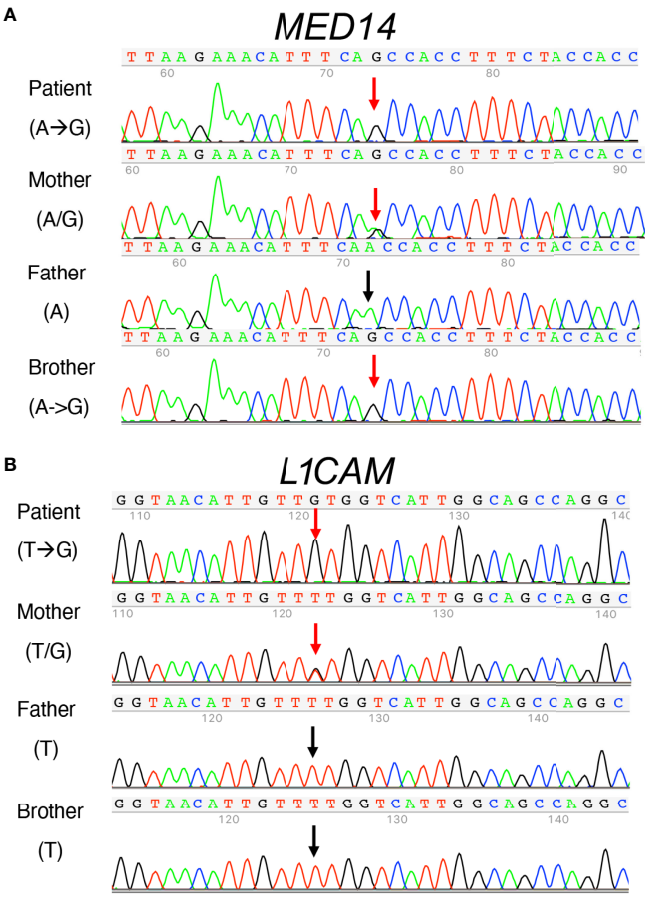

FIGURE 5
Sanger sequence analysis of MED14 V763A and L1CAM Q498H variants in the patient, his parents, and his healthy brother. **(A)** The mother carries both alleles with A and G The patient and his healthy brother inherited the same alleles with G, resulting in the same MED14 V763A variant as indicated by red arrows. The PCR-Rev primer was used for sequencing. **(B)** Patient's mother carries T and G alleles (red arrow). The patient inherited the allele with G (red arrow), resulting in the L1CAM^{Q498H} variant and his healthy brother inherited the allele with T (see black arrow). His father's allele also has a T (black arrow). PCR-Fwd primer was used for sequencing.

TABLE 3 List of potentially interesting variants identified by whole exome-sequencing.

Chr	Start	End	Ref	Alt	Gene	Location	Domain	snp138	D	ConsSites	Model	Father	Mother	Child	Brother
chrX	153134053	153134053	T	G	L1CAM	Exon11-: p.Q498H	C2	na	15.16	SOX9_B1	X-link	0%	46%	100%	0%
chr14	103571109	103571109	T	C	EXOC3L4	Exon5-: p.I440T	Sec6	na	19.66	1	AR	53%	52%	100%	50%
chr14	104436947	104436947	C	T	TDRD9	Exon6: p.R279C	DEXDc	na	20.2	na	AR	55%	64%	100%	27%
chr8	38091971	38091971	G	T	DDHD2	Exon3: p.G94W	WWE	rs202216406	17.17	na	AR	37%	58%	100%	58%
chr9	113132258	113132258	C	A	SVEP1	Exon47: p.V3547L	–	rs192794123	12.6	na	AR	52%	57%	100%	0%
chr1	55247289	55247289	C	T	TTC22	Exon7: p.G446D	–	na	27.5	na	Denovo	0%	0%	47%	0%
chrX	40541932	40541932	A	G	MED14	Exon18: p.V763A	–	na	22.5	EN1_01	X-link	0%	45%	100%	100%

Shown are either X-linked, Denovo, or autosomal recessive (AR) variants. Chr (Chromosome), Start (chromosomal start), End (chromosomal end), Ref (Refseq), Alt (alteration), Gene (gene name), Location (chromosome location and corresponding protein sequence), snp138 (dbSNP buiding 138), CADD (combined annotation dependent depletion), ConsSites (target gene per GSEA database), Model (type of variant), and the percentage of variants in each individual are shown, with 100% being homozygous and 50% or less being heterozygous.

($CD3^-CD122^+NK1.1^+$) cells in spleen (Figures 6C, D); and normal memory T cell subsets ($CD44^+CD62L^-$) (Figures 6E, F). In addition, the proliferative response to anti-CD3 plus anti-CD28 was also normal (Supplementary Figure 7C). These data showed that $L1CAM^{Q497H}$ in the mouse was not sufficient to cause a defect in the development of T cells.

Discussion

Here we describe a male proband identified through newborn screening who exhibited T-B+NK+ immunodeficiency. Informatic analysis of our initial WES suggested that a missense mutation in the X-linked *MED14* gene might be responsible for the disease. This possibility is supported by functional analysis in zebrafish. Nevertheless, subsequent introduction of the patient variant into a knock-in mouse did not replicate the baseline T cell developmental arrest observed in zebrafish, although HSPC from these mice failed to fully reconstitute peripheral T cells in the competitive transplant setting. These data were of interest because the healthy male sibling of the proband also carried the $MED14^{V763A}$ variant, and while he exhibited no signs of baseline disease, his HSPC transplanted into the patient failed to generate normal T cell numbers, leaving the patient profoundly T lymphopenic (Table 2). These findings could be consistent with the $MED14^{V763A}$ variant potentially contributing to T lymphopenia; however, because both siblings shared the $MED14^{V763A}$ variant, that variant alone was insufficient to explain the proband's disease. Consequently, variable expressivity, mediated by additional genetic variants, was considered. A search for such variants resulted in identification of a variant in the *L1CAM* gene in the proband, but not his healthy sibling. Nevertheless, introduction of the *L1cam* variant in mice had no effect on T cell development. Taken together, these data raise at least two potential etiologies in this patient. First, a distinct, yet unknown and undetected, gene mutation was actually responsible for his T cell insufficiency. Alternatively, the $MED14^{V763A}$ variant could underlie the patient's T cell insufficiency, but the developmental defect was not manifest in the sibling because of variable expressivity (43, 44, 49).

Our analysis in zebrafish supported the interpretation that the $MED14^{V763A}$ mutation damages MED14 function and prevents it from supporting the development of Lck-expressing T cell development in the thymus, most of which are αβ lineage (50). Nevertheless, baseline impairment of T cell development was not observed in mice harboring the equivalent mutation. Molecular modeling of the mouse $Med14^{V769A}$ mutation suggested it might be less destabilizing than the human orthologous variant, providing a potential explanation for why the mouse $Med14^{V769A}$ mutation does not phenocopy the baseline defect in T cell development observed in zebrafish. Importantly, the murine $Med14^{V769A}$ variant impaired the ability of HSPC to reconstitute peripheral T cells, indicating that this variant is important for supporting development or maintenance of T cells. This also provides a possible explanation for the failure of the healthy sibling's bone marrow to fully restore T cell numbers upon transfer into the proband. The mechanistic basis by which MED14 supports the function of HSPC and their capacity to fully reconstitute peripheral T cells remains unclear.

MED14 is a component of the 26-subunit Mediator Complex, a transcriptional coactivator transmitting signals from transcription factors to Polymerase II (Pol II) (13, 18, 19, 51). MED14 serves as a crucial backbone of the Mediator Complex (52). Consequently, the MED14 variant might compromise the capacity of the Mediator Complex to cooperate with transcription factors and Pol II to coactivate transcription, as exemplified by its critical role in

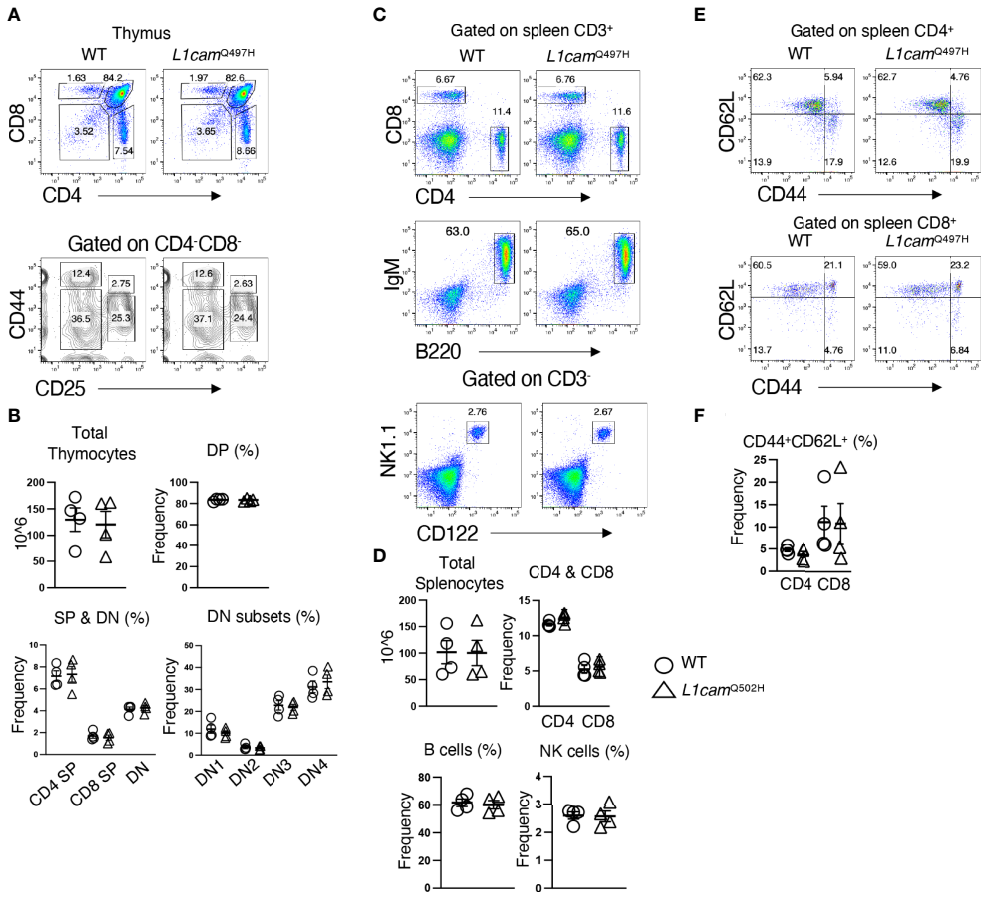

FIGURE 6
Phenotypic analysis of the *L1CAM*^{Q497H} knockin mice. **(A)** Flow cytometric analysis of the frequencies of thymocytes from wildtype (WT) and hemizygous *L1cam*^{497H} mutant mice. **(B)** The total and subpopulations of thymic cellularity are shown in bar graphs. The following populations are graphed: DN, CD4$^-$CD8$^-$; DP, CD4$^+$CD8$^+$; CD4$^+$; CD8$^+$; DN1, CD4$^-$CD8$^-$CD25$^-$CD44$^+$; DN2, CD4$^-$CD8$^-$CD25$^+$CD44$^+$; DN3, CD4$^-$CD8$^-$CD44$^-$CD25$^+$; DN4, CD4$^-$CD8$^-$CD44$^-$CD25$^-$. **(C)** Flow cytometry analysis of splenic CD4$^+$ and CD8$^+$ T cells, B (B220$^+$IgM$^+$) and NK (CD3$^-$CD122$^+$NK1.1$^+$) cells in WT and *L1cam*^{Q497H} mutant mice. **(D)** The total and subpopulations of splenic cellularity are shown in bar graphs. **(E)** Flow cytometry analysis of CD44 and CD62L staining of CD4$^+$ and CD8$^+$ T cells. **(F)** The frequency of CD44$^+$CD62L$^+$ in CD4$^+$ and CD8$^+$ T cells is shown in bar graphs. Data are representative of 2 independent experiments performed. No statistically significant differences were found in any of the indicated populations.

PPARγ-dependent and glucocorticoid receptor-dependent transactivation of targets (53, 54).

While the structural differences between mouse and human MED14 might provide an explanation for the absence of a phenotype in our mouse model, this does not explain why the patient's sibling also bears the same *MED14*^{V763A} variant and yet is healthy. One possibility is the phenomenon of variable expressivity, widely observed in genetic disorders in which distinct individuals with identical mutations manifest marked differences in disease severity, even in siblings reared in the same environment (55–57). The prevailing view is that variable expressivity occurs because different complements of modifier gene variants influence disease penetrance (49). For example, cartilage hair hypoplasia (CHH), caused by mutations in the *RMRP* gene, which encodes an untranslated multifunctional RNA gene product, can manifest immune phenotypes that range from no significant impairment to T cell deficient typical SCID (58). Importantly, this variability is even observed among patients with identical *RMRP* mutations, presumably due to differences in modifier genes (43, 55, 59–63). Likewise, differences in modifier genes might explain the distinct disease penetrance between this patient and his male sibling with the same *MED14*^{V763A} variant, assuming it is indeed responsible for the disease. The seemingly healthy sibling may also be experiencing a mild functional deficit in hematopoiesis given that his bone marrow failed to correct the T cell insufficiency upon adoptive transfer into the patient. The background variants potentially responsible are not known. As noted, a patient L1CAM variant was tested, but did not impair T cell development in a mouse model. Modifier genes underlying variable expressivity may be exceedingly difficult to identify.

In summary, the current study used newborn screening coupled with functional testing in zebrafish and in mice. While the X-linked missense mutation in *MED14* remains a potential candidate for the disease-causing allele in this patient, presumably acting together with modifier gene variants through variable expressivity, the disease might alternatively be caused by defects in another, as yet undefined gene, and perhaps could be due to a mutation in a noncoding (e.g., promoter or enhancer) element affecting gene expression rather than in a coding region. While additional investigations are required to determine the basis for the proband's disease, it is possible that further human cases of T lymphopenia associated with *MED14* variants could be found; such evidence from multiple affected individuals would provide strong supporting evidence for pathogenicity of this gene.

Data availability statement

The WES data presented in the manuscript have been deposited in dbGaP under accession numbers phs002968.v1.p1 and phs002990.v1.p1.

Ethics statement

The studies involving human participants were reviewed and approved by Research activities were performed with parental informed consent under protocols approved by the institutional review boards (IRBs) at the University of California, San Francisco and National Heart, Lung, and Blood Institute (NHLBI), National Institutes of Health (NIH), Bethesda, MD. 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 housing and handling were all performed in accordance with the approved protocols from the Fox Chase Cancer Center Institutional Animal Care and Use Committee (IACUC). Likewise, mouse experiments were performed under the auspices of IACUC-approved animal protocols, and all mouse strains were housed in accredited facilities at either Fox Chase Cancer Center or NIH. All experiments using mice at NHLBI were performed using protocols approved by the NHLBI Animal Care and Use Committee and followed NIH guidelines for use of animals in intramural research.

Author contributions

RSe, JXL, JMP, WJL, and DLW drafted the manuscript. WJL and DLW take the primary responsibility for this paper as the corresponding authors. All authors contributed to the article and approved the submitted version. JMP and CMS provided care for the patient and contributed clinical data. RSe, JXL, EM, SS and BT performed experiments. SR, MK, AS, US, MA, RMD, CL, RSr, and SEB performed data analysis.. All authors contributed to the article and approved the submitted version.

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Conflict of interest

Authors US, RA, and RS (Srinivasan) are employed by TATA Consultancy Services. SEB was a principal investigator on a research service agreement between TCS and the University of California, Berkley.

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.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.928252/full#supplementary-material.

SUPPLEMENTARY FIGURE 1
smarcal1 knockdown does not impair T cell development. **(A)** Multisequence alignment of *SMARCAL1* sequences with the patient variant (R114H) shown in a red box. **(B)** Upper panel, effect of *smarcal1* knockdown on T cell development as measured by WISH with an *lck* probe. Blue ovals mark the thymus and the numbers on the images indicate the frequencies of embryos with the depicted phenotype. Lower panel, efficacy of the *smarcal1* MO as indicated by mis-splicing of the *smarcal1* pre-mRNA by RT-PCR analysis. β-actin (*actb2)* serves as a loading control. Results are representative of at least 3 experiments.

SUPPLEMENTARY FIGURE 2
Conservation of the MED14 variant. **(A)** Multiple sequence alignment of human, mouse and zebrafish MED14. Valine 763 is boxed in red and conservation indicated (identical *, highly similar: similar.). **(B)** Schematic representation of the *med14* gene structure with the position of the Exon 3-Intron 3 MO and primers for RT-PCR analysis indicated. Blue arrows indicate primers and blue rectangle the morpholino binding site. **(C)** RT-PCR analysis of the efficacy of *med14* MO at 1 and 5 dpf of the rescue experiment. RT-PCR analysis of both *med14* and β-actin (*actb2)* are shown. Results are representative of at least 3 experiments.

SUPPLEMENTARY FIGURE 3
Effect on re-expression of the MED14 variant on generation of thymic subpopulations. A rescue experiments was performed as in Figure 2, and the resulting embryos analyzed by WISH with the indicated probes (*lck, ikaros, tcrd and foxn1*) to evaluate the ability of the MED14 variant to rescue γδ T cell development, thymic seeding, and thymic architecture. Blue ovals mark the thymus. Numbers on the figures represent the frequency of the depicted phenotype. Results are representative of at least 3 experiments.

SUPPLEMENTARY FIGURE 4
Generation and sequence validation of MED14 V769A knockin mice. **(A)** The sgRNA target sequence is shown with the sgRNA sequence indicated and the region it binds highlighted in yellow, mutations in the protospacer adjacent motif (PAM) to prevent re-cutting are colored red and newly created restriction enzyme screening site is indicated in green. The sequence trace files show PAM mutations, specific V769A mutation, and silent mutations in male founder. An Alu I digest is shown with cleaved bands indicating digestion of the mutant allele.

SUPPLEMENTARY FIGURE 5
Assessment of $Med14^{V769A/\phi}$ hematopoietic progenitor function by competitive bone marrow transplantation. $100x10^5$ allotype marked wild type (CD45.1) and $Med14^{V769A/\phi}$ mutant (CD45.2) lineage negative hematopoietic stem and progenitor cells (HSPC) were combined and transferred together into CD45.1 recipients that had been treated with 1100 rads (2x550r, 4h apart) 24h earlier. Recipient mice were placed on antibiotic-treated water (polymyxin B sulfate and neomycin) for 3 weeks and analyzed 6 weeks after transplantation. Single cell suspensions of thymus **(A)** and spleen **(B, C)** were stained with CD45.1 and CD45.2 antibodies to distinguish the genotypes of transferred HSPC and with the indicated lineage markers. Bromodeoxyuridine (BrdU) labeling was conducted by staining permeabilized cells after 24h of labeling. Gate frequencies were calculated and depicted as bar graphs of the mean +/- standard deviation. Statistical significance were determined using the t-test. P-values are indicated on the graphs. NS, not significant.

SUPPLEMENTARY FIGURE 6
Molecular modeling of the wild type and variant mouse MED14 proteins. Two views of wild type (orange) and V769A mutant (green) mouse MED14 are depicted. The right half of each panel shows a zoomed in view of aa 769 with nearby residues on the opposing helix that are capable of making contacts with the A or V769. The top panel shows wild type mouse MED14 V769 from known PDB structure 6W1S chain I, residues 643 to 890. The bottom panel shows the mouse V769A MED14 variant. Hydrophobic contacts are shown with purple lines.

SUPPLEMENTARY FIGURE 7
Construction and analysis of the the $L1CAM^{Q497H}$ knockin founder mice. **(A)** Part of human and mouse L1CAM amino acid sequences were aligned and Q498 in human and Q497 in mouse are highlighted and yellow. **(B)** The founder mouse was identified by PCR using tail gDNA and Sanger sequencing using PCR-Rev primer; black arrows indicate silent mutations introduced to prevent from subsequently cutting by Cas9, which do not change the amino acid and the red arrows indicate a G to C change to make Q to H mutation in mouse. **(C)** *In vitro* proliferation assays were performed using splenic T cells isolated from wild type littermates (WT) and $L1cam^{Q497H}$ (KI) mice, which were labeled with CFSE, stimulated with 2 µg/ml of plate-bound anti-CD3 and 1 µg/ml of anti-CD28 as indicated. Cell proliferation was determined by CFSE dilution.

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Shifting gears: Id3 enables recruitment of E proteins to new targets during T cell development and differentiation

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Shifting levels of E proteins and Id factors are pivotal in T cell commitment and differentiation, both in the thymus and in the periphery. Id2 and Id3 are two different factors that prevent E proteins from binding to their target gene cis-regulatory sequences and inducing gene expression. Although they use the same mechanism to suppress E protein activity, Id2 and Id3 play very different roles in T cell development and CD4 T cell differentiation. Id2 imposes an irreversible choice in early T cell precursors between innate and adaptive lineages, which can be thought of as a railway switch that directs T cells down one path or another. By contrast, Id3 acts in a transient fashion downstream of extracellular signals such as T cell receptor (TCR) signaling. TCR-dependent Id3 upregulation results in the dislodging of E proteins from their target sites while chromatin remodeling occurs. After the cessation of Id3 expression, E proteins can reassemble in the context of a new genomic landscape and molecular context that allows induction of different E protein target genes. To describe this mode of action, we have developed the "Clutch" model of differentiation. In this model, Id3 upregulation in response to TCR signaling acts as a clutch that stops E protein activity ("clutch in") long enough to allow shifting of the genomic landscape into a different "gear", resulting in accessibility to different E protein target genes once Id3 decreases ("clutch out") and E proteins can form new complexes on the DNA. While TCR signal strength and cytokine signaling play a role in both peripheral and thymic lineage decisions, the remodeling of chromatin and E protein target genes appears to be more heavily influenced by the cytokine milieu in the periphery, whereas the outcome of Id3 activity during T cell development in the thymus appears to depend more on the TCR signal strength. Thus, while the Clutch model applies to both CD4 T cell differentiation and T cell developmental transitions within the thymus, changes in chromatin accessibility are modulated by biased inputs in these different environments. New emerging technologies should enable a better understanding of the molecular events that happen during these transitions, and how they fit into the gene regulatory networks that drive T cell development and differentiation.

KEYWORDS

thymus, T-cell development, transcription factor, chromatin, Id proteins, E proteins

Introduction

Conventional T cells acquire their functional properties in two main phases. The first occurs in the thymus, as T cells transit through successive stages that install the gene expression programs that will run at steady state. The second phase of differentiation occurs in the periphery after exposure to signals that occur during an immune response. These signals activate accessible but latent sub-routines that are kept in check prior to the initiation of the immune response. Both processes depend on the activity of E protein transcription factors and their antagonists, the Id factors. One of the most intriguing aspects of E proteins is their context-dependent use in many different T cell lineages, and the propensity of T cell receptor (TCR) signaling and Id3 activity, in collaboration with other extracellular signals, to create those contexts. While TCR signaling is required for peripheral CD4 T cell differentiation, the specific functional pathways accessed in the periphery are very sensitive to the cytokine milieu. By contrast, the progression of T cell precursors into different pathways in the thymus appears to be driven more by TCR signal strength. In both cases, TCR-dependent upregulation of Id3 is important for allowing changes in changes in chromatin remodeling and gene expression that are needed to restrict E protein activity to the appropriate targets.

T helper cell differentiation and function

Conventional CD4 T cells emerge from the thymus as "naïve" cells ready for activation. The functional T helper cell differentiation pathways they take upon antigen encounter depends on the types of inflammatory molecules produced during the innate immune response (1) (Figure 1A). Each T helper cell subset is dependent on a specific "master regulator" transcription factor that directly induces the effector genes of each program (2). The Th17 lineage, characterized by secretion of IL-17A, IL-17F, and IL-22, is triggered by the innate response to bacteria and fungi. RORγt (*Rorc*) is the Th17 master regulator. Viruses and other intracellular pathogens induce differentiation into the T-bet (*Tbx21*) dependent Th1 pathway, leading to IL-2, TFNα, and IFNγ production. Helminth infection induces the Th2 fate, leading to secretion IL-4, IL-5, and IL-13, under the control of GATA3 (3).

Other Th subsets generated in the periphery include Bcl6-driven T-follicular helper cells (Tfh) (4), specialized for B cell help in the germinal center, and induced T-reg cells, which, like thymic-derived T-regs, depend on FoxP3 (5). In addition to playing unique roles in immunity, Th subsets also have pathogenic impacts when dysregulated (6). In general, Th1 and Th17 cells contribute to autoimmune pathology, Th2 cells are largely responsible for allergic reactions, and T-regs inhibit anti-cancer immunity (7, 8). Most Th subsets retain plasticity after activation, and some can transdifferentiate from one type to another (2). Additional Th subsets continue to be identified, including Th22, Th9, Tfh13, and Tr1 cells, suggesting that the networks controlling these effector functions are dynamic, and represent more of a physiological state than a committed fate, rendering them open to manipulation during an immune response (9–11).

Transcriptional control of Th differentiation

Differentiation of naïve CD4 T cells into the Th subsets is coordinated by several sets of signal-dependent transcription factors (12). Triggering of the αβ TCR and co-stimulatory receptors leads to activation of NFkB, NFAT, and IRF transcription family members, as well as upregulation of AP1 transcription factor family members such as BATF and Jun (13, 14). Cytokine receptor signaling leads to the activation of different sets of transcription factors, most notably members of the STAT and SMAD families (15, 16). BATF, IRF4, and the cytokine-responsive factors recruit chromatin remodeling enzymes that provide access to genes of specific Th subsets, while restricting access to genes of alternative Th subsets (13, 17). After chromatin remodeling, the master regulators are induced, providing the final key needed for functional activation during the immune response.

E proteins and Id proteins are involved in regulation of the naïve CD4 T cell state, and in the differentiation of Th2, Th17, and T-reg cells (18–20). In general, E protein activity is regulated post-translationally by Id proteins, which sequester them in inactive dimers. The requirement for E proteins for Th17 differentiation has been especially well studied. A comprehensive study conducted by the Strober group in 2013 showed that mice carrying a conditional double HEB/E2A deletion on a CD4-Cre background had a profound defect in Th17 development *in vitro*, and compromised immune function *in vivo*, using both autoimmunity and infection models (21). This study also showed that HEB and E2A can directly bind and activate the *Rorc* locus, but only in the context of Th17 cells, not in naïve CD4 T cells. Studies of Id3-deficient mice suggest that E proteins restrain the Th2 and Tfh lineages and promote the Th9 lineage, whereas Th1 cells appear to require Id proteins and to be E protein independent (21–24). Interestingly, T-regs require both Id3 and E2A in a sequential manner. TGFβ induces transient expression of Id3, which is needed to prevent repression of the FoxP3 promoter (25). This repression is not mediated directly by E proteins, but rather results from E protein-mediated upregulation of GATA3. Subsequently, E2A activity is required to directly activate the FoxP3 promoter.

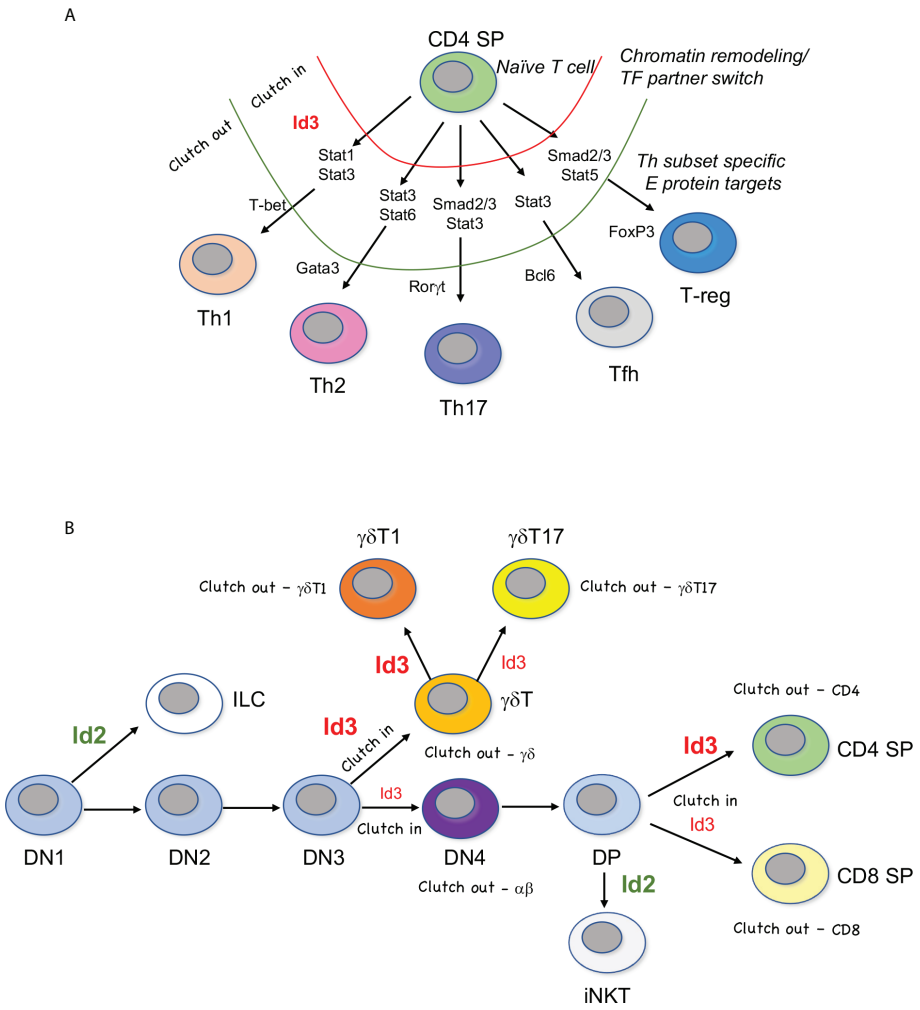

FIGURE 1
"Clutch" model of Id3-E protein mediated fate choice. **(A)** E proteins regulate a core CD4 T cell program in naïve T cells. Upregulation of Id3 causes a transient inhibition of E protein activity (red, clutch in), during which time initiating transcription factors provide access to Th subset-specific genes. Once remodeling is finished, Id3 activity ceases and E protein activity resumes (green, clutch out). E proteins can then induce master regulators and effector genes specific to each Th lineage. **(B)** Waves of graded Id3 (red) induction in response to TCR signaling pauses a subset E protein activity (clutch in) followed by reassembly of E proteins at successive stages of T cell development (clutch out). Levels of Id3 dictate lineage choice, but E proteins are often engaged in both choices downstream of lineage commitment. Id2 (green) is responsible for differentiation away from the adaptive T cell lineage and into the innate lymphoid cell lineage (ILC) or invariant natural killer T (iNKT) cell lineage. DN=double negative CD4-CD8-, DP=double positive CD4+CD8+, γδT1 = IFNγ-producing γδ T cells, γδT17 = IL-17 producing γδ T cells.

However, if E2A levels are too high, FoxP3 expression becomes unstable in T-regs, emphasizing the importance of transcription factors levels in maintaining stable outcomes (26).

The Clutch model of E protein/Id3 activity in T cell transitional states

The theme of transient Id3 expression followed by shifting E protein target gene activation suggests what we term a "Clutch" model of Th differentiation (Figure 1). In this model, Id3-mediated pausing of E protein activity would act like the clutch of a car, withholding access to the engine (E protein activity) until the appropriate gear (chromatin context) is engaged, and then allowing the engine to move the car (Th differentiation) forward in a controlled fashion (Figure 1A). E proteins bind to many effector genes in Th subsets. Therefore, it is likely that restriction of E protein binding to the "right" set of mediators within each lineage is essential for linking environmental input to functional output in Th subsets. This is clearly a strong paradigm for peripheral T cell differentiation (27). The Clutch model also applies to T cell development in the thymus, but with a twist, as described below (Figure 1B).

Moreover, the role of Id2 in thymic T cell development exhibits stark differences from Id3 during thymic development and does not conform to the Clutch model.

Id2 regulates the innate/adaptive fate choice in early T cell precursors

The earliest T cell progenitors (ETPs) to enter the thymus are not yet committed to the T-cell lineage and have alternative fates available to them depending on their access to microenvironmental signals. One of the key molecular switches that must be flipped to gain access to the T cell pathway is to increase E protein activity. This occurs in at least two different ways. The first is upregulation of E proteins at the mRNA level, and the second is the downregulation of Id2 (28). Id2 is a critical mediator of the innate/adaptive lineage split (18, 29). ETPs express "legacy genes", thus termed because they are expressed in hematopoietic stem cells (30). ETP legacy genes include Id2, the Ets protein PU.1, and the Class II bHLH factor SCL. All three of these factors can act in opposition to T-lineage commitment: PU.1 drives expression of myeloid and B cell genes (31), SCL can re-direct E proteins to stem cell gene loci and away from T cell gene loci (32), and Id2 interferes with E protein activity. E protein activity is essential for the expression of Rag recombinase genes, which are necessary for the generation of TCRs and thus T cells (33). Unlike Id3, Id2 does not appear to be under the influence of transient signals during thymocyte development but rather is subject to degradation in a cell cycle-dependent manner (34, 35). Downregulation of PU.1 and upregulation of Bcl11b in early T cell development results in the cessation of Id2 mRNA expression, which allows upregulation of T-lineage E protein target genes (36, 37). Conversely, Id2 expression is maintained in mature innate cells including ILCs, NK cells, and myeloid cells, and appears to support the maintenance of lineage fidelity.

Notch signaling shifts the E protein-Id2 balance to allow T cell development

As ETPs enter the thymus, they are exposed to Delta-like (Dll) ligands of Notch receptors, resulting in strong Notch signaling. Notch signaling is indispensable for T cell specification and lineage commitment, acting upstream of an elegant cascade of transcription factors that inhibits alternative fates and induces T-cell genes (38). While Notch regulates a wide swath of important target genes, one of the most important roles plays in T-lineage commitment is by shifting the balance between Id and E protein activity in ETPs, in three complimentary ways. First, Notch redirects PU.1 away from Id2 and towards more T-lineage friendly genes (39). Secondly, Notch upregulates the E protein HEBAlt, increasing the overall E protein availability (40). Thirdly, Notch directly upregulates Bcl11b, which downregulates Id2 at the transcriptional level (37). This delivers a one-two-three punch that directs cells permanently away from Id2-dependent ILCs and into the T-cell lineage. Thus, Id2 does not acts as a way station for changing gene availability to E proteins, but instead is more akin to a railroad switch that directs cells down one pathway or another (Figure 2).

TCR signal strength determines lineage outcomes during the intrathymic T cell lineage choices

Once cells have been switched onto the T-lineage track, they progress towards the first "checkpoint" of T cell development. There are two main checkpoints that occur during T cell development, so called because they serve as testing of the cells for functional TCR rearrangement and function (Figure 1B). During the first checkpoint, the TCRβ chain pairs with the pre-Tα chain to form a pre-TCR. The only requirement for the pre-TCR to allow "passage" through the checkpoint is for it to complex with CD3 chains and translocate to the cell membrane long enough to invoke a weak set of signaling cascades (41). Alternatively, the cell can rearrange and express a TCR composed of TCRγ and TCRδ chains. In this situation, the γδ TCR/CD3 complex is stably expressed on the surface, transmitting a stronger signal than that transduced by the pre-TCR, which directs cells away from the αβ T cell fate and into the γδ T cell fate (42, 43). After commitment to the αβ T cell lineage, cells expressing αβ TCRs are subjected to second "checkpoint" which vets these TCRs for their ability to bind to MHC/peptide and assesses the affinity of the interaction. As with the first checkpoint, this signal also serves as a lineage branchpoint, with cells experiencing lower and briefer TCR signaling adopting the CD8 fate, and cells experiencing longer and stronger TCR signaling progressing into the CD4 T cell lineage (44). This paradigm also applies to committed γδ T cells that progress along the IFNγ-producing γδT1 fate or the γδT17 fate (45) (Figure 1B). Engagement of strong γδ TCR ligands in conjunction with co-stimulatory molecules results in strong TCR signaling and the γδT1 developmental outcome, whereas a less strong TCR signal leads to the γδT17 fate (46–48). All these lineage choices are intimately associated with the balance between Id3 and E proteins (49, 50).

Translation of TCR signal strength into Id3 activity modulates E protein target gene accessibility

As in peripheral CD4 T cells, TCR signaling in early precursors leads to upregulation of Id3, and a pause in E

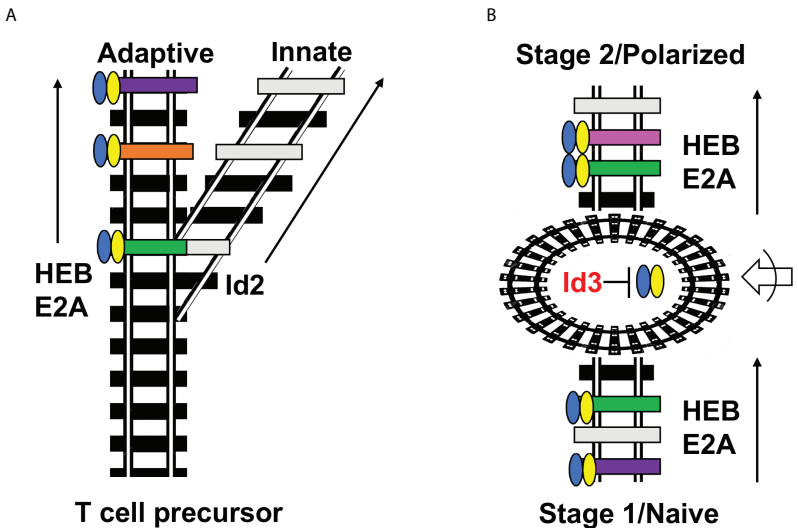

FIGURE 2
Railroad analogy of Id2 versus Id3 outcomes. **(A)** Id2 acts as a switch that diverts T cell precursors away from the adaptive fate and towards the innate fate by permanently silencing E protein activity and E protein target gene expression. **(B)** Id3 serves as a way station (roundhouse) that allows changes in accessibility of E protein target genes while E proteins are inactive, followed by E protein engagement with different E protein target genes at the next stage of development. This occurs in both CD4 T cell differentiation (Naïve/Polarized) and multiple stages of thymic differentiation (Stage 1/Stage 2). Blue-Yellow ovals = HEB/E2A. Black railroad ties = completely inaccessible genes; gray railroad ties = accessible genes lacking the proper combination of transcription factors for induction; colored railroad ties = E protein target genes bound by HEB/E2A and undergoing active transcription. Roundabout = Id3-mediated pause in E protein activity during which changes in accessibility of E protein target sites occurs. Arrow with half circle = extracellular signaling inputs that direct which genes undergo changes in chromatin accessibility.

protein activity allows chromatin remodeling and shifting of E protein target availability. There may also be a role for TCR signal strength during T helper cell differentiation, particularly in combination with cytokine signaling (51). However, there is a clear hierarchy of TCR signal strength that is induced at each checkpoint in thymic T cell development (52). During T cell development, TCR signaling may shift the balance between Id3 and E proteins to different degrees, allowing retention of E protein occupancy on some sites but not others. E2A and HEB are direct regulators of most of the genes needed for assembly of the TCR genes and formation of the pre-TCR (53, 54). Id3 is also induced in response to αβTCR signaling at the DP stage, and is necessary to overcome the gatekeeper function of E proteins at the DP to SP transition (55, 56). However, past this checkpoint, E proteins are required for the generation of CD4 SP cells (57). E proteins also regulate genes in γδ-T committed cells that dictate functional programming, including *Tcf7* (58). An elegant study by Hosoya and colleagues shed considerable light on the chromatin remodeling events that occur during αβ T cell development using ATAC-seq, which detects open chromatin and predicts the presence of transcriptional complexes (59). This study showed that the loci for both γδ-lineage and αβ-lineage genes were accessible in DN thymocytes. However, as cells transitioned from the DN to the DP stage and then to the CD4 and CD8 stages, cis-regulatory elements with predicted binding by the key γδ-lineage factor Sox13 showed a dramatic loss of accessibility. Likewise, predicted HEB sites shifted in accessibility according to the stage of αβ T cell development, consistent with Id3-facilitated chromatin remodeling at these transitions. This is doubtless just the beginning of this new phase of our journey towards a deeper understanding of T cell developmental transitions, and it will be exciting to learn how E protein genomic site occupancy changes after they are dislodged and then reassembled on different loci at progressive stages of T cell development and differentiation.

Limitations of the Clutch model of Id3-facilitated shifts in E protein targets

Like E proteins, Id3 is used widely in different contexts outside of T cell development (60, 61). Clearly, the Clutch model does not apply in all situations, but rather appears to be restricted to certain types of cells and developmental transitions. Moreover, an examination of E2A occupancy at the DN3 to DN4 transition revealed both overlapping and unique sites of E2A occupancy in both subsets, indicating that E2A was only dislodged from a subset of sites during the transition, while others were maintained (19). Release of E proteins from specific sites likely depends on both the Id3/E protein ratio and the availability of E protein binding partners.

For instance, the downregulation of Notch1 in response to pre-TCR signaling would be predicted to increase the disengagement of E proteins from sites that require both Notch factors and E proteins, but not from other sites that maintain the core T-lineage program. Importantly, E proteins themselves are important mediators of chromatin remodeling, interacting directly with both positive and negative regulators of chromatin configuration such as p300, CHD4, LSD1, and PRC2 (62–66). It is important to note that chromatin remodeling in this context does not indicate simply a shift between "open" and "closed" configurations, but also includes the transition from "poised" to "active" states (67). This may be mediated in part by fresh access to new binding partners that become available after the transition. Furthermore, the plasticity of CD4 T cell subsets suggests that lineage-specifying E protein sites remain accessible during and after CD4 T cell differentiation (68). A comprehensive understanding of global E protein occupancy changes that occur during these processes awaits further studies. Likewise, the relative contributions of E2A versus HEB to these processes are not well understood.

Discussion

While it is well understood that Id proteins inhibit E protein activity and interfere with the expression of E protein target genes, much less is known about how E protein targets shift during the developmental transitions that occur during Id3 expression, and the molecular events that underpin them. Here, the Clutch model is presented as a conceptual scaffold that will provoke questions and undergo modifications and stratification as new data is obtained revealing E protein chromatin occupancy before, during, and after T cell stages transitions, and identifying stage-specific E protein partners. Due to technical limitations, earlier studies largely relied on *in vitro* models of T cell development or differentiation such as OP9-DL co-culture derived T cell precursors or *in vitro* polarization of naïve peripheral T cells (69, 70). While these studies have provided a wealth of information into the global events that orchestrate T cell development, they cannot completely replicate the complex thymic niches that shift over time as cells migrate through different niches in the thymus, nor can they fully provide the complex medley of signals that transpire during a coordinated immune response. The advent of single cell RNA-seq, and multiomic approaches such as scRNA-seq/ATAC-seq and CITE-seq that allows that require fewer input cells are now providing unprecedented access to *ex vivo* precursors and products that arise during T cell development. Moreover, computational methods such as pseudotime modeling and RNA velocity are further advancing our understanding of transient states of development (71). Importantly, there is a fourth dimension that is rarely considered in these snapshot approaches: time. Single cell live imaging has revealed that Id3 transcription is "bursty", occurring in only a small number of cells within a population at any one time, in the B cell lineage (72). It remains to be seen whether this is true in T cell precursors, and whether TCR signaling can synchronize cells into uniformly high Id3 expressers. Alternatively, burstiness may contribute to the gradation of Id3 that mediates intrathymic T cell fate choices. By contrast, Id2 acts as a permanent switch into the innate lineage choice. This distinction highlighting the unique nature of Id3 in regulating fate choices by facilitating E protein target changes as T cells journey through development in the thymus or differentiate in the periphery during an immune response.

Author contributions

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

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Conflict of interest

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

Publisher's note

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Protein ubiquitination in T cell development

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As an important form of posttranslational modification, protein ubiquitination regulates a wide variety of biological processes, including different aspects of T cell development and differentiation. During T cell development, thymic seeding progenitor cells (TSPs) in the thymus undergo multistep maturation programs and checkpoints, which are critical to build a functional and tolerant immune system. Currently, a tremendous amount of research has focused on the transcriptional regulation of thymocyte development. However, in the past few years, compelling evidence has revealed that the ubiquitination system also plays a crucial role in the regulation of thymocyte developmental programs. In this review, we summarize recent findings on the molecular mechanisms and cellular pathways that regulate thymocyte ubiquitination and discuss the roles of E3 ligases and deubiquitinating enzymes (DUBs) involved in these processes. Understanding how T cell development is regulated by ubiquitination and deubiquitination will not only enhance our understanding of cell fate determination *via* gene regulatory networks but also provide potential novel therapeutic strategies for treating autoimmune diseases and cancer.

KEYWORDS

T cell development, thymocyte, ubiquitination, E3 ubiquitin ligase, deubiquitinating enzyme

Introduction

Ubiquitin is a highly conserved protein of 76 amino acids and a versatile posttranslational modifier that is ubiquitously expressed in all eukaryotic cells (1). Protein ubiquitination plays a crucial role in protein homeostasis, thus regulating a vast array of biological processes, such as DNA damage and repair, cell cycle progression, apoptosis and cellular signaling (2, 3). Ubiquitin is added to the protein substrate *via* a subsequent enzymatic cascade by E1 ubiquitin-activating enzymes, E2 ubiquitin-conjugating enzymes and E3 ubiquitin ligases (4). The specificity of ubiquitination is mainly achieved by E3 ligases, which are responsible for substrate recognition *via* protein interacting domains and motifs (5). Ubiquitin has seven lysine residues that can be used

to assemble polyubiquitin chains: Lys6, Lys11, Lys27, Lys29, Lys33, Lys48, and Lys63. A substrate can be polyubiquitylated or monoubiquitylated *via* polyubiquitin chains, and the impact of polyubiquitination on the target protein is greatly dependent on the type of conjugated chain (6). For example, except for Lys63, all six Lys linkages have been implicated in proteasomal degradation, with Lys48 and Lys11 being the predominant type of chains for substrate degradation in cells. Lys63-linked chains are involved in multiple nonproteolytic functions, including activation of NF-κB, DNA damage repair, and regulation of endosomal sorting pathways (7). Ubiquitination is a dynamic and reversible process, and ubiquitination induced by ubiquitin ligases can be counteracted by deubiquitinating enzymes (DUBs) to control the intensity and duration of ubiquitin signaling (8).

The thymus is the primary site for T cell development, thymic seeding progenitor cells (TSPs) arrive at the thymus from the bone marrow and initiate multistep maturation programs and checkpoints comprising lineage commitment, T cell receptor (TCR) gene rearrangement, and positive and negative selection. It is well established that thymocytes mature through ordered progression, including double-negative ($CD4^-CD8^-$, DN) stage, double-positive (DP) stage and CD4 or CD8 single-positive (SP) stages (9, 10). In the earlier DN1-3 stages, proliferation and differentiation are mainly driven by Notch signaling and cytokines such as c-kit and IL-7 (11). Then, cells successfully assembled pre-T cell receptor (pre-TCR) complexes will pass β-selection and transition from the DN3 to the DN4 stage. In DP stage, thymocytes undergo positive selection for self-human leukocyte antigen (HLA) recognition under the control of cortical thymic epithelial cells (cTECs) and negative selection to remove strong self-reactive clones based on the interaction with medullary thymic epithelial cells (mTECs) and thymic DCs (tDCs), finally becoming $CD4^+$ SP or $CD8^+$ SP cells (11). "Mature" SP thymocytes exit the thymus to the peripheral lymphoid organs (9).

Ubiquitin signaling modulates a variety of pathways involved in the T cell developmental process primarily through proteolysis-dependent mechanisms, such as Notch, pre-TCR signaling, Signal transducer and activator of transcription 3 (STAT3)-mediated signaling, Wnt signaling, and Nuclear factor κB (NF-κB) pathway (9, 12, 13). Here, we summarize the interplay between the ubiquitination system and T cell developmental programs (Figure 1). Specifically, we highlight the roles of E3 ligases and DUBs involved in these processes as well as the molecular mechanisms and cellular pathways that regulate thymocyte ubiquitination (Table 1).

E3 ubiquitin ligases in T cell development

E3 ligases are crucial components of the Ubiquitin Proteasome System. Several classes of these enzymes have been identified, known as the RING, U-box, HECT and RBR classes (45). As the last component of an enzymatic cascade, E3 ligases determine substrate specificity. Attaching ubiquitin to a protein could have profound effects on the protein's cellular localization, protein-protein interactions or stability (46). Multiple E3 ligases have been demonstrated to play a role in T cell development.

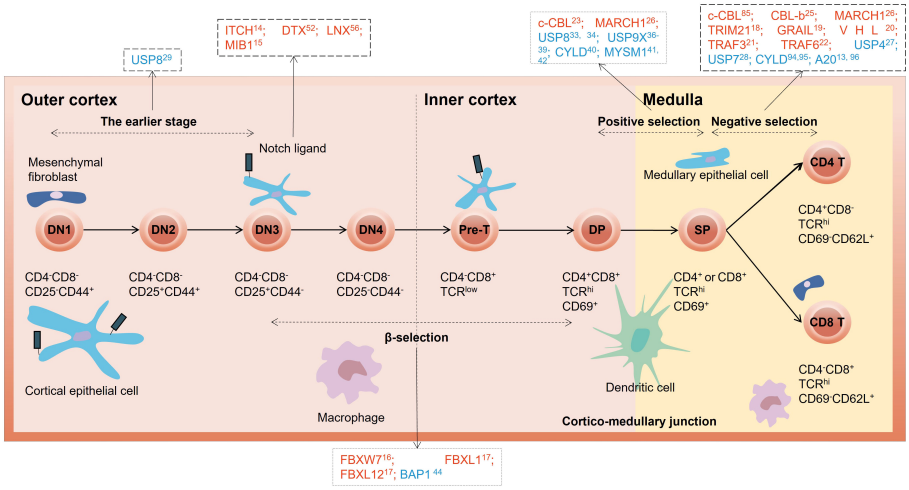

FIGURE 1
Overview of E3 ubiquitin ligases and DUBs in different stages of thymocyte development. The red letters in the black dotted box represent E3 ubiquitin ligases, and the blue letters represent DUBs. DN, double-negative; DP, double-positive; SP, single-positive.

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TABLE 1 List of E3 ligases and DUBs that modulate T cell development.

Ubiquitinase	Substrate	Cko/ko mice	Phenotype	Ref.
		E3 ligases		
Itch	Notch	Itch$^{-/-}$; Lck-Notch1 tg^{+}	-reduces DP and increases DN thymocytes -reduces apoptosis in the thymus and increases phospho-AKT signaling	(14)
Mib1	Dll1, Dll4	Mib1$^{-/-}$	-impairs Dll1 and Dll4 endocytosis -reduces DP and increases DN thymocytes	(15)
Fbxw7	c-Myc	Lck-Cre; Fbxw7$^{fl/fl}$	-promotes cell cycle exit -leads to hyperproliferation in thymocytes -increases DP thymocytes -enforces GATA3 expression	(16)
Fbxl1	Cdkn1b	Fbxl1$^{-/-}$	-resultes in an incomplete DN3-DN4 developmental block	(17)
Fbxl12	Cdkn1b	Lck-Cre; Fbxl12$^{fl/fl}$	-blocks DN3-DN4 transition	(17)
TRIM21	SOCS3	TRIM21$^{-/-}$	-increases number of thymocytes -reduces frequency of DN cells	(18)
GRAIL	TCR-CD3	GRAIL$^{-/-}$	-upregulates the function of tTregs	(19)
VHL	HIF-1α	Lck-Cre; Vhl$^{fl/fl}$	-increases cell death and caspase activity -reduces TCR-mediated Ca^{2+} signaling	(20)
TRAF3	TCPTP	Lck-Cre; TRAF3$^{fl/fl}$	-increases number of Treg cells in the thymus	(21)
TRAF6	NF-kB essential modifier (NEMO)	TRAF6$^{-/-}$	-reduces autoimmunity -reduces Aire expression -reduces Treg cells	(22)
c-Cbl	CD5, TCRζ, Zap-70, SLAP, BIM	c-Cbl$^{-/-}$	-increases TCR signaling -increases DP thymocytes -increased expression of CD3, CD5, and CD69 -enhances positive Selection of CD4^{+} T Cells	(23, 24)
Cbl-b	Foxp3, p85	Cbl-b$^{-/-}$	-regulates tTregs -reduces mature SP thymocytes	(25)
MARCH1	MHCII	MARCH1$^{-/-}$	-reduces tTregs	(26)
		DUBs		
USP4	HUWE1	USP4$^{-/-}$	-induces IR-induced apoptosis in thymus	(27)
USP7	Caspase 3	/	-regulates the apoptosis of thymocytes via interacting with caspase 3	(28)
USP8	GADS, CHMP5	CD4-cre;USP8$^{fl/fl}$	-diminishes thymocyte proliferation	(29–34)
USP9X	Themis	USP9X$^{-/-}$	-reduces thymic cellularity	(35–39)
CYLD	LCK	CYLD$^{-/-}$	-regulates DP-SP transition	(40)
MYSM1	IRF2, IRF8	MYSM1$^{-/-}$	-reduces thymus sizes and CD8^{+} T-cell numbers	(41, 42)
A20	GITR	CD4-cre;A20$^{fl/fl}$	-increases CD69 expression within NKT thymocytes	(13, 43)
BAP1	H2AK119	Rosa26^{CreERT2}; Bap1$^{fl/fl}$	-causes a block at the DN3 stage	(44)

NOTCH-regulating E3 ligases mainly regulate the early stage of T cell development

Notch signaling has been identified as a key signaling pathway involved in the regulation of T cell development, especially in thymocyte survival, proliferation and differentiation (9, 47–49). E3 ubiquitin ligases that can catalyze the ubiquitylation of Notch include Itch, Ligand of Numb-Protein X (LNX), Deltex (DTX), Mind bomb (Mib) 1, Mib2, Neuralized (Neur) 1, and Neur2 (15). Itch binds to the N-terminal of the Notch intracellular domain *via* its WW domains and promotes ubiquitination of Notch *via* K29-linked ubiquitin chains, thus promoting its lysosomal degradation (50). Itch$^{-/-}$ mice with an activated Notch1 transgene in their thymocytes show a reduction of DP and an increase of DN T cells, with a more severe autoimmune phenotype (14). Itch and Notch act in the AKT signaling concurrently in the genesis of autoimmune disease (14). In addition, Itch regulates Notch signaling *via* interacting with some molecules, such as Numb and DTX. Numb, an adapter protein, was initially identified as a negative regulator of Notch signaling. Numb binds to Itch WW domain and promotes ubiquitination and degradation of Notch1 by Itch (51). DTX, an E3 ligase, has been shown to be an itch homolog that plays a negative role in regulating Notch receptor signaling, and can cooperate with Itch to regulate NOTCH signaling *via* lysosomal degradation (52). In addition, downregulation of DTX in hematopoietic progenitors promotes T cell development in fetal thymic organ culture and *in vivo* (53). DTX antagonizes Notch1 signals by inhibiting

coactivator recruitment (54) and restores DP thymocyte survival from the glucocorticoid (GC)-induced apoptosis by repressing SRG3 promoter activity (55). LNX can also cause proteasome-dependent degradation of Numb and therefore enhance Notch signaling (56). Mib1 modulates Notch signaling by ubiquitinating the Notch receptors (Dll1 and 4), promoting their endocytosis (57). Reciprocal bone marrow (BM) transplantation experiments revealed that Notch signaling was diminished in the DN thymocytes of Mib1 conditional KO mice (15). Furthermore, knocking down Mib1 in the coculture system causes a delay in T cell growth and a failure of Dll1 endocytosis (15).

SCF complexes play crucial roles in thymic β-selection mediated cell proliferation

The SCF (Skp1-cullin-F-box protein) complex is a well-described multisubunit RING-finger E3 composed of Skp1, Cdc53/cullin, and an F box protein (58). Fbxw7 (F-box and WD-40 domain protein 7)—also known as Fbw7—is an SCF ubiquitin ligase component reported to play a role in thymocyte cell cycle progression by controlling the degradation of c-Myc, c-Jun, cyclin E, and Notch (59). Fbw7 modulates cell cycle progression by controlling c-Myc protein stability, and loss of Fbxw7 leads to hyperproliferation of thymocytes (16). Moreover, the SCF subunits Fbxl1 and Fbxl12, which are transcriptionally induced by Notch and pre-TCR signaling respectively, function identically but additively to promote the degradation of Cdkn1b and proliferation of β-selected thymocytes (17, 60). Deletion of Fbxl1 or Fbxl12 results in an incomplete DN3-DN4 developmental block and a reduced thymus size (17).

TRIM family proteins have crucial roles during negative selection

As RING-type E3 ligases, tripartite motif (TRIM) proteins have been demonstrated to regulate the innate immune response (61, 62). However, recent studies suggest that TRIM21 alters T cell development in the thymus (63). TRIM21$^{-/-}$ mice had an increased number of thymocytes and a reduced frequency of DN cells (18). TRIM21 targets suppressor of cytokine signaling-3 (SOCS3) for proteasomal degradation, thus impairing STAT3 activation in TECs (64). STAT3-mediated signaling has been shown to promote quintessential growth of mTECs (but not cTECs) (12, 65). Double-positive (DP) cells are selected by cTECs to become CD4 or CD8 SP cells (66), while SP thymocytes are further negatively selected in the medulla (67). We can surmise that TRIM21 plays a crucial role during negative selection in the thymus.

GRAIL and VHL regulate T cell development during negative selection

Gene related to anergy in lymphocytes (GRAIL) is a RING-type E3 ligase required for the initiation of CD4$^+$ T cell anergy *in vivo*. Previous studies considered GRAIL expression patterns in murine CD4$^+$ T cells as a defined anergic phenotype and a negative regulator of the immune response (68, 69). Notably, GRAIL expression is upregulated in tTregs, and its overexpression in DO11.10 T cells convert these cells to a regulatory phenotype (19). Nurieva et al. reported that GRAIL regulates Treg cell function by mediating TCR-CD3 degradation (70). Works are needed to delineate the mechanism(s) of how GRAIL mediates its suppressor activity in the thymus. The von Hippel-Lindau (VHL) is a RING-type E3 ligase that targets hypoxia-inducible factor-1α (HIF-1α) for proteasomal degradation (20). Vhl-deficient mice had a severe reduction in thymus sizes and thymic cellularity due to enhanced caspase 8 activity in the apoptotic pathway, as a result of HIF-1α accumulation (20).

TRAF family proteins regulate T cell development during negative selection

Tumor necrosis factor receptor (TNFR)-associated factor 3 (TRAF3) is a member of the TRAF family of cytoplasmic adaptor proteins and plays a role in modulating IL-2 signaling in T cells. T cell conditional TRAF3 knockout mice resulted in an increased number of Treg cells in the thymus (21) due to more efficient conversion of CD25$^+$ Foxp3$^-$ Treg precursors to CD25$^+$ Foxp3$^+$ mature Treg cells (71). TRAF6 is another adaptor E3 ligase that is involved in central tolerance by regulating the development of thymic stroma. TRAF6$^{-/-}$ fetal thymic stroma tissue fails to mediate negative selection (22). Furthermore, specific deletion of TRAF6 in TECs hinders the growth of mTECs (72). Several studies have suggested that TRAF6 regulates the establishment of thymic microenvironments through manipulating RelB (73), RANK (74) and CD40 (75) expression.

Cbl family proteins regulate multiple stages of T cell developmental processes

The Casitas B-lineage lymphoma (Cbl) family of proteins are RING-finger domain containing E3 ubiquitin ligases (76, 77). In mammals, two highly homologous adaptor proteins of the Cbl family, c-Cbl and Cbl-b, are involved in the negative regulation of the immune system (78, 79). Both c-Cbl and Cbl-b contain a highly conserved amino-terminal tyrosine-kinase binding (TKB) domain, a less conserved carboxyl-terminal proline-rich region (PRR) and a

RING finger. Through their protein-protein interaction domains, c-Cbl and Cbl-b form multiple complexes together with several signaling molecules to regulate intracellular signaling events (80). The first evidence indicating that Cbl proteins are associated with thymic selection came from experiments showing that thymocytes from c-Cbl$^{-/-}$ mice have increased signaling through the TCR and CD4^{+} CD8^{+} DP thymocytes exhibited increased expression of CD3, CD5, and CD69 in the c-Cbl knockout (KO) model (23). Moreover, c-Cbl selectively inhibits thymic-positive selection of CD4 but not CD8 T cells (23). This suggests that the positive selection of thymocytes bearing MHC class II-restricted TCRs is negatively regulated by c-Cbl. Mechanistically, c-Cbl modulates CD4^{+} T-cell development by promoting TCR-ζ lysosomal degradation. In this model, a transient trimolecular complex of TCRζ-Zap-70-Cbl is formed, and ubiquitin is then shifted from the Cbl-E2 complex to TCRζ (79, 81). In addition to Zap-70, Src-like adaptor protein (SLAP) might also act as a bridge to bond TCRζ and Cbl. In support of this, SLAP$^{-/-}$ mice were shown to have a similar phenotype to c-Cbl$^{-/-}$ mice (82–84). In addition to positive selection, c-Cbl also regulates thymocyte negative selection, probably by ubiquitinating and proteasomal degrading the pro-apoptotic molecule B-cell lymphoma 2-interacting mediator of cell death (BIM) (85). Furthermore, deactivation of c-Cbl reverses T cell developmental detention in SLP-76-deficient mice, in which T cell development is impeded at the DN3 stage (24). In conclusion, the c-Cbl protein modulates multiple stages of T cell developmental processes.

Analyses of Cbl-b KO mice resulted in no similar findings (86). Given that the expression level of Cbl-b in thymocytes is much lower than that of c-Cbl, it would not be surprising. However, Zhao Y et al. reported that Cbl-b, together with Stub1, regulates thymic-derived CD4^{+} CD25^{+} regulatory T cells (tTregs) development by targeting Foxp3 for ubiquitination and degradation in the proteasome (25). Moreover, Raberger J et al. reported that the CD4/CD8 developmental profile was noticeably altered and mature SP thymocytes were absent in Vav1$^{-/-}$ or ITK$^{-/-}$ thymocytes (87), and the signaling defects in Vav1$^{-/-}$ or ITK$^{-/-}$ thymocytes can be rescued upon deletion of Cbl-b (87). These results indicate that Cbl-b alters thymus development.

MARCH family E3 ligases modulate the development of tTregs

Membrane-associated RING-CH1 (MARCH1) is an E3 ubiquitin ligase that regulates MHCII ubiquitination (26). Thymocytes and TECs scarcely express MARCH1, while DCs in the thymus express comparatively high levels of MARCH1 (26). MARCH1 deficiency results in an elevated level of MHCII, which leads to a considerable decline in the number of thymic Treg (tTreg) cells but not conventional CD4^{+} T cells in mice (26). Another E3 ligase, MARCH8, is responsible for MHC II ubiquitination specifically in thymic epithelial cells. In MARCH8$^{-/-}$ mice, TECs express elevated levels of MHC II, but the development of conventional CD4^{+} T cells or tTreg cells remains unchanged. It is possible that tTreg development does not require MHC II ubiquitination in TECs (88).

DUBs in T cell development

In addition to E3 ligases, the ubiquitin system is also regulated by DUBs. Ubiquitin chains can be removed from the substrate by DUBs, which are essential for the dynamic regulation of the protein ubiquitination process (89, 90). Several DUBs have been identified as regulators in the T cell developmental program.

USP family proteases regulate multiple stages of T cell developmental processes

Ubiquitin-specific proteases (USPs) are the largest subfamily of DUBs and contain more than 100 members (91). Ubiquitin-specific peptidase 4 (USP4) has been shown to inhibit p53 signaling through interacting with and stabilizing ARF-binding protein 1 (ARF-BP1, also known as HUWE1), an E3 ligase for p53 (24). USP4 knockout mice are viable and fertile but exhibit enhanced ionizing radiation (IR)-induced thymocyte apoptosis (27). In addition, USP4, a DUB with dual hydrolyzing activity for K48- and K63-conjugated polyubiquitin chains, interacts with the Nemo like kinase (Nlk) and T-cell factor (TCF) 4, two known components of the Wnt pathway that are essential for cell development (92). USP7 (also known as HAUSP), which is highly expressed in the thymus, also regulates the apoptosis of thymocytes during negative selection *via* caspase-dependent signaling (28). Likewise, the processing of HAUSP does not occur in caspase 3-deficient thymocytes (28). Ubiquitin-specific protease USP8 is a deubiquitinase involved in the endosomal sorting complex required for transport (ESCRT) system (93). A recent study reported that USP8 is involved in thymocyte maturation and proliferation processes by modulating the Foxo1-IL-7Rα axis (29). Moreover, the amino-terminal SH3BM of USP8 binds with higher affinity to the TCR adaptor GADS in a caspase-dependent manner (30–32). Another study identified USP8 as a deubiquitinase for CHMP5, a component of the ESCRT complex, and uncovered the role of the CHMP5-USP8 complex in regulating thymic positive selection (33, 34). Ubiquitin-specific protease 9X (USP9X) is a member of the peptidase C19 family and encodes a protein similar in structure to ubiquitin-specific proteases. Deletion of Usp9X resulted in an overall reduction in thymic cellularity (35). Mechanistically, USP9X interacts with and stabilizes Themis, an important TCR signaling protein (36), by removing ubiquitin K48-linked

chains on Themis upon TCR stimulation, thus affecting thymic positive selection (37–39).

CYLD regulates T cell development during negative selection

Cylindromatosis (CYLD) is a lysine 63-deubiquitinating enzyme that positively regulates TCR signaling by promoting the recruitment of Lck to its substrate, Zap70, in thymocytes (40). CYLD-deficient mice displayed significantly fewer mature $CD4^+$ and $CD8^+$ single-positive thymocytes (40). Previous studies identified CYLD as a switch in T cell development during the transition from double-positive to single-positive thymocytes (40). Furthermore, S. Reissig et al. demonstrated impaired negative selection in the thymus of $CYLD^{ex7/8}$ mice, which overexpresses the naturally occurring CYLD splice variant short CYLD (sCYLD), whereas full-length CYLD (FL-CYLD) is absent (94, 95).

MYSM1, A20 and BAP1 modulate multiple stages of T cell developmental processes

Other types of DUBs involved in T cell development include Myb-like SWIRM and MPN domain containing 1 (MYSM1), A20 and BRCA1-associated protein-1 (BAP1). Conditional ablation of histone H2A deubiquitinase MYSM1 at late stages of thymic development in a mouse model showed a severe reduction in thymus sizes and $CD8^+$ T-cell numbers, indicating the critical role of MYSM1 in the positive selection of $CD8^+$ T cells (41, 42). A20, also known as TNF-α-induced protein 3 (TNFAIP3), regulates tTreg development and maturation by restraining the activation of NF-kB signaling (13, 96). T lineage cell conditional A20 knockout mice showed that tTreg cell compartments are quantitatively enlarged (13). In addition, A20 specifically limits TCR-dependent activation of NKT cells in the thymus (43). BAP1 is a member of the ubiquitin C-terminal hydrolase (UCH) subfamily of DUBs and has been shown to be involved in β-selection mediated cell expansion (44). BAP1 deletion in adult mice led to serious thymic atrophy and loss of cellularity due to defects in cell proliferation (97). Likewise, BAP1 deficiency caused a block at the DN3 stage before the pre-TCR checkpoint by facilitating the ubiquitination of histone H2A at Lys^{119} (H2AK119) (97).

Conclusion

During the past few years, several lines of evidence have shown that T cell development is regulated at multiple levels; in addition to transcriptional control, posttranslational regulation also plays a crucial role in those processes (9, 98, 99). An increasing number of studies using transgenic mouse models have demonstrated that E3 ubiquitin ligases and DUBs are involved in specific stages of thymocyte maturation by modulating the activity or stability of key proteins during cellular signal transduction cascades (98, 99). Technological advancements in single-cell proteomics, CRISPR/Cas9 mutagenesis and mass cytometry will continue adding valuable findings to this area of research. Future work on the molecular mechanisms of ubiquitination and deubiquitination in T cells will not only enhance our understanding of cell fate determination *via* gene regulatory networks but also provide potential novel therapeutic strategies for treating autoimmune diseases and cancer.

Author contributions

XLi and BZ: conceptualization and guidance. TZ: writing the original draft. KL: visualization. XLn: provide assistances. ZZ and SL: proofreading. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

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Effects of sex steroids on thymic epithelium and thymocyte development

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Sex steroid hormones have major effects on the thymus. Age-related increases in androgens and estrogens and pregnancy-induced increases in progestins all cause dramatic thymic atrophy. Atrophy can also be induced by treatment with exogenous sex steroids and reversed by ablation of endogenous sex steroids. Although these observations are frequently touted as evidence of steroid lymphotoxicity, they are often driven by steroid signaling in thymic epithelial cells (TEC), which are highly steroid responsive. Here, we outline the effects of sex steroids on the thymus and T cell development. We focus on studies that have examined steroid signaling *in vivo*, aiming to emphasize the actions of endogenous steroids which, *via* TEC, have remarkable programming effects on the TCR repertoire. Due to the dramatic effects of steroids on TEC, especially thymic involution, the direct effects of sex steroid signaling in thymocytes are less well understood. We outline studies that could be important in addressing these possibilities, and highlight suggestive findings of sex steroid generation within the thymus itself.

KEYWORDS

thymocyte development, thymocyte selection, AIRE, androgens, estrogens, progestins

Introduction

The thymus

T cells are essential in the adaptive immune response to pathogens and tumors. Many core T cell programs and characteristics underlying their responses in the periphery are set during T cell development in the thymus. The thymus, therefore, is a key determiner of quantitative and qualitative characteristics of the adaptive immune response. The thymus is an encapsulated organ that is histologically divided into a cortex and medulla. Thymic epithelial cells (TECs), dendritic cells, and fibroblasts form a stroma through which developing T cells (thymocytes) migrate as they progress through various stages.

The first step of T cell development involves the entry of circulating bone marrow-derived early T-cell progenitors (ETPs) into the thymus, *via* high endothelial venules. This entry occurs at the corticomedullary junction, and is driven by signals that include CCL25 (1). Cortical TEC (cTEC)-expressed DLL4 and IL-7 then drive T lineage commitment and proliferation of $CD4^-8^-$ (double negative, or DN) thymocytes as they migrate outward to the subcapsular zone (2, 3). DN4 thymocytes that have undergone successful T cell receptor (TCR)-β selection upregulate CD4 and CD8 to become double positive (DP) thymocytes, rearrange TCRα, and migrate inward through the cortex. TCRs are tested against thymoproteasome- and cathepsin L-generated peptides, which have unique cleavage sites and maximize the survival (positive selection) of thymocytes with functional TCRs (4–7). TCR signaling in cortical DP thymocytes upregulates TCR and CCR7 expression and trafficking to the thymic medulla. This coincides with the phenotypic transition to the $CD4^+CD8^-$ or $CD4^-CD8^+$ (single positive) phenotype. In the medulla, medullary thymic epithelial cells (mTEC) express antigens that are otherwise only found in peripheral tissues (i.e., tissue-restricted antigens, or TRAs), which are cross-presented by dendritic cells and test TCRs against a broad array of self antigens. Strongly self-reactive thymocytes that express high levels of TCR-induced proteins such as Nur77 (8, 9) undergo negative selection (death) or strong agonist selection (i.e., diversion into alternate lineages, such as Treg) (10, 11), establishing central tolerance and prevention of autoimmunity. TRA expression is driven by the transcriptional regulators Aire and Fezf2, which stochastically drive ectopic expression of thousands of genes in mTEC expressing high levels of MHC class II ($mTEC^{hi}$) (12–14).

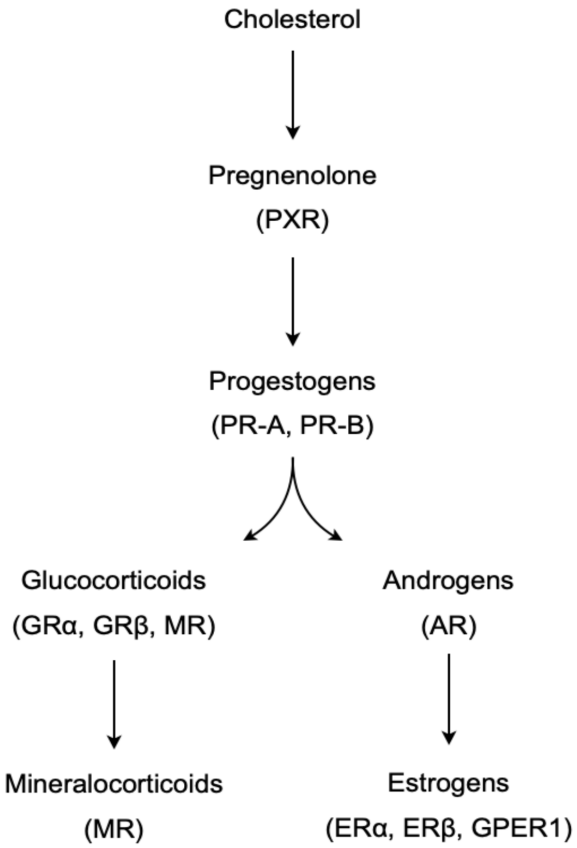

FIGURE 1
A simplified model of the steroidogenic pathway, showing the synthetic pathway of major steroid groups and their primary receptors in parentheses.

Steroids

Gonadal secretions, later identified as steroids, were among the earliest classes of signaling molecules that were recognized to have potent effects on the thymus (15–17). Steroids are small, lipophilic hormones derived from cholesterol *via* the stepwise action of a cascade of steroidogenic enzymes (Figure 1) (18). The particular suite of available and active enzymes determines the steroid products generated by a given tissue, with sex steroids (estrogens and progestins in females, androgens in males) classically produced by the gonads (female ovaries and male testes), and corticosteroids (glucocorticoids, mineralocorticoids) classically produced in the adrenal cortex. Gonad- and adrenal-secreted steroids function as classic endocrine hormones, circulating systemically to coordinate organismal development or responses to various stimuli. Steroids act on target cells primarily *via* binding to nuclear receptors, which are ligand-activated transcription factors that regulate expression of large portions of the genome. Steroids were initially categorized into groups by their major actions in the body: androgens as 'masculinizing' agents, estrogens as 'feminizing' agents, progestins as gestation-promoting hormones, glucocorticoids as energy-mobilizing hormones, and mineralocorticoids as regulators of electrolyte balance. Because these activities are mediated by genetically and functionally distinct receptors, steroids are now broadly characterized by their primary intracellular receptors, the androgen receptor (AR), estrogen receptor (ER), progesterone receptor (PR), glucocorticoid receptor (GR), and mineralocorticoid receptor (MR) (19). Each of these steroid classes can also bind membrane-associated receptors (e.g. the G protein-coupled estrogen receptor, GPER1) to induce second messenger or protein kinase cascades that have rapid nongenomic actions on target cells (20). These are generally considered to be more minor activities.

The initial identification and isolation of steroids in the 1930s was followed by studies examining their physiological actions in animals, especially when given in large doses. One of the most distinct and consistent results was the response of the thymus to progestins, androgens, estrogens, and glucocorticoids.

Large doses of these steroids resulted in a rapid and dramatic reduction in thymus size (21–23); the degree of thymus involution was even used as a bioassay to quantify steroid samples of unknown concentrations (24). This led to the notion of steroids as directly lymphotoxic molecules, which was supported by observations that endogenous sex steroid increases during puberty and pregnancy corresponded with thymic involution.

Steroids are central drivers of sexual development, and the types and concentrations of steroids in the blood are very different in females and males. These differences correspond with striking differences in immunity. Women compose the overwhelming majority of patients with autoimmune disease (>80%) (25) and are at greater risk of immune response-related pathology, whereas males are more susceptible to cancer and infection (26). This has long been attributed to the immunosuppressive actions of androgens and immunostimulatory actions of estrogens in peripheral immune responses. However, the dramatic responsiveness of thymi to steroids also raised the possibility that sex steroids might differentially affect the female and male thymus. In addition to differences in overall thymic output (27), recent data suggest there are important differences in the T cell repertoire. Given that sex steroid receptors are widely and variably expressed in thymic cell subsets (Table 1), it not surprising that steroids would have pleiotropic effects on the thymus and T cell development. In this minireview we provide an overview of the ways in which sex steroids regulate thymocyte development and the resulting composition of the peripheral T cell compartment.

Androgens

Androgens have historically been defined as steroids that stimulate the development of male characteristics, and are predominately produced by the testes. In males and females, however, androgens, especially inactive androgen precursors such as dehydroepiandrosterone (DHEA), are also produced by the adrenals and, in limited quantities, by the ovaries. Androgens act primarily by binding the AR, although androgen activation of membrane-associated receptors has been reported (28). In the periphery, androgens are generally thought of as being immunosuppressive, due in large part to suppression of cytokine production and cytotoxic effector function of activated T cells (29, 30). Furthermore, conditional deletion of AR in Treg cells reduces their number and increases Th2 cell numbers in allergic lung responses, implicating androgens as important suppressors of allergic responses in females and males (31). However, as detailed below, the immunosuppressive effects of androgens *in vivo* are likely largely due to their effects on thymocyte development and selection.

Thymic involution

Exogenous and endogenous androgens have potent effects on the thymus, with castration or inhibition of androgen synthesis causing dramatic thymic enlargement, and treatment with androgens causing atrophy (17, 21, 32). This is driven by signaling through the classical AR, since AR-deficient (ARKO) male mice have thymi that are twice the size of wild-type controls (33, 34) and are completely refractory to treatment with exogenous androgens (33, 35).

Androgen treatment *in vitro* causes DP thymocyte apoptosis; this is largely mediated through induction of DP TNFα production and is blocked by addition of anti-TNF antibodies or knockout of *Tnf* (36). However, this does not seem to be a major contributor to androgen-induced thymic atrophy *in vivo*. Rather, experiments using radiation bone marrow chimeras found that it is AR expressed by stromal

TABLE 1 Steroid receptor gene expression across different thymus cell subsets.

Steroid receptor gene (protein)	Expression level					
	DN	DP	CD4	CD8	cTEC	mTEC
Pgr (PR)	+	+	+	+	+++	++
Nr1i2 (PXR)	+	+	+	+	+	+
Ar (AR)	+	+	+	+	+++	++
Esr1 (ERα)	++	+	+	+	++	++
Esr2 (ERβ)	+	+	+	+	++	+
Gper1 (GPR30)	+	+	+	+	+++	++
Nr3c1 (GR)	++	+++	++	++	+++	++
Nr3c2 (MR)	+	+	+	+	+++	++

Gene expression of steroid receptors across major thymus cell subsets. Data are compiled from multiple sources including the Immunological Genome Project database. Relative expression across subsets is normalized individually for each gene (i.e. "+" for two different genes does not indicate equivalent expression.

cells, not thymocytes, that mediate the effect of androgens on thymus size (33, 35). These findings were confirmed using AR conditional knockout mice, in which the thymus was found to be of normal size in thymocyte ARKO and fibroblast ARKO mice, but much larger in TEC ARKO mice (33). It is the thymic epithelial compartment, therefore, that drives androgen-induced thymic involution. Of note, TEC ARKO thymi were not as large as global ARKO thymi, which may have been due to poor deletion of AR in cortical cTEC (34) or, perhaps, to contributions by another cell type. Androgen signaling in TEC appears to mediate changes in thymus size by inhibiting TEC proliferation (37) and inhibiting TEC expression of molecules that promote thymocyte survival and proliferation, such as *Ccl21* and *Il7* (33, 38).

Cortex

Androgens inhibit thymic seeding with ETPs, as there are fewer ETPs in castrated male mice (39). This was not due to effects on bone marrow hematopoiesis, as injection of T cell-depleted congenic bone marrow into castrated male mice resulted in increased numbers of DN thymocytes and later increased numbers of DP thymocytes (39). This was due to androgen inhibition of CCL25 expression by TEC, especially mTEC (38, 39). Androgens also suppress TEC expression of DLL4 (a Notch ligand critical for T lineage commitment) and IL-7 (which promotes DN survival and proliferation) (38), and in the case of the *Dll4* but not *Ccl25* and *Il7* the presence of androgen receptor binding sites in the promoter region. Chromatin immunoprecipitation and reporter plasmid experiments with the *Dll4* promoter demonstrated that binding of the liganded AR to androgen response elements was sufficient to increase gene expression (38). Finally, chemical inhibition of androgen production increased cTEC *Dll4* expression and thymocyte expression of Notch target genes, aand resulted in maximal expansion of DN, DP, CD4 +CD8-, CD4+CD8- thymocyte numbers by 1, 2, 2, and 4 weeks, respectively (38). Interestingly, castration of RAG1-deficient mice causes a dramatic increase in the numbers of DN cells but not cTECs (37), suggesting that the inhibitory roles of androgens on early thymopoiesis are mediated largely by effects on cTEC-expressed molecules and not cTEC proliferation or survival.

Investigation of TEC ARKO thymi found no change in DP thymocyte proliferation (measured by BrdU incorporation) but a reduction in DP apoptosis (proportion of Annexin V^+ cells) an elevated proportions of DP CD69hi cells, an indication of TCR-mediated signaling by self antigens (33). Together, these findings suggested that the increased cellularity of TEC ARKO thymi is due to enhanced positive selection. This was specifically tested by generating TEC ARKO mice expressing the CD4-restricted AND TCR transgene, a model of positive selection. These mice had a much larger thymus and increased proportions of DP $CD69^+$ and $CD4^+CD8^-$ cells, all consistent with enhanced positive selection (33). Female TEC ARKO mice expressing an H-Y-specific TCR, in which thymocytes are positively selected on MHC I, had a similar phenotype except that the results of selection led to increases in $CD4^-CD8^+$ cells (33). These results indicate that androgens, acting *via* as-yet unclear AR signaling pathways in TEC, inhibit antigen-specific thymocyte positive selection.

Medulla

The remarkable disparity in autoimmunity between females and males led to the idea that negative selection in the medulla might be affected by sex steroids. Early studies found that $mTEC^{hi}$ growth was especially sensitive to androgens and that they rapidly proliferated after castration (37). Subsequently a pair of intriguing studies showed that negative selection is in fact dramatically responsive to sex steroids. Comparison of female and male thymi found that expression of *Aire* and *Aire*-dependent TRAs to be higher in males than in females, both mouse and human (40, 41). Correspondingly, androgen treatment of human TEC *in vitro* or mice *in vivo* upregulated expression of *Aire* and *Aire*-dependent TRAs. Sex differences in *Aire* expression were lost when castrated males were compared with females. In cultured human cells, the liganded AR was found to bind the *Aire* promoter and directly upregulate its expression (41). Male mice are known to be less susceptible than females in many models of autoimmunity, including experimental autoimmune encephalitis (EAE) and experimental autoimmune thyroiditis (EAT). Remarkably, the induction and severity of EAE (41) and EAT (40) were the same in *Aire*-deficient male and female mice, demonstrating that, at least in these models, the sex difference in the predisposition to autoimmunity is entirely *Aire*-dependent (and therefore due to sex differences in Aire expression). Furthermore, peripheral administration of the androgen dihydrotestosterone protected against EAE in control but not *Aire*-deficient mice (41). Therefore it is androgen signaling in the thymus, and not suppression of the peripheral immune response, that appears to be the primary driver of sex differences in autoimmunity.

Estrogens

Estrogens are generally defined as steroids that regulate the development and activity of the female reproductive system and secondary sex characteristics. Estrogens, especially 17β-estradiol (estradiol), the most potent form, are primarily produced by the ovaries, although they can be produced in other tissues such as the brain (42). Estrogens signal *via* multiple receptors: the nuclear receptors ERα (*Esr1*) and ERβ (*Esr2*), and the

membrane G-protein coupled receptor GPER1 (*Gper1*). In peripheral T cells, estrogens are primarily immuno-enhancing at low concentrations inducing T cell expression of T-bet and IFNγ (43) to promote Th1 responses, and at higher concentrations inducing Gata3 and IL-4 to promote Th2 responses (44). This skewing toward Th1 *versus* Th2 is considered a central driver of the female bias in autoimmunity.

Thymic involution

Both exogenous and endogenous estrogens affect thymus size, with oophorectomy or estrogen synthesis blockade resulting in thymic hypertrophy in females and estrogen treatment causing atrophy in females and males (17, 21, 45, 46). This is primarily due to signaling *via* ERα, as *Esr1* knockout mice are partially resistant to thymic involution caused by exogenous estrogen administration (47, 48). Male and female *Esr2* KO mice, on the other hand, are similar to wild-type in their response to administered estrogen (49). *Gper1* KO mice have an intermediate phenotype, with a moderate reduction in thymus size in response to exogenous estrogens (47). It appears, therefore, that ERα and GPER1 both contribute to regulating thymus size.

As with androgens, estrogen effects on thymus cellularity appear to occur predominantly by signaling *via* the stromal compartment. Radiation bone marrow chimera experiments have shown normal thymus size in male WT recipients reconstituted with *Esr1* KO bone marrow but dramatically reduced thymus size in male *Esr1* KO recipients receiving WT bone marrow (48). Complementary radiation bone marrow chimera experiments found that exogenous estradiol caused thymic involution by signaling in both the stromal and hematopoietic compartments, as *Esr1* KO recipients of WT bone marrow had greater reduction in thymus cellularity than Esr1 KO recipients of Esr1 KO bone marrow (48). Importantly, these chimera experiments were not performed in female mice, in which endogenous estrogens would presumably contribute, especially in the absence of exogenous estradiol.

Cortex

Surprisingly, Esr1 KO male and female mice have reduced thymus size (47, 48), indicating that basal estrogens actually play a role in promoting normal thymus growth. In spite of this, exogenous estradiol inhibits early stage thymocyte development, with accumulation of DN1 thymocytes and depletion of DN2, DN3, and DP thymocytes (47). This is not mediated in the same way as by androgens, as estrogens have little or no effect on Notch signaling (50). Instead, ERα (but not ERβ or GPER1) reduces IκB phosphorylation in DN cells, promoting IκB sequestration and inhibition of NF-κB signaling (47). As NF-κB signaling promotes survival and proliferation of β-selected thymocytes, its inhibition by liganded ERα may contribute to developmental arrest at this checkpoint (50). Exogenous estradiol also increases the proportion of apoptotic DN TCRβ^{low} thymocytes, an effect that is lost in *Gper1* KO but not *Esr1* KO or *Esr2* KO mice (47). Consistent with this, activation of GPER1 by a selective estrogen agonist induced moderate thymic involution and thymocyte apoptosis, but did not lead to developmental block of DN cells (47). Therefore the nuclear and membrane estrogen receptors have distinct functions: signaling *via* ERα inhibits cortical DN development whereas signaling *via* GPER1 selectively promotes DN cell apoptosis.

Medulla

As mentioned above, *Aire* and *Aire*-dependent TRA expression are higher in males than females, and estrogen treatment downregulates expression of Aire and Aire-dependent TRA genes (40, 41). Estrogen was shown to induce methylation of the *Aire* promoter and reduce its expression whereas dihydrotestosterone had no effect (40). Dose-response studies with human TEC found that the androgen:estrogen ratio determined whether *Aire* is up- or down-regulated, and at least a 10-fold higher concentration of androgens was required to overcome estradiol-mediated downregulation (40). To extend these findings *in vivo*, fragments from the same male or female human thymus were grafted to female and male mice and relative *AIRE* gene expression was quantified. At day 4 *AIRE* expression was similar in human thymus fragments grafted to either sex. However, at 20 days *AIRE* expression in the human thymus was much lower in female than male mice. Consistent with a more potent effect of estrogens, *AIRE* expression in male recipients was similar at 4 and at 20 days, but *AIRE* expression in female recipients dropped dramatically from 4 to 20 days (40). These data show that androgen and estrogen signaling antagonize each other, with directly opposing effects on *Aire* expression and activity. Differences in antigen presentation may be further exacerbated by the fact that estrogen signaling reduces TEC expression of MHC (51).

Estrogen treatment increases disease severity in EAT, which has been attributed to signaling in peripheral T cells, in particular ERα- and ERβ-mediated induction of Th1 and Th17 responses *via* upregulation of *Tbx21* (encoding T-bet), *Rorc* (encoding RORγt), *Il17*, and *Il21* (52, 53). These in turn are proposed to drive increases in autoantibody production (52). However, thymectomy abolished the disease-enhancing effect of estrogens without affecting autoantibody titers (40). To test the specific contribution of *Aire* to EAT severity, 7-week-old male mice received intrathymic injections of recombinant adeno-associated virus (AAV) miRNA to knock down endogenous *Aire* expression. Anti-*Aire* miRNA treatment reduced *Aire* transcript abundance in the male thymus by approximately 80% compared to control

miRNA, which resulted in EAT pathology similar to that in females as quantified by autoantibody titers and numbers of thyroid-infiltrating $CD8^+$ T cells (40). Together, these data indicate that inhibition of *Aire* expression and medullary negative selection, at least in this model, is a primary mechanism of estrogen-induced immunoenhancement. An overview of androgen *versus* estrogen effects on *Aire* and thymocyte selection is shown in Figure 2.

Progestins

Progestins, in particular progesterone, are defined as steroids that support gestation. Progestins are primarily generated in the ovary and to a lesser extent in the adrenals, brain, and adipose tissue (18). During pregnancy the placenta is a major source of progesterone. Progesterone acts primarily by binding the intracellular PR, but also signals *via* membrane PRs and the cytosolic pregnane X receptor (PXR). In the periphery, progestins are well known to be immunosuppressive, antagonizing TCR signaling, suppressing expression of proinflammatory cytokines, and promoting Treg differentiation (54–56). Treg differentiation in particular appears to be a critical tolerogenic mechanism in pregnancy, with PR-deficient mice having dramatically lower maternal immune tolerance to the fetus (57).

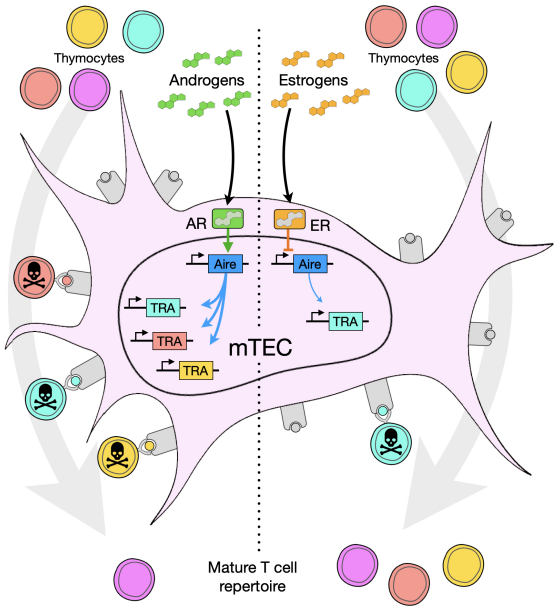

FIGURE 2
Model of steroid effects in the thymic medulla, especially on medullary thymic epithelial cells (mTEC). Androgens or estrogens bind the androgen receptor (AR) or estrogen receptor (ER), respectively, and up- or down-regulate expression of *Aire*. Aire in turn promotes, to a greater or lesser degree, expression of tissue restricted antigen (TRA) genes, generating an array of self-peptides presented on the surface of the cell bound to MHC molecules. Thymocytes with TCRs recognizing TRAs (shown as color-matched TRA, peptide antigen, and thymocyte) undergo negative selection and are absent from the mature TCR repertoire.

Thymic involution

Exogenous (21, 58) progesterone potently induces thymic involution, as does the elevated level of endogenous progesterone during pregnancy (58–60). Unlike androgens and estrogens, however, progestins do not seem to be a major contributor to age-related involution. Pregnancy-driven involution is mediated *via* signaling through the canonical intracellular PR (gene name *Pgr*), as thymi of *Pgr*-deficient mice are refractory to pregnancy-induced involution (58). Within the thymus, studies of radiation bone marrow chimeras have shown that stromal cells, rather than thymocytes, are the primary target of progesterone in driving involution (58). Very little pregnancy-induced thymic involution occurs in mice with TEC-specific *Pgr* KO, confirming that involution is almost completely mediated by PR signaling in TEC (59). Involution is global, with little or no change in the frequencies of different thymocyte subsets (59).

Cortex

Progesterone reduces homing of ETPs to the thymus (60), which appears to be at least in part due to reduction in the homing chemokine CCL25 produced by epithelial cells (60). Elevated progesterone in pregnancy downregulates *DLL4* expression (60), reducing Notch signaling and T lineage commitment of the few ETPs that do enter the thymus. Once committed, however, subsequent thymocyte developmental progresses without any major blocks (58, 59).

Medulla

Pregnancy is a unique immunological context, and maintenance of pregnancy is dependent on an appropriately selected TCR repertoire and sufficient induction of thymus- and peripheral-derived Treg cells (61). Interestingly, PR expression in the non-hematopoietic thymic compartment is necessary for normal fertility, as determined by the number of viable *versus* unimplanted and resorbed embryos (58). A few experiments have found that progesterone, like estradiol, reduces mTEC expression of *Aire in vitro* (40, 41), raising the possibility that PR-mediated decreases in *Aire* expression might reduce negative selection. However, a recent study reported that *Aire* and *Fezf2* expression were actually increased during pregnancy, and that

this increase was lost in mice with TEC-specific *Pgr* KO (59). *In vivo*, therefore, it appears that progesterone signals through PR to increase *Aire* expression, presumably enhancing negative selection and possibly even promoting agonist selection of Treg cells to result in immunological tolerance of the fetus. Whether progesterone regulation of *Aire* plays any role in fetal tolerance remains to be tested. An additional possible contributor to fetal tolerance (and the TCR repertoire in non-pregnant female and male mice) is the role of PR signaling within thymocytes themselves. Careful *in vitro* experiments using PR-deficient and GR-deficient thymocytes have shown that progesterone binds and activates the thymocyte GR (62). GR signaling is known to antagonize TCR signaling in thymocyte negative selection by opposing *Nur77* and *Helios* expression (63), and progesterone appears to antagonize thymocyte TCR signaling in a very similar way (62). Paired with high levels of progesterone within the thymus (64, 65), these data raise the intriguing possibility that progesterone can regulate antigen-mediated selection by inhibition of *Aire* expression and by antagonism of thymocyte TCR signaling. However, experiments will be necessary to specifically test each of these possibilities and their biological relevance *in vivo*.

Future directions and conclusions

The studies described above have clearly identified effects of sex steroids on thymus involution, TEC gene expression and proliferation, and thymocyte survival and apoptosis (Table 2). However, there are many important questions that remain. For example, AR directly upregulates *Tcf7* (TCF-1) (29, 30), which promotes DN thymocyte expression of *RAG* genes and TCR components as well as DP thymocyte survival. This suggests that androgens may act directly on thymocytes at early stages of commitment and differentiation. Sex steroid receptors in thymocytes may also play a role TCR signaling and thymocyte selection, although this also is largely unknown. Both AR and ERs have been shown to interact with and inhibit Nur77 (66, 67), which is induced by TCR signaling in thymocytes and promotes negative selection (8, 9). Similar interaction between the GR and Nur77 contributes to mutual antagonism between glucocorticoids and TCR signaling (63), and raises the possibility that in addition to altering *Aire* expression in mTEC, androgens and estrogens might also antagonize biological responses downstream of TCR signaling. As with glucocorticoid signaling, this could result in an altered TCR repertoire without obvious differences in thymocyte numbers. Indeed, the altered selection of the AND and H-Y transgenic TCRs in the absence of sex steroid signaling (33) is consistent with such an effect. Another interesting possibility is that of sex steroid production directly within the thymus. Thymic epithelial cells can synthesize glucocorticoids *de novo* from cholesterol (68–70), and much of the enzymatic machinery that functions in glucocorticoid synthesis also functions in synthesis of progesterone and androgen and estrogen precursors (18). Together with the finding that the thymus has locally elevated concentrations of progesterone (64, 65), this raises the intriguing

TABLE 2 Overview of steroid effects on thymus cells.

Steroid class	Cell type	Action
Progestins	cTEC	↓ CCL25, DLL4
	mTEC	↑ *Aire* & TRA expression
	thymocytes	↓ ETP homing ↓ negative selection?
Androgens	cTEC	↓ CCL25, DLL4, IL-7 expression
	mTEC	↑ *Aire* & TRA expression
	thymocytes	↓ ETP homing, T lineage commitment, positive selection ↑ negative selection
Estrogens	cTEC	?
	mTEC	↓ *Aire* & TRA expression
	thymocytes	↓ NF-κB signaling & DN development, ↑ DN apoptosis ↑ negative selection
Glucocorticoids	cTEC	?
	mTEC	?
	thymocyte	↓ TCR signaling & negative selection
Mineralocorticoids	cTEC	?
	mTEC	?
	thymocytes	?

↓, decrease; ↑, increase; ?, unknown.

possibility of sex steroid production by the thymus itself. If so, this might suggest a role for paracrine sex steroid signaling within the thymus.

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Conflict of interest

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

Publisher's note

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CD11c regulates late-stage T cell development in the thymus

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CD11c, also named integrin αX, has been deemed solely as a dendritic cell marker for decades while the delineation of its biological function was limited. In the current study, we observed in mice that CD11c deficiency led to a defect in T cell development, demonstrated by the loss of $CD4^+CD8^+$ double positive (DP) T cells, $CD4^+CD8^-$, and $CD4^-CD8^+$ single positive (SP) T cells in the thymus and less mature T cells in the periphery. By using bone marrow chimera, we confirmed that CD11c regulated T cell development in the thymus. We further showed that CD11c deficiency led to an accelerated apoptosis of CD3 positive thymocytes, but not $CD4^-CD8^-$ double negative (DN) T cells. Overall, this study added one more layer of knowledge on the regulatory mechanism of late-stage T cell development that the presence of CD11c in the thymus is critical for maintaining T cell survival.

KEYWORDS

CD11c, thymus, dendritic cell, apoptosis, T cell development

Introduction

The thymus is the primary lymphoid organ that supports T cell development consisting of three main stages (double negative (DN), double positive (DP), and single positive) (1), during which a dynamic relocation of developing lymphocytes within multiple architectural structures occurs (2). During the last two decades, it has been well elucidated that two crucial decision steps, positive and negative selections, are needed to produce functional major histocompatibility complex (MHC)-restricted T cells, while simultaneously restricting the production of auto-reactive T cells (3, 4). The traditional knowledge is that cortical thymic epithelial cells (cTECs) are involved in thymocyte positive selection, and medullary thymic epithelial cells (mTECs) and dendritic cells (DCs) are involved in negative selection (5, 6). While it is well known that events, such as T cell receptor (TCR) β chain rearrangement (7, 8), proper TCR-MHC affinity and signaling strengths (9–12), finely regulate positive and negative selections, the regulation of late-stage T cell maturation, survival, and emigration in the thymus is less studied (5).

β2 integrins are called leukocyte integrins, exclusively expressed on leukocytes (13–15). They consist of four members CD11a/CD18 (αLβ2), CD11b/CD18 (αMβ2), CD11c/CD18 (αXβ2), and CD11d/CD18 (αDβ2) (16). CD11c has been deemed primarily as a dendritic cell (DC) marker (17–19), and its physiological function hasn't been extensively explored. Our recent study revisited CD11c and discovered that it is also expressed on hematopoietic stem and progenitor cells (HSPCs), and its deficiency leads to the loss of HSPCs through an enhanced apoptosis in sepsis and bone marrow transplantation mouse models (20). In the study, we reported that CD11c (αX) knockout (KO) mice showed lower CD3 T cell counts in peripheral blood. Motivated by this clue, we further explored the biological function of CD11c, and discovered that it played a pivotal role in maintaining T cell survival at the late-stage development in the thymus.

Results

We compared the peripheral blood leukocytes in naïve wild type (WT thereafter) and CD11c KO mice, and found that, even at steady status, CD11c deficiency led to a significant loss of CD4 and CD8 T cells (Figure 1A), which was also the case in the spleen (data not shown). Since CD11c is a marker of DCs, on which MHC-II molecules are expressed to critically maintain the number of peripheral T cells (21), we examined the number of DCs. Surprisingly, although CD11c KO mice had a relatively smaller size of spleen, the number of splenic DCs including conventional DC1 (cDC1, MHC-II$^+$XCR1$^+$CD8a$^+$), cDC2 (MHC-II$^+$XCR1$^-$CD8a$^-$SIRPα$^+$CD11b$^+$), and plasmacytoid DC (pDC, PDCA$^+$CD11b$^-$Ly6C$^+$) subsets was not different from that of WT mice (Supplemental Figure 1, Figure 1A), suggesting that CD11c deficiency didn't abrogate DC development *in vivo*. We then compared the thymus, the central lymph organ for T cell development. Surprisingly, for the first time, we showed the T cell development was defective in CD11c KO mice, manifested by the smaller-sized thymus with the loss of cellularity (Figure 1B). We performed detailed phenotyping of thymic T cells at different developmental stages, which revealed that CD11c deficiency was associated with the loss of DP, CD4 SP and CD8 SP cells, but exerted no influence on the number of DN cells (Figure 1C). Further analysis showed that, although less SP CD4 and CD8 cells existed in the thymus of CD11c KO mice, they were skewed toward more mature population, demonstrated by a higher ratio of CD24^{low}Qa2^{high} cells (Figure 1D). This result indicated that immature CD4 SP and CD8 SP cells were particularly affected in the thymus of CD11c KO mice. Despite that the ratio of mature population in total CD4 SP was relatively higher, the absolute number of mature CD4 cells in thymus of CD11c KO mice was still significantly less than their WT counterpart (Figure 1D). CCR7 drives T cells from the cortex to the medulla (22, 23). CCR7 expression on CD4 and CD8 SP cells was not different between the genotypes, suggesting that the egress of T cells from the cortex to the medulla was comparable between WT and CD11c KO mice (Figure 1E). Strong TCR signal leads to negative selection, and weak signal helps to generate conventional CD4 cells (24, 25). Intermediate signal generates nTreg cells (26, 27). There was a relatively higher percentage of nTreg (CD25$^+$FoxP3$^+$CD4) cells in CD4 SP cells in the thymus of CD11c KO mice. This may indicate that nTreg pathway was less affected in CD11c KO mice compared to conventional CD4 pathway. Due to the lower number of total CD4 SP cells, however, the absolute number of nTreg in the thymus of CD11c KO mice was still less than the counterpart in WT mice (Figure 1F).

To explore the underlying mechanism that led to less thymocyte number in CD11c KO mice, we examined the apoptosis of T cells in the thymus by staining cleaved caspase-3 *ex vivo*. We found that CD11c deficiency significantly increased the apoptosis of CD3-positive subsets (DP, CD4 SP and CD8 SP cells), which are relatively more matured T cells in the thymus (Figure 2A). The more occurrence of apoptosis in CD3 positive cells was further confirmed by staining freshly isolated thymocytes with Annexin V (Figure 2B). In sharp contrast, the proliferation status was not different between the genotypes, probed by Ki-67 expression (Figure 2C).

Successful TCRβ chain rearrangement delivers proliferation signals and instructs the transition of DN cells into DP cells. This event is followed by positive selection by thymic epithelial cells (TECs) in the cortex and negative selection by DCs in the medullary region, responding to strong TCR-MHC interactions (28). Those DP cells with non-functional TCR-MHC interactions undergo death by neglect, which occurs for over 95% of DPs (29). To dissect out the cell type primarily responsible for the observed phenotype, we compared thymic DC subsets between WT and CD11c KO mice and found the number of three DC subset was comparable (Figure 3A). Although TECs are important antigen presenting cells in the thymus, no CD11c expression was detected on the surface of TECs (Figure 3B). In addition, the number of TECs between WT and CD11c KO mice was not different (Figure 3B). TECs contain two subpopulations; Cortical thymic epithelial cells (cTECs, Ly51$^+$), which are the primary cell type involved in thymocyte positive selection, and medullary thymic epithelial cells (mTECs), which are involved in negative selection. We compared these two subpopulations by probing Ly51 expression and didn't observe the difference between the genotypes (Figure 3B). Thus, TECs were excluded from the potential contributor to the phenotype observed in CD11c KO mice. CD11c was highly expressed on DCs, as expected (Figure 3B). CD11c was not detected on DN, DP, and SP T cells (data not shown). To further verify whether DCs in the thymus were responsible for the T cell maturation defect in

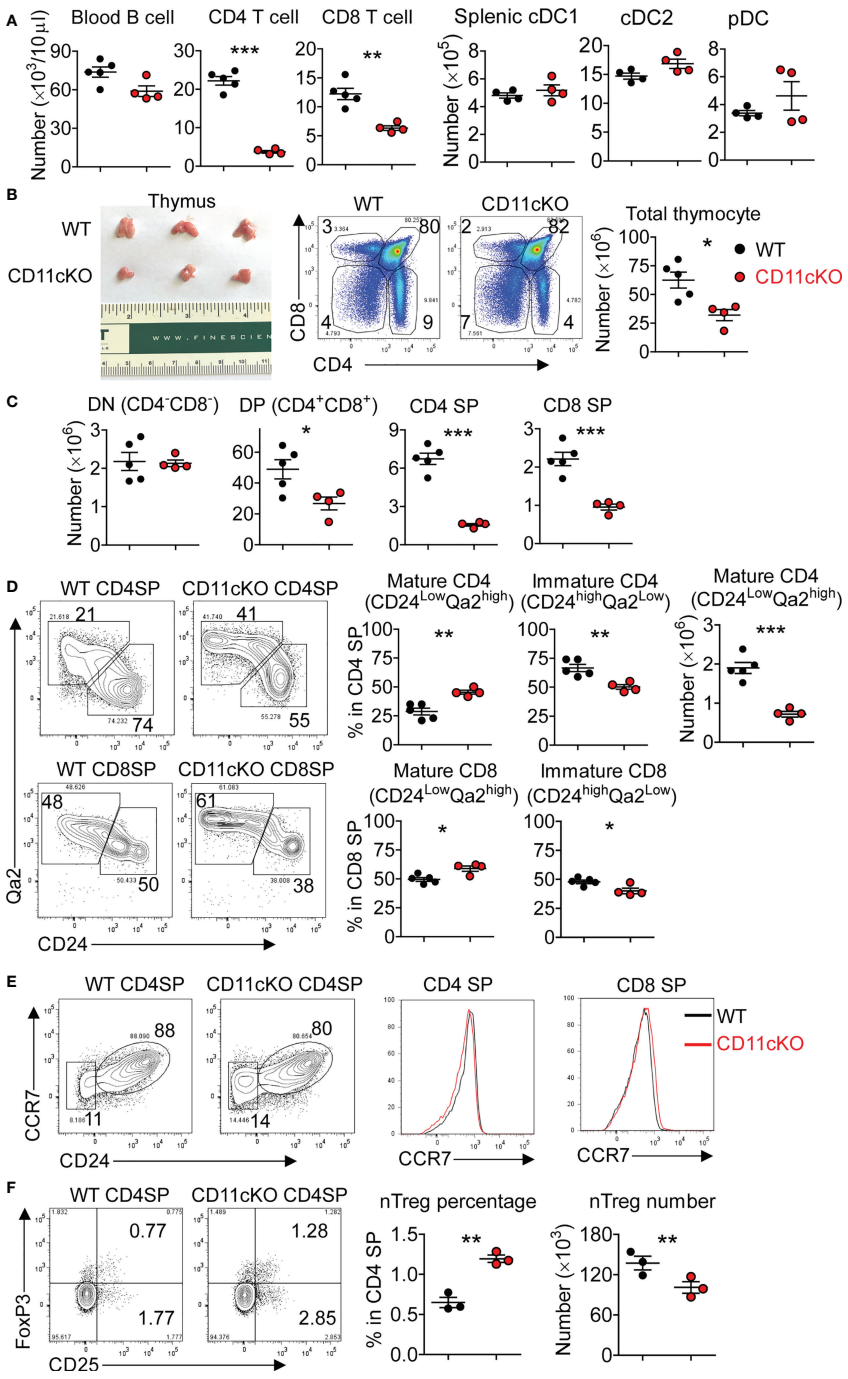

FIGURE 1
Thymic atrophy in CD11c KO mice. **(A)** Left: T and B cell counting in the peripheral blood in naïve WT and CD11c KO mice; Right: dendritic cell subset counting in the spleen. cDC1 was gated as MHC-II$^+$XCR1$^+$CD8a$^+$, cDC2 as MHC-II$^+$XCR1$^-$CD8a$^-$SIRPα$^+$CD11b$^+$, and pDC as PDCA$^+$CD11b$^-$Ly6C$^+$. **(B)** Left: thymus image; Middle: representative FACS data, gated on total thymocytes of WT and CD11c KO mice; Right: dot plots of thymocyte number. **(C)** Thymic T cell subset numbers. DN was gated as CD4$^-$CD8$^-$, DP as CD4$^+$CD8$^+$, CD4 SP as CD4$^+$CD8$^-$, and CD8 SP as CD4$^-$CD8$^+$. **(D)** Left: representative FACS data showing maturation status of thymic CD4 SP and CD8 SP cells of WT and CD11c KO mice; Middle: dot plot showing percentage of immature and mature subsets in total CD4 and CD8 SP cells; Right: dot plot showing absolute number of mature CD4 SP cells; **(E)** Representative FACS data showing CCR7 expression on thymic CD4 SP and CD8 SP cells of WT and CD11c KO mice; **(F)** Left: representative FACS data showing natural regulatory T cells (nTreg) in thymic CD4 SP cells of WT and CD11c KO mice; Right: dot plots of both percentage and absolute number. nTreg cells were gated as CD25$^+$FoxP3$^+$CD4$^+$ cells. Experiments were repeated at least 2-3 times with the same pattern. Student t test was performed for statistical analysis. *p < 0.05, **p < 0.01, ***p < 0.001.

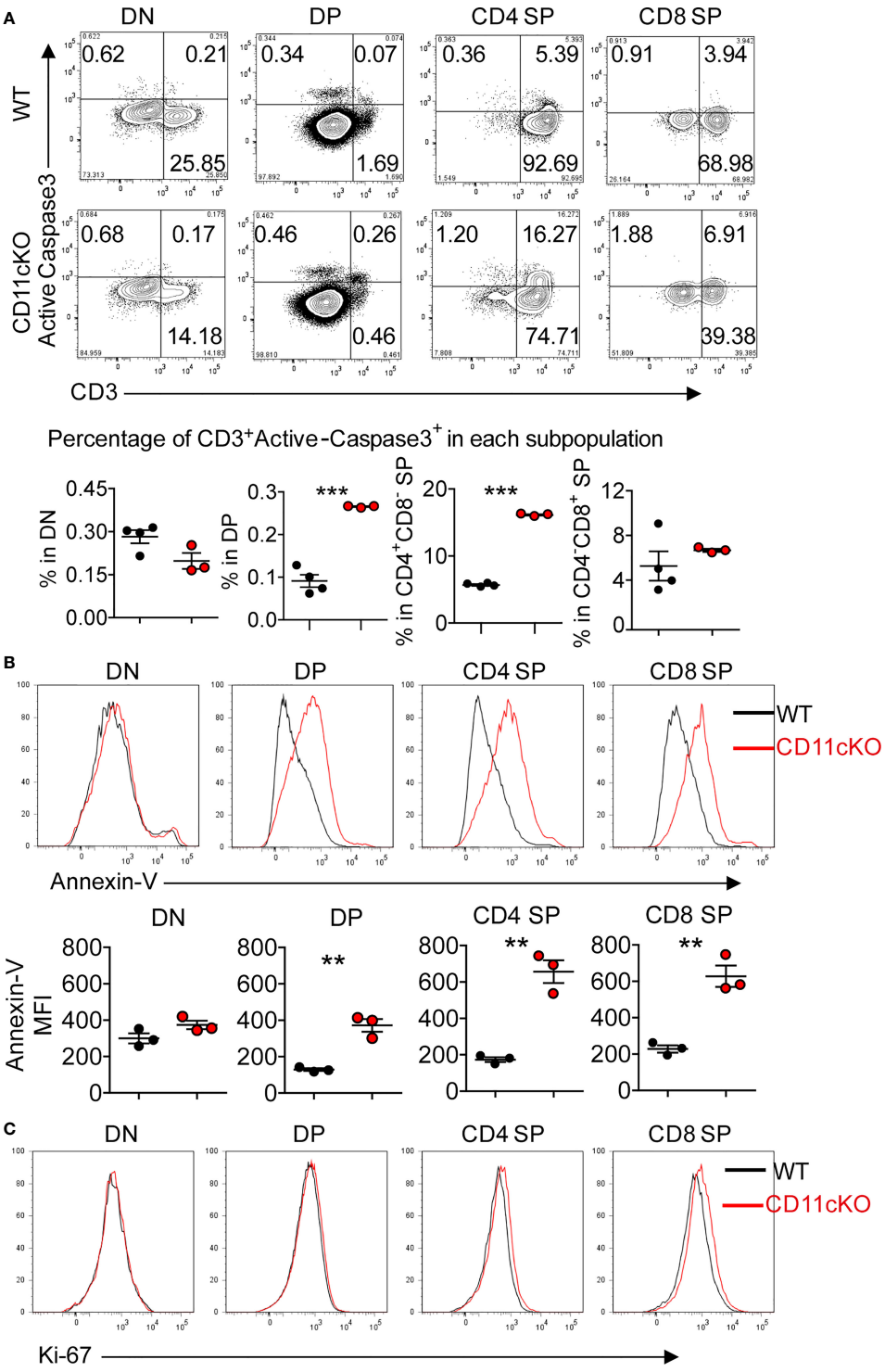

FIGURE 2
Apoptosis and proliferation analysis in CD11cKO mice. **(A)** Up panel: representative FACS data gated on different thymic T cell subsets with active cleaved caspase 3 expression; Bottom panel: Dot plots of percentage of $CD3^+$Active-$caspase3^+$ cells in indicated subsets. **(B)** Up panel: representative Annexin-V staining overlay analysis, gated on different thymic T cell subsets; Bottom panel: MFI. **(C)** Representative Ki-67 staining overlay analysis, gated on different thymic T cell subsets. Each symbol represents an individual mouse. Experiments were repeated at least 3 times with the same pattern. **(A)** and **(B)**, Student t test was used for statistical analysis. $^{**}p < 0.01$, $^{***}p < 0.001$.

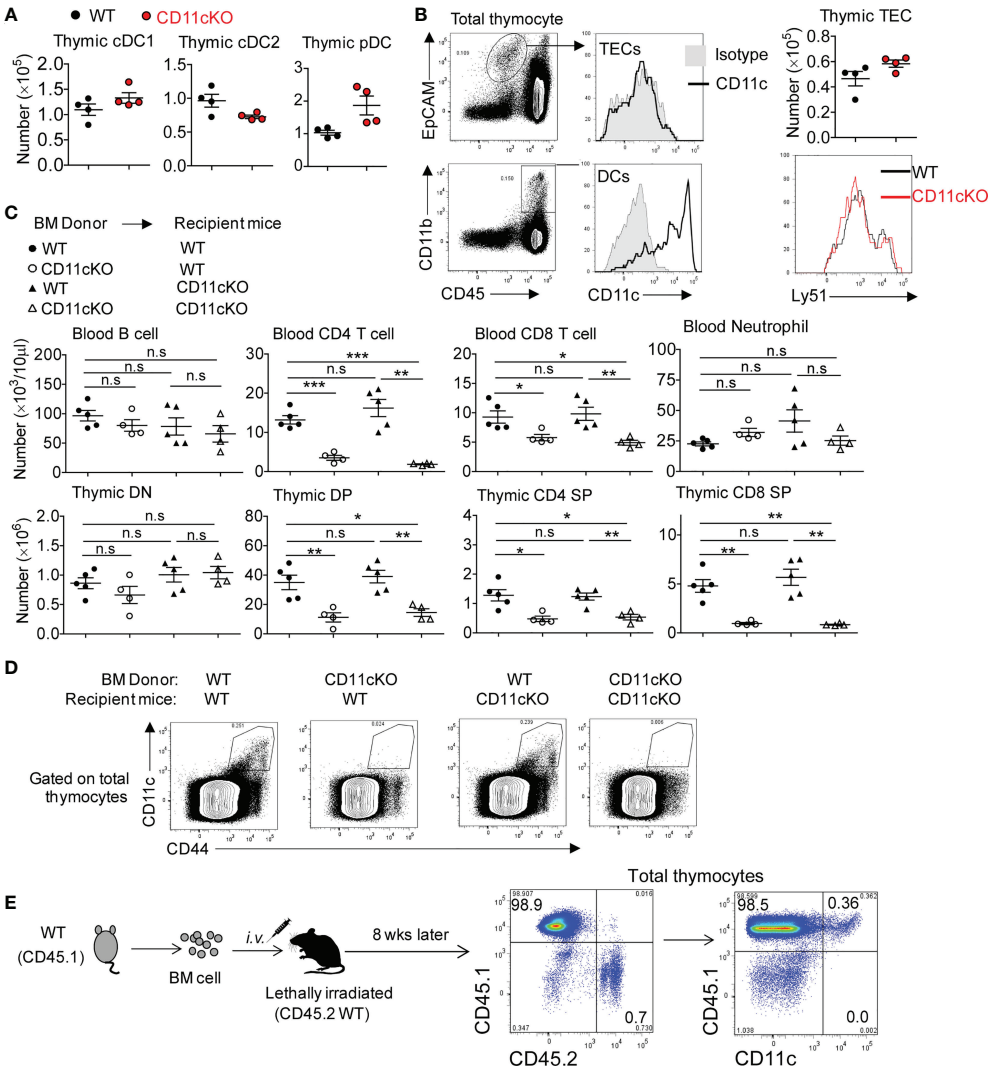

FIGURE 3
The role of CD11c in irradiation-sensitive hematopoietic cells in thymus. **(A)** thymic DC subset cell counting. cDC1 was gated as MHC-II$^+$XCR1$^+$CD8a$^+$, cDC2 as MHC-II$^+$XCR1$^-$CD8a$^-$SIRPα$^+$CD11b$^+$, and pDC as PDCA$^+$CD11b$^-$Ly6C$^+$. Gating strategy could be found in Supplement Figure 1. **(B)** Left panel: representative FACS data showing CD11c expression pattern; Right-up panel: thymic TECs counting; Right- bottom panel: Ly-51 staining overlay analysis of TECs. **(C)** Blood (up panel) and thymic (bottom panel) analysis of bone marrow chimeric mice. **(D)** Representative FACS data showing CD11c expression pattern in thymocytes of chimeric mice described in **(C)**. **(E)** Representative FACS data showing CD11c expression pattern in thymocytes of chimeric mice by transferring CD45.1 WT bone marrow cells into lethally irradiated CD45.2 WT recipient mice. Each symbol represents an individual mouse. Experiments were repeated at least 3 times with the same pattern. **(A, B)**, Student t test was used for statistical analysis; **(C)** One-way ANOVA with Bonferroni *post hoc* analysis was performed. *p < 0.05, **p < 0.01, ***p < 0.001. n.s., no significant difference.

CD11c KO mice, we created bone marrow (BM) chimera. Recipient mice, either lethally irradiated WT or CD11c KO mice were transplanted with either WT-derived or CD11c KO-derived bone marrow cells. Six weeks after the transplantation, peripheral blood leukocytes were monitored to ensure the success reconstitution of hematopoietic system. Mice were sacrificed at 8 weeks post BM transplantation. As shown in Figure 3C, as long as the donor BM cells were derived from CD11c KO mice, T cell development defect was observed regardless of the background of recipient mice, consistent with what we observed in CD11c KO mice. CD11c expression was done in the thymus of chimeric mice (Figure 3D). To further solidify our finding that CD11c-expressing cells in the thymus is irradiation sensitive and also to exclude the error due to mouse background, we also made the chimera mice in an opposite way. We used CD45.1 WT as donor and CD45.2 WT as recipient mice. We confirmed that all CD11c positive cells are donor (CD45.1) derived ones (Figure 3E). Thus, combined with the

result of CD11c expression analysis in the thymus of chimeric mice (Figures 3D, E), we concluded that CD11c in irradiation-sensitive hematopoietic cells unexpectedly played an essential role in maintaining T cell survival in the thymus.

Discussion

The current study discovered that CD11c was essential in regulating thymic T cell development by maintaining the survival of T cells at later stages of the development, which adds additional nodes to both T cell biology and DC function in the thymus. While positive and negative selections are well studied, late-stage T cell maturation in the thymus and its emigration into the periphery are less examined. Herein, we discovered that CD11c was critical in maintaining the survival of T cells, preferably CD3-positive ones, thus adding one more layer of knowledge on the regulatory mechanism of T cell maturation in the thymus.

Regarding the role of DCs in thymic T cell development, controversial reports have been made. On one side, thymic DCs have been deemed as a major player that mediates negative selection to induce the apoptosis of DP cells (28, 30, 31); on the other side, thymic DCs were also reported to be involved in positive selection, thus to maintain the survival of DP cells (32, 33). The discovery that CD11c plays an essential role in maintaining the survival of T cells suggests that, in addition to MHC-II molecule, DCs could use CD11c to maintain T cell survival. Interestingly, we found that $CD24^{hi}$ immature CD4 T cells were selectively depleted in CD11c KO mice. $CD24^{hi}$ immature CD4 SP cells are defined as "semi-mature" and susceptible to apoptosis when triggered through TCR (1, 34). Thus, our data is in line with the previous reports describing that $CD24^{hi}$ immature CD4 SP cells are more sensitive to apoptosis over $CD24^{lo}$ mature CD4 SP cells.

Overall, this study highlights the role of CD11c as a functional molecule to maintain the survival of T cells in the thymic late-stage T cell development.

Methods

Mice

Animal studies were approved by the Institutional Animal Care and Use Committee of Boston Children's Hospital. Wild type mice on the C57BL/6J background were purchased from Jackson laboratory and acclimated in our animal facility before use. CD11c germline knockout mice (CD11cKO mice) on the C57BL/6J background were kindly given by Dr. Ballantyne (Baylor University), as described in our previous publication (20). For experiments, 7~10 week-old mice were used. Flow cytometry, and cell counting were performed as previously described (35). Regarding TEC and DC detection, the thymus was digested by type IV collagenase (0.5 mg/ml) and DNase I (50 unit/ml) in RPMI-1640 containing 5% FCS for 30 minutes at 37° C, followed by washing and resuspension.

Chimera experiment

To generate single bone marrow chimeras, recipient mice on the C57BL/6 background were irradiated with two doses of 550 rad with 4-hour intervals. WT or CD11c KO derived bone marrow cells (total of 5×10^6 cells) were injected into the tail vein of lethally irradiated recipients (WT or CD11c KO mice). Mice were evaluated for the reconstitution of the immune compartment after bone marrow transplantation. To prevent bacterial infection, the mice were provided with autoclaved drinking water containing sulfatrim for 1 week prior to and for 4 weeks after irradiation.

Apoptosis analysis

Annexin-V staining method: Thymocytes were stained with fluorochrome conjugated antibodies to surface marker including CD3, CD4, CD8, and Annexin-V in the presence of Annexin-V binding buffer. After washing, cells were resuspended in Annexin-V binding buffer and collected freshly.

Active-caspase-3 method: Thymocytes were stained with fluorochrome conjugated antibodies to surface marker including CD3, CD4 and CD8. After washing, cells were fixed, permeabilized and stained intracellularly with fluorochrome-conjugated anti-active caspase 3-by using fixation/permeabilization reagents and protocols from BD Bioscience. In certain situation, intracellular Ki-67 staining was done together with active caspase-3 staining.

Antibodies

Fluorochrome-conjugated antibodies or cell death related dyes are: from Biolegend: FITC- or PE-Cy7-anti-mCD3 (145-2C11), Pacific blue- or PE-Cy7- anti-mCD45.1 (A20), Pacific blue- or FITC- anti-mCD45.2 (104), Pacific blue- or PE-anti-mCD45 (30-F11), Pacific blue- or PE-Cy7- or APC- anti-mCD4 (GK1.5), APC-Cy7-anti-mCD8 (53-6.7), PE-Cy7-anti-mCCR7 (4B12), PE-Cy7-anti-mCD11b (M1/70), FITC-anti-mLy6C (HK1.4), Pacific blue- anti-mI-A/I-E (M5/114.15.2), APC-anti-mQa2 (695H1-9-9), APC-anti-mLy51 (6C3), PE-anti-mCD326 (Ep-CAM, clone G8.8), PE-anti-mCD25 (3C7), Alexa Fluor488-antiFoxP3 (FJK-16s). From eBioscience: PE-Cy7-anti-Ki67 (S01A15). From BD

Biosciences: FITC-rabbit-anti-active caspase3 (C92-605), FITC-Annexin V, Annexin V staining buffer, and BD Cytofix/Cytoperm buffer. Cell counting was done by applying Sphero AccuCount beads (ACBP-50-10; Spherotech Inc, Lake Forest, IL). Data were acquired on a Canto II cytometer (BD Biosciences) and analyzed using FlowJo software (Tree Star).

Statistical analysis

Statistical analyses were performed using Prism 4 (Graphpad Software). Student's t-test, unpaired and paired, and one-way ANOVA were used according to the type of experiment. P value < 0.05 was considered significant.

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 authors.

Ethics statement

The animal study was reviewed and approved by Institutional Animal Care and Use Committee of Boston Children's Hospital.

Author contributions

Both authors designed research, did experiment, analyzed data and wrote the manuscript. All authors contributed to the article and approved the submitted version.

Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.1040818/full#supplementary-material

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Development and function of natural TCR$^+$ CD8αα$^+$ intraepithelial lymphocytes

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The complexity of intestinal homeostasis results from the ability of the intestinal epithelium to absorb nutrients, harbor multiple external and internal antigens, and accommodate diverse immune cells. Intestinal intraepithelial lymphocytes (IELs) are a unique cell population embedded within the intestinal epithelial layer, contributing to the formation of the mucosal epithelial barrier and serving as a first-line defense against microbial invasion. TCRαβ$^+$ CD4$^-$ CD8αα$^+$ CD8αβ$^-$ and TCRγδ$^+$ CD4$^-$ CD8αα$^+$ CD8αβ$^-$ IELs are the two predominant subsets of natural IELs. These cells play an essential role in various intestinal diseases, such as infections and inflammatory diseases, and act as immune regulators in the gut. However, their developmental and functional patterns are extremely distinct, and the mechanisms underlying their development and migration to the intestine are not fully understood. One example is that Bcl-2 promotes the survival of thymic precursors of IELs. Mature TCRαβ$^+$ CD4$^-$ CD8αα$^+$ CD8αβ$^-$ IELs seem to be involved in immune regulation, while TCRγδ$^+$ CD4$^-$ CD8αα$^+$ CD8αβ$^-$ IELs might be involved in immune surveillance by promoting homeostasis of host microbiota, protecting and restoring the integrity of mucosal epithelium, inhibiting microbiota invasion, and limiting excessive inflammation. In this review, we elucidated and organized effectively the functions and development of these cells to guide future studies in this field. We also discussed key scientific questions that need to be addressed in this area.

KEYWORDS

intraepithelial lymphocytes (IELs), CD8αα$^+$, intraepithelial lymphocytes precursors (IELps), thymus, TCRαβ$^+$ CD8αα$^+$ IELs, TCRγδ$^+$ CD8αα$^+$ IELs

Introduction

Intestinal intraepithelial lymphocytes (IELs) are embedded within the intestinal epithelial layer of many species, including fish, pigs, mice, and humans (1, 2), although their quantity and distribution varies among species (3). These cells were initially described in 1847 as round cells within the epithelium of the small intestine and were defined as

nutrition-absorbing cells (4). Later research suggested that they are predominantly composed of T cells and play a role in dealing with antigens from the intestinal lumen (4, 5). IELs were previously divided into conventional and unconventional subsets, with the former originating from $CD4^+$ or T cell receptor (TCR)$\alpha\beta^+$ $CD8\alpha\beta^+$ T cells and migrating from peripheral lymphoid tissues, and the latter arising from $CD4^-$ $CD8\alpha\beta^-$ double-negative cells and migrating from the thymus (5). Further studies have identified several subsets of TCR-negative cells and revealed that IELs are a heterogeneous cell population that contains diverse TCR-positive and TCR-negative subsets (6).

TCR^-IELs have been classified in recent years, including innate lymphoid (ILC)-like cells, iCD8α cells, and other $iCD3^+$ cells (iCD8α cells are a special subtype of $iCD3^+$ cells that express CD8α homodimers) (6–9). TCR^+ IELs are classified as induced and natural IELs. Induced IELs are mostly either $CD4^+$ or $CD8\alpha\beta^+$, with a minority of $CD8\alpha\alpha^+$ (6, 10); natural TCR^+ IELs comprise $TCR\alpha\beta^+$ and $TCR\gamma\delta^+$ T cells along with CD8α homodimers, instead of CD4 or CD8αβ (10). $TCR\alpha\beta^+$ $CD4^-$ $CD8\alpha\beta^-$ $CD8\alpha\alpha^+$ (hereafter called $TCR\alpha\beta^+$ $CD8\alpha\alpha^+$ IELs) and $TCR\gamma\delta^+$ $CD4^-$ $CD8\alpha\beta^-$ $CD8\alpha\alpha^+$ (hereafter called $TCR\gamma\delta^+$ $CD8\alpha\alpha^+$ IELs) cells are two subtypes of natural IELs that decrease with age, also named natural CD8αα IELs, because CD8αα is regarded as their hallmark (11).

Substantial evidence indicates that CD8αα IELs share specific phenotypes, developmental pathways, migration patterns, gene profiles, and functions with other IELs subsets. Although the two CD8αα IELs subsets share multiple characteristics, and thus, can sometimes be classified into the same population, several significant differences were observed. To the best of our knowledge, $TCR\alpha\beta^+$ $CD8\alpha\alpha^+$ IELs and $TCR\gamma\delta^+$ $CD8\alpha\alpha^+$ IELs are the two major cell populations within the intestinal epithelium and account for the majority of IELs. Recent studies have also partly uncovered their role in immune surveillance, immune response, mucosal epithelial protection and restoration, immune homeostasis, systemic metabolism, and immune regulation in the local environment of the intestine. This review focuses on $TCR\alpha\beta^+$ $CD8\alpha\alpha^+$ and $TCR\gamma\delta^+$ $CD8\alpha\alpha^+$ IELs and aims to reveal the unique pathways of their development and functional characteristics.

Classification of IELs

TCR- IELs

TCR^+ IELs have been investigated for several decades; nevertheless, TCR^- IELs have been recently discovered and shown to comprise several cellular subsets (Figure 1). $NKp44^+$ $CD103^+$ ILC1 populations that express CD160 and CD101 (markers of intraepithelial lymphocyte) are embedded not only within the intestinal epithelium of humans but their counterparts have been identified in mice as cell populations expressing CD160, NKp46, and NK1.1 (8). In addition, partial $CD3^-$ IELs express CD56, NKp44, IL-23R, RORγt, and gut-homing chemokine receptor CCR6, thus displaying the characteristics of three cell subsets: NK cells, ILC1, and ILC3 (12). In a subsequent study, a more comprehensive strategy for characterizing ILC was established by suggesting that these are closely associated with NK cells and are described as ILC-like cells (13).

In addition to ILC-like subsets, other special cell populations of TCR^- IELs have been recently identified: $iCD3^+$ and $iCD8\alpha^+$ populations. iCD8α cells comprises a new innate TCR^- IELs population expressing CD8α as homodimers and was discovered in both humans and mice (9). Similar to $TCR\alpha\beta^+$ $CD8\alpha\alpha^+$ IELs and TCR $\gamma\delta^+$ IELs, the development of iCD8α cells also requires IL-15 and $E8_I$ enhancers (9). Another subset of TCR^- -IELs was further identified to reside in both humans and mice. These cells display hybrid characteristics of ILCs and T cells, express intracellular CD3, and are named iCD3 cells (7),. This evidence suggests that iCD8α cells might belong to the group of iCD3 cells (7).

TCR^+ IELs

TCR^+ IELs are a well-characterized population of cells (6) and include diverse $TCR\alpha\beta^+$ and $TCR\gamma\delta^+$ cells (Figure 1). They can be classified into induced and natural IELs based on different developmental origins and phenotypes (14). Induced IELs primarily express CD4 or CD8αβ, derive from conventional TCR $\alpha\beta^+$ T cells of peripheral lymphoid tissues, and include $TCR\alpha\beta^+$ $CD4^+$, $TCR\alpha\beta^+$ $CD8\alpha\beta^+$, $TCR\alpha\beta^+$ $CD4^+$ $CD8\alpha\alpha^+$, and $TCR\alpha\beta^+$ $CD8\alpha\beta^+$ $CD8\alpha\alpha^+$ IELs (5, 6). In contrast to induced IELs, natural IELs comprise $TCR\alpha\beta^+$, $TCR\alpha\beta^+$ $CD8\alpha\alpha^+$, $TCR\gamma\delta^+$, and $TCR\gamma\delta^+$ $CD8\alpha\alpha^+$ cells, and originate from TCR $\alpha\beta^+$ $CD4^-$ $CD8\alpha\beta^-$ and $TCR\gamma\delta^+$ $CD4^-$ $CD8\alpha\beta^-$ double-negative cells, respectively. The latter are able to migrate to the intestinal epithelium after undergoing thymic development and subsequently acquire the CD8αα phenotype (5). Furthermore, TCR^-IELs belong to natural IELs. In addition to distinct developmental pathways, induced IELs are absent at birth and increase with age, while natural IELs are present at birth and decrease with age (5, 6). This suggests that the reduction in natural IELs may be due to an increase in induced IELs. $TCR\alpha\beta^+$ $CD8\alpha\alpha^+$ and $TCR\gamma\delta^+$ $CD8\alpha\alpha^+$ IELs are two important subsets of TCR^+ IELs, which comprise a large proportion of IELs and play critical roles in the intestinal immune response and tolerance.

Development of natural $CD8\alpha\alpha^+$ IELs

$TCR\alpha\beta^+$ $CD8\alpha\alpha^+$ IELs

$TCR\alpha\beta^+$ $CD8\alpha\alpha^+$ IELs are first identified in mice and the existence of them in humans remains controversial (4). Some studies suggested that this population is present in gestation and

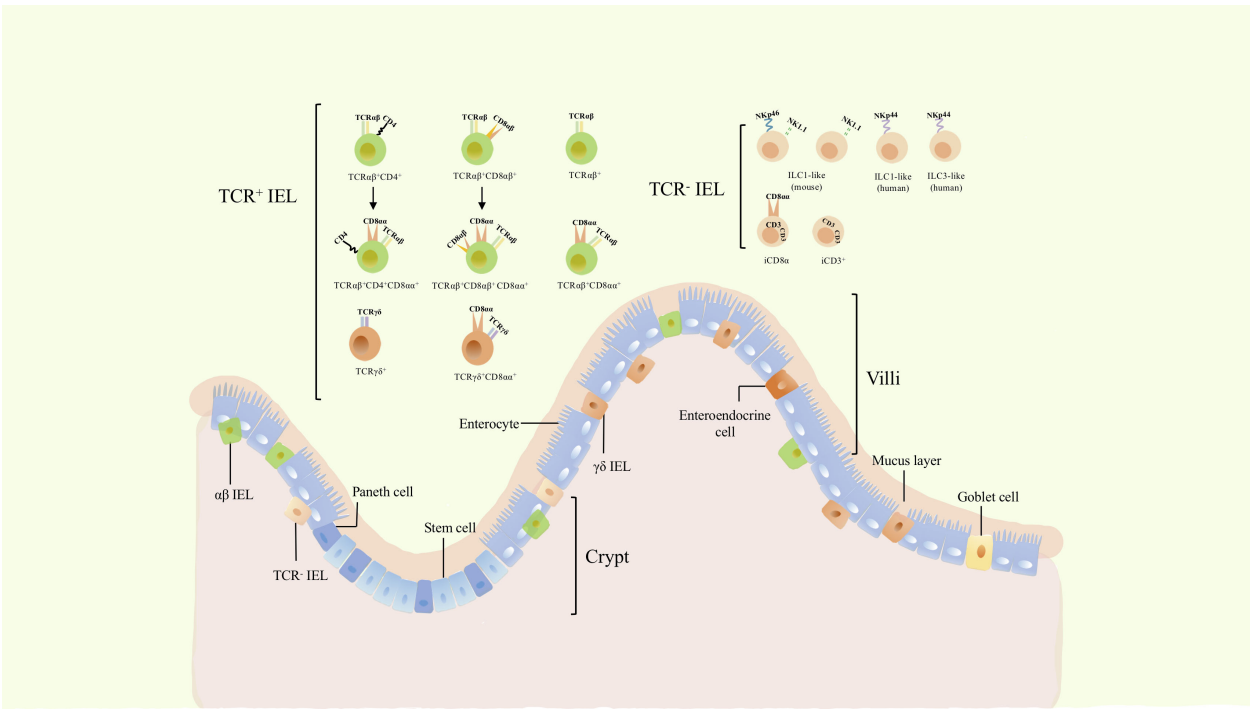

FIGURE 1
The classification and location of intraepithelial lymphocytes (IELs). Gut epithelium is composed of a single layer of enteroendocrine cells (intestinal epithelial cells). IELs are a group of heterogenous cells embedded within intestinal epithelium. Dependeing on the expression of TCR, they can be divided into TCR$^+$ and TCR$^-$ IELs. TCR$^+$ IELs include αβ and γδ T cells. The former includes TCRαβ$^+$ CD4$^+$, TCRαβ$^+$ CD8αβ$^+$, as well as induced TCRαβ$^+$ CD4$^+$ CD8αα$^+$ and TCRαβ$^+$ CD8αβ$^+$ CD8αα$^+$ cells. TCRαβ$^+$, TCRαβ$^+$ CD8αα$^+$, γδ IELs and TCR$^-$ IELs cells are natural cellular subsets. γδ IELs consists of TCRγδ$^+$ and TCRγδ$^+$ CD8αα$^+$ cells, while TCR$^-$ IELs comprises ILC1-like, ILC3-like, and iCD3$^+$ cells including its special subset, iCD8α.

rare in adult humans (4, 6). This group of cells are one of the predominant populations in diverse IELs subsets. Nonetheless, TCRαβ$^+$ CD8αα$^+$ IELs have a contentious origin. It was initially thought that development and differentiation occur in the thymus, but further studies reported the presence of TCRαβ$^+$ CD8αα$^+$ IELs in irradiated, neonatally thymectomized, and athymic mice, thus suggesting that not all IEL populations are developed by a functional thymus (15). In subsequent studies, some researchers proposed that TCRαβ+ CD8αα+ IELs are generated independently of the thymus, whereas the generation of other subsets of IELs, including CD8 αβ$^+$ and CD4$^+$CD8αα$^+$, is thymus-dependent (16). Meanwhile, precursors of CD8αα$^+$ IELs are present in the gut, making some researchers believe that the development and differentiation of CD8αα$^+$ IELs occur in the intestinal region (17). In subsequent studies on the identification of iCD8α IELs, the hypothesis that the precursors of conventional IELs were TCR$^-$ CD8α$^+$ cells in the intestinal epithelia, was controversial. Furthermore, substantial evidence has indicated that both TCRαβ$^+$ CD8αα$^+$ and TCRγδ$^+$ CD8αα$^+$ IELs originate from thymic cells, suggesting that the potential precursors reside in double-negative thymocytes. Meanwhile, athymic mice had a lower number of TCRαβ$^+$ CD8αα$^+$ IELs which could be restored after transplanting the fetal thymus, confirming that the majority of TCRαβ$^+$ CD8αα$^+$ IELs arose from the thymus, while the extrathymic pathway may also provide such cells in adults (Figure 2) (18–20).

Until now, thymus-dependent development of TCRαβ$^+$ CD8αα$^+$ IELs was mostly agreed upon, as the thymus is an important organ for self-antigen recognition and selection of T cells. After induction by TCRβ, pre-TCR-CD3 signaling, and other signaling molecules, a small fraction of CD4$^+$ CD8αβ$^+$ CD8αα$^+$ thymocytes (i.e., TP cells), were the post-selection precursors of TCRαβ$^+$ CD8αα$^+$ IELs (21), which retained the expression of CD8αα at the stage of positive selection (21). The noncoding region of *Cd8* gene, E8$_I$, as well as the combination of E8$_I$ and E8$_{II}$ (both CD8α enhancers) are also involved in the expression of CD8αα and the suppression of the expression of CD8αβ in immature thymocytes (22–24). Recently, the specific precursors of TCRαβ$^+$ CD8αα$^+$ IELs have been identified. Two subsets of precursors of TCRαβ$^+$ CD8αα$^+$ IELs (hereafter called IELps) were identified from the TCRβ$^+$ CD5$^+$ CD122$^+$ H-2Kb$^+$ CD4$^-$ CD8$^-$ thymocytes: PD-1$^+$ T-bet$^-$ cells (hereafter called PD-1$^+$ IELps) and T-bet$^+$ PD-1$^-$ cells (hereafter called T-bet$^+$ IELps)

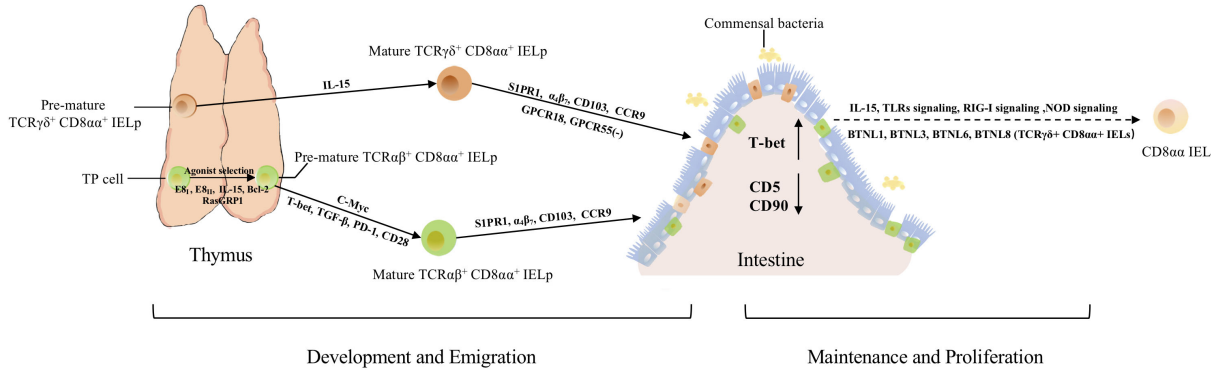

FIGURE 2
The development, migration, maintenance, and proliferation of TCRαβ$^+$ CD8αα$^+$ and TCRγδ$^+$ CD8αα$^+$ IELs. Both types arise from thymic IELps. TP cells become DN cells by regulation from E8I, E8II, IL-15, Bcl-2, and RasGRP1. E8I and E8II can suppress the expression of CD8αβ. RasGRP1 contributes to the transmission of weak TCR signals in the process of selection. Besides, c-Myc controls the development of TCRαβ$^+$ CD8αα$^+$ IELps *via* IL-15 and Bcl-2. After agonist selection, pre-mature TCRαβ$^+$ CD8αα$^+$ IELps further develop with the help of T-bet, TGF-β, and PD-1. Mature TCRαβ$^+$ CD8αα$^+$ IELps migrate to the intestine directly with the help of S1PR1, α4β7, CD103, and CCR9. Besides these molecules, TCRγδ$^+$ CD8αα$^+$ IELps also require GPCR18 and GPCR55 for localization and regulation of their accumulation. After IELps arrive in the intestine, the expression of CD5 and CD90 is downregulated, while the expression of T-bet is upregulated, exhibiting the phenotype of CD8αα. Meanwhile, the crosstalk between commensal bacteria, IECs, and CD8αα IELs contributes to the maintenance and proliferation of CD8αα cells, *via* NOD2 signaling, TLRs signaling, RIG-I signaling, IL-15, and other signaling pathways. In addition, BTNL1, BTLN3, BTNL6 and BTNL8 could promote the maturation and expansion of γδ IELs.

(25). PD-1$^+$ IELps are localized in the cortex and restricted by classical major histocompatibility complex (MHC) molecules. They are nascent and self-reactive, whereas T-bet$^+$ IELps are located in the medulla and restricted by non-classical MHC I molecules, and their number increases with age (25). Meanwhile, only T-bet$^+$ IELps expressed the memory marker CD44 and chemokine receptor CXCR3, while neither PD-1$^+$ IELps nor T-bet$^+$ IELps expressed CCR7 (25). Although two kinds of IELps could give rise to TCRαβ$^+$ CD8αα$^+$ IELs, evidence indicates that T-bet$^+$ IELps are preferentially retained in the thymus, and PD-1$^+$ IELps are the main precursors of TCRαβ$^+$ CD8αα$^+$ IELs (25). In a subsequent study, CD122$^+$ PD-1$^+$ α4β7$^+$ CD103$^-$ IELps and CD122$^+$ PD-1$^-$ $\alpha_4\beta_7^-$ CD103+ IELps were identified, and it was proposed that the former subset was congruent with PD-1$^+$ IELps, whereas the latter was represented by T-bet$^+$ IELps (26). This further proves the presence of two types of thymic IELps. In a recent study, researchers found a group of killer innate-like T cells (ILTCks) could mediate cancer immunity, whereas showed αβILTCk-TCR expressing thymocytes co-expressed PD-1 and CD122, which is similar to IELps, revealed the αβILTCk-TCR thymocytes could also differentiate into IELs (27).

Furthermore, IL-15 might participate in the differentiation of TP precursors (21). The maturation of IELps is accompanied by the upregulation of MHC class I molecules H-2Kb and CD122 (25, 28). Jiang et al. proposed that c-Myc regulates the development of IELps *via* IL-15- and Bcl-2-dependent survival (29). Agonist selection and IL-15 receptor signaling can induce T-bet expression, indicating that T-bet, TGF-β, and PD-1 are all involved in the development of CD8αα$^+$ IELs (Figure 2) (25, 30, 31). The development of thymic IELps does not depend on IL-15 (25, 32). Although researchers have defined several characteristics of IELps, their maturation, localization, and emigration patterns are still not fully understood.

The development of different T cell lineages requires TCR signals. Similar to regulatory T cells, TCRαβ$^+$ CD8αα$^+$ IELs are self-reactive and require exposure to self-agonists in the thymus (26, 33). PD-1$^+$ IELps express PD-1, CD69, Nur77, and Egr2, display signs of elevated TCR signaling (34), and are capable of self-reactivity after undergoing positive agonist selection (35, 36). However, the high affinity of TCRs for self-antigens or MHC is removed to maintain self-tolerance. The number of PD-1$^+$ IELps increased in Bim-deficient mice, suggesting that IELps may also be produced by clonal deletion (37). However, the mechanism by which IELps escape deletions is not fully understood. Some DP thymocytes survive by downregulating the expression of CD8β and upregulating the expression of CD8αα, CD8αα$^+$ cells, which would also activate an altered gene expression program (21, 38–41). These results indicate a possible mechanism by which IELps survive. Furthermore, RAS Guanyl Releasing Protein 1 (RasGRP1), a Ras activator required to transmit weak TCR signals, is also an essential molecule for the survival of TCRαβ$^+$ CD8αα$^+$ IELps during agonist selection (26). In addition, CD28-deficient mice have more PD-1$^+$ IELps (25), and PD-1 can inactivate CD28 signaling (42), suggesting that PD-1 and CD28 may play roles in the survival and differentiation of IELps. Meanwhile, the anti-apoptotic protein Bcl-2 promotes the survival of IELps and TCRαβ$^+$ CD8αα$^+$ IELs by antagonizing Bim (43).

Although recent evidence has shed light on the development of TCRαβ$^+$ CD8αα$^+$ IELs, the different signals, gene programs,

and molecules involved in the development of these cells are not fully understood.

TCRγδ$^+$ CD8αα$^+$ IELs

γδ T cells reside in various organs such as the intestine, skin, vagina, gingiva, uterus, and tongue (44–48). Meanwhile, more γδ T cells reside in the intestinal intraepithelial tissue than in other tissues. TCRγδ$^+$ CD8αα$^+$ IELs are present in both humans and mice. In humans, only 13% of IELs are γδ T cells (49), whereas in mice, the proportion of γδ T cells is around 50-60% (6, 10, 49, 50). Most γδ IELs expressed CD8αα homodimers (hereafter TCRγδ$^+$ IELs referred to both TCRγδ$^+$ IELs and TCRγδ$^+$ CD8αα$^+$ IELs).

The TCR specificity of TCRγδ$^+$ CD8αα$^+$ IELs is unknown, but seems similar to that of conventional peripheral γδ T cells (6). Comparable to TCRαβ$^+$ CD8αα$^+$ IELs, the origin and development of TCRγδ$^+$ CD8αα$^+$ IELs have been controversial (Figure 2). Previous studies indicated that they developed in the absence of the thymus, while others proposed they originate from the thymus. Although the thymic precursors and development of TCRγδ$^+$ CD8αα$^+$ IELs remain poorly understood, their development and differentiation are very similar to those of TCRαβ$^+$ CD8αα$^+$ IELs, for example, in terms of the expression of CD8αα as well as the suppression of CD8β. Additionally, they may require the same molecules and programs to develop, differentiate, and survive. Nonetheless, in contrast to TCRαβ$^+$ CD8αα$^+$ IELs, the repertoire and development of TCRγδ$^+$ CD8αα$^+$ IELs seemed to be unaffected by MHC antigens and RasGRP1 (26), and were independent of microbial and food antigens (51).

Butyrophilin-like proteins (BTNL; members of the B7 superfamily of costimulatory receptors) are expected to act as co-stimulators of IEL receptors. However, the functions of BTNL members have not yet been elucidated. BTNL1, BTNL3, BTNL6, BTNL8, BTN3A1, BTN3A2, and Skint1 are involved in the regulation of TCR γδ $^+$ cells, with BTNL1, BTNL4, and BTNL6 being widely expressed in the mouse gut (52). The number of TCRγδ$^+$ IELs is reduced in Btnl1$^{-/-}$ mice, suggesting that BTNL1 expressed by the epithelial cells of small intestinal villi, promotes the maturation and expansion of TCRγδ$^+$ IELs (51). In addition, BTNL1 together with BTNL6 can induce TCR-dependent stimulation of γδ$^+$ T cells (51). Further experiments confirmed that BTNL6 and BTNL1 are required for the development of TCR γδ $^+$ IELs (53). Additionally, BTNL3 and BTNL8 expressed in the human gut epithelium can regulate the development of TCR Vγ4 (51). Furthermore, Skint, a Btnl gene expressed by thymic epithelial cells and suprabasal keratinocytes, drives the maturation of progenitors of dendritic epidermal T cells (DETCs) (54, 55), suggesting that this gene may also facilitate the maturation of TCRγδ$^+$ IELs. However, this is debatable, because Skint genes are only expressed in γδ T cells residing in the skin and thymus (55). Collectively, these results suggest that intestinal epithelial cells (IECs) may facilitate the development and function of TCRγδ$^+$ CD8αα$^+$ IELs.

Migration and maintenance of natural CD8αα$^+$ IELs

Conventional T cells arise from lymphoid precursors, which are derived from pluripotent stem cells in the marrow and migrate to the thymus. In the thymus, within the cortex, T cell progenitors undergo positive selection and migrate to the medulla for further differentiation, selection, and maturation, which imply a delicate regulatory program. For example, the expression of CCR7 is upregulated to facilitate migration. In addition, TGF-β-activated kinase 1 (TAK1) facilitates the functional maturation of T cells, and NF-κB signaling is required for cell proliferation and egress (56, 57). After acquiring the competence to proliferate and migrate, T cells move from the perivascular spaces into the vasculature in response to sphingosine-1 phosphate binding to sphingosine-1 phosphate receptor 1 (S1PR1; G-protein-coupled receptor) (58–63). Like conventional T cells, IELps also express S1PR1, indicating that they may employ a similar mechanism of egress from the thymus (Figure 2). Mature IELps express S1PR1 (59, 62, 63), confirming the hypothesis that IELps depend on S1PR1 to enable thymic egress (64). After migrating from thymus to vasculature, lymphocytes roll alone the endothelial cells, then adhere to them and migrate across the endothelium to emigrate from the vasculature into tissues (65). Previous studies exhibited that $\alpha_4\beta_7$ is a receptor to MAdCAM-1, while MAdCAM-1 is expressed by mucosal venules to help lymphocyte traffic into Peyer's patches and the intestinal lamina propria (LP), suggested $\alpha_4\beta_7$ mediates the adherence of IELs to intestinal epithelial (65–67). Integrins $\alpha_4\beta_7$ and $\alpha_E\beta_7$ (i.e., CD103, a hallmark of tissue-resident T cells), CD122, CD160, and 2B4 are common molecules associated with gut-homing and retention of cells (48, 66, 68–71); the expressions of $\alpha_4\beta_7$, CD103, and CCR9 direct competent IELps migrate, entry and firmly attach to the gut epithelium (Figure 2) (14, 25, 30, 72, 73). Meanwhile, recent study showed that transcription factor LRF could promote the expression of integrin α4β7, control the late differentiation and facilitate the gut-homing process of CD8αα IELp (74). Meanwhile, mice lacking the vitamin D receptor showed low expression of CCR9 (75), indicating that vitamin D is also a factor affecting the migration of CD8αα$^+$ IELs. Furthermore, orphan receptor G protein-coupled receptor 18 (GPCR18) is required for the localization of CD8αα IELs, especially TCRγδ$^+$ CD8αα$^+$ IELs (Figure 2) (76). GPCR 55 negatively regulates the accumulation of TCRγδ$^+$ CD8αα$^+$ cells (Figure 2) (77).

During the agonist-selection process, TP cells express high levels of CD5 and CD90, indicating that these cells receive high TCR activation signals and then become DN αβT cells (30).

After CD8αα$^+$ IELs arrive in the gut, the expression of CD5 and CD90 is downregulated and the expression of CD103 and CD8αα is upregulated, and CD8αα$^+$ IELs become resident cells (Figure 2) (21, 30, 78). Meanwhile, CD8αα$^+$ IELs also upregulate the expression of T-bet, which could induce the expression of CD8αα homodimers (Figure 2) (30). IL-15 is a critical molecule that mediates the expression of T-bet and CD5, and there is evidence that IL-15 is involved in the maintenance and expansion of CD8αα$^+$ IELs instead of their induction (Figure 2) (21).

The development, survival, and maintenance of CD8αα$^+$ IELs is affected by diverse molecules and factors (Figure 2). Exposure to external food antigens or pathogens and different gut environments can shape and maintain CD8αα$^+$ IELs. Gut bacteria can shape the differentiation of diverse T cells (79–84). Cervantes-Barragan et al. showed that *Lactobacillus reuteri* (L. reuteri) produced indole derivatives of tryptophan which activate the aryl hydrocarbon receptor, allowing downregulation of the expression of T-helper-inducing POZ/Krueppel-like factor (ThPOK), which is implicated in the differentiation of CD4$^+$ CD8αα$^+$ double-positive IELs (DP IELs) (85). This result suggests that ThPOK plays a role in regulating the expression of CD8α and that microbial factors or specific diets could promote the differentiation and maintenance of IELs.

NOD2 signaling helps maintaining the homeostasis of CD8αα+ IELs *via* the recognition of gut microbiota and IL-15 production (86). This further demonstrates that the gut microbiota promotes the retention of CD8αα$^+$ IELs. Meanwhile, Yu et al. suggested that MyD88-dependent signaling contributed to the maintenance of the number of TCRαβ$^+$ CD8αα$^+$ IELs and TCRγδ$^+$ IELs *via* IL-15 production, which was influenced by the interaction between commensal bacteria and IECs *via* TLRs signaling (87). As c-Myc regulates the development of IELps *via* IL-15, and IL-15 mediates the expression of T-bet to induce the expression of CD8αα homodimers and help maintain the homeostasis through NOD2 and MyD88-dependent signaling, IL-15 is considered to be involved in the development and maintenance of TCRαβ$^+$ CD8αα$^+$ IELs. Meanwhile, as another study showed that IECs, macrophages and DCs in the intestine could express IL-15 (86), and enterocytes express BTNLl1, BTNL3, BTNL6, and BTNL8 of the BTNL family to promote the expansion of TCRγδ$^+$ CD8αα$^+$ IELs (51), these results indicated that IECs and other cells in intestine may help the maintenance and expansion of TCRαβ$^+$ CD8αα$^+$ and TCRγδ$^+$ CD8αα$^+$ populations *via* expression of IL-15 and of BTNL molecules. Commensal viruses and retinoic acid-inducible gene I (RIG-I) signaling are essential for the homeostasis of IELs (88). Furthermore, the thymus leukemia antigen, which is confined to the surface of IECs, functions as an effective effector in modulating the IEL response (89). These results suggested that multiple cells and viruses in the intestine contribute to the survival and maintenance of CD8αα$^+$ IELs.

Konijnenburg et al. revealed that the dynamic localization and distribution, migration, scanning patterns, and energy utilization of TCRγδ$^+$ IELs are driven by microbial density through the sensing of IECs (3), which is a consequence of epithelial-immune crosstalk. In a subsequent study, Jia et al. identified commensal bacteria that contributed to γδ IELs surveillance (90). Furthermore, the development and homeostasis of TCRαβ$^+$ CD8αα$^+$ IELs requires β2m expression, not of classical class I molecules K and D (70). Moreover, a recent study indicated that the development and maintenance of CD8αα$^+$ IELs partly depend on low oxygenic conditions (91).

Function of various IELs in gut epithelium

The gut epithelium is a unique immunological compartment that is in contact with numerous external microorganisms and environmental antigens and as well as with the internal environment. The gut epithelium comprises a single layer of IECs, with diverse IELs embedded between these cells, and provides the first line of defense. This suggests that these cells may undertake potentially essential functions, despite the small total proportion of IELs. Considering this characteristic, the gut mucosal immune system requires a delicate program to respond to pathogens, while maintaining tolerance to innocuous antigens. In mice, studies showed that IELs increase in the late disease process of enteropathies such as CeD, graft vs. host disease, allograft rejection, autoimmune (4). In human, TCRαβ$^+$ CD8αβ$^+$ IELs and innate-like IEL lacking surface TCR expression were involved in the development of villous atrophy in patients with refractory CeD (4). CD8αα homodimers decreased antigen sensitivity of the TCR and acted as repressors to negatively regulate T cell activation (92). CD8αα IELs are related to inflammatory bowel disease (IBD) and infection and play a critical role in protection against pathogens, as well as in controlling bacterial overgrowth. This indicates their involvement in the promotion of mucosal defense and epithelial homeostasis (89, 93–96). Besides, recent study showed that integrin β7 deficiency protects mice from metabolic syndrome and against atherosclerosis, whereas IELs in the small intestine had the highest expression of β7, revealed that β7$^+$ natural IELs could modulate systemic metabolism and accelerate the progression of cardiovascular disease (97). Although most of these functions are shared, the functions of the different subsets of IELs differ slightly (Figure 3).

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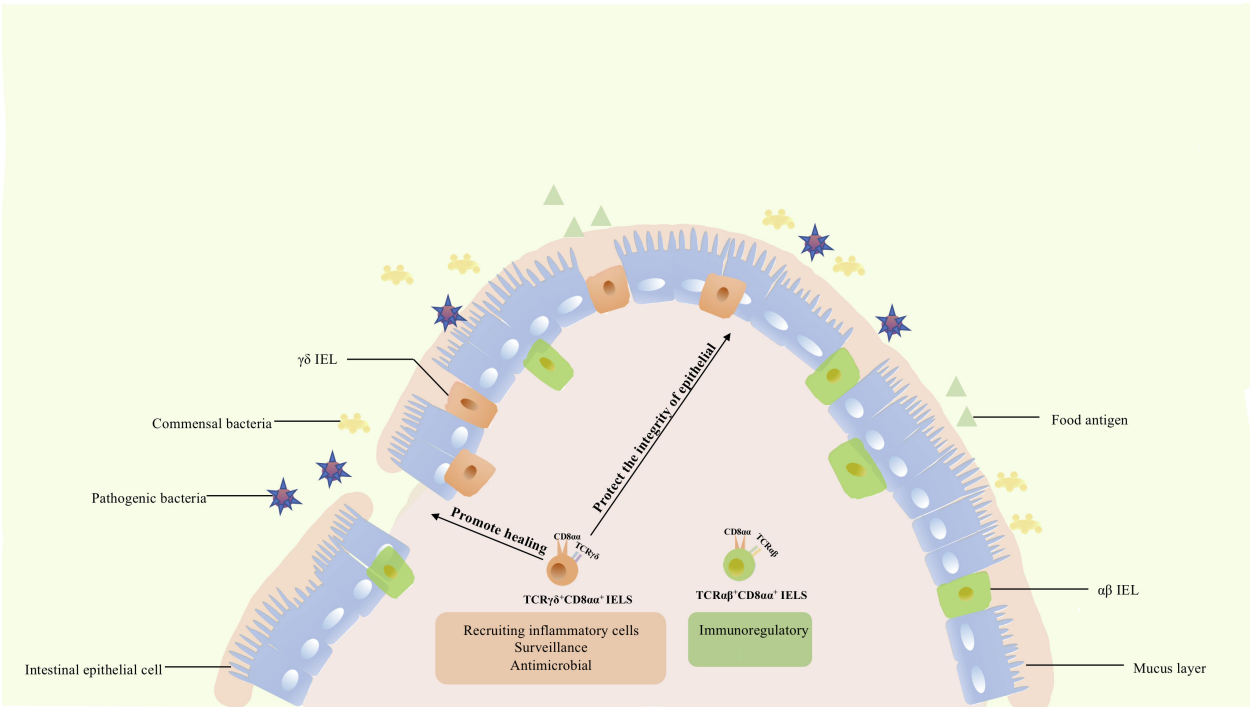

FIGURE 3
The functions of CD8αα IELs. TCRαβ$^+$ CD8αα$^+$ IELs express Ly49A, Ly49C, Ly49E, and other genes of the Ly49 family, as well as 2B4, fibrinogen-like protein-2, TGF-β3, and LAG-3, being involved in immune regulation. In contrast to TCRαβ$^+$ CD8αα$^+$ IELs, TCRγδ$^+$ IELs express TGFβ1, TGFβ3, prothymosin β4, heat shock proteins, chemokine KC, and βig-h3, being involved in injury healing and protection of the integrity of epithelium. Meanwhile, these cells could also express cytokines KC, IL-1β, MIP2α, Cxc19, and Cxc116 thus recruiting various inflammatory cells. Besides, they are characterized by a specific dynamic pattern to surveil and respond to pathogen invasion, undertaking diverse roles in the intestine.

Functions of TCRαβ$^+$ IEL

The function of TCRαβ$^+$ CD8αα$^+$ IELs has not been completely elucidated. In general, IELs expressing TCRαβ can respond to pathogens. Global analysis revealed that this population expressed NK receptor-related genes, such as Ly49A, Ly49C, and Ly49E of the Ly49 family, and genes that were expected to down-modulate their reactivity (70). These cells also express fibrinogen-like protein-2, TGF-β3, LAG-3, and genes associated with corresponding inhibitory or activation functions, such as 2B4 (70). TCRαβ$^+$ CD8αα$^+$ IELs and NK cells share similar characteristics, and TCRαβ$^+$ CD8αα$^+$, TCRαβ$^+$ CD8αβ$^+$, and TCRγδ$^+$ CD8αα$^+$ IELs have significantly different functions. TCRαβ$^+$ CD8αα$^+$ IELs might have suppressive and regulatory roles. Besides, this cellular population prevents induced colitis, a role mediated by IL-10. This method of protection is unique and differs from that of TCRγδ$^+$ and TCRαβ$^+$ CD8αβ$^+$ IELs (70, 98). Collectively, these results indicate that TCRαβ$^+$ CD8αα$^+$ IELs contributes to the maintenance of intestinal immunity and immune regulation.

Functions of TCRγδ$^+$ IEL

TCRγδ$^+$ IELs were scattered predominantly in the central and upper locations of the villi (3). Although TCRγδ$^+$ and TCRαβ$^+$ CD8αα$^+$ IELs share similar developmental pathways and expression of specific genes, these subsets are significantly different. In contrast to TCRαβ$^+$ CD8αα$^+$ IELs, the TCRγδ$^+$ population did not show a significantly high expression of NK receptor-related genes or of the other genes mentioned previously (70).

Unlike αβ T cells, γδ T cells commonly contribute to the maintenance and restoration of body-surface integrity. Boismenu et al. proposed that activated TCRγδ$^+$ IELs produce keratinocyte growth factor (an epithelial cell growth factor belonging to the fibroblast growth factor family) and stimulate the differentiation, regeneration, and migration of epithelial cells, whereas TCRαβ$^+$ IELs do not (99). Furthermore, a substantial amount of TCRγδ$^+$ IELs was enriched around the injured region in dextran sodium sulfate (DSS)-induced mouse colitis (100). TCRγδ$^+$ IELs upregulated the expression of

cytoprotective factors such as heat shock proteins, chemokine KC, and βig-h3 to promote keratinocyte proliferation and wound healing during DSS treatment (101). In addition, TCRγδ$^+$ IELs secrete TGFβ1, TGFβ3, and prothymosin β4 which protect the intestinal epithelium (14). These studies further confirmed that TCR γδ$^+$ IELs resolved inflammatory lesions by secreting multiple factors. However, although studies have shown that TCRγδ$^+$ IELs help maintain and restore the integrity of intestinal epithelia in IBD (100, 102), the function of TCRγδ$^+$ IELs in this pathology is not fully understood. TCRγδ$^+$ IELs also secrete proinflammatory factors which can induce or aggravate colitis (103, 104). Park et al. showed that activation of TCRγδ$^+$ IELs by commensal bacteria induces spontaneous colitis (105). Nevertheless, this also indicates that T regulatory cells could suppress TCRγδ$^+$ IELs *via* IL-10 to maintain intestinal homeostasis (105).

In addition, TCRγδ$^+$ IELs upregulated the expression of chemotactic molecules such as cytokines KC, IL-1β, MIP2α, and Cxc19, for various inflammatory cells, and the expression of microbial pattern recognition receptors such as TLR1 and CD4 in DSS-induced colitis (101). Meanwhile, they are accompanied by increased complement components 1qa, 1qb, and lysozyme, which are bactericidal proteins, and by increased expression of RegIIIγ (a pancreatitis-associated protein) (101). MyD88 is also required for regulation of RegIIIγ expression, and commensal bacteria could regulate the response of TCRγδ$^+$ IELs to mucosal damage through MyD88-dependent and MyD88-independent pathways (101, 106). TCRγδ$^+$ IELs could also recruit inflammatory cells, respond to bacteria, and be associated with commensal bacteria. Activated TCRγδ$^+$ IELs could limit bacterial penetration of resident microbiota or new organisms from the environment (106).

In addition, several studies have revealed the cytotoxic properties of activated TCRγδ$^+$ IELs. These cells produce interferons, TNF-α, and antimicrobial proteins in response to viral or bacterial infections (1, 107). At the same time, the immune surveillance of TCRγδ$^+$ IELs follows a dynamic migration pattern: they survey pathogen invasion by shifting along the basement membrane, migrate into the lateral intercellular space between two adjacent enterocytes and change the pattern when pathogen invasion occurs (48). Additionally, these cells facilitate tumor necrosis factor-mediated shedding of apoptotic enterocytes with the help of CD103-mediated extracellular granzyme release (108).

Collectively, although the functions and detailed molecular mechanisms of TCRγδ$^+$ IELs have not been fully defined, current evidence indicates their roles in preserving and restoring the integrity of the intestinal epithelium, recruiting inflammatory cells, surveilling, responding to enteric infection, maintaining mucosal homeostasis, and facilitating pathological epithelial cell shedding. These functions indicate the importance and delicate regulatory traits of TCR γδ $^+$ IELs.

Conclusion and unanswered questions

The gut is an essential nutrient absorption organ that directly encounters multiple antigens in the gastrointestinal tract and contains various immune cells with distinct functions and distributions. IELs are a small number of heterogeneous cells residing in the intestinal epithelium, undertaking the role of the first line of defense of the immune system. Their functions also include maintaining immune homeostasis, other possible competencies. Besides, studies exhibited IELs are associated with multiple disease such as CeD, tropical sprue and parasite infections. Natural TCRαβ$^+$ CD8αα$^+$ and TCRγδ$^+$ CD8αα$^+$ IELs are two special populations of IELs that exhibit phenotypes and characteristics that are different from conventional T cells or other subsets of IELs. TCRαβ$^+$ CD8αα$^+$ IELs are capable of immune regulation, whereas TCRγδ$^+$ CD8αα$^+$ IELs can protect the integrity of intestinal epithelia, heal injured mucosal epithelia, maintain homeostasis of the resident microbiota, inhibit microbiota invasion, respond to pathogens, and limit excessive inflammation. Meanwhile, recent study revealed the role of natural IELs in dietary metabolism, showed the potential research value of these cells. In brief, a number of studies have highlighted the importance of TCRαβ$^+$ CD8αα$^+$ and TCRγδ$^+$ CD8αα$^+$ IELs, indicating the possibility of taking advantage of these cells to strengthen the understanding of intestinal immunity, metabolism and cure diverse associated illnesses or infections.

However, the development, function, gene profiles of these cells, as well as the regulatory mechanisms underlying their effect against different conditions require further exploration. For instance, although previous studies of TCRαβ$^+$ CD8αα$^+$ IELs identified two thymic progenitors and revealed their distinct features, migrating patterns, and some specific gene profiles, the proportions and potential functional or phenotypic differences between the two IELps are not fully understood. TCRαβ$^+$ CD8αα$^+$ and TCRγδ$^+$ CD8αα$^+$ IELs have various roles under normal or infectious/inflammatory conditions, their existence being essential in organisms. However, the specific molecules regulating their function are not clear, although several critical transcription factors, cytokines, chemokines, and other molecules involved in their development, maturation, migration, and function, were identified. These unanswered questions should be the focus of future research.

Author contributions

YG drafted the manuscript. HC, JZ, and HX edited the manuscript. JH and DZ supervised the work and edited the manuscript. All authors contributed to the article and approved it for publication.

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Conflict of interest

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

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Effective differentiation of double negative thymocytes requires high fidelity replication of mitochondrial DNA in an age dependent manner

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One of the most proliferative periods for T cells occurs during their development in the thymus. Increased DNA replication can result in increased DNA mutations in the nuclear genome, but also in mitochondrial genomes. A high frequency of mitochondrial DNA mutations can lead to abnormal mitochondrial function and have negative implications on human health. Furthermore, aging is accompanied by an increase in such mutations through oxidative damage and replication errors. Increased mitochondrial DNA mutations cause loss of mitochondrial protein function, and decrease energy production, substrates, and metabolites. Here we have evaluated the effect of increased mitochondrial DNA mutations on T cell development in the thymus. Using mice carrying a mutant mitochondrial DNA polymerase γ (PolG) that causes increased mitochondrial DNA mutations, we show that high fidelity replication of mitochondrial DNA is pivotal for proper T cell development. Reducing the fidelity of mitochondrial DNA replication results in a premature age-dependent reduction in the total number of CD4/CD8 double negative and double positive thymocytes. Analysis of mitochondrial density in thymocyte subpopulations suggests that this may be due to reduced proliferation in specific double negative stages. Taken together, this work suggests that T cell development is regulated by the ability of mitochondria to faithfully replicate their DNA.

KEYWORDS

DNA polymerase γ, mutator mice, T cell development, thymus, mitochondria, aging, proliferation

Introduction

T lymphocytes are a critical part of the adaptive immune response, needed for protection from non-self, maintenance of self-tolerance, and removing abnormal cell growth in an antigen specific manner. T cells originate from stem cells in the bone marrow, which migrate to the thymus to mature, and egress into the periphery where they can perform their effector functions. Fully functioning T cells can then proliferate and differentiate according to appropriate immune responses. One of the most proliferative periods for T cells occurs during development in the thymus. Maturation of lymphoid precursors can occur in various stages marked by CD4 and CD8 expression, i.e., $CD4^-CD8^-$ double negative (DN), $CD4^+CD8^+$ double positive (DP), $CD4^+CD8^-$ single positive or $CD4^-CD8^+$ single positive (SP). In addition, the DN stage can be further compartmentalized into four major stages based on expression of CD25 and CD44: $CD44^+CD25^-$ (DN1), $CD44^+CD25^+$ (DN2), $CD44^-CD25^+$ (DN3), and $CD44^-CD25^-$ (DN4). These different stages are defined based on different phases of the T cell receptor β (TCRβ) gene selection where the most proliferative stage is just prior to the DP stage when productive TCRβ gene arrangement occurs (1). Such responses require synchronous metabolic support through glycolysis and oxidative phosphorylation which occur in the cytoplasm and the mitochondria, respectively (2).

The mitochondrion is made up of around 1,000 different proteins encoded by mitochondrial and nuclear genes (3). Approximately ~99% of the proteins in this semi-autonomous organelle are transcribed from nuclear genes. However, mitochondrial function is dependent on the mitochondrial genome. The mitochondrial genome is highly regulated to meet cellular demands and makes copies irrespective of the cell replication and in differentiating cells (4–6). Access to these genes is imperative for normal mitochondrial function as they encode for 37 different mitochondrial proteins, all of which play a critical role in normal mitochondrial physiology (7). As part of the aging process mutations accumulate in these genes, and over time these modifications of the mitochondrial genome can cause proteins to lose their functional capacity. Robust mutation repair mechanisms guard the nuclear genome of the cell (8). In contrast, there are fewer mechanisms for correcting mitochondrial gene mutations, the most important of which is carried out by the N-terminal "proofreading" exonuclease domain of the mitochondria DNA (mtDNA) polymerase γ (PolG) (9, 10). PolG corrects mismatched mitochondrial nucleotides, which if not corrected can result in increased somatic mtDNA mutations and decreased production of energy, substrates, and metabolites by the mitochondria (10–12). Immune cells, including T cells, are highly dependent on these products as they are some of the most dynamic cells in the body, being among the most proliferative cells, requiring multiple rounds of DNA replication, both nuclear and mitochondrion (13, 14).

In this study, we have evaluated the effect of increased mtDNA mutations on T cell development. Using mice carrying the *PolGD257A* mutation in the mtDNA polymerase γ (PolG) that causes increased mtDNA mutations (up to 500-fold higher mutation burden than WT mice) (15), we show that high fidelity replication of mtDNA is pivotal for proper T cell development. Reducing the fidelity of mtDNA replication results in a premature age-dependent reduction in the total number of CD4/CD8 double positive and negative thymocytes (16). Analysis of mitochondrial density in thymocyte subpopulations suggests that this may be due to reduced proliferation in specific double negative stages. Taken together, this work suggests that T cell development is regulated by the ability of mitochondria to faithfully replicate their DNA.

Results

Low fidelity mtDNA polymerase impairs thymocyte development in an age and genotype dependent manner

To determine the effects of increased mtDNA mutations on T cell development, we examined the low fidelity *PolGD257A* mouse model at different ages, analyzing age groups previously shown in the literature to represent young (1-3 months), mature (6-8 months) and old (11-13 months) mice (Figure 1A) (17, 18). We weighed the thymi and counted the total number of thymocytes from the three different age groups (young, mature, old) and genotypes; wildtype ($PolG^{+/+}$ or WT), heterozygous ($PolG^{D257A/+}$), and homozygous ($PolG^{D257A/D257A}$). Consistent with previously published results, we found that there is decreased weight and total number of thymocytes (Figures 1B, C) in the $PolG^{D257A/D257A}$ relative to WT in the mature mice (15). However, these differences were not observed in the young or old groups. Furthermore, we found that the $PolG^{+/D257A}$ mice shared the same phenotype as WT mice in all age groups, suggesting that some WT polymerase may be sufficient for the development of these cells. Examination of RNA-Sequencing data for PolG expression in thymus samples of neonatal and adult mice by RNA-revealed no age-related difference in expression (Supplemental Figure 1).

We next used CD4 and CD8 to differentiate the thymocyte stages utilizing flow cytometry (Figures 2A) (19). There was an increase in the percent of the DN population in the mature and old $PolG^{D257A/D257A}$ mice relative to WT mice (Figures 2B, C). Furthermore, the total cell numbers also indicated that there was fewer total number of DP and SP $CD4^+$ and $CD8^+$ T cells in the mature $PolG^{D257A/D257A}$ group relative to the WT (Figure 2C). Taken together, this suggests that dysfunctional PolG causes a block at the DN developmental stage.

We sought to further investigate the DN populations with the goal of determining the stage at which these cells are being prevented from passing into the more mature stages, including the DP stage. Therefore, we investigated the four identifiable double negative populations: DN1-4, utilizing the expression patterns of CD25 and CD44 as markers (Figures 2A, D). As expected, we observed the greatest differences in the DN3 or DN4 populations, the most proliferative stages during T cell development (Figures 2E, F). We saw an increase in cell percentages of DN1, and decrease in DN3 stages, in the mature $PolG^{D257A/D257A}$ (Figure 2F). Furthermore, we saw a decrease in the total number of DN2 and DN3 populations in mature $PolG^{D257A/D257A}$ mice relative to WT

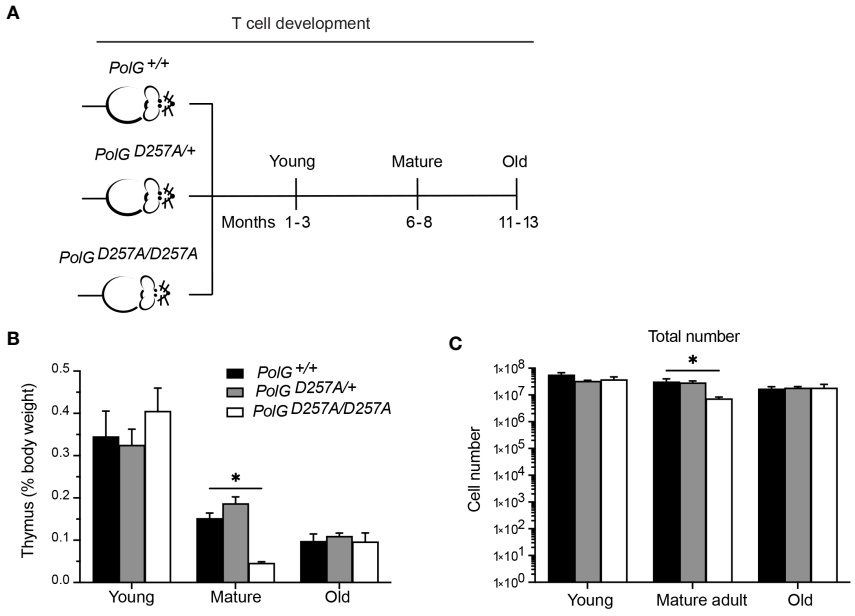

FIGURE 1
Fidelity of mtDNA replication is important for normal thymocyte numbers. **(A)** Experimental outline of $PolG^{D257A/D257A}$ genotypes and age groups. **(B)** Thymi from young, mature, and old $PolG^{D257A/D257A}$ mice were weighed and plotted as a percentage of body weight. **(C)** Thymocyte numbers from young, mature, and old $PolG^{+/+}$ and $PolG^{+/D257A}$ were determined and plotted. Data representative 11 independent experiments, n=5-24 mice per group. *p<0.05-.01.

mice, as well as curiously, in young $PolG^{+/D257A}$ mice but not in young $PolG^{D257A/D257A}$ mice. We also noted an increase in the number of the DN4 population in old $PolG^{D257A/D257A}$ mice relative to WT mice (Figure 2F). There was also what seemed like a corresponding increase in the percentage of the DN1 population, and a reduction in the percentage of DN3 population in mature $PolG^{D257A/D257A}$ mice relative to WT mice. The old $PolG^{D257A/D257A}$ mice had reduced percentages of DN1, DN2, and DN3 populations and an increase in DN4 populations. This data suggests that the presence of a low fidelity mtDNA polymerase resulted in an overall decrease in the number of DN populations and stalled T cell development, likely at the DN3 population in mature mice.

Expression of TCR transgene partially rescues the DN population in $PolG^{D257A/D257A}$ mice

To interrogate the effects of the fidelity of mtDNA replication in a model of T cell development expressing a fixed TCR, we crossed *PolGD257A* mice to OT-II transgenic mice which carry rearranged TCR alpha and beta chains that are restricted to major histocompatibility complex II (MHCII) A^b (and recognizes the peptide 323-339 from the protein Ovalbumin) (20, 21). The presence of the already rearranged TCR accelerates the developmental stages through the DN3 when gene segments encoding the TCR alpha and beta chains would usually rearrange (22, 23). Using this transgenic T cell receptor (TgTCR) model, we tracked the T cell development in the thymus, focusing on young and mature groups given their differences in T cell development.

We collected thymi from young or mature groups and analyzed thymic weights and thymocyte number. We observed a strong concordance between the TgTCR model and the non-TgTCR $PolG^{D257A/D257A}$, where we saw no significant differences in the young mice, but a reduced thymic weight of mature OTII/$PolG^{D257A/D257A}$ mice relative to the control (Figures 3A, B). However, when we analyzed the percentage of DN and DP populations in the OTII/$PolG^{D257A/D257A}$ mice relative to the control, we observed an increased overall percentage of the DN population in the OTII/$PolG^{D257A/D257A}$, as was found in the non-TgTCR model, along with a decrease in the percentage of the DP population (Figure 3C). Closer examination of the DN populations revealed a trend towards an overall decrease in the percentage of the cells in the DN1-3 stages (significantly decreased in DN1), unlike what we observed in the non-TgTCR model, but an increase in the percentage of the DN4 population relative to the control (Figure 3D). This data suggests that the presence of a low fidelity mtDNA polymerase resulted in stalled T cell development at the DN3 stage in mature mice, and this is altered in the transgenic model with accelerated development through these stages.

Mature transgenic TCR T cells with low fidelity mtDNA polymerase have decreased mitochondrial density in DN subpopulations and exhibit less proliferation

We next wanted to explore whether the block in T cell development observed in mature non-transgenic and OTII/

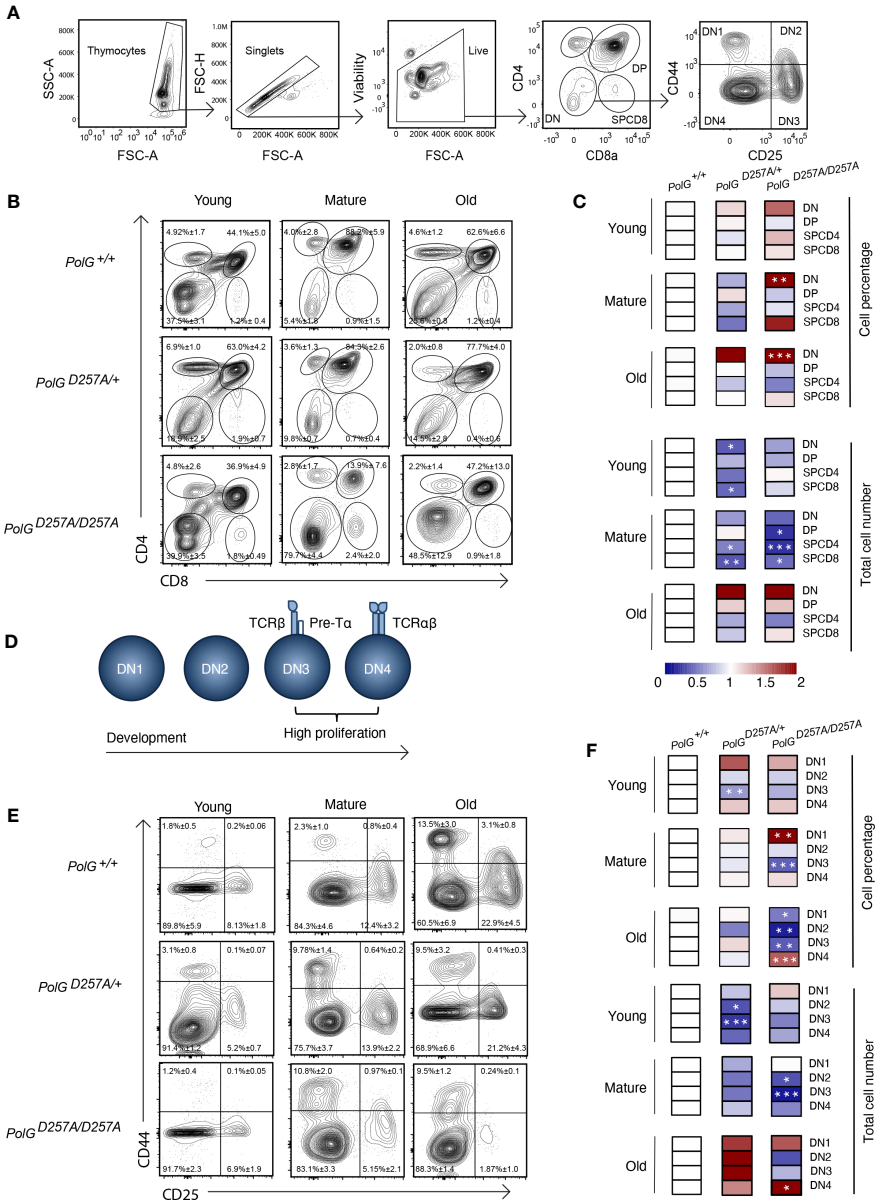

FIGURE 2
Error prone mtDNA replication impairs thymocyte development in mature $PolG^{D257A/D257A}$ mice. **(A)** Gating scheme used to determine the percentage (and number) of developing T cell populations. DN= double negative, DP= double positive, and SP=single positive. **(B, C)** Thymi from young, mature, and old $PolG^{+/+}$, $PolG^{+/D257A}$, and $PolG^{D257A/D257A}$ mice were stained for CD4 and CD8 surface markers. The gating shows DP, $CD4^+$ and $CD8^+$ SP thymocytes. Heatmaps are a representative quantification that are normalized by the mean of the control, where red indicates an increase and blue indicates a decrease. **(D)** Cartoon schematic showing the progression of T cell development in the DN stage. **(E, F)** Thymocytes from $PolG^{+/+}$, $PolG^{+/D257A}$, and $PolG^{D257A/D257A}$ mice were stained for CD25 and CD44 to differentiate DN subpopulations, and percentages of the DN1-4 stages determined. Data are displayed as mean ± standard error of mean and representative 11 independent experiments, n=7-24 mice per group, where *p<0.05-.01, **p<0.01-0.001, ***p<0.001 based on multiple t tests.

$PolG^{D257A/D257A}$ mice was associated with alterations in mitochondrial density within the double negative population. We first compared thymocytes from mature non-transgenic mice, and used a mitotracker stain, to determine mitochondrial density, gating on double negative subpopulations (DN1-4) (24). We found that the DN1 stage exhibited and 4 stages exhibited elevated mitochondrial density, while the DN2 and 3 stages exhibited reduced mitochondrial density in $PolG^{D257A/D257A}$ (Figures 4A, B). This correlated with the percentage of cells in each of these stages, and for the DN2 and 3 stages, the number of these developing $PolG^{D257A/D257A}$ thymocytes (see Figures 2F, E)

Since these DN populations are known to be highly proliferative, we wanted to explore whether decreased mitochondrial density negatively impact these highly proliferative stages of T cell development in the mature non-transgenic mice (25–27). Figure 4D shows an analysis that determines proliferation (based

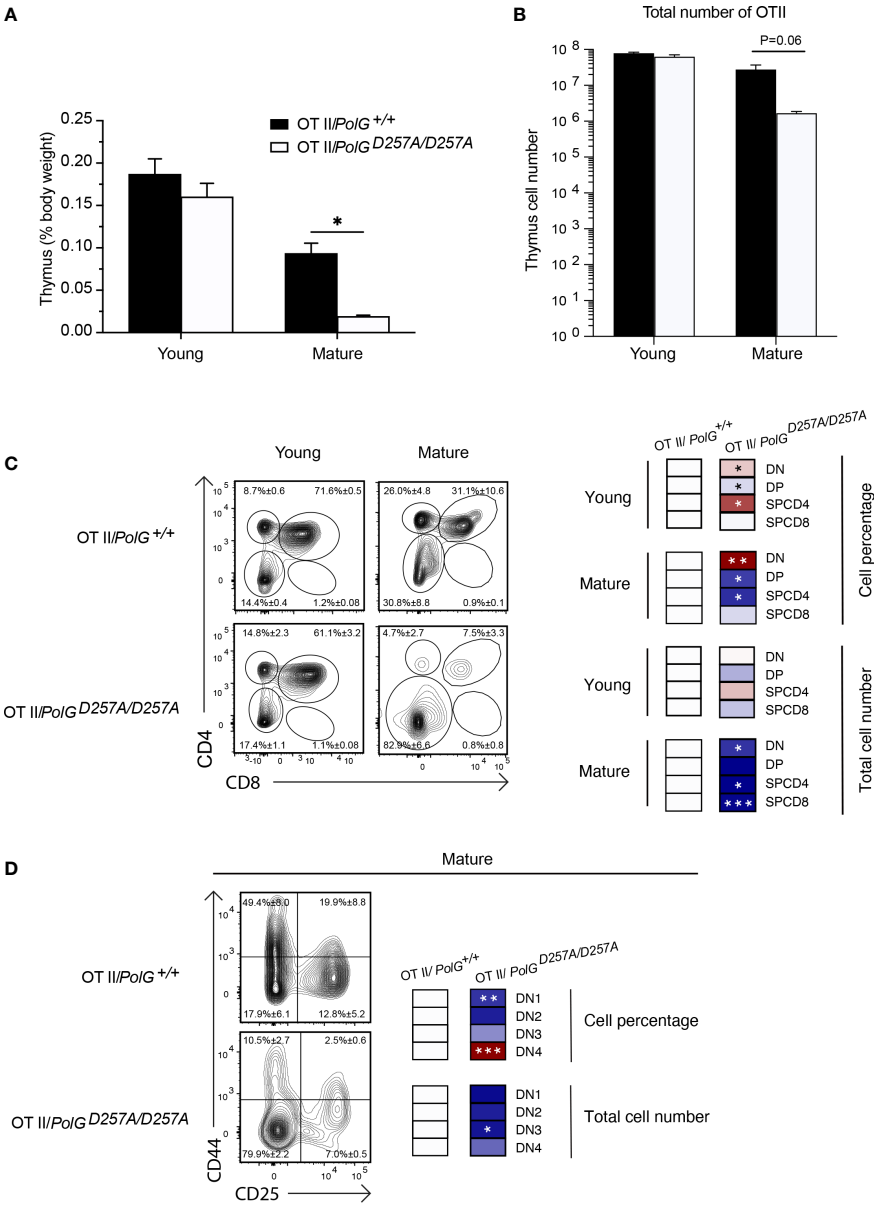

FIGURE 3
Mature OTII/*PolG*$^{D257A/D257A}$ mice have decreased thymi weight and increased DN populations relative to controls. **(A)** Thymi from mature OTII/*PolG*$^{+/+}$ OTII/*PolG*$^{D257A/D257A}$ mice were weighed and plotted as a percentage of body weight. **(B)** Thymocyte numbers from mature OTII/*PolG*$^{+/+}$ OTII/*PolG*$^{D257A/D257A}$ mice were determined and plotted. p=0.06. **(C)** Thymocytes from mature OTII/*PolG*$^{+/+}$ and OTII/*PolG*$^{D257A/D257A}$ mice were stained for CD4 and CD8 and analyzed for CD4/CD8 expression. **(D)** Thymocytes from OTII/*PolG*$^{+/+}$ and OTII/*PolG*$^{D257A/D257A}$ mice were stained for CD25 and CD44 to identify DN1-4 subpopulations, and percentages of the DN1-4 stages determined. Heatmaps are a representative quantification that are normalized by the mean of the control, where red indicates an increase and blue indicates a decrease. Data are displayed as mean ± standard error of mean and representative of 2 independent experiments, n=3-5 mice per group, where *p< 0.05-.01, **p< 0.01-0.001, ***p<0.001 based on multiple t tests.

on cell size using FSC-A, since the larger cell size is associated with cell cycle progression) (27, 28), where populations *a* and *b* are low proliferation populations, and population *c* is high (Figure 4C). The proportion of large (proliferating) cells (as determined by the increase in cell size), particularly among the DN3 cells in mature thymocytes was drastically decreased in *PolG*$^{D257A/D257A}$ mice relative to the control (Figure 4D). Additionally, there was a decrease in the mitochondrial density (shown as an increase in the cells in gate *a*) in the DN3 population in *PolG*$^{D257A/D257A}$ mice relative to WT (Figures 4D, E).

We next analyzed mitochondrial density within the double negative population of mature OTII/*PolG*$^{D257A/D257A}$ mice, again, using Mitotracker to determine mitochondrial density, gating on double negative subpopulations (DN1-4) (24). Here, we found that all four DN stages exhibited reduced mitochondrial density in mature OTII/*PolG*$^{D257A/D257A}$ mice (Figure 5A), with the

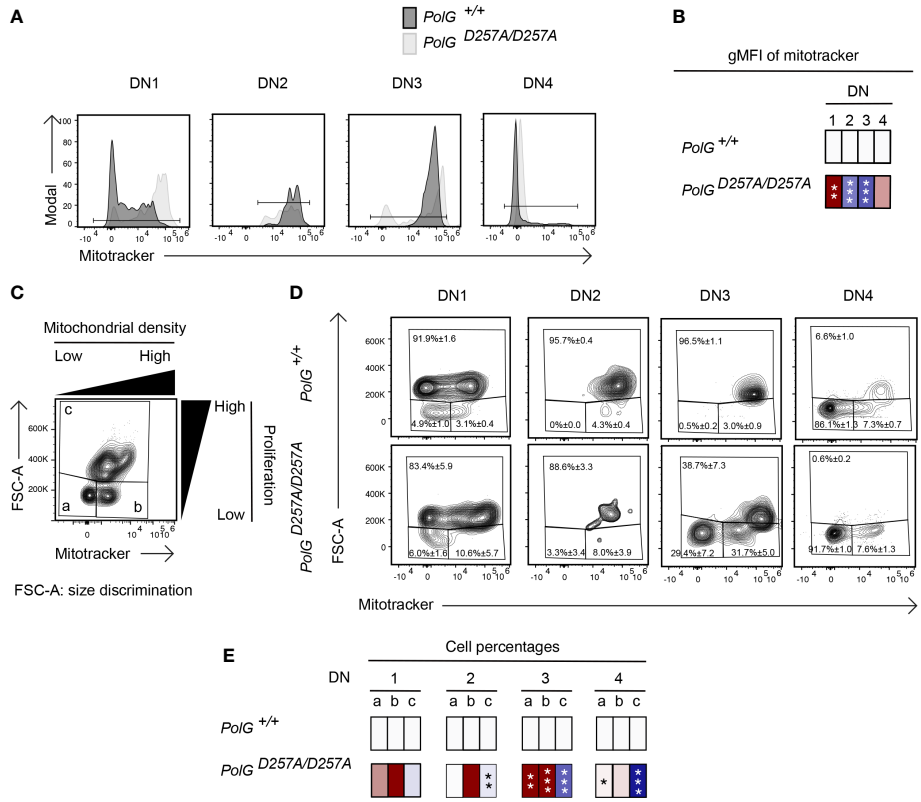

FIGURE 4
Reduced mitochondrial density and proliferation in DN thymocytes in mature $PolG^{D257A/D257A}$ mice. **(A)** Thymocytes from $PolG^{+/+}$ and $PolG^{D257A/D257A}$ mice were stained for mitochondria, along with CD25 and CD44. Histograms depict mitochondria density in gated double negative subpopulations: DN1, DN2, DN3, and DN4. **(B)** Geometric mean fluorescence intensity (gMFI) of mitochondria density staining in DN1-4 thymocytes from $PolG^{+/+}$ and $PolG^{D257A/D257A}$ mice. **(C)** Illustration of gating strategy used to discriminate larger proliferating cells and mitochondrial density and **(D, E)** frequencies of size (FSC-A) and mitochondrial density (mitotracker stain) in DN1-4 populations as indicated. Heatmaps are a representative quantification that are normalized by the mean of the control, where red indicates an increase and blue indicates a decrease. Data are displayed as mean ± standard error of mean and representative of 1 independent experiment, n=3 mice, where *p< 0.05-.01, **p< 0.01-0.001, ***p<0.001 based on multiple t tests.

difference increasing in the more mature stages towards DN4 (Figure 5B). Together this suggests that as thymocytes develop along the DN1-4 stages, reduced fidelity of mtDNA replication in the $PolG^{D257A/D257A}$ background may lead to progressively reduced mitochondrial density.

We also analyzed these DN populations to determine whether decreased mitochondria also negatively impact this highly proliferative stage of T cell development in the mature OTII/$PolG^{D257A/D257A}$ mice (25–27). Figure 5D shows a similar analysis that determines proliferation (based on cell size using FSC-A, since the larger cell size is associated with cell cycle progression) (27, 28), where populations *a* and *b* are low proliferation populations, and population *c* is high (see Figure 5C). The proportion of large (proliferating) DN3 and DN4 cells in OTII/$PolG^{D257A/D257A}$ thymocytes (as determined by the increase in cell size in gate c) was drastically decreased relative to the control (Figure 5D). Additionally, there was a decrease in the mitochondrial density (shown as an increase in the cells in gate *a*) in the OTII/$PolG^{D257A/D257A}$ DN3, and again more drastically in DN4 populations relative to OTII/$PolG^{+/+}$ (Figures 5D, E).

Mature OTII/$PolG^{D257A/D257A}$ T cells have decreased mitochondrial density and proliferation at the SP CD4$^+$ T cell stage

Lastly, we wanted to determine whether the aberrant effects observed in the mature thymocytes continued through the rest of T cell development in the thymus. To determine this, we looked at more mature DP and SPCD4 T cell populations in the thymus. There was a decrease in the proliferating cells (larger cells in gate *c*) in the OTII/$PolG^{D257A/D257A}$ relative to the control (Figure 6A), although this was not seen in the non-transgenic $PolG^{D257A/D257A}$ DP cells. Examination of the more mature DP and SPCD4 OTII/$PolG^{D257A/D257A}$ T cell populations in the thymus revealed decreases in the mitochondrial density (gates *a* and *b*), in CD4SP cells in both non-transgenic $PolG^{D257A/D257A}$ and OTII/$PolG^{D257A/D257A}$ thymocytes, which was less apparent in DP thymocytes (cf. Figure 6B, vs A). Similar results were observed in CD4SP non-transgenic $PolG^{D257A/D257A}$ thymocytes (Figure 6C. note that OTII thymocytes develop into CD4SP, not CD8SP).

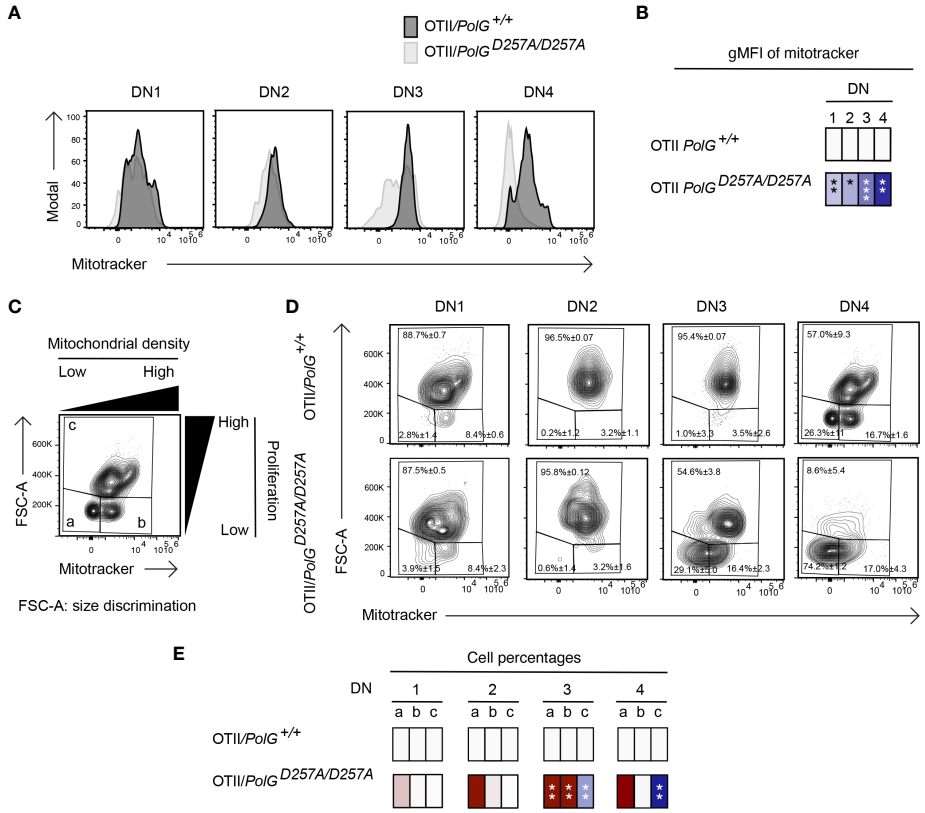

FIGURE 5
Reduced mitochondrial density and proliferation in DN thymocytes in mature OTII/*PolG*$^{D257A/D257A}$ mice. **(A)** Thymocytes from OTII/*PolG*$^{+/+}$ and OTII/*PolG*$^{D257A/D257A}$ mice were stained for mitochondria, along with CD25 and CD44. Histograms depict mitochondria density in gated double negative subpopulations: DN1, DN2, DN3, and DN4. **(B)** Geometric mean fluorescence intensity (gMFI) of mitochondria density staining in DN1-4 thymocytes from OTII/*PolG*$^{+/+}$ and OTII/*PolG*$^{D257A/D257A}$ mice. **(C)** Illustration of gating strategy used to discriminate larger proliferating cells and mitochondrial density and **(D, E)** frequencies of size (FSC-A) and mitochondrial density (mitotracker stain) in DN1-4 populations as indicated. Heatmaps are a representative quantification that are normalized by the mean of the control, where red indicates an increase and blue indicates a decrease. Data are displayed as mean ± standard error of mean and representative of 1 independent experiment, n=3 mice, where *p< 0.05-.01, **p< 0.01-0.001, ***p<0.001 based on multiple t tests.

Discussion

The role of mitochondria in T cell development has only recently been intensely investigated, and understanding the effects of mtDNA mutations on this process is even more limited (2). Here, we have explored the effects of reduced mtDNA replication fidelity, which leads to increase in mtDNA mutations in mice carrying the mutant *PolGD257A*, on T cell development. We found that reducing the fidelity of mtDNA replication results in premature age-dependent reduction in the total number of CD4/CD8 double positive and negative thymocytes. This is likely due to reduced proliferation in the highly proliferative double negative stages due to reduced mitochondrial density and the accompanying effect on mitochondrial products needed for appropriate function. Taken together, this work suggests that T cell development is regulated by the ability of mitochondria to faithfully replicate their DNA.

We examined the importance of the fidelity of the mtDNA replication in T cell development and the significance of this process in an age dependent manner, given that over time mtDNA mutations accumulate naturally in WT mice and at an accelerated rate (>500 fold) in *PolGD257A* mice (15). Others have previously shown that homozygous mutations in the exonuclease region of *PolGD257A* result in premature aging phenotypes, including the thymus, in 9-13 month old mice (15). Interestingly, 3 month old mice have an increase in apoptosis in total thymi, suggesting that there might be fewer thymocytes because of increased cell death (15). Considering the age gap between 3- and 9-month-old mice, we sought to determine whether these differences are detectable between these ages, and whether these findings depend on the dose of the PolGD275A mutant. Similar to previous findings, we saw decreased weight and total cell number of thymocytes in the mature *PolG*$^{D257A/D257A}$ mice (15, 29). That this observation was not detected in the heterozygous group suggests that one functional copy of the PolG gene may be sufficient to otherwise ameliorate negative effects.

We further investigated the effects on various T cell developmental stages. The DN stage includes TCR β chain-selection, survival, differentiation, proliferation, and allelic exclusion at the TCRβ locus (30). We predicted that we would see an increase in the DN stage in the mature *PolG*$^{D257A/D257A}$ mice relative to the control because there would be higher mutations due to the high proliferation rates, and the findings supported this

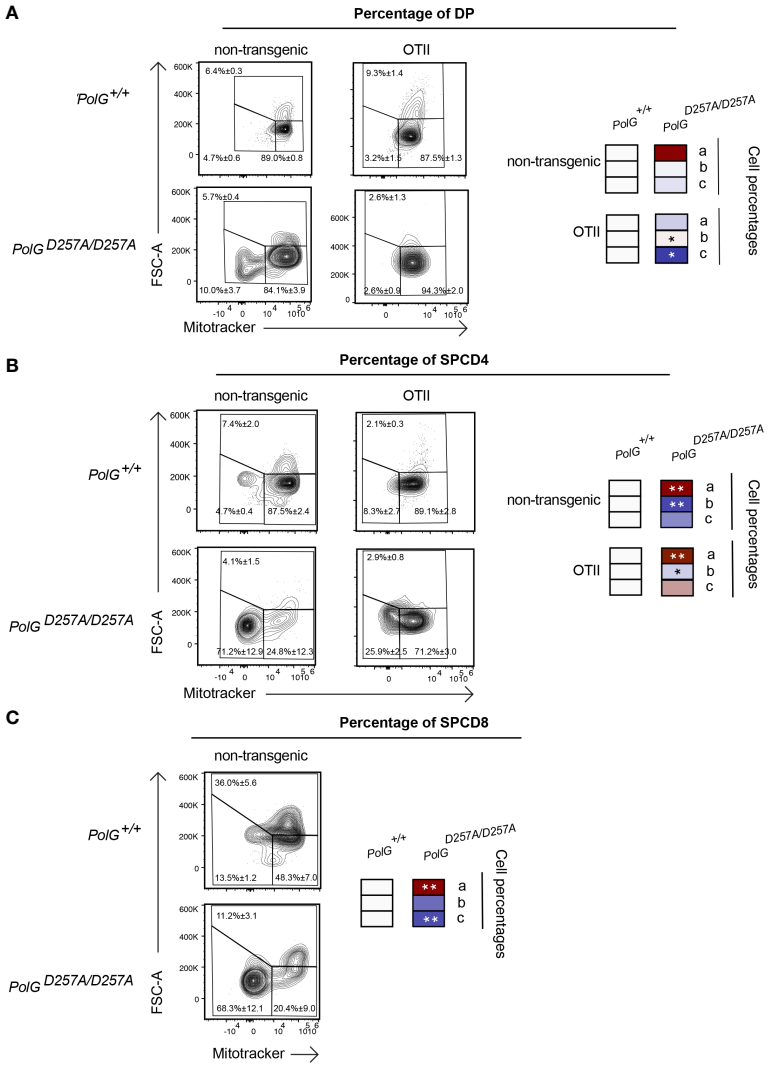

FIGURE 6
Single positive CD4$^+$ T cells in mature OTII/*PolG*$^{D257A/D257A}$ mice have decreased mitochondrial density. **(A)** Thymocytes from *PolG*$^{+/+}$, *PolG*$^{D257A/D257A}$, OTII/*PolG*$^{+/+}$ and OTII/*PolG*$^{D257A/D257A}$ mice were stained for mitotracker, along with CD4 and CD8, and the frequencies of size (FSC-A). The histograms represent mitochondrial density (mitotracker stain) in CD4/CD8 DP, **(B)** CD4 SP, or **(C)** or CD8 SP population plotted population plotted (the *PolG*$^{+/+}$ and *PolG*$^{D257A/D257A}$ mice only in **(C)**). Data are displayed as mean ± standard error of mean and representative of 1 independent experiment, n=3 mice, where *p< 0.05-.01, **p< 0.01-0.001, based on multiple t tests.

prediction. There are different factors that distinguish progression through the DN stage, discriminated between DN1-4 stages. DN1-2 stages or early thymic precursors are the earliest clearly identifiable intrathymic stages (23). We observed an increase in the DN1 population in the mature *PolG*$^{D257A/D257A}$ mice relative to the controls. This suggests that there may be a developmental stall in the DN1 stage, thus reducing progression to the subsequent stages and affecting further T cell development.

T cell commitment occurs at the end of the DN2 prior to entry into the DN3, and during this stage they traverse a significant proliferative expansion (31). We observed a decrease in the cell number and percentage of DN3 population in the *PolG*$^{D257A/D257A}$ mice, suggesting that the fidelity of mtDNA replication significantly affects the highly proliferative cells in the DN3 stage. A possible reason for the reduction in the number of these cells is increased cell death, although this requires further investigation. This finding highlights the importance of functional mitochondria and mitochondrial products in this highly proliferative stage, which may be more sensitive to higher mtDNA mutational burden in the *PolG*$^{D257A/D257A}$ mice.

The DP stage, which follows the highly proliferative DN stage, is where the CD4 and CD8 co-receptors are upregulated and expression of the rearranged TCRα and β chains occur (30). Following which, T cells go through positive selection where the TCRs are tested for having appropriate avidity for the MHC/peptide. These stages do not require high proliferation like the DN stage. Therefore, we predicted that we would not see a difference in the percentage of these cells, but a decrease in the cell number in the *PolG*$^{D257A/D257A}$ mice relative to the control, and this conclusion aligned with the data.

We also examined the effect of increased mtDNA mutations on T cell development in OTII/*PolG*$^{D257A/D257A}$ transgenic mice. The

rationale for our analysis of OTII/*PolG*$^{D257A/D257A}$ mice was that the presence of the already rearranged TCR accelerates the progress through the DN developmental stages, when gene segments would normally rearrange and encode the TCR alpha and beta chains. Unlike the non-transgenic mice, T cell development in the transgenic mice do not have to go through TCR gene rearrangement in the same manner as non-transgenic mice, since unlike the non-transgenic mice, T cells in the transgenic mice do not have to go through TCR VDJ recombination at the alpha and beta loci during their development (32). Thus, if the increased proliferation during these stages was inordinately affected by the low fidelity mtDNA replication, then these stages could potentially be less affected in these transgenic thymocytes. Notably, we did not see the same trend of increased numbers of thymocytes in the DN stage in the OTII/*PolG*$^{D257A/D257A}$ mice. However, we found that there was a block later in the DN stage, with an increase in the percentage of DN4 in the mature OTII/*PolG*$^{D257A/D257A}$ mice relative to the control.

We explored potential mechanisms for these observed defects in T cell development in the mutant mice by analysis of mitochondrial density and cell size, as a proxy for cell division. Abnormal mtDNA replication can lead to altered expression of mitochondrial proteins which are important for the electron transport chain and can cause decreased oxidative phosphorylation and activate mitochondrial degradation through mitophagy. If there are fewer mitochondria to make appropriate substrates for cell replication, this can alter metabolism and cause deleterious effects to cell proliferation (2).

Further analysis of the mitochondrial density indicated that there was decreased in the DN2 and 3 populations in *PolG*$^{D257A/D257A}$ mice compared to the controls. Furthermore, there is a notable reduced proliferation in the DN3 population in the *PolG*$^{D257A/D257A}$ relative to WT. This could be due to a buildup of mtDNA mutations relative to the control at this stage. Furthermore, it is possible that increased mtDNA mutation could result in decreased mitochondrial function and increased mitophagy. By contrast, the OTII/*PolG*$^{D257A/D257A}$ DN1-4 populations exhibited decreased mitochondrial density compared to the controls. Furthermore, there is a notable reduced proliferation in DN3 and DN4 populations in the OTII/*PolG*$^{D257A/D257A}$ relative to WT. This trend appeared to increase over differentiation stage, where the largest differences between mitochondrial density and proliferation were observed at the DN4 stage. This could also be a due to a larger buildup of mtDNA mutations relative to the control at this stage, and increased mitophagy. One major limitation to our findings is that it is unclear whether they are influenced by non-T cell dependent factors given that *PolG*$^{D257A/D257A}$ and OTII/*PolG*$^{D257A/D257A}$ models are globally mutated. Therefore, future experiments will need to be conducted to determine whether our observations are due to T cell intrinsic effects.

Finally, our investigation of T cell developmental stages beyond the DN stage in non-transgenic and OTII transgenic mice revealed that while proliferation of DP thymocytes in mature non-transgenic *PolG*$^{D257A/D257A}$ is not obviously affected compared to WT mice, this is impaired in OTII/*PolG*$^{D257A/D257A}$ compared to WT OTII mice. This suggests that the *PolG*$^{D257A/D257A}$ mutant cells are not all selectively inhibited from further development given their presence, but that the rearranged transgene may affect their progression, as it does in the DN stage. Notably however, there increased percentage of low mitochondrial density in the mature non-transgenic *PolG*$^{D257A/D257A}$ and OTII/*PolG*$^{D257A/D257A}$ SPCD4 (as well as SP CD8 in non-transgenic mice, note that OTII thymocytes develop into CD4 SP cells due to the nature of the rearranged TCR) with respect to the controls. The observed low mitochondrial density may be an indicator that there is a decreased capacity to quickly make new mitochondrial due to possibly aberrant mitochondrial function. There were also decreases in the total number of $CD4^+$ and $CD8^+$ T cells in the lymph node of mature *PolG*$^{D257A/D257A}$ mice, but interestingly, not in the spleen (Supplemental Figure 2). These findings suggest that the mature T cells may be affected by reduced fidelity of mitochondrial DNA replication, in an organ specific manner.

There are drugs with reported side effects of decreased mtDNA content and mitochondrial functional impairment similar to what is observed in the PolG mouse model (33, 34). One common observation is mitochondrial toxicity seen with the use of reverse transcriptase inhibitors which can resemble purines (adenosine and guanosine) and pyrimidines (cytidine, thymine, and uridine), known as nucleoside-analogue reverse-transcriptase inhibitor. These inhibitors are widely utilized as an antiviral treatment with patients who have viral infections (35). It is currently unclear whether such drugs induce similar alterations as we observe in the PolG mutant mice.

Altogether our data revealed that the fidelity of mtDNA replication is critical to T cell development, affecting proliferation in specific DN stages. This reduced proliferation and block in the DN stages may be due to decreased mitochondrial density, resulting in reduced proliferation and development of T cells. This study contributes to our understanding of PolG and the fidelity of mtDNA replication in T cell development, providing new insight into how mitochondria affect this process.

Materials and methods

Mice

All mice were maintained and housed in specific pathogen-free facilities and experiments were performed in accordance with protocols that were approved by the Institutional Animal Care and Use Committee at University of Cornell University. *PolG*$^{D257A/D257A}$ mice have been previously described and obtained from the Jax labs (15). Male *PolG*$^{+/D257A}$ and *PolG*$^{D257A/D257A}$ were backcrossed with WT C57BL/6J, mice, to generate male and female heterozygous and homozygous littermates which were then crossed and used for this study. The presence of *PolGD257A* knock-in mutation was determined by PCR using the following primers in genotyping (5' to 3'; reverse common: AGT CCT GCG CCA ACA CAG; wildtype forward: GCT TTG CTT GAT CTC TGC TC; mutant forward: ACG AAG TTA TTA GGT CCC TCG AC). OTII transgenic mice were also obtained from the Jax labs and crossed with *PolG*$^{D257A/D257A}$ and then the OTII/*PolG*$^{D257A/+}$ were crossed with OTII/*PolG*$^{D257A/+}$ and the subsequent generation were used for experiments.

Antibodies for flow cytometry

The following antibodies were used for flow cytometry analysis: PerCP-Cy5.5-anti-CD25, PE-anti-CD24, CD44-FITC, PE-CF594-anti-CD4 (BD Biosciences, Inc.), PacificBlue-anti-CD4, PacificBlue-anti-CD8a, eF506-viability dye (eBioscience Inc.), and AF700-anti-CD8a, Pecy7-anti-TCRβ (BioLegend). Mitotracker green (Cell Signaling Technology) was used to detect mitochondrial density.

Flow cytometry

Organs were mechanically dissociated through 70 μM screen placed in complete RPMI media while on ice. For surface staining, cells were stained, washed, and all flow cytometry analysis was conducted in the Cornell University Flow Cytometry Core Facility using the Thermo Fisher Attune NxT and data was analyzed in FlowJo (Tree Star, Ashland, OR) where all data were performed gating on doublet excluded viable cells.

Data extraction and statistical analysis

Flow cytometry data was extracted and analyzed using code through RStudio 2022.07.1 Build 554 (GitHub repository TMPierpont/tmisBasic/Heatmap/R/timsDataLoader.R 2022). This code was used RStudio to export percentages from FlowJo analysis and to calculate total cell numbers. The flow cytometry gates show average cell percentages, and heatmaps are a representative quantification that are normalized by the mean of the control. Statistical analyses were carried out utilizing Prism 9 (GraphPad, San Diego CA) and RStudio. Statistical significance was set at $p<0.05$, and all data are reported as a mean, plus and minus standard error of the mean (SEM). Age and genotype were compared by multiple t-tests.

Data availability statement

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

Ethics statement

The animal study was reviewed and approved by Institutional Animal Care and Use Committee at Cornell University.

Author contributions

AA conceived the project, supervised experiments, and analyzed data. CL, NB, DZ, UC, ANV, and WH performed experiments. CL analyzed the data. AA and CL wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

AA receives research support from 3M Company, WH receives research support from MegaRobo.

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.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2023.1128626/full#supplementary-material

SUPPLEMENTARY FIGURE 1
No difference in PolG mRNA expression in neonatal an adult thymus. RNA-Sequencing data from neonatal (6-8 days old) and adult gBT-1 thymocytes (2-4 months old, GSE80597) was analyzed for expression of polg. Results not statistically significant.

SUPPLEMENTARY FIGURE 2
Reduced number of T cells in Lymph nodes, but not spleen, in mature $PolG^{D257A/D257A}$ mice. Lymph node and spleen cells were analyzed for number of $CD4^+$ and $CD8^+$ T cells.

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