THE ROLE OF AIRE, MICRO-RNAs AND CELL-CELL INTERACTIONS ON THYMIC ARCHITECTURE AND INDUCTION OF TOLERANCE

EDITED BY: Geraldo Aleixo Passos, Daniella Areas Mendes-da-Cruz and Ernna Hérida Oliveira

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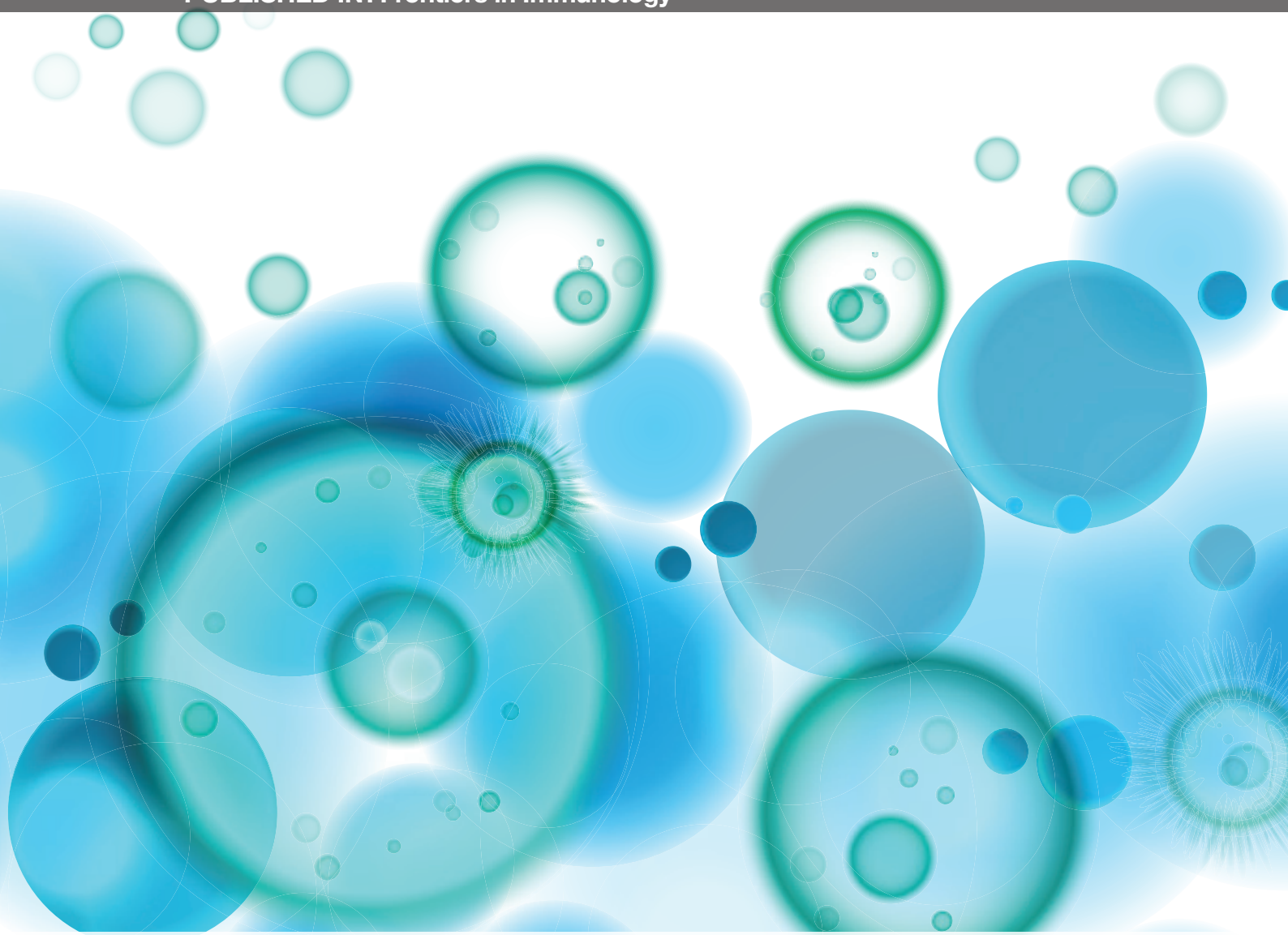

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THE ROLE OF AIRE, MICRO-RNAs AND CELL-CELL INTERACTIONS ON THYMIC ARCHITECTURE AND INDUCTION OF TOLERANCE

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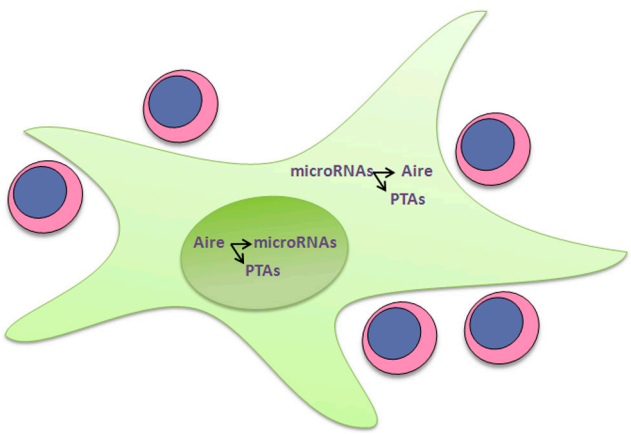

A medullary thymic epithelial cell (mTEC) (here depicted in green) interacts with developing thymocytes (here depicted with blue nucleus and pink cytoplasm), which through MHC-II presents the self peripheral tissue antigens (PTAs). In the nucleus of mTECs, the autoimmune regulator (Aire) protein plays its role as an very important transcriptional regulator of PTAs and microRNAs. Once within the cytoplasm, microRNAs act as posttranscriptional regulators of Aire and PTA mRNAs.

Image taken from: Passos GA, Mendes-da-Cruz DA and Oliveira EH (2015) The thymic orchestration involving Aire, miRNAs, and cell–cell interactions during the induction of central tolerance. Front. Immunol. 6:352. doi: 10.3389/fimmu.2015.00352

The focus of this eBook is to bring new insights into central immune tolerance. To fulfill that, much has been discussed about the master in the regulation of tolerance, the autoimmune regulator (Aire) gene the main thymus cell type that expresses this gene, the medullary thymic epithelial cells (mTECs).

It includes one Editorial and 12 other excellent contributions in the format of mini reviews or original research papers covering one or more of these aspects: promiscuous gene expression (PGE), epigenetics, miRNAs, association of the Aire gene and miRNAs, thymocyte–TEC interaction, coxsackievirus and type 1 diabetes, exosomes in the thymus, thymic crosstalk, thymic B cells, T cell

development, chemokines and migration of T cells, miRNAs and the thymic atrophy, cell–cell interactions, and thymus ontogeny.

Authors raised hypothesis, discuss concepts, and show open questions. The remaining important issues to resolve questions within the central tolerance research are briefly discussed below.

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Our group contributed with a mini review focusing on cell–cell interactions within the thymus involving TECs and thymocytes and the role of the Aire gene on the induction of central tolerance throughout the modulation of TRA expression in mTECs. In addition, we discuss the recent evidence that Aire also regulate the expression of miRNAs in these cells. On its turn, the Aire-dependent miRNAs might exert control over TRAs. We raise issues that besides the transcriptional control exerted by Aire, PGE could also be being controlled through posttranscriptional mechanism involving miRNAs.

A very pertinent question raised by Gabriel Skogberg and cols is on the role of exosomes on TRA presentation by TECs to thymocytes and its implication in the thymocyte selection. Exosomes may be liberated by TECs to the extracellular milieu and transport TRAs as well as MHC molecules, establishing intercellular communication to enhance antigen presentation to developing thymocytes. Authors discuss how intercellular communication via exosomes within the thymus could have consequences on TRA presentation and finally on central tolerance.

The thymic crosstalk, i.e., the reciprocal control by the close contact between TECs and thymocytes, which influences the differentiation of both types of cells was elegantly reviewed by Noëlla Lopes and cols. Authors discuss the role of dendritic cells (DCs) subsets in the process of deletion of autoreactive T cells and the generation of natural Tregs and raise questions how hematopoietic cells may control the organization of the thymic medulla.

Thymus is an organ composed of different cell types including TECs, DCs, macrophages among other cell types, and of course thymocytes. Recently, researchers have identified an unexpected cell type formed by B cells, which may be originated from intrathymic B lymphopoiesis or immigration from the periphery. Tomoyoshi Yamano and cols contributed with a mini review discussing the role of thymic B cells expressing MHC-II, CD80, and Aire, in the crosstalk with

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The regulation of T cell development is apparently well resolved; however, several unsolved questions remain. This important aspect is represented in this Research Topic through the mini review by Iris Caramalho and cols. Authors show new questions on the beginning of Treg lineage commitment, their spatial localization within the human thymus and their molecular components.

Cell migration within the thymus is crucial for the central tolerance. Developing thymocytes migrate throughout the thymus being exposed initially to the cortex and then to the thymic medulla were they respectively undergo positive and negative selection. Chemokines represent key regulators for thymocyte migration. Zicheng Hu and cols argue the role of chemokines in the thymic cell migration and induction of central tolerance.

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EDITORIAL
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Editorial: The Role of Aire, microRNAs and Cell–Cell Interactions on Thymic Architecture and Induction of Tolerance

Geraldo Aleixo Passos[1,2], Daniella Arêas Mendes-da-Cruz[3] and Ernna Hérida Oliveira[1]*

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Keywords: Thymus gland, central tolerance, Aire gene, miRNAs, cell-cell interactions, *Coxsackievirus*, exosomes, chemokines

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AUTHOR CONTRIBUTIONS

DM, EO and GP wrote the Editorial.

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Promiscuous gene expression in the thymus: a matter of epigenetics, miRNA, and more?

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The induction of central tolerance in the course of T cell development crucially depends on promiscuous gene expression (pGE) in medullary thymic epithelial cells (mTECs). mTECs express a genome-wide variety of tissue-restricted antigens (TRAs), preventing the escape of autoreactive T cells to the periphery, and the development of severe autoimmunity. Most of our knowledge of how pGE is controlled comes from studies on the autoimmune regulator (Aire). Aire activates the expression of a large subset of TRAs by interacting with the general transcriptional machinery and promoting transcript elongation. However, further factors regulating Aire-independent TRAs must be at play. Recent studies demonstrated that pGE in general and the function of Aire in particular are controlled by epigenetic and post-transcriptional mechanisms. This mini-review summarizes current knowledge of the regulation of pGE by miRNA and epigenetic regulatory mechanisms such as DNA methylation, histone modifications, and chromosomal topology.

Keywords: mTEC, promiscuous gene expression, Aire, epigenetic, miRNA, tolerance, tissue-restricted antigen

INTRODUCTION

The establishment of central tolerance to all organs of the body is to a large extent mediated by the unique ability of medullary thymic epithelial cells (mTECs) to express a vast variety of self-antigens. This so-called promiscuous gene expression (pGE) encompasses a genome-wide selection of tissue-restricted antigens (TRAs), and so far no involvement of tissue-specific transcription factors in their regulation has been observed in the thymus (1, 2). pGE sets the scope of self tolerance, i.e., clonal deletion and Treg induction, and faulty thymic expression of even a single TRA can precipitate organ-specific autoimmunity (3–5); however, we still lack a coherent model incorporating and explaining all the intricacies of pGE.

Most of our knowledge of the molecular control of pGE comes from studies on autoimmune regulator (Aire) (6, 7). Mutations in the *AIRE* gene cause a rare monogenic autoimmune disorder autoimmune polyendocrinopathy–candidiasis–ectodermal dystrophy (APECED), affecting multiple organs with a preference for endocrine glands (8, 9). The Aire-deficient mouse model recapitulates the autoimmune phenotype observed in human patients (10).

Autoimmune regulator controls the expression of a subset of TRAs by interacting with the general transcriptional machinery and promoting transcript elongation. Aire does not have a dedicated DNA recognition motif, and it is unclear how it is targeted to an exclusive set of TRA-encoding genes, which is largely conserved across species (11). Depending on the cellular context, Aire can induce the expression of different sets of genes (12), suggesting that the epigenetic landscape of mTECs plays a role in defining Aire targets. Moreover, many TRAs are expressed in TECs in an Aire-independent manner implying that additional factors also regulate pGE. Noteworthy, a set of cell-lineage-specific TFs has proven dispensable for promiscuous transcription of the corresponding target genes in the thymus (13–16). Thus, the likelihood of tissue-specific TFs responsible for the Aire-independent gene regulation in mTECs or TFs acting in concert with Aire remains an open question.

Recent studies documented a role for epigenetic and post-transcriptional mechanisms in regulating pGE in general and the function of Aire in particular (14, 17–19). Indeed, both APECED patients (8) and mouse mutants (20) display variability in the disease severity and the organs affected depending on different genetic backgrounds, indicating that other genetic or epigenetic components define the exact course of the individual disease. Here, we briefly review our current knowledge of how DNA methylation, histone modification, and miRNA may influence pGE and mTEC maintenance.

EPIGENETIC REGULATION OF pGE

Transcription factor-triggered gene expression is cross-regulated by a number of enzymes, which modify the DNA itself (DNA methylation) or the histones (histone post-translational modifications). Recent studies showed that DNA and histone modifications can alter the promoter structure and accessibility to the extent of adding new TF binding sites, thus shaping the level and pattern of gene expression and, consequently, developmental decisions (21). Accumulating evidence suggests that all these modifications might also be involved in regulating pGE.

DNA METHYLATION

Methylation of cytosines in CpG dinucleotides is essential for the regulation of embryonic development, cell lineage progression, gene expression, and chromatin structure (22) and is implicated in several human diseases (23). The majority of CpGs in the human genome are methylated, whereas CpG islands at the transcription start sites of housekeeping genes are hypomethylated

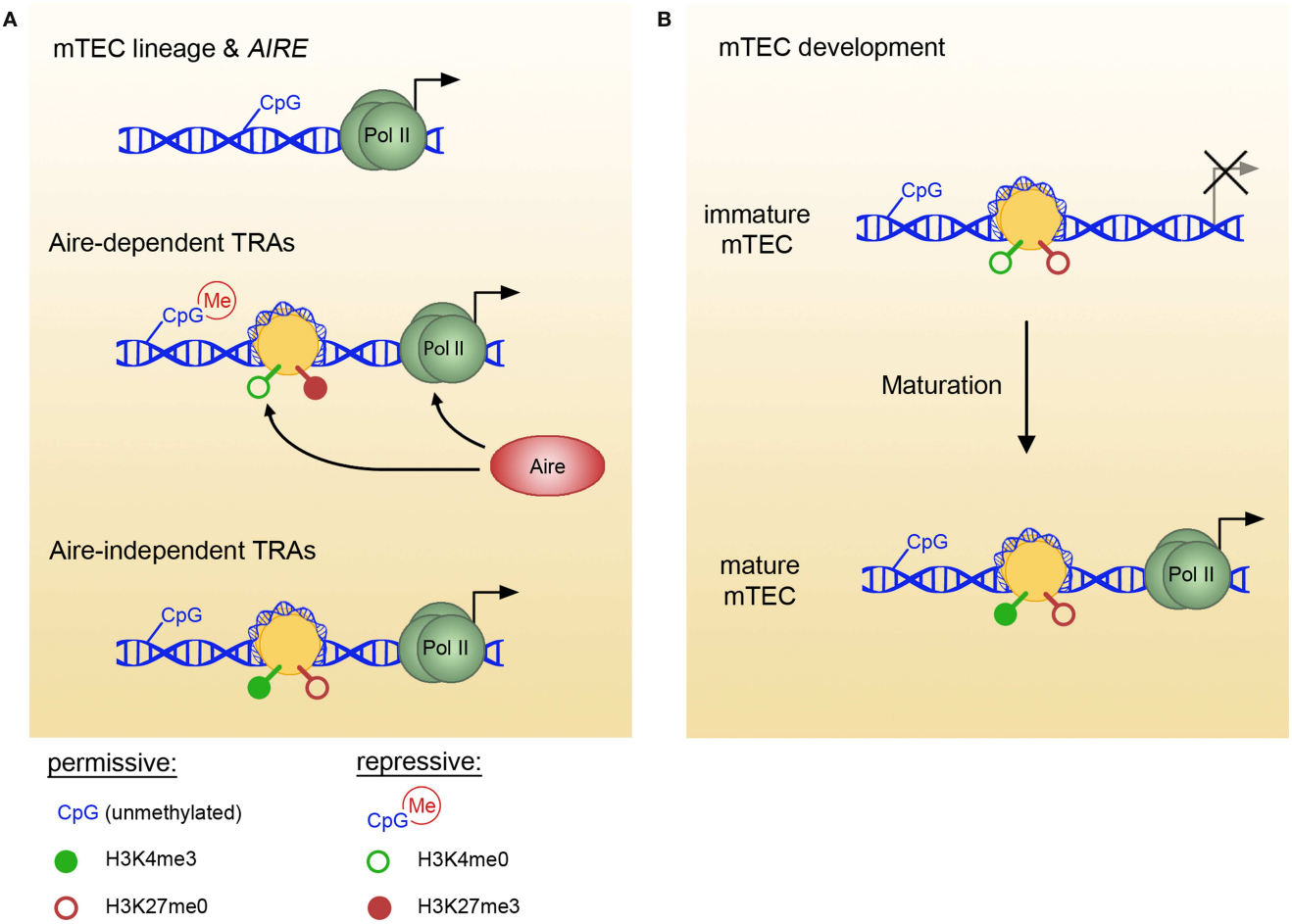

FIGURE 1 | Epigenetic marks in mTECs vary between TRA pools and with mTEC maturation. **(A)** In mTECs, the loci of TEC lineage-specific genes and various TRA pools bear different epigenetic marks. Thus, lineage genes and *AIRE* (top) are characterized by low DNA methylation content (permissive), Aire-dependent genes (middle) favor repressive marks, whereas Aire-independent genes (bottom) are maintained in a permissive state. Aire specifically binds H3K4me0, facilitating polII recruitment and transcript elongation. **(B)** Aire-independent TRA loci undergo epigenetic changes with mTEC maturation, indicating that permissive marks are acquired in a stepwise fashion and maintained before the onset of gene expression. For example, in immature mTECs, *Csn2* genomic locus exists in an epigenetically neutral state, and the appearance of permissive H3K4me3 modification coincides with mTEC maturation and the onset of transcription. TRAs, tissue-restricted antigens; mTEC, medullary thymic epithelial cell.

(24). As a rule, DNA methylation inversely correlates with gene expression level: a high degree of DNA methylation at promoter regions prevents the binding of transcription factors to their DNA-binding motives and results in transcriptional silencing (21, 22). DNA methylation pattern reflects the developmental status of cells with respect to lineage commitment/progression. Thus, the gene loci of myeloid-specific TFs and their binding sites are hypermethylated in the cells of the lymphoid lineage (25). Consistently, demethylation of promoter regions facilitates lineage-specific gene expression, e.g., $CD8^+$ T cell markers are specifically unmethylated and highly expressed in T cells committed to the CD8 lineage (25). Comparison of the methylation patterns of stem/progenitor cells and lineage-committed cells of various tissues demonstrated that the changes in methylation occur at certain lineage-specific gene promoters rather than in extended chromosomal clusters; moreover, this specific methylation pattern is maintained in cells and defines their identity (25–27).

Several recent reports on DNA methylation profiles in mTECs suggest that this epigenetic modification might also pertain to the control of mTEC lineage commitment and pGE; however, the exact specificity and significance of DNA methylation remains unclear (**Figure 1A**). A number of TEC-specific genes are hypomethylated in mTECs in contrast to other thymic cell types and peripheral tissues (28), suggesting that this pattern arises during lineage commitment and defines mTEC cellular identity. *In vitro* and *ex vivo* studies demonstrated that the *AIRE* gene promoter is hypomethylated in mTECs (29, 30), which indicates its transcription-permissive state. In contrast, the *Fgg* (fibrinogen gamma chain) gene, which encodes a liver-specific protein promiscuously expressed in an Aire-dependent manner, is hypermethylated in both Aire-expressing and Aire-deficient

mTECs (28). Further intriguing results were obtained from the comparison of the DNA methylation state of the casein locus and the *Gad1* (Gad67) promoter between immature and mature adult mTECs (14). The DNA methylation pattern of both regions exists in a permissive state already in immature mTECs, presumably allowing rapid promoter activation after mTEC maturation (**Figure 1B**). Interestingly, the *Csn2* promoter showed progressive demethylation in mature mTECs during embryonic development, which preceded the onset of gene expression (14). Together with the reported hypermethylation of *Fgg* locus (28), these observations suggest that DNA methylation pattern of TRA loci in mTECs does not reflect the promoter activity in the same way as in other peripheral tissues. In summary, although mTECs seem to adapt the "peripheral" DNA methylation pattern for their lineage-specific genes, they might employ a different strategy for genes expressed promiscuously.

HISTONE MODIFICATION

Post-translational modifications of histone N-terminal tails are known to be essential in the regulation of transcription, chromatin structure, DNA repair and replication, and alternative splicing. They control gene expression by both recruiting effector proteins, the so-called readers of histone marks, and through changing the compaction state of the chromatin (21, 31). Histone modifications fall into active (promoting transcription) and repressive marks and are associated with different chromatin compaction states (32). Active histone marks at promoter and enhancer regions influence polII assembly and elongation through interaction with the basic transcriptional machinery: H4 acetylation and H3 trimethylation at K4 are recognized by TFIID and mediate polII assembly at promoters (33, 34).

In some instances, active and repressive marks can co-exist within the same region. Thus, embryonic stem cells display overlapping repressive and permissive histone modifications at developmental genes, maintaining their inactivity in steady state, but allowing for rapid activation when differentiation starts (35). Other examples of a poised, bivalent histone code at lineage-specific gene promoters have been observed in $CD4^+$ T cell lineages (36). The coexistence of active (H3K4me3) and repressive (H27K4me3) marks at promoters of lineage-defining transcription factors GATA3, Tbet, Rorc, and Foxp3 endows different $CD4^+$ T cell subtypes with the plasticity to rapidly cross-differentiate into another subtype in response to environmental stimuli (37).

Studies of histone modifications in mTECs indicate that the histone code plays a role in the regulation of pGE, although the exact mechanisms employed might differ for different sets of TRAs (**Figure 1A**). Aire-dependent genes in mTECs are characterized by a lack of H3K4 trimethylation and enrichment in repressive H3K27me3 (17, 38). Aire has been shown to specifically bind to unmethylated H3K4, targeting genes in a state of low H3K4me3 or H3 acetylation (17). Aire binding was correlated with an increase in H3K4me3 and polII recruitment to Aire-dependent gene promoters, implying that Aire facilitates the establishment of active histone marks (17). After polII assembly, Aire assists in the transcriptional elongation through facilitating p-TEFIIb recruitment (39, 40). Since p-TEFIIb recruitment requires active histone marks at the enhancer regions (41), involvement of Aire in reading enhancer histone code remains an intriguing possibility.

In contrast to Aire-dependent genes, Aire-independent TRAs seem to favor permissive histone modifications (38). In a recent study, Kyewski and colleagues assessed the chromatin state in mature and immature mTECs at two specific loci encoding Aire-independent TRAs, namely *Csn2* and *Gad1* (14). Both gene promoters were characterized by permissive histone marks; furthermore, in the case of the *Csn2* promoter, H4 acetylation and H3K4me3 marks increased with mTEC maturation (**Figure 1B**). Interestingly, repressive H3K27me3 marks were absent from *Csn2* promoter in both immature and mature mTECs, suggesting that at least some TRA promoters maintain a neutral rather than repressed steady state in immature mTECs (14). Whether a similar poising of chromatin occurs in Aire-dependent gene loci early in mTEC lineage progression remains to be determined.

In summary, the histone code of TRA promoters in mTECs can exist in either permissive (Aire-independent genes) or bivalent (Aire-dependent genes) states. One of many functions of Aire seems to be reading and modifying the histone marks, but it is still unclear how their initial deposition is regulated. To this end, functional studies of chromatin modifiers in TECs should shed new light on the mechanisms mTECs employ to achieve and maintain a transcriptionally poised state of TRA-coding genes. It is also pertinent to understand the epigenetic differences between Aire-dependent and -independent genes, which might lead to the identification of factors controlling Aire-independent pGE.

EPIGENETIC LANDSCAPE FLEXIBILITY IN mTECs

Recent studies revealed that DNA methylation and histone modifications function in a cooperative manner to re-shape chromatin (21, 42), and promoter activity can be predicted by the combination of both (43). The stability of transcriptionally active sites largely depends on the cellular and developmental context; more changes in the epigenetic landscape occur during development and lineage progression than in terminally differentiated cells (41). Is this also the case for pGE? On the population level, mTECs express thousands of genes promiscuously, but their global DNA methylation profile is not significantly different from that of peripheral tissues (28). One should, however, consider that individual TRAs are expressed by only a minor fraction of mTECs [1–3% on average; (38, 44)]. This might result in an under-representation of TRA-specific epigenetic marks in a population analysis. In this respect, studies of single TRA loci within the mTEC subpopulations expressing that specific TRA will be more informative than global epigenomic approaches. Given that pGE increases in its complexity during mTEC maturation and that even in mature mTECs the expression of individual TRAs seems to be transient (45), the epigenetic landscape in the thymus might turn out to be more flexible and dynamic than in other tissues.

Thus, in the case of the casein locus DNA methylation pattern is established before the histone code and both exist in a permissive state even before Csn2 mRNA can be detected [Ref. (14); **Figure 1B**]. Since Csn2 seems to be somewhat special with regard to its expression frequency in mTECs (44), the sequential establishment and cooperation of CpG context and histone marks should be examined in other, less frequent TRA loci at

different stages of mTEC development. The interplay between histone marks and DNA methylation pattern can result in the organization of actively transcribed loci in transcriptional factories (46), demonstrating the influence of epigenetic marks on genome topology. Recently, emerging evidence suggests that higher order interactions between chromosomal regions in cis and trans might impose further influence on gene expression (47, 48). Importantly, stochastic interchromosomal interactions can account for gene expression heterogeneity in a population: for example, co-localization of β-globin gene and its enhancer was observed in 5–10% of cells in a population and correlated with a ~100-fold increase of β-globin expression in these "jackpot" cells (49). The fact that only a small proportion of mTECs expresses a given TRA at a given time together with a recent observation of TRA loci co-localization (45) suggests that such higher order organization might regulate patterns of gene co-expression in single mTECs in the context of pGE.

POST-TRANSCRIPTIONAL REGULATION OF pGE

miRNA represents a class of small (≈22 nt) RNA molecules involved in the post-transcriptional control of gene expression, acting as switches and fine-tuners of translation (50). Primary miRNA transcripts are polII-dependent and undergo two steps of post-transcriptional processing: by Drosha and DGCR8 in the nucleus and by Dicer and TRBP in the cytoplasm [Ref. (51); **Figure 2A**]. The mature miRNA are incorporated into the RNA-induced silencing complex (RISC), binding of which to the target mRNA causes a translational block and subsequent mRNA decay (52, 53). Target recognition depends on a sequence-specific interaction between the target mRNA 3′UTR and the seed sequence of miRNA (54). More than half of all protein-coding genes in mammals are regulated by miRNA, and many of them have binding sites for several unrelated miRNA in their 3′UTR. Additional complexity arises from the fact that a single miRNA can affect the expression of multiple targets. Studies of miRNA function in various tissues revealed complex balanced miRNA–mRNA interaction networks, regulating tissue homeostasis, cell fate decisions, and disease progression (55).

Several recent studies suggest that miRNA may be involved in the regulation of pGE. We showed that a number of miRNA exhibit subset-specific expression in TECs isolated from murine or human thymus; a substantial overlap between miRNA signatures of both species suggests that miRNA expression profiles in TECs are evolutionarily conserved (18). We also demonstrated that maturation-dependent expression of certain miRNA in mTECs correlates with Aire expression (18). Furthermore, changes in miRNA signature have been reported upon Aire knockdown in cell culture (56) and in Aire null mutant thymi (18). Whether Aire directly regulates transcription of miRNA-encoding genes in mTECs and whether such a regulation is a part of stochastic pGE remains to be determined. Since miRNA-encoding genes can use alternative promoters and many miRNA are located in introns (57, 58), Aire might be involved in the direct control of miRNA transcription as well as in the miRNA biogenesis coupled to host mRNA processing. Interestingly, Aire has been implicated in Lin-28-dependent regulation of let-7 miRNA in ES cells (59). As for Aire being regulated by miRNA, a recent report showed that Aire expression could be controlled by miRNA-220b in an artificial cell culture system (60). It is unclear whether this regulation occurs in human or mouse mTECs *in vivo*, and no conserved miRNA target sites in Aire mRNA have been predicted *in silico* by the currently available target prediction tools.

miRNA expression in the thymic epithelium is indispensable for the establishment of central tolerance. Thus, TEC-specific ablation of Dicer or DGCR8 (and therefore all mature miRNA) leads to premature thymic involution, diminished T cell output and increased susceptibility to autoimmune disease (18, 61–63). The lack of Dicer in TECs leads to a dramatic decline in pGE – a possible underlying cause of the breach in central tolerance. Interestingly, pGE decline affects both Aire-dependent and -independent TRAs in mTECs and cTECs (18), and precedes the loss of TEC cellularity (18, 62). The premature involution phenotype of Dicer and DGCR8 mutants is recapitulated in the mouse model lacking miR-29a (61). However, these latter mutants do not exhibit the defects in epithelial organization that result from the loss of all canonical miRNA (61, 63) and show only mild delayed impairment of Aire-dependent pGE (18), suggesting that miRNA other than miR-29a play a role in TEC maintenance and function. Further investigation of the mTEC-specific miRNA and their targets will be needed to comprehend the miRNA-dependent regulation of pGE.

How do post-transcriptional inhibitors facilitate pGE? First, in rare cases, miRNA were shown to activate rather than repress gene expression, e.g., through binding to the 5′-UTR [Ref. (64); **Figure 2A**]. Gene expression activation can also happen indirectly after the miRNA-mediated downregulation of proteins involved in transcriptional repression or RNA decay (53, 65). Finally, miRNA could affect pGE indirectly by promoting the maturation of mTECs. Indeed, FoxN1–Cre-mediated loss of Dicer causes alterations in mature mTEC surface antigen profiles (18) and might impair the early stages of mTEC lineage progression (**Figure 2B**). The fact that mTEC lineage progression and terminal differentiation seem to be unaffected in miR-29a mutants (18) suggests that other miRNA play a role in these processes. Of note, stemness, differentiation, and senescence of keratinocytes seem to be controlled by a complex network of p63 and several miRNA (66). Given the close parallels between keratinocyte and mTEC differentiation (67) further studies on miRNA function in the thymus should reveal whether a similar network determines turnover, maintenance, and function of mTECs.

Apart from being required for mTEC development and pGE, TEC-specific miRNA might play a role in other mechanisms of central tolerance establishment. One of these mechanisms involves a transfer of TRAs from mTECs to dendritic cells (68). Though it is unclear by which precise means the antigens are shared, exosome transfer is a possible route (69). Intriguingly, a recent study showed that human thymic exosomes contain TRAs and TEC-specific miRNA (70). miRNA transfer via exosomes was shown to be functionally relevant in various settings. Thus, T cells share their miRNA by this pathway with antigen-presenting cells and other T cells (71, 72). Whether transfer of miRNA from mTECs to dendritic cells indeed takes place via exosomes and the functional significance of this exchange will be clarified in future studies.

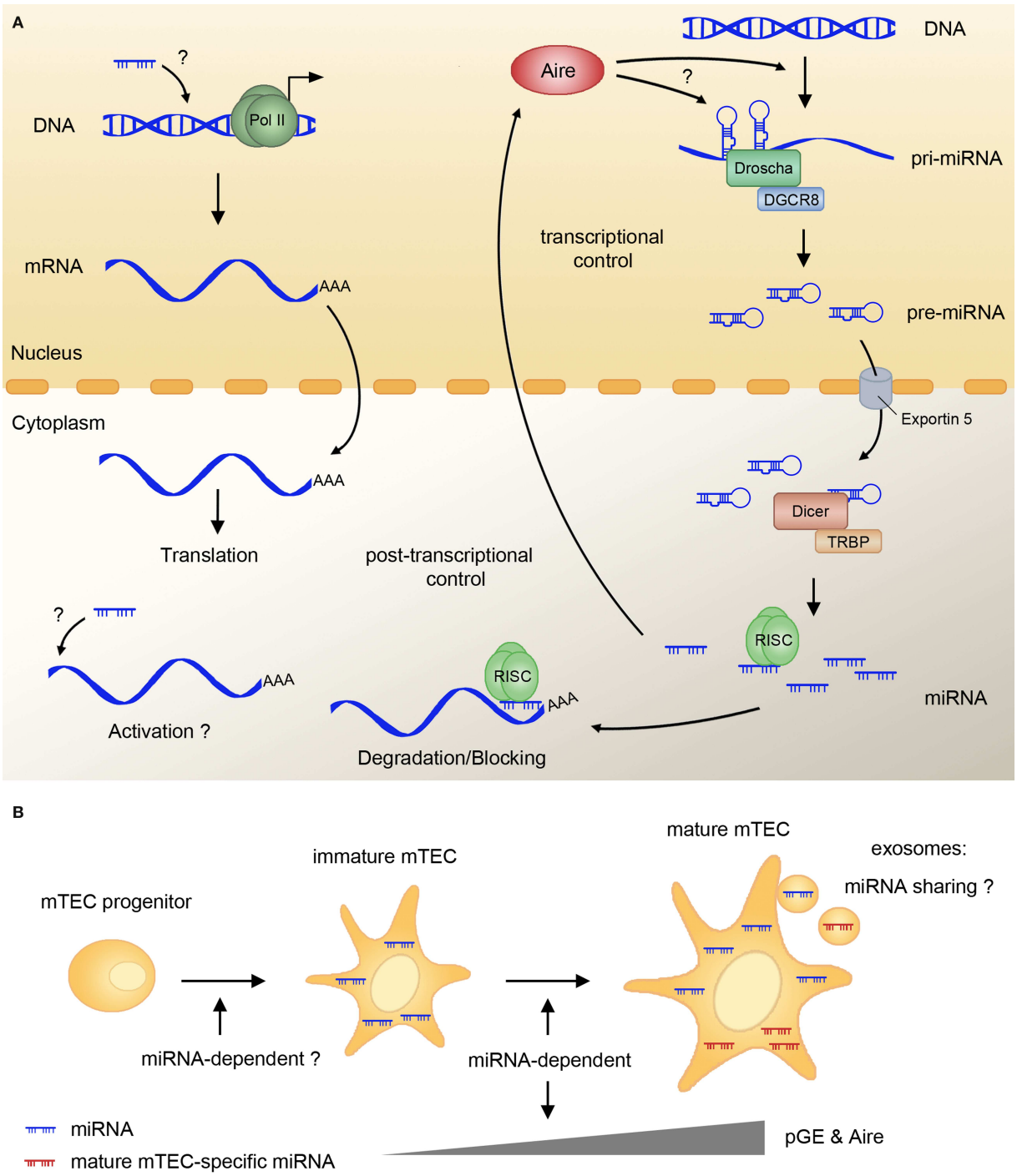

FIGURE 2 | miRNAs regulate pGE and mTEC maturation. **(A)** The majority of miRNA primary transcripts (pri-miRNA) are generated by polII and further processed by Drosha/DGCR8 in the nucleus to the stage of pre-miRNA. Pre-miRNA are exported into the cytoplasm by Exportin 5, and further cleaved by the Dicer complex, resulting in mature miRNA. These can be incorporated into the RNA-induced silencing complex (RISC), which binds the target mRNA and usually mediates translational block and/or mRNA degradation. In mTECs, miRNA are indispensable for the expression of Aire and Aire-dependent and -independent TRAs. The mechanism of miRNA action in pGE is not known, and it might involve activation of transcription or translation of TRAs. Aire might be involved in regulating the expression of miRNA-encoding genes and in the generation of miRNA precursors from the so-called miRtrons. **(B)** mTEC maturation and pGE rely on an intact miRNA pathway, as Dicer deletion in TECs blocks different stages of mTEC lineage progression. Several miRNA are specifically upregulated upon mTEC maturation; however, their exact function and the influence they exert on TRA and Aire expression remains to be determined. The fact that mTEC-specific miRNA are found in human thymic exosomes suggest the possibility of mTECs sharing these small regulators with other antigen-presenting cells in the course of central tolerance induction. RISC, RNA-induced silencing complex; mTEC, medullary thymic epithelial cell; pGE, promiscuous gene expression.

CONCLUDING REMARKS

Promiscuous expression of peripheral antigens in the thymus keeps autoimmunity at bay; grasping its exact molecular mechanism will lead to a better understanding of how central tolerance is established and maintained. Transcription factor-mediated gene expression has been shown to go hand in hand with epigenetic and post-transcriptional regulation in many peripheral tissues. Recent studies of these modes of regulation in mTECs suggest that epigenetic marks are deposited and interpreted in an unconventional way in the course of pGE, and that miRNA play an important role in maintaining TRA expression. The future challenge lies in finding out how exactly mTECs utilize ubiquitous epigenetic and post-transcriptional mechanisms to achieve and maintain their extraordinarily broad expression profiles. Will pGE eventually turn out to employ a unique scenario of gene regulatory modes for the sake of preserving tolerance?

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Eph/ephrins-mediated thymocyte–thymic epithelial cell interactions control numerous processes of thymus biology

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Numerous studies emphasize the relevance of thymocyte–thymic epithelial cell (TECs) interactions for the functional maturation of intrathymic T lymphocytes. The tyrosine kinase receptors, Ephs (erythropoietin-producing hepatocyte kinases) and their ligands, ephrins (Eph receptor interaction proteins), are molecules known to be involved in the regulation of numerous biological systems in which cell-to-cell interactions are particularly relevant. In the last years, we and other authors have demonstrated the importance of these molecules in the thymic functions and the T-cell development. In the present report, we review data on the effects of Ephs and ephrins in the functional maturation of both thymic epithelial microenvironment and thymocyte maturation as well as on their role in the lymphoid progenitor recruitment into the thymus.

Keywords: thymocytes, thymic epithelium, Eph, ephrin, thymic cell seeding

Introduction

The thymus is a lymphoid organ engaged in the production and homeostatic maintenance of functionally mature T cells, in which developing thymocytes interact sequentially with an epithelial network whose three-dimensional architecture is essential for the process. Thymocyte–thymic epithelial cell (TEC) interactions are, therefore, key for thymus functioning (1), and Eph and ephrins, two groups of molecules involved in these cell-to-cell contacts, have emerged as novel elements governing numerous thymic processes (2). Eph represent the largest group of receptor tyrosine kinases; they bind to surface ligands, ephrins and, according to their sequence homology and affinity for ephrins, are divided into EphA (10 members), which preferentially binds ephrins-A (6 members), ligands bound to the membrane through glycosylphosphatidylinositol, and EphB (6 members) that bind ephrins-B (3 members) that contains a transmembrane domain and a short cytoplasmic tail (3).

Eph/ephrins constitute an ubiquitous system due to the high number of members and their promiscuity, such that a single receptor can bind different ligands and vice versa, albeit with distinct affinities (4). Eph/ephrin-mediated interactions result in bidirectional signaling in the expressing cells, *forward* signals transmitted by Eph, and *reverse* in the ephrin-expressing cell (5), providing different cell responses depending on the multiple combinations and the direction of signaling (4). Eph/ephrins activate numerous signaling pathways that regulate cytoskeleton and cell adhesion but also gene transcription (6).

Eph and/or Ephrin are Expressed in the Thymus and Their Absence Results in Profound Thymic Hypocellularity

Eph and ephrins, particularly those of the B group, are expressed widely in both thymocytes and TECs, frequently the same cell co-expressing the two types of molecules. They appear early in the thymic primordium (7–9) and a lack of these results in decreased numbers of both thymocytes (9–11) and TECs (12). The thymic hypocellularity of Eph/ephrin-deficient mice correlates with concomitant increased apoptosis affecting distinct thymocyte subsets (9–11). However, in all these Eph/ephrin-defective mice, it is difficult to establish conclusive correlations between thymic cellularity and thymocyte proliferation. Even in thymocyte-conditioned ephrin-B1/B2 thymuses, despite the evident reduced cellularity, there are increased proportions of proliferating DP thymocytes (11), suggesting some attempt to recover the thymic cell content (13).

Thymic epithelial cells (TECs) have been proposed to have a limited expanding capacity and the number of endodermal progenitors that organize the early thymic primordium could determine the final size of embryonic and adult thymus (14). Indeed, there is little information on the control of TEC survival and proliferation in general, and by the EphB group in particular. Developing thymuses of EphB2- and/or EphB3-deficient mice show increased TEC apoptosis largely affecting immature $EpCAM^+MTS20^+$ cells and $EpCAM^+Ly51^+$ cTECs, and *in vitro* activation of either EphB or ephrin-B signaling decreases the proportions of apoptotic WT TECs, whereas its disruption in RTOCs resulted in increased TEC death. Importantly, RTOCs established only with EphB-defective TECs yielded higher proportions of apoptotic cells than those observed when RTOCs were established with TECs and total thymocytes, suggesting that TEC survival is governed to a greater extent by Eph–ephrin-mediated thymocyte–TEC interactions (12).

On the other hand, decreased seeding of lymphoid progenitors, which periodically colonize the thymus, could also contribute to organ size and cellularity. Reduced lymphoid seeding into the thymus can be achieved by a reduction in the colonizing progenitor numbers and/or altered mechanisms of migration. Although, EphB2-deficient mice show decreased proportions of early BM hematopoietic progenitors compared to WT mice (15) their contribution to thymic seeding is a matter of discussion (16). On the contrary, BM cells expressing molecules known to be involved in thymus seeding (i.e., CCR7, CCR9, CXCR4, PSGL1) (17, 18) neither exhibit significant changes in EphB-deficient mice (15) nor in the numbers of fetal liver $CD45^+PIRA/B^+$ precursors (unpublished data). Therefore, the lack of EphB affects the migratory capacity of progenitor cells rather than the proportions of colonizing cells.

In vivo and *in vitro* assays have demonstrated that the lack of EphB in either lymphoid progenitors or thymic stroma reduces thymic seeding in both fetal (19, 20) and adult mice (15). *In vitro* colonization of WT FTOCs by $EphB2^{-/-}$ or $EphB3^{-/-}$, but not EphB2-LacZ, Lin^- BM cells was significantly reduced compared to that shown by WT cells (19) and adult WT mice showed lower seeding into the thymus after *in vivo* injection of EphB-deficient Lin^- BM cells than WT ones (15). This inability of progenitors to enter both fetal and adult thymus seems to be dependent, at least partially, on a direct role of Ephs in regulating cell migration, as previously reported for the cell migration of peripheral lymphocytes (21–23). *In vitro* migration of mutant progenitor BM cells was significantly reduced through fibronectin, laminin, or chemokine gradients, with a more severe reduction in EphB2-deficient cells than in EphB2-LacZ counterparts. Moreover, EphB2 stimulation by coated ephrin-B1Fc proteins inhibited laminin- and fibronectin-governing migration as well as CXCL12, CCL21, and CCL25-induced chemotaxis, but EphB2-LacZ cells did not exhibit reduced migration (19). This indicates that the extracellular domain of EphB promotes migration ligand independently while forward signaling promotes cell arrest.

In both experimental approaches, all tested BM progenitors, included WT ones, showed decreased migration into the EphB-deficient thymus, particularly the $EphB2^{-/-}$ one, which indicates the relevance of the thymic microenvironment in the process (15, 19). In fact, in both adult and fetal thymuses, decreased migration correlated with reduced production of ECM components, such as fibronectin and laminin, and chemokines (i.e., CXCL12, CCL21, CCL25) (15, 19). Furthermore, P-selectin involved in progenitor cell migration into the adult thymus (17), showed reduced expression on endothelial cells of both EphB2- and EphB3-deficient thymuses, but not of those of EphB2-LacZ cells, and decreased migration in $EphB2^{-/-}$ thymuses also correlated with reduced endothelial expression of ephrin-B1 and ephrin-B2, whereas in $EphB3^{-/-}$ thymuses, the reduction only affected ephrin-B1, reinforcing the idea that forward signals mediated by the pair EphB2/ephrin-B1 are particularly important for intrathymic lymphoid recruitment (15).

All these results, therefore, support a role for reduced thymic seeding in the thymic hypocellularity found in Eph/ephrin mutants. However, increased apoptosis of both thymocytes and TECs seems to be more relevant because, whereas thymuses deficient in both CCR7 and CCR9 with profoundly altered lymphoid colonization later recover normal thymocyte numbers (24), EphB2- and EphB3-deficient thymuses do not show that compensatory property; on the contrary, they increase their hypocellularity by increasing the death of DN and DP cells (9).

Thymic Alterations Observed in Eph/Ephrin-deficient Mice Reflect the Relevance of Thymocyte–TEC Interactions

Eph/ephrin deletion results in specific phenotypic alterations in both thymocytes and TECs. The lack of EphA4 results in a blockade of T cell maturation that results in a drop in DP cell proportions (10) and blockade of Eph/ephrin-A interactions in FTOCs treated with fusion proteins affects the maturation of immature $CD4^-CD8^+$ thymocytes (2). In correlation, these thymuses show a profound collapse of the cortical epithelial network that significantly reduces the number of cell layers and their organization whereas immature $K5^+K8^+$ TECs and areas devoid of epithelial cell marker expression increase. Apparently, the epithelial defects determine the lymphoid phenotype because mutant FTOCs grafted under the WT kidney capsule produce decreased

proportions of DP thymocytes (10) while mutant thymocytes in a WT stroma do not reproduce these changes. On the contrary, in Eph/ephrin-B-deficient mice, alterations are very important in the epithelial component but less severe in the developing thymocytes (9, 11, 25). EphB2- and EphB3-deficient thymuses exhibit minimal changes in the T-cell subset proportions, with an increased percentage of total DN cells and reduction of DN3 ($CD44^{-}CD25^{+}$) cells (9). However, in this case, EphB acts cell-autonomously on T-cell differentiation as grafted EphB-deficient alymphoid fetal thymus lobes colonized by WT lymphoid progenitors exhibit normal T-cell differentiation (26), while chimeric thymuses generated with $EphB2^{-/-}$ and $EphB2/B3^{-/-}$ Lin^{-} BM cell progenitors injected into SCID mice showed a blockade of T-cell maturation at DN stage and chimeras established with $EphB3^{-/-}$ progenitor cells showed a partial blockade at this same point that resulted in low numbers of DP cells (27). Therefore, both EphB2 and EphB3 autonomously control thymocyte development at DN to DP transition. Both molecules are also necessary for the maturation of DP cells to SP thymocytes as demonstrated in reaggregates (RTOCs) formed with EphB2- or EphB3-deficient DP thymocytes and WT TECs (28). Eph expression on thymocytes is also important for thymocyte survival as in all these chimeric SCID mice there were increased proportions of apoptotic thymocytes, principally DP and SP $CD4^{+}$ cells. Both thymocyte differentiation and survival seem to be dependent on Eph/ephrin-mediated thymocyte–TECs interaction and regulated by both forward and reverse signals, as SCID mice receiving EphB2-LacZ cells showed DP cells but did not produce SP thymocytes and did not show increased apoptosis. Therefore, although Eph forward signaling on thymocytes is necessary for thymocyte development, reverse signaling on interacting cells, presumably thymic epithelium, partially rescues DN cell progression to the DP cell compartment, and is important for cell survival (27). Accordingly, conditional deletion of ephrin-B1 and/or ephrin-B2 in TECs also affects the T-cell development and the lack of ephrin-B2 is presumably the most important, although ephrin-B1 also contributes, as double mutants show a more severe affectation (11). In addition, specific deletion of these ephrins in thymocytes results in a partial blockade of T-cell maturation at the DN3 stage (11, 29, 30) and increased thymocyte apoptosis (11). The phenotype is similar in single and double mutants suggesting that both molecules have a cooperative rather than redundant role in thymocyte maturation (11). A similar phenotype, however, has not been found when EphB2- or EphB3-deficient thymocytes are developed in a WT stroma in a bone marrow transplantation experiment into SCID mice (26).

Eph and ephrin signaling also affect thymic epithelium development and organization as in both EphB- and ephrin-B-mutant mice there is a profound transformation of thymic epithelium that exhibits altered TEC phenotypes (i.e., immature $K5^{+}K8^{+}MTS10^{+}$ medullary epithelial cells (mTECs), cortical $K5^{-}K8^{-}MTS20^{+}$ cells and $K5^{+}K8^{+}$ cells) and altered 3D organization. This change provokes a 2D structure that results in increased epithelial cysts, collapsed epithelium, and large areas devoid of epithelial cell markers (11, 25, 31). These latter areas, of unknown significance, also exist in WT thymuses and in other mice with defects in molecules, such as Foxn1, Kremen 1, or Stat3, involved in TEC maturation (32–34), but are specially developed in EphB-deficient thymuses. They contain thymocytes and blood vessels, frequently surrounded by enlarged sheaths of connective tissue, and are different in cortex and medulla: the former ones contain thymocytes and some sheathed blood vessels, whereas in the medulla mTECs delimit areas with enlarged blood vessels, increased numbers of ER-$TR7^{+}$ fibroblasts, components of the ECM (collagen IV, fibronectin, and laminin) (35), dendritic cells (36), and thymocytes in some areas (37–39).

Cortical areas devoid of epithelium have been described by others (37–39), receiving the name of epithelial-free areas (EFAs). EFAs are MHC class-II negative, little vascularized areas that contain abundant thymocytes frequently in division (39) reported as accumulations of DP thymocytes that do not undergo positive selection and will die subsequently by apoptosis (40). On the contrary, medullary epithelium-free areas that express several connective tissue markers could have a mesenchymal condition (35) and represent areas in which Eph-deficient TECs have undergone an epithelial–mesenchymal transition, losing their epithelial cell markers and acquiring a mesenchymal nature. In Eph mutant mice, EFAs could arise as a consequence of impeded intermingling and mutual exclusion of thymocytes and TECs caused by the lack of Eph–ephrin signaling as known in other systems (41).

Presumably, TEC maturation is autonomously governed by EphB2 and EphB3 expressed on TECs, as some of the phenotypical alterations found in EphB2- or EphB3-deficient mice can be reproduced in grafted mutant lobes colonized by WT host thymocytes. However, EphB expressed on thymocytes can also play a non-autonomous role since the epithelial phenotype of these grafted mutant lobes was not exactly the same as that found in EphB-deficient thymuses (26), and chimeric SCID thymuses receiving EphB-deficient thymocytes showed altered histological organization (27).

Selective deletion of ephrin-B1 and/or ephrin-B2 genes in thymocytes or TECs permits to determine the relevance of Eph/ephrin signaling in distinct thymic components (11). In all ephrin-B-deficient mice, but particularly in the double mutants with ephrin-B1 and ephrin-B2 deleted in TECs, the thymuses are small, with scarcely developed cortex and medulla, high numbers of $K5^{+}K8^{+}$ cells, and numerous epithelial cysts. Ephrin-B2 deletion in TECs causes altered distribution of $Ly51^{+}$ cortical (c) TEC subsets defined as $Ly51^{hi}$ cells that express DLL4, and would constitute the cortical niche of DN thymocytes and $Ly51^{lo}$ cTECs that would represent that of DP cells. Thus, ephrin-B1 deletion in TECs but, also, the lack of ephrin-B1 and B2 in thymocytes induce a cortex in which rounded groups of cTECs express Ly51 homogeneously, suggesting that ephrin-B1 and B2 expressed in both thymocytes and TECs cooperate in regulating the differentiation and distribution of cortical niches (11). Furthermore, ephrin-B deletion from both thymocytes and TECs affects medulla organization. The medulla of thymuses bearing a deletion of ephrin-B1 or B2 in thymocytes, or those without ephrin-B1 or ephrin-B1/B2 in TECs, shows increased numbers of large monolayered epithelial cysts formed largely by immature $K5^{+}K8^{+}$, sometimes $MTS20^{+}$, TECs, but rarely containing mature $UEA1^{+}$ or $MTS10^{+}$ cells that would represent an arrest of medulla development at an immature stage. They also contain numerous $UEA1^{hi}$ cells that form small cysts surrounded by a thin rim of

UEA1$^{lo/-}$MTS10^{+} cells that would represent a certain blockade of medulla organization at a late stage of development, in which medullary TEC subsets UEA1^{hi}MTS10^{-} and UEA1^{lo}MTS10^{+} develop but MTS20^{+} cell expansion and 3D organization are affected (31).

Interestingly, these studies support that ephrin-B1 and ephrin-B2 deletion in TECs result in different TEC phenotypes similar to those generated by ephrin-B1 or ephrin-B2 deletion in thymocytes. This, once again, indicates that Eph/ephrin-mediated thymocyte–TEC interactions are also important for TEC development and arrangement. Although, these molecules also mediate homotypic interactions (thymocyte–thymocyte; TEC–TEC), presumably their involvement in thymocyte–TEC interactions is more important to explain their role in the thymus. However, there are no complementary phenotypes when the effects of ephrin deletion in TECs are compared with those observed in thymuses with EphB-deficient epithelium, or when Eph mutant or ephrin mutant phenotypes are compared. This indicates that in the thymus, as in other systems (4), the final balance of *forward* and *reverse* signals in thymocytes and/or TECs would be more relevant than the mere presence/absence of certain Eph or ephrins. Besides, other factors must contribute to the complexity of the system as phenotypes in different mutant models are more severe in mice with C57/Bl6–CD1 mixed background than in the non-inbreed strain C57/Bl6 (10, 11).

In vitro experiments also clearly support the relevance of Eph/ephrin-mediated thymocyte–TEC interactions in thymus biology: ephrin-B1Fc proteins added to RTOCs, formed by fetal TECs and DP thymocytes, disorganize the 3D thymic epithelial network, prevent thymocyte–TEC association, and alter TCR$\alpha\beta$ signaling (28). Numbers and timing of the establishment of cell conjugates also change when they are established with EphB-deficient DP thymocytes (27).

On the other hand, proper T-cell maturation occurs thanks to the movement of developing thymocytes throughout the thymic parenchyma, facilitating their interactions with distinct niches favoring the necessary thymocyte–TEC crosstalk (42). Analysis by confocal microscopy of the positioning of EphB2-deficient or WT Lin^{-} BM progenitors in reconstituted FTOCs demonstrated that higher numbers of WT cells reached the central area of WT lobes than of EphB2-deficient cells (19). Furthermore, EphB2$^{-/-}$ total thymocytes migrate less efficiently through laminin or fibronectin or in response to CXCL12, CCL21, or CCL25, than WT cells. More importantly, when *forward* EphB2 signals were activated by ephrin-B1Fc protein treatment, there was a significant reduction in the migration of all EphB2$^{-/-}$, but not EphB2-LacZ, thymocyte subsets. Therefore, together with chemokines and ECM molecules, the migration of developing thymocytes throughout the thymic stroma could be promoted by inactivated EphB2 receptors, and negatively modulated by EphB2/ephrin-B interactions (19).

Remarkably, these profound phenotypic alterations observed in mice deficient in distinct Eph or ephrins do not correlate with immune deficiencies and/or pathological processes. EphA4$^{-/-}$ thymuses (10) and those with deleted ephrin-B1 and/or ephrin-B2 in TECs (11) show decreased proportions of both DP TCR$\alpha\beta^{hi}$ cells and CD69^{+} cells that could reflect an inefficient TCR$\alpha\beta$ selection. However, peripheral lymphoid organs of both EphA4$^{-/-}$ mice (10) and EphB-deficient mice (9) show decreased numbers of total T cells, but not significant changes in the proportions of distinct T-cell subsets. In addition, there are no changes in the central and peripheral TCR$\alpha\beta$ repertoire expressed on CD4^{+} T cells of EphB2- and/or EphB3-mutant mice, except for an increased proportion of Vβ3^{+}CD4^{+} cells in both thymus and lymph nodes of the three mutants (43).

In summary, Eph and ephrins are molecules that through mediating thymocyte–TEC interactions are involved in numerous processes occurring into the thymus, including cell migration into and through thymus, T-cell differentiation, and TEC maturation.

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How does thymus infection by coxsackievirus contribute to the pathogenesis of type 1 diabetes?

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Through synthesis and presentation of neuroendocrine self-antigens by major histocompatibility complex proteins, thymic epithelial cells (TECs) play a crucial role in programing central immune self-tolerance to neuroendocrine functions. Insulin-like growth factor-2 (IGF-2) is the dominant gene/polypeptide of the insulin family that is expressed in TECs from different animal species and humans. *Igf2* transcription is defective in the thymus of diabetes-prone bio-breeding rats, and tolerance to insulin is severely decreased in *Igf2*$^{-/-}$ mice. For more than 15 years now, our group is investigating the hypothesis that, besides a pancreotropic action, infection by coxsackievirus B4 (CV-B4) could implicate the thymus as well, and interfere with the intrathymic programing of central tolerance to the insulin family and secondarily to insulin-secreting islet β cells. In this perspective, we have demonstrated that a productive infection of the thymus occurs after oral CV-B4 inoculation of mice. Moreover, our most recent data have demonstrated that CV-B4 infection of a murine medullary (m) TEC line induces a significant decrease in *Igf2* expression and IGF-2 production. In these conditions, *Igf1* expression was much less affected by CV-B4 infection, while *Ins2* transcription was not detected in this cell line. Through the inhibition of *Igf2* expression in TECs, CV-B4 infection could lead to a breakdown of central immune tolerance to the insulin family and promote an autoimmune response against insulin-secreting islet β cells. Our major research objective now is to understand the molecular mechanisms by which CV-B4 infection of TECs leads to a major decrease in *Igf2* expression in these cells.

Keywords: enterovirus, coxsackievirus, thymus, self-tolerance, type 1 diabetes, insulin family, insulin-like growth factor 2

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Introduction

The major genetic determinants of type 1 diabetes (T1D) are the class II major histocompatibility complex (MHC) on chromosome 6 – which accounts for almost 50% of the genetic susceptibility – as well as a number of non-MHC genes, including the variable number of tandem repeat (VNTR) alleles upstream of the *INS/IGF2* (*IDDM2*) locus, *PTPN22*, *CCR5*, *IL2RA*, *IL10*, and *CTLA4*. However, only 10% of the individuals bearing a genetic predisposition will develop T1D, and more than 50% of

monozygotic twins are discordant for the disease, which illustrates the implication of environmental influences in T1D pathogenesis (1) as for all autoimmune diseases.

Type 1 diabetes occurrence has been related to a number of viruses but epidemiological studies have provided the strongest evidence that enteroviral infections, in particular, by coxsackievirus B (CV-B), are frequent events preceding T1D onset (2–7). Human enteroviruses include human pathogens, such as poliovirus, CV-B, rhinovirus, and echovirus. Using RT-PCR detection, CV-B genome was detected in 5 out of 12 (42%) newly diagnosed T1D patients and in 1 of 12 (8%) patients during the course of T1D. None of T2D patients and none of 15 healthy controls had enterovirus sequences in their blood (8). CV-B4 E2 can persistently infect human β cells (9) and a CV-B4 variant infects β cells leading to a disturbance of proinsulin synthesis and insulin secretion (10). The mechanism most accredited to explain the link between CV-B infection and T1D is a specific tropism of the virus for insulin-secreting islet β cells (11) – that is, mediated by their expression of the specific virus receptor – and a bystander activation of autoreactive T cells by antigens released by β cells after their damage caused by CV-B infection (12). Another crucial study has shown that CV-B4 is able to infect β cells in patients with T1D and that such infection is associated with both inflammation and severe β-cell functional disturbance (13). The persistent aspect of enterovirus infection is also an important factor to take into account [for a complete review, see Ref. (14)]. Very recently, this scenario received a strong support through the Diabetes Virus Detection (DiViD) study that detected a low-grade enteroviral infection in the islets of Langerhans collected from living patients newly diagnosed with T1D (15). This study does not prove a causal relationship between enterovirus infection and T1D, but is the first to detect enterovirus in pancreatic islets from patients close to the time of their diagnosis of T1D. The association between T1D and viral infections has also been previously reinforced by a genetic linkage between T1D susceptibility and host determinants of the antiviral response, such as the antiviral oligoadenylate synthase (OAS1) and the interferon-induced helicase (IFIH1), which intervene in innate immunity by recognition of RNA genome of picornaviruses, such as enteroviruses (16, 17). Besides this pancreotropism of CV-B, we have been exploring for a long time another mechanism that could play an essential and complimentary role in the development of the diabetogenic autoimmune response, namely, thymus infection.

Thymus-Dependent Central Self-Tolerance to Islet β Cells

As previously demonstrated that the thymus epithelium plays a unique role in programing central self-tolerance to neuroendocrine functions [complete reviews in Ref. (18–20)], as well as to many tissue-related antigens (21). Following gene transcription in the thymus, neuroendocrine precursors are processed not according to the classical model of neurosecretion but for presentation by, or in association with, the thymic MHC machinery. In the thymus, MHC presentation of neuroendocrine self-peptides promotes two intimately associated but paradoxical events: (1) negative selection and deletion of self-reactive T cell clones and (2) Generation of self-specific regulatory T (tTreg) cells that are able to inhibit in the periphery those "forbidden" self-reactive T cells that escaped thymic clonal deletion. The AutoImmune REgulator (AIRE) protein controls intrathymic transcription of neuroendocrine genes, including all the members of the insulin gene family (22) that are transcribed in the murine thymus according to the following hierarchy: *Igf2* > *Igf1* > *Ins2* > *Ins1*. Thymic self-antigen expression and AIRE function are also regulated by epigenetic and post-translational mechanisms (23).

There is now mounting evidence that a defect in intrathymic negative selection is implicated in the development of autoimmune endocrine diseases, such as T1D (24–27), although this is still discussed for the non-obese diabetic (NOD) thymus (28, 29). Contrary to *Igf1* and *Ins2*, *Igf2* transcription is defective in the thymus of diabetes-prone of bio-breeding (BB) rats (30), one of the two animal models of T1D with the NOD mouse. In humans, *INS* transcripts are measured at a lower level in the thymus from fetuses with short class I VNTR alleles, the second genetic trait (*IDDM2*) of T1D susceptibility (31, 32). Both VNTR alleles and AIRE determine the concentration of *INS* transcripts in the human thymus (33). In the mouse, *Ins2* is predominantly transcribed in the thymus, while *Ins1* expression is dominant in islet β cells, which leads to a higher immunological tolerance to *Ins2*. This explains why the breeding of $Ins2^{-/-}$ mice onto the NOD background accelerates insulitis and diabetes onset (34), whereas insulitis and diabetes are markedly inhibited in $Ins1^{-/-}$ congenic NOD mice (35). There is now firm evidence that *Ins1* codes for the primary insulin-derived autoantigenic epitopes tackled by the autoimmune diabetogenic process (36, 37). In addition, there is a very rapid onset of autoimmune diabetes after a thymus-specific *Ins1* and *Ins2* deletion resulting from the crossing of $Ins1^{-/-}$ mice with mice presenting a specific *Ins2* deletion in *Aire*-expressing medullary thymic epithelial cells (TECs) (38). The insulin transactivator *Mafa* also regulates *Ins2* transcription in the thymus and targeted *Mafa* disruption induces appearance of anti-islet antibodies (39).

Tolerogenic Properties of IGF-2: Multiple Facets

Given the direct relationship between the expression level of a protein/peptide in the thymus and the immunological tolerance to this protein/peptide (40), the hierarchical profile of the intrathymic expression of insulin-related peptides (IGF-2 > IGF-1 > insulin) suggests that tolerance to insulin-like growth factor-2 (IGF-2) is high and that tolerance to insulin is low. This is indirectly supported by the fact that insulin is the primary autoantigen of T1D (36, 37) while no autoimmune response against IGF-2 has ever been reported. Conversely, the highly immunogenic properties of insulin might actually be related to its very low expression in rare medullary (m) TEC subsets. Recently, the alternate variant INS–IGF-2 has been identified as a novel autoantigen in T1D (41), but there is still no data about the expression of this hybrid protein in thymic epithelium. Spontaneous autoimmune diabetes does not develop in $Igf2^{-/-}$ mice although these mice display

a marked lower tolerance to insulin, which evidences that *Igf2* expression mediates cross-tolerance to insulin and is required for the programing of a complete immunological tolerance to this protein (42). The homologous sequences Ins B9-23 and IGF-2 B11-25 compete for binding to the MHC-II DQ8 allele, and their presentation to PBMCs isolated from $DQ8^+$ T1D adolescents induce distinct cytokine profiles with a regulatory profile for IGF-2 B11-25 that is not observed for Ins B9-23 (43). Two recent studies have further evidenced the tolerogenic properties of IGF-2 by enhancement of Treg cell functions in an experimental model of food allergy (44), as well as promotion of antigen-specific Breg cell properties (45).

Our studies have also shown that the blockage of IGF-mediated signaling in the thymus severely interferes with T-cell growth and differentiation blocks T-cell differentiation (46), which was further confirmed by the demonstration that an antibody to CD222 (the IGF-2 receptor, an endosomal transporter that regulates protein trafficking) plays a central function in the initiation of T-cell signal transduction (47).

Therefore, the predominant expression of IGF-2 in the thymus is not only associated with a higher immunological tolerance to this protein but also seems to confer significant tolerogenic properties to IGF-2- and IGF-2-derived antigen sequences. On these experimental bases, we have proposed the novel concept of "negative self-vaccination" that is under current development through DNA vaccine methodology (48).

Thymus Infection by Enteroviruses

Given the programing of self-tolerance to islet β cells in the thymus and its defect in the development of the autoimmune diabetogenic response, we investigated the question of a putative role played by an enteroviral infection in an acquired dysfunction of the three major properties of this primary lymphoid organ: thymopoiesis, establishment of central self-tolerance, and generation of self-antigen-specific tTreg cells. A persistent replication of CV-B4 E2 (a "diabetogenic" CV-B strain) and JBV (a prototype CV-B strain) in primary cultures of human TECs was demonstrated by detection of positive- and negative-strand viral RNA in extracts from cell cultures, by immunofluorescence staining of the VP1 capsid protein, and by release of infectious particles up to 30 days after culture inoculation without any apparent cytolytic effect. The persistence of CV-B4 infection was associated with an increased rate of TEC proliferation and with an increase in the secretion of the cytokines IL-6, LIF, and GM-CSF in the supernatants. CV-B4 replication was not restricted to the CV-B4 E2 strain and did not depend on the genetic background of the host. However, cytokine secretion in human TEC cultures infected with CV-B4 E2 was higher than in cultures infected with CV-B4 JBV (49). Therefore, although they are considered as cytolytic viruses, enteroviruses can infect persistently some tissues, such as thymus and pancreas.

Coxsackievirus B4 E2 is also able to infect human fetal thymic organ cultures (FTOC). Viral RNA was detected by quantitative RT-PCR in CV-B4 E2-infected human FTOC, which supported high yields of virus production, as well as in flow-sorted thymic T cell populations for 7 days after infection. In FTOC, double positive $CD4^+CD8^+$ thymocytes were the principal target cells of infection and were progressively and severely depleted with no sign of apoptosis. Of note, massive thymic depletion of developing T cells and the subsequent $CD4^+CD25^+$ tTreg cells was shown previously to result in systemic autoimmunity (50). CV-B4 E2 replication caused a major up-regulation of MHC class I expression on thymic T cells and TECs. This MHC class I up-regulation was correlated with markers of CV-B4 infection (viral RNA quantification, release of infectious particles), and this was the result of a direct infection rather than caused by production of soluble factors, such as interferon-α (51). Interestingly, Krogvold et al. also reported an overexpression of MHC class I in the islets of all the patients included in their recent study (15). CV-B4 E2 was similarly shown to disturb T-cell differentiation in infected murine FTOC (52). In concordance with previous observations (53), CV-B4 oral inoculation of outbred mice results in a systemic spreading of viral RNA and a detection of viral RNA in thymus, spleen and blood up to 70 days after inoculation (54). Finally, CV-B4 infection of a murine mTEC line induces a dramatic decrease in *Igf2* transcription and IGF-2 production in long-term cultures of this cell line, while *Igf1* transcripts were much less affected and *Ins2* transcripts were not detected in these experimental conditions (55). Inoculation of the mTEC line with CV-B3, CV-B4 JVB, or echovirus 1 also induced a decrease in IGF-2 production, while herpes simplex virus 1 stimulated IGF-2 production. As already cited, a defect of *Igf2* expression in the thymus was suggested to play a role in the development of autoimmune diabetes in the diabetes-prone BB rat (30). Although these effects need to be reproduced *in vivo*, they strongly support our hypothesis that CV-B4 infection of the thymus could disrupt central self-tolerance to the insulin family, and could also enhance CV-B4 virulence through induction of central immunological tolerance to this virus. We are currently investigating the molecular mechanisms responsible for the CV-B-induced decrease of thymic IGF-2 expression in this mTEC line and *in vivo* after oral inoculation of CD1 mice. Since the CV-B-mediated effects in mTEC line are more pronounced on IGF-2 protein than on *Igf2* transcription, we concluded that post-transcriptional and/or post-translational mechanisms could be both involved.

As previously discussed by Zinkernagel (56), fetal exposure to maternal enterovirus infections should also be taken into account. One study has shown that enterovirus infection during the first trimester of pregnancy is not associated with a higher risk for T1D in the childhood (57), but another one has evidenced that such maternal enterovirus infection was a risk factor in offspring diagnosed with T1D between 15 and 30 years of age (58). More recently, a study has investigated that the effects of CV-B4 E2 oral inoculation of CD1 mice at days 4, 10, or 17 of gestation. Severe inflammation of the pancreas and higher glucose blood levels were observed only when dams were previously infected and, in particular, at day 17, thus, in the late phase of pregnancy (59). CV-B4 E2 oral inoculation of pregnant mice is also associated with fetal thymus infection and disturbance of T-cell differentiation (Jaïdane, personal communication). Obviously, the question of materno-fetal transmission of enterovirus infection highly deserves to be further investigated.

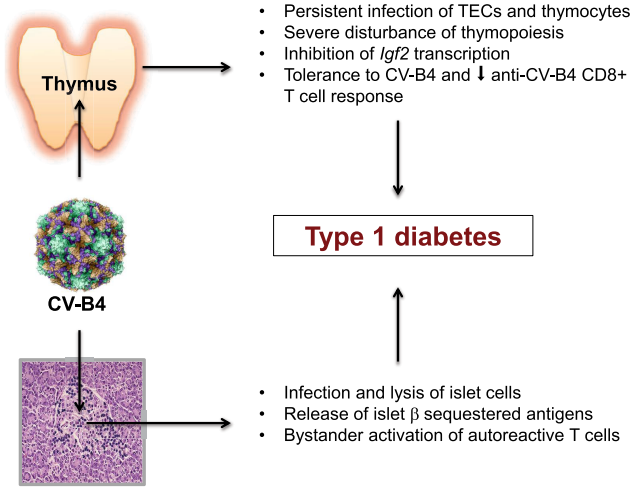

FIGURE 1 | Coxsackievirus B4 persistent infection of thymus and pancreatic islets are closely associated and implicated in T1D pathogenesis.

Conclusion: A Model Associating CV-B-Induced Dysfunction of Central Tolerance and Peripheral Bystander Activation

In addition to the necessity of standardization for the serological and RT-PCR detection of CV-B infection as recommended by Gale and Atkinson (60), there is also an urgent need for a thorough investigation of the relationships between CV-B and the host immune system (**Figure 1**). What is our current knowledge about this point? CV-B4 is able to persistently infect α and β cells in human pancreatic islets, and to cause functional impairment and β-cell death characterized by nuclear pyknosis. The CV-B4-induced damage to the islet cells causes release and presentation of sequestered islet antigens. Through bystander activation, autoreactive T cells initiate the diabetogenic autoimmune process. Now, with regard to the origin of these autoreactive T cells, more and more experimental evidence points to the generation in the thymus of "forbidden" T cell clones due to a failure of the central tolerogenic mechanisms. This thymus defect results in a progressive enrichment of the peripheral T cell repertoire with self-reactive T cells and a decreased generation of self-antigen tTreg cells. From our collaborative work, it appears that CV-B4 is also able to persistently infect the epithelial and lymphoid compartments of the thymus. CV-B4 infection of the thymus leads to increased secretion of diverse cytokines synthesized in TECs, to a severe depletion of double positive $CD4^+CD8^+$ thymocytes, and to marked up-regulation of MHC class I molecules expressed by TECs and double positive thymic T cells. Moreover, CV-B4 infection of a murine mTEC line induces a marked decrease in *Igf2* transcription and IGF-2 production. Therefore, a CV-B4 persistent infection of the thymus may lead to significant thymus and immune dysregulation that associates:

- A significant impairment of thymus-dependent self-tolerance issued from the decrease in the presentation of insulin family related self-antigens, and putatively a direct viral interference with self-antigen presentation (61).
- An induction of central tolerance to CV-B4 and a secondary decrease of anti-CV-B4 $CD8^+$ T-cell mediated response, so that further exposure to the virus could promote more severe damage to the peripheral target tissues.

If further research confirmed such rational assumption based on our new knowledge of thymus functions, then an anti-CV-B4 vaccination could be considered as a strategy for T1D prevention in regions with a high incidence of this disease such as in Scandinavian countries (62).

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The thymic orchestration involving Aire, miRNAs, and cell–cell interactions during the induction of central tolerance

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Developing thymocytes interact sequentially with two distinct structures within the thymus: the cortex and medulla. Surviving single-positive and double-positive thymocytes from the cortex migrate into the medulla, where they interact with medullary thymic epithelial cells (mTECs). These cells ectopically express a vast set of peripheral tissue antigens (PTAs), a property termed promiscuous gene expression that is associated with the presentation of PTAs by mTECs to thymocytes. Thymocyte clones that have a high affinity for PTAs are eliminated by apoptosis in a process termed negative selection, which is essential for tolerance induction. The Aire gene is an important factor that controls the expression of a large set of PTAs. In addition to PTAs, Aire also controls the expression of miRNAs in mTECs. These miRNAs are important in the organization of the thymic architecture and act as posttranscriptional controllers of PTAs. Herein, we discuss recent discoveries and highlight open questions regarding the migration and interaction of developing thymocytes with thymic stroma, the ectopic expression of PTAs by mTECs, the association between Aire and miRNAs and its effects on central tolerance.

Keywords: AIRE, miRNA, MTEC, thymus gland, thymocytes, cell adhesion, promiscuous gene expression, central tolerance

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Introduction

The induction of central immune tolerance is an increasingly complex and intricate process that occurs within the thymus (1, 2). Inside this organ, immature thymocytes interact sequentially and in a three-dimensional architecture with two distinct structures: the cortex and the medulla. In the cortex, the double-negative (DN) and double-positive (DP) thymocytes interact with cortical thymic epithelial cells (cTECs), allowing MHC-mediated self-peptide presentation to DP thymocytes expressing the α/β T cell receptor (α/β TCR), featuring intermediate affinity/avidity. Positive selection is a result of this interaction, which causes DP thymocytes to differentiate into mature single-positive (SP) thymocytes (3, 4).

The DP thymocytes that do not undergo positive selection are eliminated through death by neglect. Thereafter, the surviving SP and DP thymocytes migrate to the thymic medulla, where they interact with medullary thymic epithelial cells (mTECs). These cells are very peculiar because they ectopically express a large set of peripheral tissue antigens (PTAs) (5–8). Therefore, it is possible to

find insulin, a PTA that represents pancreatic beta cells, and a myriad of other autoantigens in the thymus.

The immunological significance of this property, which was termed promiscuous gene expression (PGE) (9–12), is associated with the presentation of PTAs by mTECs to SP and DP thymocytes. Thymocyte clones that express α/β TCR with a high affinity for PTAs are eliminated by apoptosis in a process termed negative selection or clonal deletion, which is essential for central tolerance induction (13–16). This process prevents the passage of autoreactive T cell clones to the periphery, which could provoke aggressive autoimmunity.

Therefore, the migration of thymocytes within the thymus enables the physical association of these cells with different thymic microenvironments (15, 17). Immunologists are interested in elucidating which chemotactic factors and/or adhesion molecules are involved in this process (18, 19).

Another very important factor in central tolerance is the autoimmune regulator (Aire) gene that controls the expression of a large set (but not all) of PTAs in mTECs (20, 21). Mutations in these gene that lead to a loss of Aire function can result in autoimmune polyendocrinopathycandidiasis-ectodermal dystrophy (APECED), an autoimmune disease characterized by hypoparathyroidism, candidiasis (yeast infection), and adrenal insufficiency (22–24). The mechanism of the Aire gene as a transcriptional regulator of Aire-dependent PTAs and the effect of point mutations found in the Aire gene sequence on clinical phenotypes (APECED or other autoimmune diseases) have received attention in recent years (25–27).

In addition, researchers have observed that in addition to PTAs, Aire controls the expression of microRNAs (miRNAs) in mTECs (28). In turn, miRNAs are important for the organization of thymic architecture and act as posttranscriptional controllers of PTAs (29, 30).

In this mini-review, we briefly discuss (1) the main aspects of three-dimensional thymus architecture, focusing on the migration and interaction of developing thymocytes with the thymic stroma and positive and negative selection; (2) the ectopic expression of PTAs by mTECs and role of the Aire gene; and (3) the current evidence for the link between Aire and miRNAs in thymic architecture and the induction of central tolerance.

Thymus Architecture, Migration of Thymocytes and the Induction of Central Tolerance

Developing thymocytes interact with the thymic microenvironment while they migrate and differentiate within the organ. This microenvironment is subdivided into two main regions, and each region is composed of different cell types that produce soluble and non-soluble molecules that can modulate thymocyte migration and maturation (31, 32). Thymic lobules are divided into cortical and medullary regions that are connected by a cortico-medullary junction. The cortex microenvironment is filled with cTECs, thymic nurse cells (TECs-thymocyte-forming lymphoepithelial complexes), macrophages, migratory dendritic cells (DCs), and fibroblasts. The medullary region contains mTECs, macrophages, resident and migratory conventional DCs, plasmacytoid DCs, fibroblasts, and B cells (16) (**Figure 1A**). Both regions are filled with a network of extracellular matrix (ECM) molecules, such as type I and IV collagens, fibronectin, and laminin. Soluble molecules, such as hormones, cytokines, growth factors, chemokines, and sphingolipids, are also found in the thymus and are produced by the lymphoid and non-lymphoid compartments. These soluble moieties can be present in the ECM and mediate cell–ECM and cell–cell interactions (33–35).

Thymocyte differentiation and migration occur simultaneously in the thymic microenvironment. T cell progenitors enter the cortico-medullary region via post-capillary venules (36) and rapidly migrate through the cortex toward the subcapsular zone, where DN thymocytes are primarily located. Subsequently, thymocytes migrate to the middle cortex and begin expressing both CD4 and CD8 co-receptors, becoming DP cells. During this stage, cells are selected based on the rearrangement of TCR genes, which leads to the membrane expression of productive TCRs. Cells that do not express productive TCRs undergo apoptosis, whereas cells expressing productive TCRs continue the differentiation process. Then, cells with TCRs that interact with high avidity with MHC-presented self-antigens expressed by mTECs and DCs undergo apoptosis in a process termed negative selection (37). The presentation of self-antigens by mTECs is controlled by Aire and guarantees the deletion of autoreactive T-cell clones, supporting central tolerance (38). In this context, cells with TCRs that interact with low/median avidity with MHC-presented self-antigens survive and continue the maturation process. Survival signals mediated by TCRs and CD4/CD8 co-receptors lead to the down-regulation of a co-receptor, and thymocytes become mature $CD4^+CD8^-$ or $CD8^+CD4^-$ SP cells (**Figure 1A**).

Thymocyte localization and guidance are controlled by ECM molecules and chemokines, among others molecules, and their respective receptors. For example, the entrance of T-cell progenitors in the thymus is controlled by CCL21/CCR7 and CCL25/CCR9 (chemokine/chemokine receptor) interactions (39). Migration of immature cells within the thymus is controlled by CXCL12/CXCR4 and CCL20/CCR6 interactions (40, 41), and CCR7 signaling is essential for the migration of DP thymocytes to the medulla (42). Moreover, CCR7 is involved in thymocyte egress, which is also controlled by sphingosine-1-phosphate receptor 1 signaling (43). The absence of such molecules in the thymus not only abrogates thymocyte development but also induces changes in the histological organization of the organ (44).

Thymic architecture and organization are essential for proper T-cell development and depend on both the lymphoid and non-lymphoid compartments. Alterations in one compartment can affect the other and consequently modify T-cell development and the repertoire of exported mature T cells to peripheral lymphoid organs. For example, Rag mutations substantially impair thymocyte development and consequently affect the distribution and maturation of TECs, diminishing the proportion of mTECs and inducing a lack of AIRE protein expression (45, 46). Lack of AIRE expression can in turn directly affect negative selection and break central tolerance. Interestingly, Aire deficiency can modulate the intrathymic expression of chemokines as a control mechanism of thymocyte development (47). In this context, one can

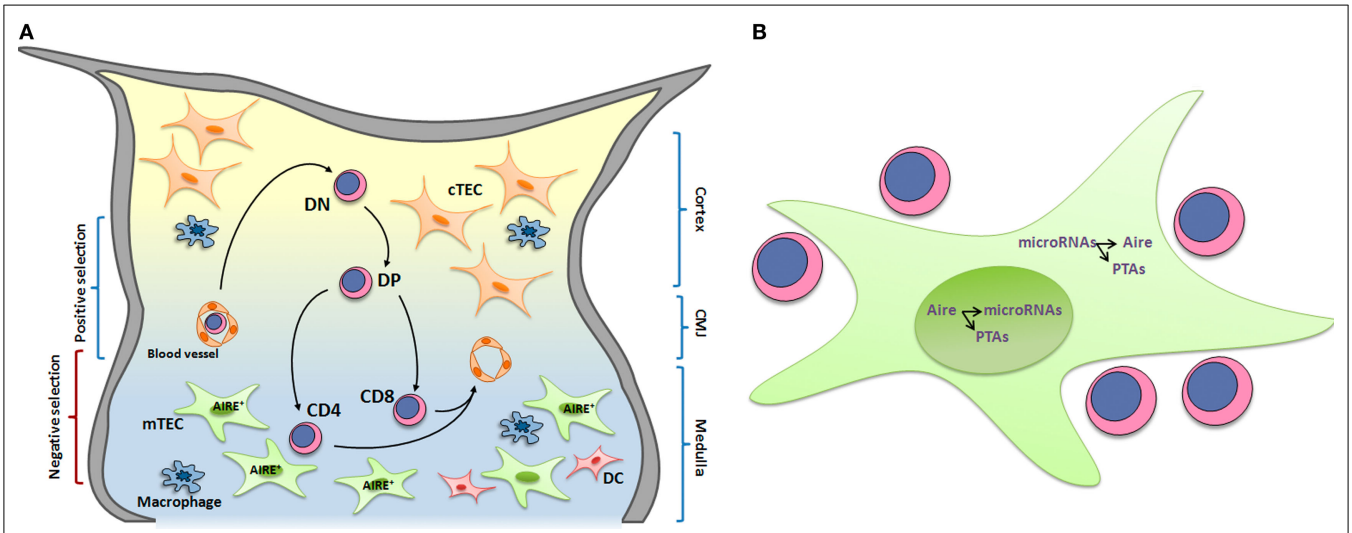

FIGURE 1 | (A) Thymocyte development and interactions with microenvironmental cells. T cell development occurs while cells migrate and interact with thymic microenvironmental components, including thymic epithelial cells, dendritic cells, and macrophages. These interactions are responsible for the selective processes, which lead to the formation of a self-restricted T-cell repertoire. In this context, medullary thymic epithelial cells (mTECs) play an essential role by expressing AIRE, which control the expression of a set of peripheral tissue antigens (PTAs) that are presented to developing thymocytes. High avidity of MHC-PTAs presented by mTECs with TCRs lead to thymocyte death by apoptosis and consequently avoid self-reactive T cell maturation. DN, double-negative $CD4^-CD8^-$ thymocytes; DP, doublepositive $CD4^+CD8^+$; CD4, single-positive $CD4^+CD8^-$; CD8, single-positive $CD8^+CD4^-$ thymocytes; cTEC, cortical thymic epithelial cell; mTEC, medullary thymic epithelial cell. CMJ, cortico-medullary junction. **(B)** The transcriptional and posttranscriptional control pathway of promiscuous gene expression. The peripheral tissue antigens (PTAs) expressed in medullary thymic epithelial cells (mTECs) are transcriptionally controlled by Aire within the nuclear compartment, which also controls the expression of miRNAs. Within the cytoplasm, the miRNAs control Aire and PTAs.

argue that chemokines and other molecules controlling thymocyte migration (such as ECM molecules) could also modulate Aire expression.

The Role of Aire in the Ectopic Expression of PTAs in the Thymus

During the induction of central tolerance in the thymus, self-reactive regulatory T cells (T_{reg}) are negatively selected, even if these cells play a role in the periphery. In fact, all sets of antigen-presenting cells, including cTECs, mTECs, and thymic DCs, act as self-antigen peptide presenting cells (6–9, 48–53). Thymic DCs present only the PTA peptides that were expressed and processed by mTECs (38).

The expression of PTAs by mTECs is a key process of (auto)immune representation. Due to the wide-ranging diversity of PTAs expressed by these cells, this phenomenon has been termed PGE (5, 9, 12, 48, 54–62).

The primary implication of this type of gene expression, which is heterogeneous and ectopic, is associated with the maintenance of immune homeostasis and controlling the reactivity and self-aggressive autoimmune diseases.

Notably, cTECs and mTECs are essential but not sufficient for these selection events (55). The cTEC-derived signals may regulate the positive selection of thymocytes that recognize the MHC-peptide complexes themselves; however, mTECs that express AIRE help ensure tolerance to self-antigens (63).

A subset of mTECs express the Aire gene (chromosome 10C1 in mice and 21q22.3 in humans) (64) and the claudin proteins (Cld3 or Cld4) on their surface. In these cells, AIRE and the claudin proteins act as adhesion molecules and represent the major proteins that contribute to the molecular architecture of cell junctions. All $Cld3^+$, $Cld4^+$, and $Aire^+$ adult TEC cells strongly express MHC class II and CD80 molecules on their surface (51).

"Immature" $CD80^-$/MHC-II^- mTECs express a limited set of PTAs, whereas "mature" $CD80^+$/MHC-II^+ mTECs exhibit greater PTA diversity, including PTAs whose expression is Aire dependent (11). These findings have led some researchers to propose "the terminal differentiation model"; i.e., mTECs undergo a continuous process of differentiation similar to the skin or intestinal epithelium, and the full complement of PGE is contingent upon this process (55). mTEC cells are very peculiar due to their unique gene expression pattern. They are capable of expressing more than 19,000 protein-coding genes, including "ectopic" genes that correspond to PTAs. Currently, no other known cell type expresses such a large set of genes (65).

We next sought to determine whether these cells also have unique machinery for gene expression control.

Although the transcriptional control of PGE is partially exerted by Aire, mutations in this gene cause severe autoimmunity that involves various organs and tissues in both mice and humans. In humans, this disease is a syndrome termed APECED, and patients have mutations along the Aire sequence, suggesting that mutations in Aire trigger aggressive autoimmunity (22, 66).

However, the existence of APECED patients who lack Aire mutations (67–69) suggests that other factor(s) may be involved

in controlling aggressive autoimmunity. These observations led us to wonder if temporal changes and a slight deregulation of wild-type Aire expression during development could contribute to autoimmunity.

Variation of Aire expression might disturb Aire-dependent PTAs in the thymus and consequently trigger aggressive autoimmunity, a hypothesis that was previously tested by our group (58). This hypothesis is a promising subject for further research and is currently being studied in humans with thymic cells isolated from Down syndrome patients, which feature trisomy of chromosome 21, providing a unique opportunity to evaluate the effect of natural Aire gene dosage in humans (70–72).

The functional role of Aire has been demonstrated using knockout (KO) mice. Aire in mice and humans encodes a protein with affinity for DNA that functions as a positive transcription factor regulating the expression of PTAs mTECs, but AIRE can also act as a negative regulator of other genes (8, 10, 73–76).

The link between Aire expression and the induction of thymocyte apoptosis, a biological process crucial for negative selection, has been demonstrated (37, 77). However, according to our best knowledge, no investigation has assessed this link considering the possible effect of variations in Aire expression in mTECs on adhesion with thymocytes and the induction of apoptosis. Alternatively, Aire-deficient mTEC cells may lose their adhesion ability. This question is still open for further research.

Interestingly, the AIRE targets low-transcribed genes. It interacts with hypomethylated promoter regions in the chromatin through its PHD1 domain (62, 78–82).

However, recent evidence has demonstrated that the AIRE acts indirectly in regulating PTA transcription. According to Giraud et al. (83), AIRE can be considered an unusual transcription factor because it does not appear to function as a typical transactivator. These authors demonstrated that AIRE activates PTA transcription by releasing stalled RNA Pol II from blockage at the promoter region of its target genes. This model suggests that AIRE acts during the elongation stage of transcription rather than at transcription initiation (83). The "promiscuity" of Aire on a large set of downstream PTA genes might be due the unspecific mode of action of RNA Pol II on different promoter regions, but this remains to be determined.

A new exciting possibility for the Aire mechanism is its influence in controlling alternative splicing of PTA genes in mTECs. Aire has been shown to increase the amount of measurable exons per gene and enables the production of PTAs from these exons (84); these properties might significantly increase the diversity of PTA isoforms in mTECs and consequently increase the range of self-representation.

It is possible that aggressive autoimmunity is associated with an imbalance of PTAs isoforms in mTECs.

The Link Between Aire and miRNAs

In our view, not only Aire but also miRNAs may play a role in central tolerance. This hypothesis is plausible considering the vast range of action of miRNAs, which affect more than half of all mRNAs originating from protein-coding genes in human or murine cells (85).

This range of action is expected to reach mRNAs encoding proteins involved in the central tolerance mechanism, including PTA mRNAs and Aire mRNA itself.

First, researchers evaluated the role of the endoribonuclease Dicer, a key enzyme implicated in miRNA maturation, on thymic function. They found that Dicer-KO mice exhibit progressive degeneration in thymic architecture and function, provoking alterations in T cell differentiation and peripheral tolerance, pinpointing miRNA-29a as a specific miRNA participating in this process (29).

Then, mice lacking Dicer expression in the thymic epithelia were found to exhibit a set of abnormalities, including alterations in the expression profiling of cTEC and mTEC mRNAs. T cells obtained from a Dicer-deficient thymus were pathogenic and produced aggressive autoimmunity (86). These finding were instrumental for further research on the role of miRNAs in central tolerance induction.

Moreover, thymic epithelial cells isolated from murine or human thymuses feature overlapping of miRNA signatures, suggesting evolutionary conservation of miRNA expression profiles (87). These authors also demonstrated that Aire expression is associated with maturation-dependent expression of miRNAs.

However, a direct demonstration that Dicer and consequently miRNAs play a role in TEC-thymocyte adhesion, which is crucial for positive and negative selection, is still lacking. This question is open for further investigation.

As discussed above, the AIRE acts in close association with RNA Pol II (83). Because this polymerase transcribes miRNAs in addition to mRNAs (88–92), Aire may affect miRNA expression.

Our group was the first to directly demonstrate this possibility (28). We showed that in murine mTECs, Aire controls the transcription of miRNAs located within a genomic region that encompasses an open-reading frame (ORF of Gm2922 mRNA).

This finding enabled further evaluation of the role played by Aire-dependent miRNAs in the posttranscriptional control of PTAs. Thus, we reconstructed miRNA–mRNA interaction networks from mTECs isolated from BALB/c (non-autoimmune) or non-obese diabetic (NOD) (autoimmune) mice. As expected, dozens of PTA mRNAs interacted with miRNAs. Interestingly, none of the classical Aire-dependent PTAs (e.g., Ins2) interacted with miRNAs, strongly suggesting that they are somewhat resistant to posttranscriptional control (30).

What would be the consequences of a lack of miRNA action on these PTAs? Could this lack of action aid autoantigen synthesis by mTECs, consequently inducing tolerance? What causes these Aire-dependent PTAs to be "resistant" to miRNA action? Could changes in their 3′UTRs (length or mutations) or imbalance in miRNAs expression levels (or both) cause this resistance? We have suggested that there may be changes in length of the 3′UTR sequence of Aire-dependent PTAs expressed in mTECs (30). Researchers including our group and the group of Mathieu Giraud in Paris are now challenged to evaluate the structure of mRNAs in general and/or the 3′UTR of mRNAs of PTAs expressed in mTECs compared with other cell types.

Based on these recent results, is possible to drawn a pathway for the transcriptional and posttranscriptional control of PGE in mTECs (**Figure 1B**). Within the nuclear compartment, the AIRE

controls PTA and miRNA transcription (Aire-dependent PTAs and miRNAs). Once in the cytoplasm, miRNAs play a role in the posttranscriptional control of Aire and PTA mRNAs. Would PTA mRNAs with altered 3′UTRs be refractory to the action of miRNAs?

Although these aspects have only recently begun to be explored, they represent new, exciting questions for present and future research on the molecular genetic basis of immune tolerance.

Concluding Remarks

The molecular genetic control of central tolerance remains an open question in immunology. The identification and cloning of the Aire gene was instrumental in studying the molecular genetics of this process. As the primary controller of PTA expression in mTECs, Aire is the master pillar of central tolerance. Aire expression is common in the thymus; and this observation led to the idea of PGE. However, Aire did not fit well as a classic transcription factor. The AIRE operates in conjunction with various other partner proteins in the release of RNA Pol II shortly after the initiation of PTA gene transcription. This property enabled better understanding of the vast range of AIRE activity. Recently, miRNAs have been found to be the modulators of post-transcriptional controllers in the thymus. Researchers are now challenged with deciphering the transcriptional and post-transcriptional control pathway of PGE involving Aire and miRNAs.

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Exosomes in the thymus: antigen transfer and vesicles

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Thymocytes go through several steps of maturation and selection in the thymus in order to form a functional pool of effector T-cells and regulatory T-cells in the periphery. Close interactions between thymocytes, thymic epithelial cells, and dendritic cells are of vital importance for the maturation, selection, and lineage decision of the thymocytes. One important question that is still unanswered is how a relatively small epithelial cell population can present a vast array of self-antigens to the manifold larger population of developing thymocytes in this selection process. Here, we review and discuss the literature concerning antigen transfer from epithelial cells with a focus on exosomes. Exosomes are nano-sized vesicles released from a cell into the extracellular space. These vesicles can carry proteins, microRNAs, and mRNAs between cells and are thus able to participate in intercellular communication. Exosomes have been shown to be produced by thymic epithelial cells and to carry tissue-restricted antigens and MHC molecules, which may enable them to participate in the thymocyte selection process.

Keywords: exosome, thymic epithelial cell, tolerance, tissue-restricted antigen, miRNA

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Introduction

Exosomes are small (30–100 nm) vesicles released by cells into the extracellular space. They are a subgroup of extracellular vesicles (EVs) formed by inward budding of membranes in the late endosomes, thus creating a multi-vesicular body (MVB), which may dock to the outer membrane of the cell and release its content of exosomes (1, 2). In hindsight, the recycling of the transferrin receptor back to the cell membrane from an endocytic route in developing erythrocytes was the original description of exosomes in 1983 (3). One particularly striking finding, which in recent years has heavily influenced exosome research, was the identification of microRNA (miRNA) and mRNA in exosomes (4). Exosomal transfer of functional miRNA and mRNA has been demonstrated to result in regulation of gene expression through miRNA as well as translation of proteins through mRNA in recipient cells (5, 6). Exosomes are abundant in, and fairly easy to purify from, bodily fluids such as saliva (7), peripheral blood (8), bronco alveolar lavage fluid (9), and urine (10, 11), and one area of extensive research is the use of exosomes as diagnostic biomarkers for various pathologic conditions (12).

Exosomes have also shown therapeutic promise, e.g., in a study by Zitvogel and co-workers it was demonstrated that tumor peptide-pulsed dendritic cells (DCs) released exosomes that carried MHC I and II as well as co-stimulatory molecules and that these exosomes primed cytotoxic T-cells and suppressed the growth of established tumors (13). Native antigens on tumor-derived exosomes can be taken up by DCs and cross-presented to tumor-specific cytotoxic T-cells (14).

Other studies have revealed that exosomes are capable of presenting antigens to T-cells. In 1996, Raposo et al. (15) reported that both human and murine B-cell derived exosomes could induce an

antigen-specific MHC II-restricted T-cell response. Exosome-like structures, named tolerosomes, are released from epithelial cells of the small intestine and have been shown to induce specific tolerance to fed antigens (16). As with the B-cell derived exosomes, tolerosomes seem to deliver antigens in a MHC dependent manner (17).

The thymus is the organ responsible for the establishment of an immune competent but yet self-tolerant T-cell population. While the extreme diversity of the mature effector T-cell specificities is a prerequisite for an effective defense against invading pathogens/infectious agents, the negative selection of self-reactive effector T-cells and the positive selection of T regulatory cells ensure tolerance to self-structures in order to avoid autoimmunity. To ensure a self-tolerant peripheral population, thymocytes are selected against a comprehensive set of self-antigens (18) of which many are produced and presented by medullary thymic epithelial cells (mTECs) (19) under control of the autoimmune regulator (AIRE) (20). The importance of a functional thymic antigen expression has been validated in models where even a defect expression of a single antigen may lead to development of peripheral organ-specific autoimmunity (21). Of equal importance is the selection of regulatory T-cells (Tregs) within the thymus, foremost from clones with a somewhat elevated TCR avidity for self-antigens (22). Proper thymic Treg development is also dependent on AIRE, and in particular for the functionally important Tregs that are induced during the perinatal period (23).

Since thymocytes outnumber TECs by several orders of magnitude, and the fact that each individual TEC only expresses a small subgroup of self-antigens (18, 24), a possible dissemination of antigen from TECs has been discussed in order to aid individual TECs to cover an extended volume of the thymic microenvironment (25). This would increase the number of possible antigen–thymocyte interactions and allow additional impact of the thymic DC populations. Antigen transfer could potentially occur by different means such as apoptotic bodies, nanotubes, and/or exosomes. Here, we review the current literature and argue that thymic exosomes have a potential role in T-cell maturation and selection.

Antigen Transfer and Indirect Antigen Presentation in the Thymus

Clearly, direct cell–cell contacts in the thymus are pivotal for the development of a functional T-cell population (26). The addition of antigen transfer between thymic cell populations could, however, optimize thymic cell communication by making antigens more available to the pool of developing thymocytes. Through the last two decades, a number of studies performed under different experimental conditions have demonstrated the transfer of antigens from TECs to DCs. Already in 1994, Kyewski and co-workers observed intercellular transfer of $E\alpha_{52-68}$ (a-chain of the MHC class II allele I-E^d, amino acids 52–68) from TECs to thymic DCs in a unidirectional fashion (27). They proposed that this mechanism "may enhance the efficacy of tolerance induction by spreading self-antigens" (27). Subsequent experiments with OVA-specific TCR-transgenic mice (RIP-mOVA model) revealed that transfer of antigen to and presentation by hematopoietic cells also applies to MHC class I-restricted epitopes, since it resulted in deletion of both MHC class I- and MHC class II-restricted OVA-specific thymocytes when OVA was expressed only by mTECs (28). The identity of the hematopoietic cells in the RIP-mOVA study was unclear but further studies have revealed that deletion of CD11c+ cells using CD11c-Cre mice crossed with mice expressing diphtheria toxin under the control Rosa Locus containing a loxP-flanked STOP cassette results in increased frequencies of CD4+ thymocytes and increased CD4 T-cell infiltration into peripheral tissues (29). Likewise, Aschenbrenner and colleagues have observed that DCs capture mTEC-derived antigens and take part in deletional tolerance (30). This observation was strengthened by a study by Koble and Kyewski who demonstrated a presentation of TEC antigens by DCs. In their study, thymic but not peripheral DCs presented TEC-derived OVA to OVA-specific T-cells and were constitutively provided with mTEC-derived proteins (31). Further, unidirectional antigen transfer from mTEC to DCs was shown to also apply for native endogenous self-antigens *in vivo* (31). Non-redundant contribution of DCs and mTECs is further suggested based on simultaneous hematopoietic MHC class II deficiency and reduced MHC II expression on mTECs; this combination has an additive worsening effect on negative selection compared to either of the single deficiencies alone (32).

Also, the Treg formation seem to be dependent of DC-TEC cross-talk, which was elegantly demonstrated in a study on bone marrow chimeras in which CD28/B7 signaling was disrupted on either hematopoietic-derived antigen-presenting cells (APCs) or on TECs. The results showed that when B7 was restored in the hematopoietic-derived APCs this was enough to restore Treg numbers, hence hematopoietic-derived APCs and TECs can independently contribute to Treg development (33). The transfer of material in this study was unidirectional toward DCs, and the discussed mechanisms were primarily exosomes and apoptotic bodies. Transfer of material from TECs to DCs has also been demonstrated in the work by Hubert and co-workers in which OT-II restricted thymocytes were deleted by a soluble form of OVA that required presentation by bone marrow-derived cells (34). Using tetramer staining and transfer of bone marrow with ablated expression of MHC II, Taniguchi and co-workers found an abolished negative selection of T-cells specific for the AIRE-controlled self-antigen retinoid-binding protein (35). They concluded that intercellular transfer of the interphotoreceptor retinoid-binding protein peptide epitope of amino acids 277–290 from AIRE-expressing mTECs to bone marrow-derived APCs is important for negative selection of the investigated peptide. In re-aggregated thymic organ cultures, both the thymic epithelium and conventional DCs (as opposed to plasmacytoid DCs) have been shown capable of eliminating autoreactive CD4 thymocytes and to support natural Treg (nTreg) development on their own (36). In addition, Perry and co-workers recently reported that CD8 α+ DCs preferentially acquire and present AIRE-dependent antigens to developing Treg cells (37). They also showed that bone marrow-derived APCs and mTECs play non-overlapping roles in shaping

of the T-cell receptor repertoire in terms of deletion and Treg selection (37).

T-Cell Stimulation by Exosomes, With or Without DCs

The capacity of exosomes to directly stimulate target cells has been debated. Some studies have suggested that there is an absolute need for DC presence for efficient exosomal stimulation of T-cells (38–40), while others have shown that exosomes are able to directly stimulate T-cells without any aid from DCs (15, 41–44). Models in which the efficiency of exosomal T-cell stimulation increases by DC presence have also been put forward (45). Interestingly, thymic exosomes carry ICAM-1 (46), which is both required for efficient T-cell responses (40) and involved in exosome binding to DCs (47). In addition to ICAM-1, thymus exosomes carry the opsonin MFGE8, which indicates that thymic exosomes would readily be engulfed by APCs such as DCs (46). TEC exosomes are also strongly positive for HLA-DR, which suggest a possibility that they contribute with antigens not only indirectly via, e.g., DCs but also directly to developing thymocytes (48). The presence of co-stimulatory molecules on exosomes may be important for their potential to affect the maturation of nTreg precursors (49). However, whether TEC exosomes that carry antigen presentation molecules and antigens need APCs or not to participate in thymocyte selection *in vivo* is not known. Possibly, thymic exosomes could take part in negative selection both directly by interacting with the thymocytes or indirectly by delivering antigens to APCs such as thymic DCs (**Figures 1A,B**).

Characteristics of Thymic Exosomes

The first observation of exosome-like structures in thymic tissue was made by Wang and co-workers (50). These mouse thymic exosomes were characterized by high content of TGF-beta, CD9, and MHC II. In human beings, thymic exosomes were originally characterized with the use of explant cultures (46). The thymic exosomes shared features with exosomes from other sources, such as a size distribution of 30–100 nm for a majority of the vesicles, density peaking at 1.18–1.19 g/ml, which is less than the typical density of histone dense apoptotic bodies (51), and presence of typical exosomal proteins such as TSG101, CD9, CD81, and HLA-DR. Since these thymic exosomes were isolated from whole thymic tissue, the cellular source could not be determined, and the vesicles were most probably a mix of exosomes from different sources, e.g., thymocytes, TECs, and DCs. Even so, tissue-restricted antigens (TRAs), defined by protein-expression allowed in a maximum of five tissues in the human protein atlas (HPA) (52) were identified in the exosomes (2′,3′-cyclic-nucleotide 3′phosphodiesterase, reticulon 3, tropomyosin 3, and the GNAS protein), which suggest that a portion of the exosomes originates from the thymic epithelium (46). These four identified TRAs are possible candidates to participate in the selection/maturation processes within the human thymus. With the exception of one study that address thymic expression of 2′,3′-cyclic-nucleotide 3′phosphodiesterase (53), the four TRAs are hitherto unaddressed in thymic research. Interestingly, 2′, 3′-cyclic-nucleotide 3′phosphodiesterase is recognized by IgG autoantibodies in multiple sclerosis patients (54). In addition, tropomyosin 3 was suggested to be a candidate antigen in endometriosis (55).

Other traits seem to be specific for thymic exosomes compared to exosomes from other sources. One is the massive yield of ~1 mg of thymic exosomes per gram of thymic tissue grown in an explant culture (46). Other characteristics typical for thymic exosomes are the low expression of CD63 and the high expression of TSG101 on their surface (46). However, low levels of CD63 could have functional implications for thymic exosomes since it has been reported that siRNA mediated knockdown of the tetraspanin CD63 in a B-lymphoblastoid cell line (LCL) resulted in an increased CD4+ T-cell recognition as evaluated by IFN-γ production. The increase in T-cell response could not be explained by changes in antigen processing or MHC II-expression (56). Instead, equal amounts of exosomes from $CD63^{low}$ LCL cells and control LCL cells stimulated the T-cells to comparable degrees, but the $CD63^{low}$ LCL cells produced more exosomes, which in the end enhanced the total T-cell-stimulatory capacity of the $CD63^{low}$ LCL cells.

Formal proof that TECs are able to produce exosomes was provided with the use of an approach in which primary cultures of TECs were established under selective conditions to eliminate the presence of thymocytes, DCs, fibroblasts, and peripherally produced exosomes (48, 57). The results showed that TECs produce exosomes and that these exosomes contained TRAs and a number of known autoantigens (48). Among the identified autoantigens in TEC exosomes were myelin basic protein (58), collagen type II (59), TITIN (60), heat shock protein 60 [connected with various autoimmune diseases (61)], transglutaminase 2 (62), desmoglein 1, and desmoglein 3 (63). In addition to autoantigens, previously reported mTEC-enriched TRAs were present in TEC exosomes, e.g., glutathione S-transferase M3 (GSTM3), LDL receptor, monocarboxylate transporter 4 (SLC16A3), mucins (MUC5B and MUC18), and myosin 1B (MYO1B) (48).

The observation that TEC exosomes have a higher fraction of proteins classified as TRAs (24%) compared to the fraction of TRAs in the cultured TECs (21%) could argue for a directed loading of TRAs into the exosomes (48).

Exosomes isolated directly from thymic tissue and exosomes isolated from TEC-cultures share a set of TRAs, such as 2′,3′-cyclic-nucleotide 3′ phosphodiesterase, which strengthens that thymic explant exosomes are partly of epithelial origin and that their TRA-content can be analyzed with whole thymic tissue as starting material. See **Figure 1C** for a schematic summary of the TEC-exosomal proteome. In addition, the presence of antigen-presenting molecules together with TRAs indicates that exosomes could transfer intact functional peptide-MHC complexes.

RNA Transfer by Exosomes

Exosomes have been increasingly recognized for their ability to transfer functional miRNAs and mRNAs between cells (4), and recently, it was shown that Tregs utilize exosomes for transfer of miRNA in order to functionally silence effector T-cells (64). The importance of miRNAs in the thymus has rendered an increased interest, and miRNAs have been shown to affect promiscuous gene expression under the influence of AIRE (65, 66), to be involved in

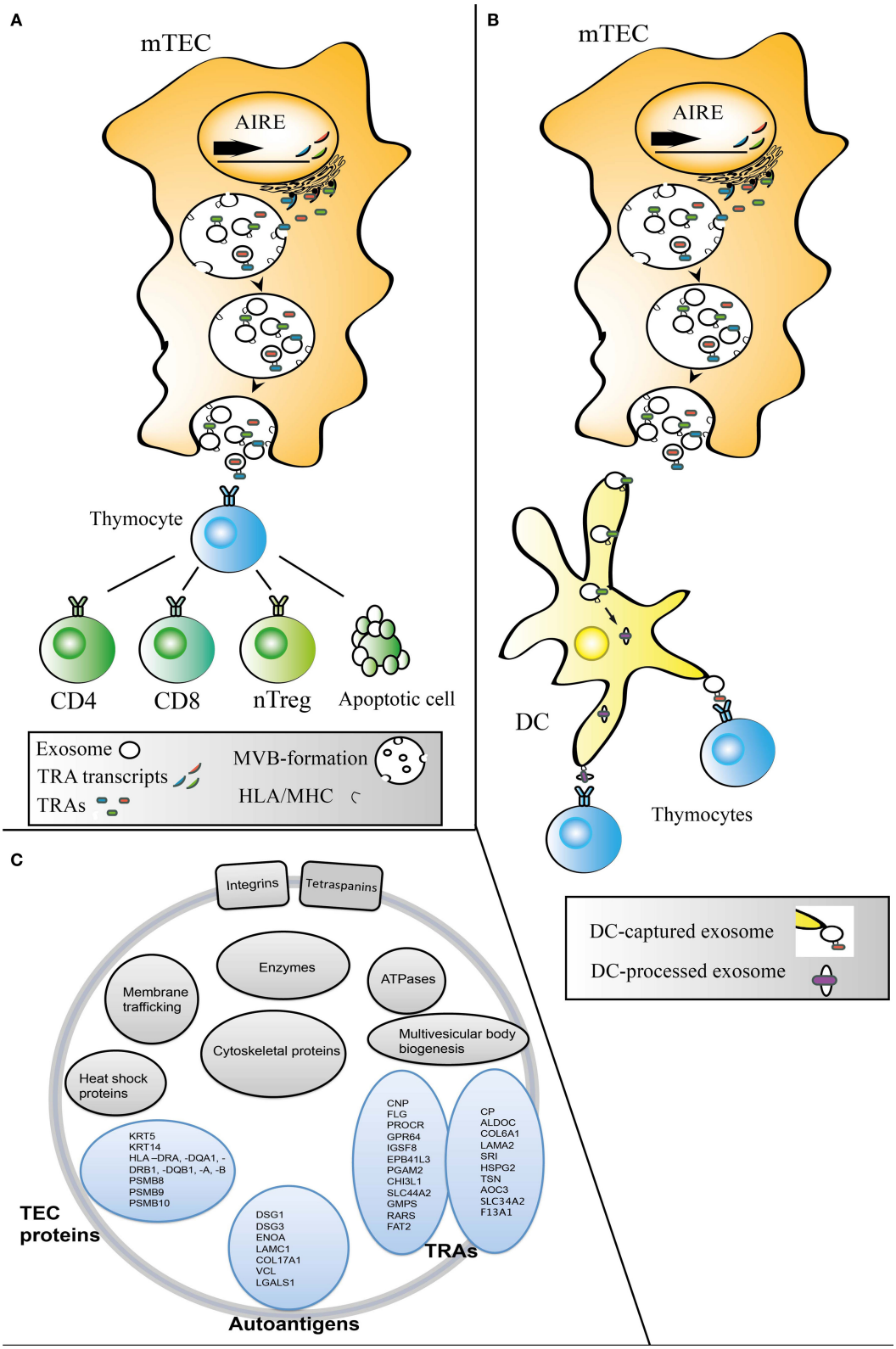

FIGURE 1 | Models for transfer of exosomal material from TECs to other thymic cell populations. Transcription and translation of AIRE-dependent TRAs followed by TRA loading of exosomes, MVB fusion with the mTEC plasma membrane leading to exosome release from mTECs. The exosomes could then take an intercellular route from mTECs directly **(A)** to CD4+, CD8+, and developing nTregs and/or indirectly **(B)** via thymic DC or other APCs. **(C)** The proteome of thymic exosomes typically include exosomal markers, e.g., tetraspanins, TEC associated proteins, TRAs, and autoantigens (46, 48).

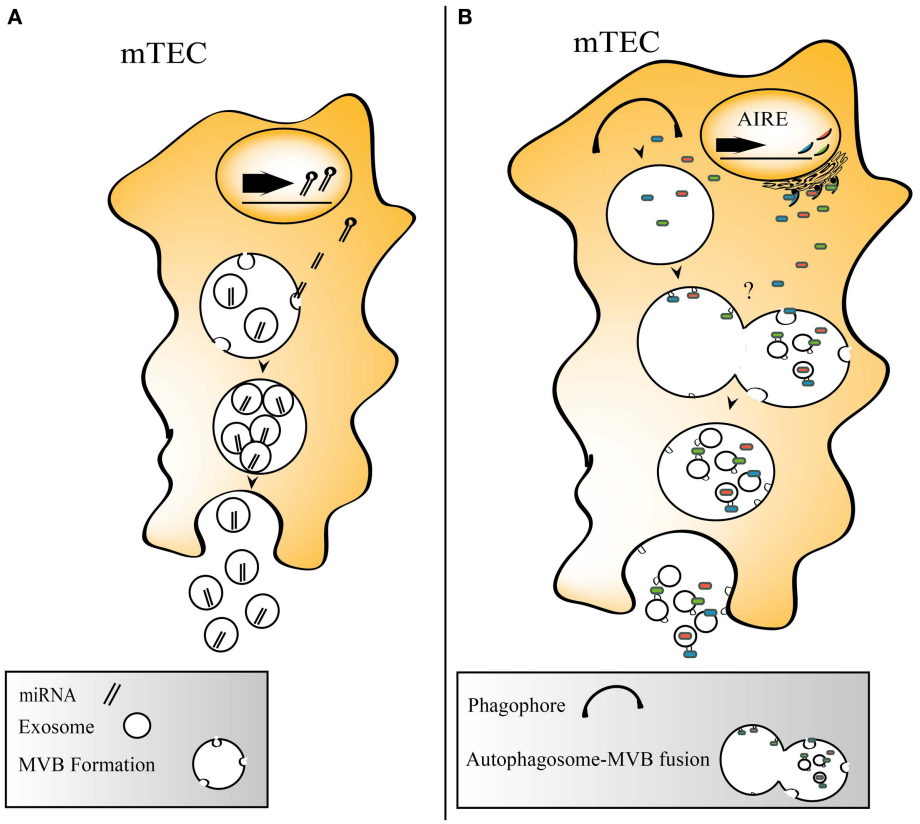

FIGURE 2 | (A) Thymic exosomes contain miRNAs that could be taken up by adjacent cells. **(B)** Suggested model describing two principally different routes for TRA loading onto exosomes. TRAs could be engulfed by phagophores, which are processed to autophagosomes that fuse with MVBs where TRA carrying exosomes are formed. Alternatively, TRAs are directly directed into MVBs.

thymic involution (67) and the maintenance of thymic epithelia (68). Thymic exosomes contain a number of miRNAs, and among them is the highly TEC enriched miRNA hsa-miR-149 (66). The role of thymic miRNAs has been thoroughly reviewed recently (69). Possible roles for miRNA sharing within the thymus by exosomes include control of TEC development and regulation of TRA expression. See **Figure 2A** for a schematic view of miRNA incorporation into TEC exosomes.

Exosomal Biogenesis – Crossroads with Autophagy?

Autophagy may serve as a way to generate self-peptides from endogenously produced proteins that allows loading onto MHCII, and the constitutive autophagy process present in the thymus has been suggested to be involved in the negative selection and Treg formation (70). Thymic exosomes carry proteins involved in autophagy, such as autophagy related protein 7 (46). Two interesting observations are that a subgroup of TECs is rich in MVBs (71), and that at the same time, TECs have a constitutive autophagic activity (72). The simultaneous appearance of autophagic ultrastructure elements and multi-vesicular bodies in TECs support this notion (71). The intersection between autophagy and exosome formation is illustrated by the regulation of both autophagy and exosome biogenesis by GAIP interacting protein C terminus in pancreatic cancer cell lines (73). Exosomal release is also impaired in mouse embryonic fibroblasts lacking ATG12-ATG3, and immature autophagosomes are shown to fuse with MVBs in these cells (74). Whether this also occurs in TECs is so far unaddressed, but this route could potentially make antigens that are processed in the autophagic machinery available for export on exosomes and at the same time enhance the loading of endogenous antigens onto MHC class II molecules (**Figure 2B**).

Concluding Remarks

Tissue-restricted antigen presentation within the thymic micromileus is pivotal to establish central tolerance. Antigen transfer between thymic cell populations, e.g., from mTECs to thymocytes and DCs is an established phenomenon that is poorly investigated from a mechanistic point of view. To get a more comprehensive understanding of central tolerance, the role of antigen transfer and the responsible vectors, e.g., exosomes, need to be studied and understood in more depth.

The abundant presence of TRA-containing exosomes in thymic tissue and the many observations of antigen transfer from mTECs to DCs lead us to speculate that this antigen transfer is, at least partly, mediated by exosomes. However, such a mechanism has not yet been formally proven. Although this review has been focused on possible antigen transfer by mTEC-derived exosomes

to DCs and thymocytes, it is not excluded that also cTECs produce exosomes and that exosomes are shuttling antigens between different TEC populations. Other questions also remain regarding thymic exosomes that are equally important to answer in future studies; do exosomes exist *in vivo* in enough quantities to be biologically functional in the context of tolerance induction? If so, which is the primary route, direct interaction with thymocytes or indirect via APCs? What is the importance of exosomes for nTreg induction? Also, does miRNA content of the exosomes affect the development and maturation of thymic cells? Examples of experimental approaches that could be used to address these questions are TEC specific inhibition of the ESCRT machinery using the FOXN1-cre system or microinjection of thymic exosomes isolated from wild type mice into AIRE$^{-/-}$ thymii. The outcome of this kind of experiments may give hints to whether a therapeutic use of tailor made exosomes to induce antigen-specific tolerance may be possible in the future.

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REVIEW
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Thymic crosstalk coordinates medulla organization and T-cell tolerance induction

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The thymus ensures the generation of a functional and highly diverse T-cell repertoire. The thymic medulla, which is mainly composed of medullary thymic epithelial cells (mTECs) and dendritic cells (DCs), provides a specialized microenvironment dedicated to the establishment of T-cell tolerance. mTECs play a privileged role in this pivotal process by their unique capacity to express a broad range of peripheral self-antigens that are presented to developing T cells. Reciprocally, developing T cells control mTEC differentiation and organization. These bidirectional interactions are commonly referred to as thymic crosstalk. This review focuses on the relative contributions of mTEC and DC subsets to the deletion of autoreactive T cells and the generation of natural regulatory T cells. We also summarize current knowledge regarding how hematopoietic cells conversely control the composition and complex three-dimensional organization of the thymic medulla.

Keywords: autoimmune regulator, dendritic cells, medulla, medullary thymic epithelial cells, natural regulatory T cells, negative selection, T-cell tolerance, thymic crosstalk

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Introduction

Healthy individuals mount effective T-cell immune responses directed against pathogens while avoiding autoimmune attacks directed toward self-antigens. The random generation of the T-cell receptor (TCR) repertoire results in the production of autoreactive TCRs, which necessitates their selection in the thymus (1). Anatomically, the thymus is compartmentalized into an outer region called the cortex and an inner region called the medulla. The cortex supports early stages of T-cell differentiation, including the positive selection of $CD4^+$ and $CD8^+$ thymocytes. Nonetheless, the cortex also supports a substantial loss of DP thymocytes that are specific for ubiquitous self-antigens (2, 3). The medulla sustains the induction of T-cell tolerance, which is established by two distinct main mechanisms: negative selection (also known as clonal deletion) of potentially hazardous autoreactive T cells, and the production of natural regulatory T (nTreg) cells. Negative selection consists of the deletion of immature T cells bearing TCRs, which are highly reactive against self-antigens (4, 5). Although this process is remarkably efficient, it cannot completely purge the TCR repertoire of self-reactive specificities and thus allows potentially hazardous T cells to reach the periphery. To control potential deleterious effects of autoreactive T cells that have escaped the negative selection process, the thymus produces a specific subset of T cells called nTregs. This cell type belongs mainly to the $CD4^+$ T-cell lineage and specifically expresses the transcription factor forkhead box P3 (FOXP3), which is essential for their development and function (6). The induction of T-cell tolerance is established within the medullary microenvironment, which is composed of a dense 3D network of

antigen-presenting cells (APCs), namely thymic dendritic cells (DCs) and medullary thymic epithelial cells (mTECs) (**Figure 1A**). In this review, we discuss our current knowledge regarding the phenotypic features of the different subsets of thymic DCs and mTECs as well as their relative contribution to the induction of T-cell tolerance. We also summarize recent progress in our understanding of the thymic crosstalk that sustains the composition and complex three-dimensional (3D) organization of the medulla.

Thymic Medullary APCs Involved in T-Cell Tolerance Induction

Features of Thymic DCs

In the thymus, DCs represent only approximately 0.5% of the total thymic cells, which is less than that in other lymphoid organs. Although peripheral DCs have been long described as heterogeneous, only recently thymic DCs have also been shown to constitute a heterogeneous cell population. It is now accepted that thymic DCs comprise three distinct subsets: two conventional DC (cDC) subsets and plasmacytoid DCs (pDCs) (**Figure 1B**) (7). The two subsets of cDCs, which express high levels of CD11c, have different origins and can be distinguished based on specific cell surface markers. The $CD11b^{-}CD8\alpha^{hi}Sirp\alpha^{-}$ (signal regulatory protein α) cDCs develop intrathymically and are commonly termed intrathymic or resident cDCs. In contrast, the $CD11b^{+}CD8\alpha^{lo}Sirp\alpha^{+}$ cDCs have a myeloid origin and continuously migrate from the periphery via the blood circulation into the thymus (8). They are referred to as extrathymic or migratory cDCs. Under steady-state conditions, resident and migratory cDCs represent two-thirds and one-third of the thymic

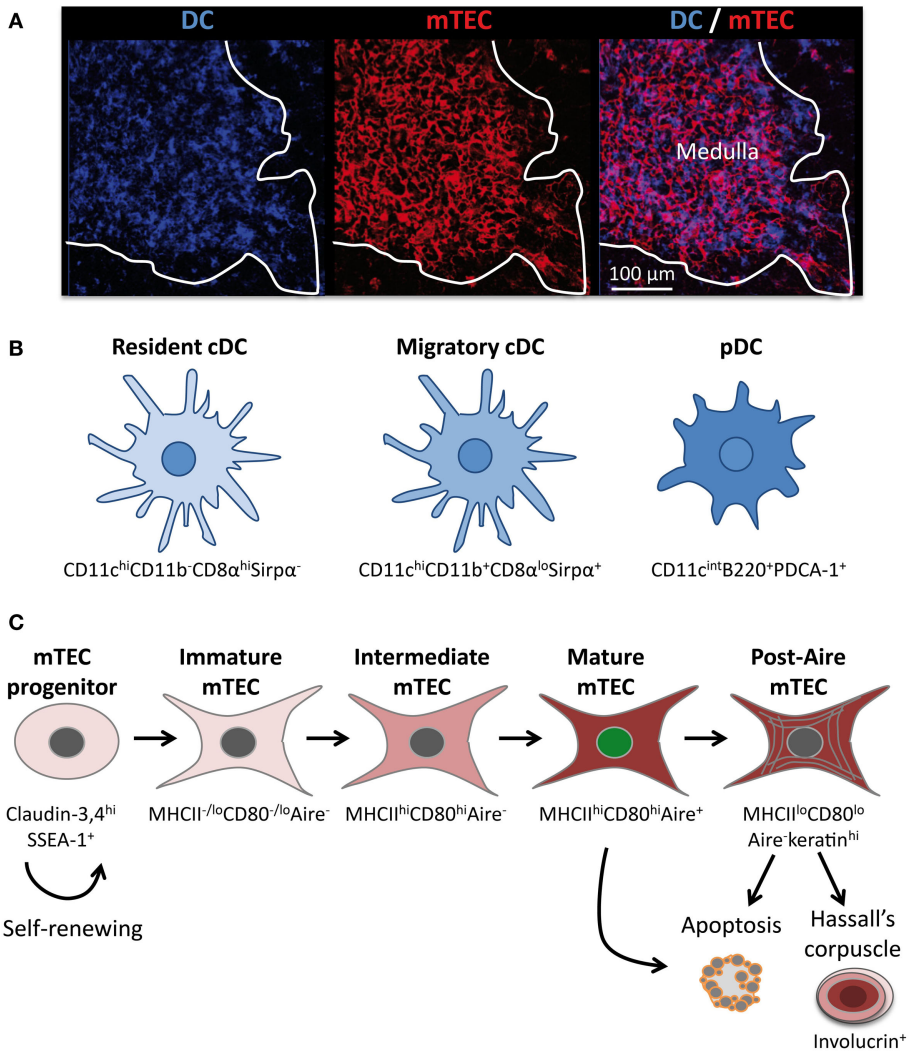

FIGURE 1 | The thymic medulla is composed of a dense network of distinct subsets of DCs and mTECs. (A) Confocal micrograph of a mouse thymic section stained with antibodies against the DC-specific marker CD11c (blue) and the mTEC-specific marker K14 (red). **(B)** Three distinct subsets of DCs are located mainly in the medulla: resident cDCs ($CD11c^{hi}CD11b^{-}CD8\alpha^{hi}Sirp\alpha^{-}$), migratory cDCs ($CD11c^{hi}CD11b^{+}CD8\alpha^{lo}Sirp\alpha^{+}$), and pDCs ($CD11c^{int}B220^{+}PDCA\text{-}1^{+}$). **(C)** Schematic representation of mTEC differentiation. mTECs arise from a pool of self-renewing mTEC progenitors. Distinct stages of mTEC maturation can be identified based on the differential expression of MHCII, CD80, and Aire. The end stages of maturation can lead to the emergence of post-Aire mTECs, apoptosis, or to the development of Hassall's corpuscle.

cDCs, respectively (7). Resident cDCs arise from a common T/DC precursor and reside exclusively in the thymus throughout their long life (7, 9, 10). They express CD8α mRNA and display CD8αα homodimers at their surface. In contrast, migratory cDCs do not synthesize CD8α mRNA, and the low expression level of CD8α observed at the surface of this cell type is a consequence of the uptake of cell surface CD8αβ heterodimers from thymocytes (11). Strikingly, following their migration in the thymus, migratory cDCs upregulate CD80 and CD86 costimulatory molecules as well as CD11c and MHCII molecules (8). In addition, in contrast to resident cDCs, migratory cDCs proliferate extensively and mature in interdigitating cDCs. Consequently, migratory cDCs overall exhibit a more activated phenotype compared with their resident counterparts (12).

The third subset of thymic DCs corresponds to pDCs, which continuously migrate to the thymus via the bloodstream. They are defined as $CD11c^{int}B220^{+}PDCA\text{-}1^{+}$ and represent approximately 30% of the total thymic DCs (**Figure 1B**). Like their immature counterparts in the periphery, thymic pDCs present a plasmacytoid morphology rather than a dendritic morphology. Upon migration in the thymus, pDCs enlarge and adopt a semi-mature phenotype via the upregulation of CD11c and MHCII molecules (8). Moreover, they express high levels of Toll-like receptors (TLR) 7 and 9 and low levels of TLR 2, 3, and 4 (13). Overall, migratory DCs, i.e., pDCs and cDCs, represent 50% of the total thymic DCs. Parabiosis experiments have shown that migratory DCs are localized in the medulla and at the cortico-medullary junction (CMJ) (8). Antigen-loaded peripheral pDCs were found to be localized preferentially at the CMJ upon their migration in the thymus (14). Intriguingly, both pDCs and migratory cDCs change their phenotype shortly after entering the thymus, suggesting that the medullary microenvironment provides specific factors that contribute to the functional specification of these DC subsets. The identity of these factors that drive the maturation as well as the extensive proliferation of migratory DCs remains elusive.

Features of mTECs

Similarly to DCs, mTECs also constitute a heterogeneous cell population that represent less than 1% of the total thymic cells (15). Histologically, mTECs are commonly identified by the expression of cytokeratin-5, 14, MTS10, and ERTR5 markers as well as by reactivity with the lectin Ulex Europaeus Agglutinin 1 (UEA-1) (16–19). However, it is not completely clear whether these markers stain the bulk of mTECs or whether they preferentially detect some specific subsets. However, the whole mTEC compartment can be identified by flow cytometry and is generally defined as $CD45^{-}EpCAM^{+}$ (epithelial cell adhesion molecule) $Ly51^{-/lo}$. mTEC subsets can be further defined with respect to other markers, including the levels of cell surface MHCII and CD80 expression as well as of the transcription factor Aire (**Figure 1C**). Recent advances have established the relationship between these different cell subsets by demonstrating that mTEC differentiation proceeds along distinct maturational stages. RTOC experiments have shown that $MHCII^{-/lo}CD80^{-/lo}Aire^{-}$ immature mTECs give rise to $MHCII^{hi}CD80^{hi}Aire^{+}$ mature mTECs (20–22). Consistently during embryogenesis, $MHCII^{-/lo}CD80^{-/lo}Aire^{-}$ immature mTECs appear prior to the emergence of $MHCII^{hi}CD80^{hi}Aire^{+}$ mature mTECs (20, 22). Mature mTECs are thus believed to derive from immature mTECs via an intermediate stage that is $Aire^{-}$ but has acquired high levels of MHCII and CD80 expression (**Figure 1C**). $Aire^{+}$ mature mTECs were initially described to be post-mitotic and short-lived and were thus thought to represent the last stage of mTEC differentiation (20, 21). Apoptosis of this cell type has been proposed to be induced by Aire itself and to be favorable for the diffusion of self-antigens within the medullary microenvironment (21). Recent studies of cell fate mapping, allowing the permanent labeling of Aire-expressing cells even after the termination of transcription, have challenged this concept by demonstrating the existence of a post-Aire stage (23, 24). Approximately half of $Aire^{+}$ mature mTECs seems to further progress to this post-Aire stage, which does not express Aire and expresses MHCII and CD80 molecules at reduced levels, thereby generating $MHCII^{lo}CD80^{lo}Aire^{-}$ mTECs (**Figure 1C**) (24, 25). This end-stage maturation of mTECs closely resembles that of keratinocytes (25). Finally, mTECs lose their nuclei to form Hassall's corpuscles that can be detected by the expression of markers such as involucrin, cytokeratins 6/10, desmogleins 1/3, and lympho-epithelial kazal type related inhibitor (LEKTI) (25, 26).

Interestingly, all mTEC subsets are simultaneously present in the post-natal thymus (**Figure 1C**). In addition, the turnover period for mature mTECs is estimated to be between 2 and 3 weeks (20, 21). These observations suggest that the mature mTEC population is continuously replenished by differentiation from an mTEC progenitor. Consistent with this notion of perpetual renewal, recent studies have demonstrated the presence in adults of thymic epithelial progenitors and/or stem cells that are capable of generating both mature cortical and medullary lineages in a stepwise fashion (27, 28). Furthermore, a novel transitional progenitor stage characterized by the expression of cTEC markers such as CD205, β5t, and high levels of IL-7 has been identified in the embryonic thymus and shown to have the potential to generate mTECs (29–31). Moreover, an mTEC-specific stem cell capable of ensuring lifelong mTEC subsets was recently found within the $claudin\text{-}3,4^{hi}SSEA\text{-}1^{+}$ (stage-specific embryonic antigen 1) population (**Figure 1C**) (32). Of note, adult mTEC stem cells have a lower regenerative capacity than their embryonic counterparts. At the current stage of knowledge, the relationships among the common thymic epithelial stem cells (27, 28), the transitional progenitor that harbors cTEC-properties (29–31) and $claudin\text{-}3,4^{hi}$ $SSEA\text{-}1^{+}$ mTEC stem cells (32) remain unknown. Thus, further investigations are needed to clarify the relationship among these cells as well as their relative contributions to medulla formation and homeostasis within the embryonic and adult thymus. The identification of specific markers that allow distinct discrimination between these cell types would be helpful to evaluate their respective regenerative capacity. Such studies could aid in identifying clinical applications, notably for improving thymic function in the context of elderly or cytoablative treatments. Taken together, these findings have revealed that the medullary epithelium is not static but, in contrast, is much more dynamic than previously considered.

Tight Collaboration Between Medullary APCs for the Establishment of T-Cell Tolerance

Medullary thymic epithelial cells play a privileged role in the induction of central T-cell tolerance through their ability to express a broad range of tissue-restricted self-antigens (TRAs) (33). A recent study has shown, by using deep transcriptome sequencing, that mature mTECs express 19,293 genes, i.e., approximately 85% of the mouse genome (34). Thus, mTECs constitute the only cell type described that expresses such a large number of genes. The transcription factor Aire is the only regulator known to date that drives the expression of many TRAs (35). Aire alone regulates 3,980 genes (34). The importance of Aire in the induction of T-cell tolerance is illustrated by the fact that mutations in this gene are responsible for the development of the human autoimmune syndrome autoimmune polyendocrinopathy syndrome-1 (APS-1), which is also known as autoimmune polyendocrinopathy candidiasis ectodermal dystrophy (APECED) (36, 37). Similarly to humans, Aire-deficient mice show signs of autoimmunity characterized by inflammatory infiltrates and serum autoantibodies (38). The mechanisms by which Aire controls the transcription of TRAs have been extensively reviewed elsewhere (39–41). In contrast, although Aire-independent TRAs represent approximately 80% of the genes expressed in mTECs, the mechanisms that regulate them are largely unknown. The participation of other regulatory factors as well as epigenetic regulation thus remains to be identified.

Cross-Presentation of mTEC-Derived TRAs by Resident cDCs

Tissue-restricted self-antigens expressed by mTECs, independently of their subcellular origin, were described to be cross-presented by resident cDCs, which reside in close proximity to mTECs (42–45) (**Figure 2**). This unidirectional transfer of self-antigens is thought to be favored by a high mTEC turnover, which might allow the subsequent uptake of materials by cDCs. Although several potential mechanisms of intercellular material transfer have been proposed, such as the uptake of apoptotic bodies, gap junctions, exosome transfer, and membrane exchange ("nibbling"), experimental evidence is still lacking, and the precise underlying mechanisms remain unclear (46, 47). However, a recent study found that human TECs produce exosomes that carry antigen-presentation molecules and TRAs, suggesting that TEC-derived exosome could be involved in TRA cross-presentation (48). Given that a particular TRA is expressed only by a minor fraction of mTECs (1–3%), this phenomenon of intercellular antigen transfer likely ensures efficient scanning of TRAs by developing SP thymocytes (49). Furthermore, two-photon imaging experiments have shown that SP thymocytes are extremely mobile and make frequent and transient contacts with DCs, which might greatly contribute to the efficient selection of T cells during their 4- to 5-day residency in the medulla (50, 51). Proper localization of resident cDCs in the medulla is controlled by the expression of the chemokine receptor XCR1 and its Aire-dependent associated chemokine XCL1 (52). XCL1-deficient mice show fewer medullary DCs and defective generation of nTreg cells, suggesting that medullary cDCs contribute to nTreg cell development (**Figure 2**). Consistent with this observation, resident cDCs have been found to play an important role in the generation of nTregs via their ability to acquire and present Aire-dependent TRAs (53).

mTECs Act as *Bona Fide* APCs

Medullary thymic epithelial cells have thus been initially recognized to play a privileged role in T-cell tolerance because they constitute an "antigen reservoir" that mirrors the peripheral self (33). However, the use of transgenic mouse models that mimic TRA expression in the thymus have shown that mTECs can efficiently induce the clonal deletion of $CD8^+$ T cells (42, 54). Recent studies have demonstrated that they also act as *bona fide* APCs to $CD4^+$ T cells. mTECs have the ability to autonomously present endogenously expressed TRAs via MHCII molecules by using an unconventional endogenous pathway called macroautophagy, which allows the shuttling of cytoplasmic constituents into lysosomes (55, 56). $Aire^+$ mTECs can induce both the negative selection of autoreactive T cells as well as the generation of nTreg cells (**Figure 2**) (53, 57–60). The induction of nTreg cells was found to be mTEC-dependent because mTECs have the ability to foster the development of $Foxp3^-CD25^+$ nTreg precursors (61). In accordance with these findings, mice showing an enhanced mTEC compartment display increased production of nTreg cells (62, 63). Conversely, mice showing a reduced mTEC compartment exhibit a reduction of nTreg cells (64, 65). Interestingly, a recent study has shown that a large proportion of thymic Tregs corresponds to peripheral recirculating Tregs (66). The participation of mTECs to this phenomenon of recirculation to the thymus remains to be examined. Interestingly, post-Aire mTECs were found to maintain intermediate TRA expression (24). Thus, it is plausible that this newly identified mTEC subset plays a role in the establishment of T-cell tolerance. Further studies, based for instance on cell-specific ablation, are needed to address this issue. Moreover, although $MHCII^{-/lo}CD80^{-/lo}Aire^-$ and $MHCII^{hi}CD80^{hi}Aire^-$ mTECs express fewer genes compared with $Aire^+$ mTECs (34), only a few thousands genes are differentially expressed, which suggests that these immature subsets could have a non-redundant function in the induction of T-cell tolerance. In addition, these distinct mTEC subsets express different levels of MHCII and costimulatory molecules, which may significantly impact T-cell selection. Consistent with these observations, *in vivo* knock-down of MHCII molecules specifically in $Aire^+$ mTECs leads to an increased proportion of $CD4^+$ SP and an enhanced selection of nTregs (59). These findings suggest that there is an underlying division of labor within mTEC subsets, with immature mTECs likely providing more potent induction of nTregs and mature mTECs preferentially prone to negative selection. Of note, the *in vivo* dynamics of the interactions of $CD8^+$ and $CD4^+$ T cells with mTECs remain unknown to date. It would be very informative to compare the interactions of medullary $CD8^+$ and $CD4^+$ T cells with $Aire^-$ and $Aire^+$ mTECs to determine to what extent the frequency and duration of these interactions influence T-cell outcomes. Two-photon imaging experiments assessing fresh thymic slices are expected to achieve this goal in the near future and may reveal a complex choreography between SP thymocytes and mTECs.

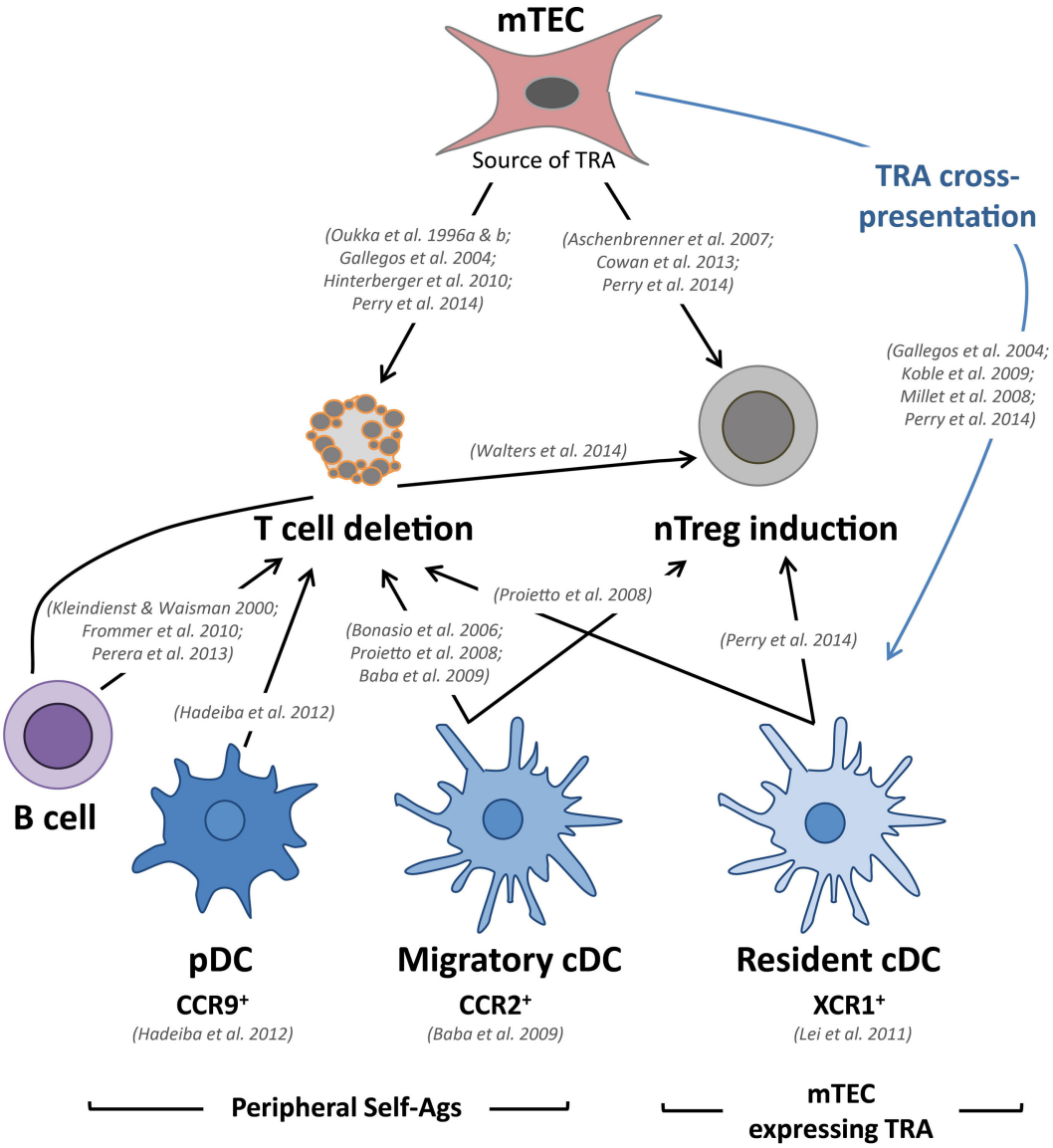

FIGURE 2 | mTECs and DCs tightly collaborate to delete autoreactive T cells and to induce the generation of nTreg cells. Relevant *in vivo* studies are indicated in this figure. Tissue-restricted self-antigens (TRAs) expressed and presented by mTECs can lead to the deletion of autoreactive T cells and the induction of nTregs. These self-antigens can also be transferred to and presented by resident cDCs, resulting in T-cell deletion and the induction of nTregs. Furthermore, migratory cDCs and pDCs also reinforce the establishment of central T-cell tolerance via the presentation of antigens captured in the periphery. Migratory cDCs are also involved in T-cell deletion and the induction of nTregs, whereas pDCs only contribute to the deletion of autoreactive T cells in mice. Thymic B cells have also been shown to participate in the deletion of autoreactive T cells and the generation of nTregs.

Migratory DCs Reinforce the Presentation of Self-Antigens

Although mTECs express a diverse repertoire of TRAs that largely contribute to the induction of T-cell tolerance, they cannot encompass the spectrum of all peripheral self-antigens. Migratory DCs have been shown to reinforce the deletion of autoreactive thymocytes by sampling peripheral self-antigens that would otherwise be undetectable to developing thymocytes. Studies based on Rag2$^{-/-}$ OTII TCR-transgenic mice have shown that migratory cDCs induce the negative selection of autoreactive CD4^{+} thymocytes (12, 67). Interestingly, in co-culture assays, Sirpα^{+} cDCs efficiently convert CD4^{+}CD25^{-} thymocytes into CD4^{+}CD25^{+}Foxp3^{+} nTregs (12, 68). Migratory cDCs were also found to efficiently induce nTreg cells *in vivo* (12). Thus, in the steady state, migratory cDCs have the ability to transport antigens captured in the periphery and contribute to the establishment of tolerance by deleting autoreactive CD4^{+} thymocytes and inducing nTreg cells (**Figure 2**). These studies have mainly focused on MHCII-restricted TCR-transgenic models, and consequently, the role of migratory cDCs in CD8^{+} T-cell deletion remains unclear. Migratory cDCs home to the thymus in a CCR2-dependent manner (69). CCR2-deficient mice display a decreased number of

migratory cDCs in their thymus and exhibit defective negative selection against blood-borne antigens (69). However, the deficiency in CCR2 does not completely alter the migration of these cells, suggesting the potential involvement of other chemokine receptors. Of note, activated cDCs exhibit a reduced ability to home to the thymus, thus preventing the inappropriate deletion of cells capable of recognizing pathogen-derived antigens (67).

A third subset of DCs, namely pDCs, has recently been described to participate in the induction of T-cell tolerance. Until recently, the function of pDCs in the thymus has remained largely enigmatic, although it was suggested that they could protect the thymus against viral infections via their ability to produce type I interferon (7). In the periphery, in addition to secreting large amounts of type I interferon in response to viral infections, it became evident that pDCs can also function as *bona fide* APCs that are capable of modulating T-cell responses (70). Recent advances have demonstrated that pDCs possess tolerogenic properties in specific contexts, primarily through the induction or the proliferation of nTreg cells (71–74). Consistent with these tolerogenic functions observed in the periphery, pDCs were shown to colocalize with Foxp3$^+$ Tregs and to promote the generation of nTreg cells from immature thymocytes via CD40–CD40L and interleukin-3 in the human thymus (75). Similarly, thymic stromal lymphopoietin (TSLP)-activated human pDCs induce the generation of nTregs (76). However, in mice, thymic pDCs do not efficiently induce the generation of nTregs from immature thymocytes *in vitro* (12, 68). *In vivo*, no role of thymic pDCs was observed in the conversion of thymocytes into the nTreg cell lineage (14). These studies suggest that in contrast to their human counterparts, murine thymic pDCs are intrinsically inefficient at inducing nTreg cells. Murine thymic pDCs, however, were shown to transport peripheral antigens to the thymus, inducing the deletion of autoreactive CD4$^+$ thymocytes (14) (**Figure 2**). Their role in the deletion of CD8$^+$ thymocytes remains unclear. The migration of pDCs in the thymus was found to be dependent on CCR9, a chemokine receptor that is also involved in T-cell progenitor homing (14, 77, 78). Importantly, pDCs that are activated by TLR ligands lose their ability to home to the thymus by downregulating CCR9, thus preventing the unwanted induction of T-cell tolerance toward pathogens (14). Under normal conditions, CCR9 deficiency does not completely block the recruitment of pDCs in the thymus, suggesting that other chemokine receptors could be involved in this process. Interestingly, transgenic mice overexpressing CCL2 in the thymus under the myelin basic protein (MBP) promoter exhibit a massive thymic recruitment of pDCs, which express CCR2 (79, 80). The thymic migration of pDCs could be mediated via both CCR9 and CCR2. The generation of double knockout mice for CCR9 and CCR2 should reveal whether these two chemokine receptors are sufficient for directing the thymic recruitment of pDCs.

A New Player: Thymic B Cells

In the medulla, in addition to mTECs and DCs, a third type of APC, namely the B cell, has also been implicated in the induction of T-cell tolerance (**Figure 2**). The vast majority of thymic B cells develop within the thymus from Rag-expressing progenitors, whereas recirculating B cells represent a minority (81). Thymic B cells display unique phenotypic hallmarks in comparison to peripheral B cells. They express high levels of MHCII and costimulatory molecules, supporting their robust antigen-presenting capacity (81). Of note, a recent report has shown that thymic B cells express Aire and display tolerogenic properties upon migration into the thymus (82). An original study using transgenic mice on an I-E-deficient background, in which B cells specifically express I-E MHCII molecules, established the capacity of thymic B cells to mediate the negative selection of CD4$^+$ but not CD8$^+$ T cells (83). Similarly, transgenic B cells, which exclusively present an antigen derived from the myelin oligodendrocyte glycoprotein (MOG), efficiently induce the deletion of MOG-specific CD4$^+$ T cells (84). A recent study has also suggested that thymic B cells capture self-antigens through their B-cell receptors and delete autoreactive T cells by presenting peptides derived from these self-antigens (81). Furthermore, thymic B cells also contribute to the generation of nTreg cells (85).

Therefore, mTECs, DCs and B cells participate in the induction of T-cell tolerance through the negative selection of autoreactive T cells and the generation of nTreg cells (**Figure 2**). Interestingly, a recent study using deep sequencing in a fixed TCRβ chain model comparing different genetically modified mice has shown that bone marrow-derived APCs and mTECs play non-redundant roles in shaping the TCR repertoire (53). Roughly half of the Aire-dependent deletion or nTreg induction processes require antigen presentation by bone marrow cells (53). Moreover, the origin of the tissue antigens captured in the periphery and transported in the thymus by migratory cDCs and pDCs remains unclear. Additional studies are needed to determine the degree of the spectrum of overlap among antigens presented in the thymus by these two cell types. In addition to peripheral tissue antigens, although migratory DCs are suspected to participate in T-cell tolerance toward inoffensive foreign antigens derived from food or the commensal gut flora, experimental evidence is still lacking (86). Thus, it is possible that specific thymic DC subsets capture distinct sets of self-antigens and, consequently, could differentially impact the TCR repertoire. Additional studies performed at the polyclonal TCR level are required to elucidate this important issue.

Involvement of Thymic Crosstalk in the Composition and Patterning of the Medulla

The thymic medulla plays a pivotal role in the selection of SP thymocytes. In turn, the expansion and organization of the medulla is governed by developing SP thymocytes. These reciprocal interactions between these two cell types is referred to as "thymic crosstalk" (87). Mice exhibiting a block in thymocyte development at the DP stage, such as TCRα$^{-/-}$ and ZAP70$^{-/-}$ mice, show prominent defects in medulla formation (88, 89). The transplantation of wild-type bone marrow cells in SCID mice lacking TCR-positive cells restores medulla formation and mTEC maturation, indicating that hematopoietic cells control the development of the medullary epithelium (90). Subsequent studies have established that TCR-bearing mature T cells control medulla formation (88, 91, 92). Thus, these pioneer studies indicated that SP thymocytes provide instructive signals that are critical for controlling the expansion and organization of the medulla. Recent advances have

facilitated our understanding of the underlying molecular and cellular participants that are responsible for these crucial processes in the establishment of T-cell tolerance.

AIRE$^+$ mTEC Differentiation in the Embryonic Thymus

In the embryonic thymus, lymphoid tissue inducer (LTi) cells identified as CD3$^-$CD4$^+$IL-7Rα^+ were found to regulate the first cohort of Aire$^+$ mTECs, which emerge around embryonic day 16 of gestation (**Figure 3A**) (20, 22, 93). LTi cells are present during embryogenesis at a time that correlates with the appearance of Aire$^+$ mTECs, before the development of SP thymocytes (22). The emergence of Aire$^+$ mTECs is controlled by a member of the tumor necrosis factor (TNF) superfamily: receptor activator of nuclear factor kappa-B (RANK), which is expressed by mTECs, and its corresponding ligand, RANKL (also known as TRANCE), which is expressed by LTi cells (**Figure 3A**). Strikingly, mice that are deficient for RANK or RANKL show an absence of Aire$^+$ mTECs in the embryonic thymus, indicating that this TNF member regulates the emergence of Aire$^+$ mTECs (22, 94). In accordance with these findings, the exposure of 2-deoxyguanosine-treated fetal thymus organ cultures (FTOCs) to recombinant RANKL or an agonistic antibody to RANK induces the appearance of mature mTECs (22, 95, 96). Conversely, the addition of osteoprotegerin, a soluble decoy receptor for RANKL, or the recombinant RANK-Fc protein, impairs Aire$^+$ mTEC differentiation (94, 97). Importantly, LTi-deficient Rorc$^{-/-}$ mice do not show a complete absence of Aire$^+$ mTECs, suggesting that other embryonic cell types play a role in the development of the medullary epithelium (98). An additional cellular contributor, the invariant Vγ5$^+$TCR$^+$ dendritic epidermal T-cell progenitor, which also expresses RANKL, has likewise been recently implicated in the emergence of Aire$^+$ mTECs in the embryonic thymus

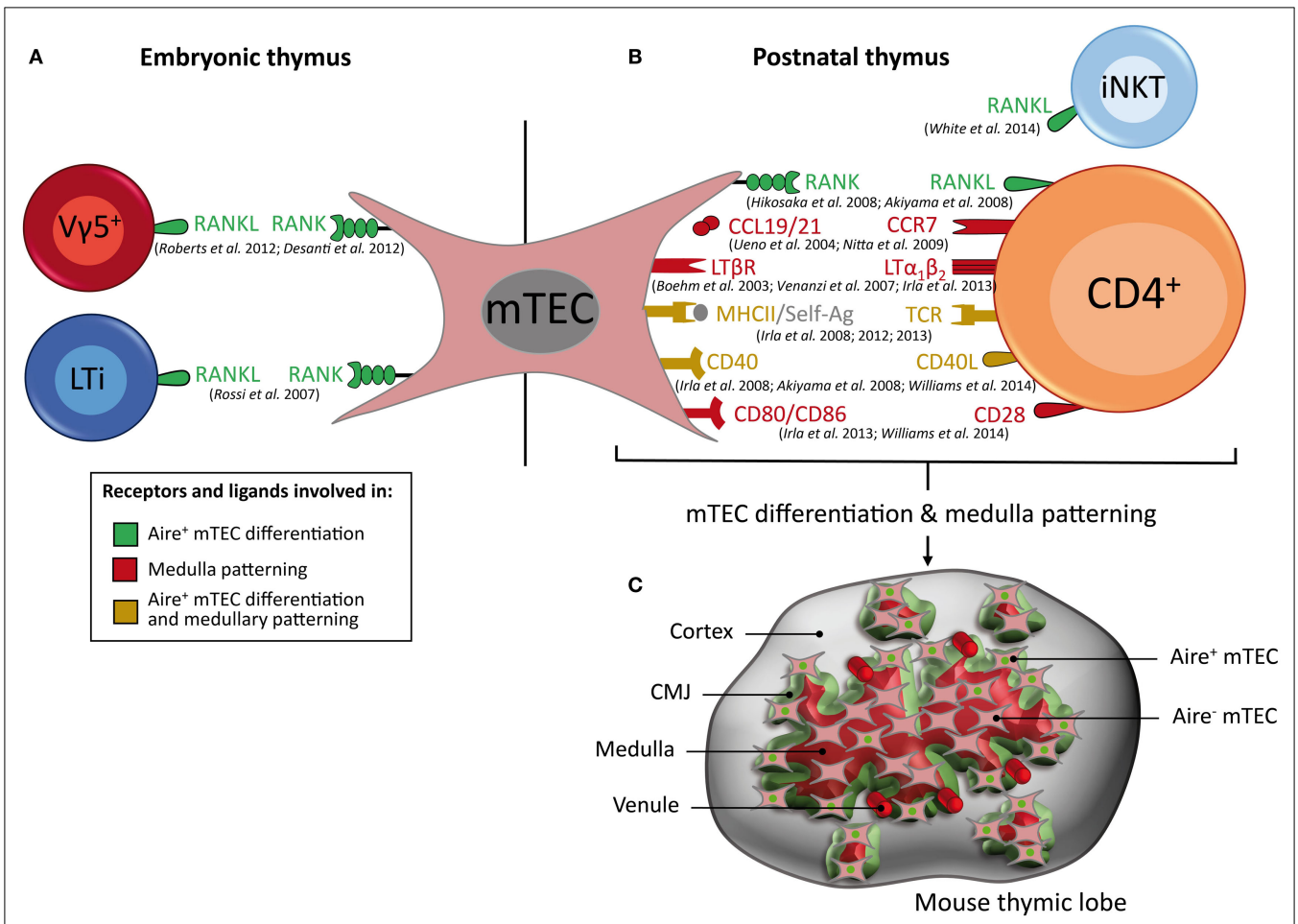

FIGURE 3 | Key cell types, receptors, and ligands that contribute to Aire$^+$ mTEC differentiation and medulla patterning. (A) RANKL, which is expressed by Vγ5$^+$ T cells and LTi cells in the embryonic thymus and **(B)** by CD4$^+$ thymocytes and iNKT cells in the post-natal thymus, induces Aire$^+$ mTEC differentiation. In the post-natal thymus, crosstalk between mTECs and CD4$^+$ thymocytes via CCL19/21–CCR7, LTβR/LTα1β2, and CD80/86–CD28 controls medulla patterning, whereas MHCII/self-antigen–TCR complexes and CD40–CD40L contribute to both the differentiation of Aire$^+$ mTECs and patterning of the medulla. Receptors and ligands involved in Aire$^+$ mTEC differentiation are represented in green, in medulla patterning in red, and in both processes in yellow. **(C)** Schematic representation of 3D medullary organization in the post-natal thymus. Aire$^+$ mTECs (denoted by a green nucleus) and venules are preferentially localized at the cortico-medullary junction (CMJ).

(**Figure 3A**) (97). The addition of purified Vγ5$^+$ thymocytes or LTi cells in reaggregate thymus organ culture (RTOC) experiments induces similar proportions of Aire$^+$ mTEC differentiation. Interestingly, Vγ5$^+$ thymocytes and LTi cells are both present in individual Aire-expressing medullary environments, suggesting that they act collectively to influence mTEC maturation. Mice that are deficient in both LTi and γδ T cells (Rorc$^{-/-}$ × Tcrd$^{-/-}$ mice) show a further decreased number of fetal Aire$^+$ mTECs compared with mice that are deficient in either LTi or γδ T cells alone. However, Rorc$^{-/-}$ × Tcrd$^{-/-}$ double-deficient mice do not show a complete absence of Aire$^+$ mTECs, which suggests that other cell type(s) that remain(s) to be identified could also be involved in this differentiation process. Therefore, the two innate immune cells, Vγ5$^+$ thymocytes and LTi cells, both of which express RANKL, drive the emergence of Aire$^+$ mTECs in the embryonic thymus.

AIRE$^+$ mTEC Differentiation in the Post-natal Thymus

In the post-natal thymus, the RANK–RANKL axis also plays a crucial role in the differentiation of mature mTECs (**Figure 3B**). The absence of RANK or RANKL expression leads to a drastic reduction of Aire$^+$ mTECs and TRA expression (22, 94, 99). Conversely, mice that are deficient for osteoprotegerin, a soluble decoy receptor for RANKL, display a large medulla with many Aire-expressing mTECs (99). In contrast to the embryonic thymus, Aire$^+$ mTECs are partially reduced in the post-natal thymus from RANK$^{-/-}$ or RANKL$^{-/-}$ mice, which suggests that after birth, additional signal(s) are involved in the differentiation and maintenance of mature mTECs. These observations led to the identification of a second member of the TNF superfamily, namely CD40, which is involved in this process (**Figure 3B**). CD40$^{-/-}$ and CD40L$^{-/-}$ mice show more subtle defects in mTEC subsets compared with those observed in RANK$^{-/-}$ or RANKL$^{-/-}$ mice (94, 100). However, these defects were markedly increased in RANK$^{-/-}$ × CD40$^{-/-}$ double-deficient mice compared with RANK$^{-/-}$ mice, demonstrating that RANK and CD40 cooperate to promote mTEC differentiation in the post-natal thymus (94). Moreover, Aire and TRA expression are dramatically affected in these double-deficient mice, which consequently develop severe autoimmune manifestations. Taken together, these findings provide strong support for a model in which the emergence of Aire$^+$ mTECs during embryogenesis involves RANK signaling, whereas the subsequent mTEC differentiation in the post-natal thymus involves cooperation between the RANK and CD40 signals (**Figures 3A,B**).

Several groups have investigated the cellular sources of RANKL and CD40L in the post-natal thymus. Although SP thymocytes were initially found to promote the organization and maturation of the medulla, it remained to be determined whether the instructive signals were provided in a different manner by CD4$^+$ and/or CD8$^+$ thymocytes. RANKL was found to be expressed by both CD4$^+$ and CD8$^+$ thymocytes, with a preferential expression by CD4$^+$ thymocytes (101). In contrast, CD40L was found to be exclusively expressed by CD4$^+$ thymocytes (99, 100). The simultaneous analysis of RANKL and CD40L proteins revealed a sequential acquisition of first RANKL on CD69$^+$ semi-mature CD4$^+$ thymocytes and then of CD40L on CD69$^-$ mature CD4$^+$ thymocytes, suggesting that RANKL and CD40L are delivered by distinct CD4$^+$ subsets (101). The respective role of CD4$^+$ and CD8$^+$ thymocytes in mTEC differentiation was explicitly addressed through the use of knockout mice lacking either CD4$^+$ or CD8$^+$ thymocytes (100). The numbers of CD80^{hi}Aire$^+$ mature mTECs are essentially unaffected in mice lacking CD8$^+$ thymocytes (β2m$^{-/-}$ mice), which suggests that they are dispensable for this process. In contrast, the numbers of CD80^{hi}Aire$^+$ mature mTECs are strongly reduced in mice that lack the positive selection of CD4$^+$ thymocytes, such as *H2-Aa*$^{-/-}$ and *CIIta*$^{IV-/IV-}$ mice (100). Thus, CD4$^+$ thymocytes play a dominant role in promoting the development of the mature mTEC compartment (102). Nevertheless, in mice lacking CD4$^+$ thymocytes, a minor population of CD80^{hi}Aire$^+$ mature mTECs is still detectable, suggesting that another cell type is also involved in the acquisition of a mature phenotype. Even if a rare number of LTi cells is present in the post-natal thymus, it is unlikely that these cells contribute significantly to mTEC differentiation after birth because Id2$^{-/-}$ mice, which lack LTi cells, exhibit normal mature mTEC cellularity (99). Similarly, TCRγδ-deficient mice do not exhibit any obvious defect in mature mTECs (99). Thus, LTi and TCRγδ cells seem to be dispensable for mTEC differentiation during post-natal life. A recent study has suggested that invariant NKT cells that also express RANKL participate in Aire$^+$ mTEC differentiation in adult mice (103). Thus, it is likely that CD4$^+$ thymocytes and invariant NKT cells cooperate to drive mTEC differentiation (**Figure 3B**). Although CD4$^+$ thymocytes play a dominant role in mTEC differentiation, it remained to be determined whether they influence this differentiation process via the release of soluble mediators or by directly engaging in physical interactions with mTECs. The generation of transgenic mice lacking MHCII molecules specifically in mTECs has shown that TCR–MHCII-mediated contacts between the two cell types are required for normal mature mTEC cellularity (100). Furthermore, mTEC differentiation occurs only when CD4$^+$ thymocytes recognize their cognate antigen on mTECs (96, 100). Taken together, these findings revealed distinct molecular and cellular mechanisms that sustain the generation of mTECs that display a mature phenotype in the embryonic and post-natal thymus.

Three-Dimensional Organization of the Thymic Medulla

The 3D reconstruction of wild-type thymic lobes has revealed that the medulla is highly complex, consisting of a major central compartment surrounded by ~100 islets (**Figure 3C**) (104–106). Interestingly, individual medullary islets initially derive from a single progenitor (107). Thus, during thymic development, some growing mTEC islets likely fuse together, leading to the emergence of larger islets and ultimately to a major central compartment. Additional studies of the 3D organization of the thymic lobes during thymus development from fetal to adult stage would be extremely informative to further understand the formation of the medullary architecture. Similar studies performed during aging should also reveal fundamental mechanisms of thymic involution. The recent development of multicolor fate mapping systems based on Cre-lox technology are expected to unravel the dynamics of

the development and remodeling of the medulla during a lifetime (108). Importantly, such transgenic systems should aid in determining whether individual medullary islets are indeed derived from a single progenitor or, alternatively, whether they are derived from several clones. The discovery of the co-existence of a major medullary compartment and a hundred distinct smaller islets with a broad volume distribution raises questions regarding the functional relevance of individual islets compared to the central medulla. We estimate that individual islets may contain from only a few to as many as several thousand cells, with an average of a few tens or a few hundreds of cells (unpublished observations). Thus, for the smallest islets, it remains to be determined whether this low number of cells expresses a TRA array large enough to induce T-cell tolerance or, in contrast, whether these few cells do not express sufficient TRA and may permit the escape of potentially hazardous autoreactive T cells. It would be interesting to determine whether the large medulla and small individual islets display similar sets of TRAs. In addition, to be functional, small medullary islets must be vascularized, which remains to be further investigated using 3D reconstruction (106). Thus, further investigation is required to improve our understanding of the functional implications of the medullary topology. Of note, the medulla is not smooth at all, but on the contrary exhibits a highly folded/convoluted shape, with a complex contour at any scale, ranging from the total structure to the cellular level. Such multi-scale complexity is described best by fractal geometry, which affords a high area of interface for a given volume (105). Such characteristics are also typically found in the lungs or intestinal microvilli, which have a large surface area to maximize the exchange of oxygen or nutrients, respectively. In the case of the thymic medulla, this fractal shape ensures a large interface area between the cortex and the medulla, which is referred to as the CMJ. This fractal geometry also ensures that the average distance from any location within the cortex to the nearest medulla remains reasonably low (105). By comparison, the distances from any location within the cortex to the nearest medullary location are significantly reduced compared with the distances that would be obtained for the simplest shape, i.e., a spherical medulla. The CMJ likely plays a critical role in the function of the thymus because it constitutes the site where T cells go through at three critical steps of their journey through the thymus: (i) T-cell progenitors enter the thymus from venules preferentially located at the CMJ, travel outward in the cortex and subsequently migrate inward from the cortex to the medulla, undergoing positive selection; (ii) they cross the CMJ and migrate through the medulla, undergoing negative selection; (iii) they ultimately leave the thymus and enter the periphery, again via venules located at the CMJ (109–111). Indeed, the CMJ exhibits a high density of large venules, representing a privileged site for thymocyte homing/export, by extra/intravasation through venule walls, respectively (**Figure 3C**). Remarkably, the CMJ is also particularly dense in Aire$^+$ mTECs, which is expected to favor the encounter with SP thymocytes that are migrating from the cortex to the medulla (93, 105). This distribution is strikingly pronounced in neonates compared to adults. This observation is consistent with the finding that Aire is important during the perinatal period to prevent the emergence of autoimmune disorders (112). Therefore, the CMJ represents not only a privileged site of T-cell progenitor homing and export of mature T cells but also a privileged region that favors the encounter of SP thymocytes with Aire$^+$ mTECs. A first wave of negative selection is thus expected to occur in this region, which could play a more important role in the induction of T-cell tolerance than previously thought.

Cellular and Molecular Crosstalk in Medulla Organization

Alterations in the cortico-medullary migration of SP thymocytes result in marked defects in the medullary organization. This is well illustrated in mice that lack CCR7 expressed by SP thymocytes or its two ligands CCL19 and CCL21 expressed by mTECs, which are responsible for the migration of SP thymocytes from the cortex to the medulla (113). CCR7- and CCR7 ligand-deficient mice show an arrest of thymocyte migration in the cortex and abnormal medulla formation characterized by small medullary regions that are sparsely distributed throughout their thymi (113, 114). The complex 3D organization of the medulla is preferentially controlled by positively selected CD4$^+$ thymocytes (105, 115). H2-Aa$^{-/-}$ mice lacking CD4$^+$ thymocytes are devoid of any large medullary compartment, leading to a reduced medullary volume. In these mice, the numbers of mTECs are severely reduced, affecting CD80^{hi}Aire$^-$ and CD80^{hi}Aire$^+$ subsets (96). However, the formation of the medulla is less severely affected in mice lacking CD4$^+$ thymocytes than in mice lacking SP thymocytes such as TCR$\alpha^{-/-}$ and ZAP70$^{-/-}$ mice. These observations suggest that other cell type(s) participate in the expansion of the medulla. Although invariant NKT cells have been implicated in Aire$^+$ mTEC differentiation, their role in the organization of the medulla remains to be defined. CD8$^+$ thymocytes seem to play a minor role compared with CD4$^+$ thymocytes because β2m$^{-/-}$ mice, which lack CD8$^+$ thymocytes, do not exhibit defects either in the 3D organization of the medulla or in the composition of the mTEC subset (96, 105). These observations suggest that CD4$^+$ thymocytes are prominently required for the development and 3D organization of the medulla by controlling mTEC cellularity. Furthermore, the organization of the medulla is also dependent on antigen-specific TCR–MHCII-mediated interactions between autoreactive CD4$^+$ thymocytes and mTECs displaying autoantigen–MHCII complexes (96). Several MHCII-restricted TCR-transgenic mice lacking expression of the cognate antigen, such as OTII-Rag2$^{-/-}$, B3K508-Rag1$^{-/-}$, and female Marilyn-Rag2$^{-/-}$ mice, show severe impairment in medulla formation. In contrast, this defect is restored when the cognate antigen is expressed by mTECs, as for example in OTII-Rag2$^{-/-}$ mice crossed with Rip-mOVA mice (in which the Rip-mOVA transgene drives the synthesis of membrane-bound OVA specifically in mTECs), or provided exogenously, as for example in OTII-Rag2$^{-/-}$ mice injected with OVA$_{323–339}$ peptide. Moreover, RTOC experiments in which OTII-Rag2$^{-/-}$ thymocytes are reaggregated in the presence or absence of OVA$_{323–339}$ peptide have demonstrated that the addition of the cognate antigen restores the numbers of mTECs similarly to those induced by WT thymocytes (96). These antigen-specific interactions between mTECs and CD4$^+$ thymocytes also require the engagement of the CD28–CD80/86 and CD40–CD40L costimulatory axes (**Figure 3B**). Defects in the CD28–CD80/86 or CD40–CD40L costimulatory pathway alone have a slight effect on the architecture of the medulla (105, 116). In contrast, the combined absence of CD28–CD80/86 and CD40–CD40L results

in a drastic impairment in medulla formation (116). These different experimental results thus favor a model in which autoreactive $CD4^+$ thymocytes control the formation and organization of the medulla in an antigen-dependent manner that involves the CD28–CD80/86 and CD40–CD40L costimulatory pathways. Interestingly, two-photon microscopy experiments have revealed that autoreactive thymocytes do not directly undergo cell death after encountering a negative selecting ligand but instead remain viable and motile for some time in the medullary microenvironment (51). They adopt a confined migration pattern during which they likely provide to mTECs instructive signals that would be necessary for both mTEC differentiation and organization.

A third member of the TNF superfamily, namely the lymphotoxin β receptor (LTβR) expressed by mTECs, and its ligand, the heterotrimer LTα1β2 expressed by SP thymocytes, was found to orchestrate the organization of the medulla (105, 117, 118). A deficiency in LTβR leads to a disorganized medullary architecture and alterations in mTEC subsets, notably in UEA-1^+ mTECs and terminally differentiated involucrin$^+$ mTECs (117–119). LTβR signaling also regulates the expression of Aire-independent TRAs and CCL19 in mTECs (120, 121). These defects are associated with the appearance of signs of autoimmunity, which suggests that LTβR signaling is required for the establishment of central tolerance (117, 120). Of note, mice that are deficient for LTβR ligands, such as LT$\alpha^{-/-}$, LT$\beta^{-/-}$, or LIGHT$^{-/-}$ mice, exhibit an intermediate phenotype compared with that observed in LTβR$^{-/-}$ mice. Consequently, the contribution of lymphotoxin signaling to mTEC development is only partially understood (117, 118). The 3D reconstruction of LT$\alpha^{-/-}$ thymic lobes has revealed that LT$\alpha^{-/-}$ mice are devoid of any large medullary compartment, leading to a substantial reduction of the medulla volume. Of note, the absence of LTα results in a less drastic phenotype compared with that observed in mice lacking $CD4^+$ thymocytes, which suggests that other(s) mediator(s) contribute to the effect mediated by $CD4^+$ T cells (105). Interestingly, the absence of Aire results in morphological changes in mTECs (26). However, it remains unclear whether Aire affects the 3D organization of the medulla in terms of the numbers and volumes of the medullary islets. Further investigations including the identification of other molecular participants in the topology of the medulla as well as the determination of the 3D distribution of specific mTEC subsets are required. Indeed, recent findings have revealed a differential distribution of mTEC subsets throughout the medulla. Aire$^+$ mTECs were found to be preferentially positioned at the CMJ, whereas post-Aire mTECs were described to be localized toward the center of the medulla (24, 93, 105). A 3D map of distinct mTEC subsets, including mTEC stem cells, may thus reveal a subtle compartmentalization of these specific cell types within the thymic medulla.

Importantly, this cellular crosstalk between mTECs and autoreactive $CD4^+$ thymocytes regulates a cascade of events that control the expression of TNF superfamily members that are essential for both the differentiation and organization of mTECs. In this context, autoantigen-specific interactions between mTECs and $CD4^+$ thymocytes, involving the CD40–CD40L and CD28–CD80/86 axes, lead to the upregulation of lymphotoxin ligands in autoreactive $CD4^+$ thymocytes (96, 116). Then, LTβR signaling induces RANK expression in mTECs (95, 96), and subsequently, RANK signaling induces the upregulation of CD40 in mTECs (101). This cellular crosstalk with autoreactive $CD4^+$ thymocytes is likely to fine-tune the homeostasis of the medulla, allowing the thymus to adapt optimally for the establishment of T-cell tolerance.

Concluding Remarks

Thymic crosstalk is the indispensable interplay between medullary APCs and developing T cells that coordinates the induction of T-cell tolerance. DCs, B cells, and mTECs have all been shown to control the selection of SP thymocytes. DCs reinforce the induction of T-cell tolerance by cross-presenting mTEC-derived TRAs and by displaying peripheral self-antigens captured in the periphery. Furthermore, thymic B cells can also express Aire and act as APCs. Nevertheless, mTECs are the lead player in T-cell tolerance induction due to their constitutive expression of TRAs. At the molecular and cellular levels, studies conducted over the last decade have furthered our understanding of the thymic crosstalk that sustains mTEC differentiation as well as the organization of the medulla. However, the precise consequences of thymic crosstalk on mTEC differentiation, proliferation, and survival remain to be defined. Additional studies are needed to identify the downstream target genes induced in mTECs by crosstalk signals in both the embryonic and the post-natal thymus. Future work can be expected to elucidate how thymic crosstalk shapes the T cell repertoire. Such studies would be extremely informative for further delineating the mechanisms that govern the establishment of T-cell tolerance. This knowledge is expected to pave the way toward novel therapeutic strategies aimed at preventing the development of autoimmunity and controlling age-associated thymic involution.

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Thymic B cells and central T cell tolerance

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Central T cell tolerance is believed to be mainly induced by thymic dendritic cells and medullary thymic epithelial cells. The thymus also harbors substantial numbers of B cells. These may arise though intrathymic B lymphopoiesis or immigration from the bloodstream. Importantly, and in contrast to resting "mainstream" B cells in the periphery, thymic B cells display elevated levels of MHC class II and constitutively express CD80. Arguably, their most unexpected feature is the expression of autoimmune regulator. These unique features of thymic B cells result from a licensing process that involves cross-talk with CD4 single-positive T cells and CD40 signaling. Together, these recent findings suggest that B cells play a more prominent role as thymic APCs than previously appreciated.

Keywords: central tolerance, B cells, antigen presentation, germinal center, CD40, class-switching, Aire

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Introduction

B cells represent approximately 0.3% of the thymic cellularity. Although their absolute number may, in fact, exceed that of thymic dendritic cells, their role a APCs for central tolerance induction is not well understood, and thymic B cells have often been regarded as "innocent bystanders." Recent data suggest that this view may need to be revised. Here, we will provide a short overview of novel insights into distinct features of thymic B cells and how these may predispose thymic B cells to support T cell tolerance.

The Origin of Thymic B Cells

Intrathymic B Cell Development

The early thymic progenitor (ETP), i.e., the cell type that gives rise to the T cell lineage, retains some B cell potential (1). Notch signaling is essential for T lineage specification in ETPs, so that precursors lacking Notch-1 fail to generate T lineage cells in the thymus. There are increased numbers of B cells in the thymus of conditional Notch-1 knockout mice (2), and this was interpreted to indicate that in the absence of Notch signaling, ETPs undergo B cell differentiation as a "default cell fate" (3, 4). However, subsequent experiments argued that most of the accumulation of thymic B cells under these conditions is not a cell intrinsic effect of Notch-deficiency, but may stem from immigration of peripheral B cells as a result of excessive niche availability (5). This notion is supported by the observation that perturbations in T cell differentiation downstream of the loss of B lineage potential result in effects on thymic B cell numbers that are reciprocal to their effects on T cell numbers. Specifically, TCR$\beta^{-/-}$ mice, in which T cell development is blocked prior to pre-TCR expression, harbor a drastically diminished overall T cell compartment (arrested at the DN3 stage), yet have a 10-fold increase in thymic B cell numbers, whereas TCR$\alpha^{-/-}$ mice, in which T cell differentiation is arrested at the DP stage, display slightly decreased numbers of B cells in the thymus (6, 7).

Does intrathymic B cell differentiation occur under non-perturbed *steady state* conditions? There were early reports on the existence of cells within the thymus whose phenotype – surface (s)IgM$^-$B220$^+$CD43$^+$ – resembled that of B cell progenitors in the bone marrow (BM). When these cells were purified and injected intrathymically (i.t.), they gave rise to mature B cells within the thymus (8). Akashi et al. estimated that concomitant to the release of about 1×10^6 T cells, the thymus also exports around 3×10^4 B cells each day (6). In sum, there is good evidence that part of the thymic B cell population arises through differentiation within the thymus.

Immigration of Peripheral B Cells

Using more conclusive surface marker combinations, we recently revisited the issue whether the thymus harbors significant numbers of B cell precursors (9). Among CD19$^+$IgM$^-$IgD$^-$ BM cells, pre- and pro-B cells are commonly identified as CD2$^+$c-Kit$^-$ and CD2$^-$c-Kit$^+$ cells, respectively. We found that around one-third of thymic CD19$^+$ cells were surface IgM$^-$IgD$^-$, and thereby resembled B cell precursors in the BM. However, pro-B cells (CD19$^+$IgM$^-$IgD$^-$CD2$^-$c-Kit$^+$) were essentially undetectable in the thymus. Moreover, most thymic CD19$^+$IgM$^-$IgD$^-$CD2$^+$c-Kit$^-$ cells expressed surface sIgG. Thus, the majority of CD19$^+$IgM$^-$IgD$^-$ cells in the thymus (unlike their phenotypic counterparts in the BM) are class-switched mature B cells and not B cell precursors. Based upon the paucity of B cell precursors in the thymus, we wondered whether peripheral B cells enter the thymus in the *steady state*. In order to address this, we intravenously injected bulk splenic B cells into syngeneic hosts. Seven days later, donor B cells were detectable in both spleen and thymus, whereby the relative abundance among host B cells in the thymus was about 5- to 10-fold lower as compared to the spleen. Although at first glance, this suggests that thymic immigration is a fairly efficient process, any comparison of its efficacy in relation to homing to the spleen is blurred by the fact that upon entering the thymus, B cells undergo several cell divisions. The capacity to enter the thymus does not seem to be restricted to any particular activation state, since purified naïve B cells (IgM$^+$IgD$^+$) also entered the thymus.

Taken together, it is reasonable to assume that both intrathymic B lymphopoesis and immigration of BM-derived B cells contribute to the thymic B cell pool. However, we lack a precise understanding of the relative contribution of either pathway. On the one hand, the virtual absence of pro- and pre-B cells may render intrathymic differentiation an unlikely source of the majority of thymic B cells. On the other hand, thymic B cells in parabiosed mice do not equilibrate to the same extent as is observed for splenic B cells, insinuating a substantial contribution of intrathymic B cell differentiation (10). Unraveling the lineage relation between peripheral "mainstream" B cells and thymic B cells remains experimentally challenging. Ultimately, this issue is linked to the questions whether thymic B cells display distinct features, and whether these features are manifestations of a hard-wired "thymic B lineage differentiation program" or result from extrinsic cues.

Intrathymic B Cell Licensing

The Unusual Phenotype of Thymic B Cells

Some phenotypic features (e.g., CD5 expression) had suggested that thymic B cells may be related to the fetal liver-derived B1 lineage (8). However, whereas *bona fide* B1 cells in the peritoneal cavity are restored only by reconstitution with fetal liver cells, but not BM cells, the thymic B cell pool is efficiently generated from both precursors (10). Thus, thymic B cells clearly are genealogically related to the B2 "mainstream" B cell lineage.

Unlike resting B cells in spleen and lymph node, thymic B cells express high levels of MHC class II and the co-stimulatory molecules CD80 and CD86 (9–11). Moreover, a substantial fraction of thymic B cells have class-switched, whereby the distribution of isotype classes is remarkably stereotypic from mouse to mouse. Perhaps the most unusual feature of thymic B cells is their expression of the autoimmune regulator (Aire) gene. Aire is known to be crucial for "promiscuous gene expression" (pGE) of peripheral self-antigens in medullary thymic epithelial cells (mTECs) (12). The only cell-type other than mTECs that had so far been reported to express Aire is rare cells in the lymph node which have been termed as extrathymic Aire expressing cells (eTACs) (13). eTACs are of hematopoietic origin, yet their exact lineage identity remains elusive (14). Using Aire-reporter mice, we noted a reporter-positive population of non-mTEC cells in the thymus and subsequently identified these cells as thymic B cells (9). Faithful expression of the Aire-reporter was confirmed by RT-PCR and intracellular protein staining. Aire protein was detectable in nuclear dots in around 2–3% of thymic B cells, whereby protein levels were substantially lower than in mTECs. A comparison of gene expression profiles in WT versus Aire$^{-/-}$ thymic B cells revealed that several hundred genes are differentially expressed. Very few of these had previously been reported to be Aire dependent in mTECs or eTACs, indicating that Aire's function as a transcriptional regulator is cell context dependent. Of note, whereas in mTECs the expression of several thousand genes is modulated by Aire, only a few hundred genes are controlled by Aire in thymic B cells or eTACs. Furthermore, it remains to be established whether Aire-dependent expression of any tissue-restricted antigen in thymic B cells is essential for T cell tolerance.

Are these distinctive features of thymic B cells an inherent feature of B cells that arise through intrathymic B lympopoiesis? To address this question, we followed the fate of i.v. injected IgM$^+$IgD$^+$ B cells, which are MHCII^{intermediate}, CD80$^-$ and Aire$^-$. Seven days after injection, donor cells in the spleen had retained their initial phenotype. In contrast, cells that had immigrated into the thymus recapitulated all features of *steady state* thymic B cells, indicating that the unique phenotype of thymic B cells is imprinted by extrinsic cues in the thymic microenvironment, and we referred to this microenvironmental programing as "thymic B cell licensing" (9).

Thymic B Cell Licensing Requires CD40

The transition from a MHCII^{int}CD80$^-$Aire$^-$ stage to a MHCII^{hi}CD80$^+$Aire$^+$ phenotype during thymic B cell licensing is strikingly reminiscent of mTEC "maturation." However,

whereas mTEC maturation is orchestrated by RANK signals (15–17), thymic B cell licensing crucially requires another TNF family member, CD40. Treatment of splenic B cells with agonist anti-CD40 antibody emulated B cell licensing, including induction of Aire (9). Thymic B cells in *Cd40*$^{-/-}$ and *Cd40lg*$^{-/-}$ mice are substantially diminished, consistent with a role of CD40 signals in thymic B cell homeostasis (7). Strikingly, the phenotype of thymic B cells in the absence of CD40 signaling is that of resting peripheral B cells. Thus, distinct signaling axes control the expression of Aire and the acquisition of a MHCII^{high}CD80^{+} phenotype in mTECs or thymic B cells.

Cross-Talk with Autoreactive CD4 T Cells Regulates Thymic B Cell Numbers and Licensing

Fujihara et al. showed that thymic B cell numbers are regulated by CD4 single-positive (SP) thymocytes (7). Similarly, we found that thymic B cells from TCR$\alpha^{-/-}$ mice, which lack SP thymocytes, express low levels of MHCII, are negative for co-stimulatory molecules, do not undergo class-switching and fail to up-regulate Aire (9). Strikingly, although CD4 SP thymocytes in "monoclonal" OT2 TCR transgenic mice on TCR$\alpha^{-/-}$ background express normal levels of CD40L, B cells in these mice are not licensed, indicating that a polyclonal repertoire of CD4 SP cells is necessary. The requirement for diverse TCRs seems to reflect a critical role of autoreactivity within the nascent CD4 SP compartment. Along these lines, B cell licensing can be mimicked *in vitro* when splenic B cells are pulsed with cognate antigen and co-cultured with specific CD4 SP cells. Moreover, MHCII-deficient B cells do not undergo licensing upon immigration into the thymus. Together, these observations suggest that cognate interactions through direct antigen presentation by B cells provide a platform for CD40 signaling and thereby initiate licensing (**Figure 1**). This tolerogenic feed-forward loop represents a striking parallel to the very similar cross-talk between mTECs and CD4 SP cells that is thought to bolster the tolerogenic features of mTECs (18).

Thymic B Cells and Central T Cell Tolerance

Evidence for a Non-Redundant Contribution of Thymic B Cells to Central Tolerance

Several studies have shown that thymic B cells can contribute to negative selection under particular experimental conditions. Forced expression of the I-E MHCII molecule exclusively on B

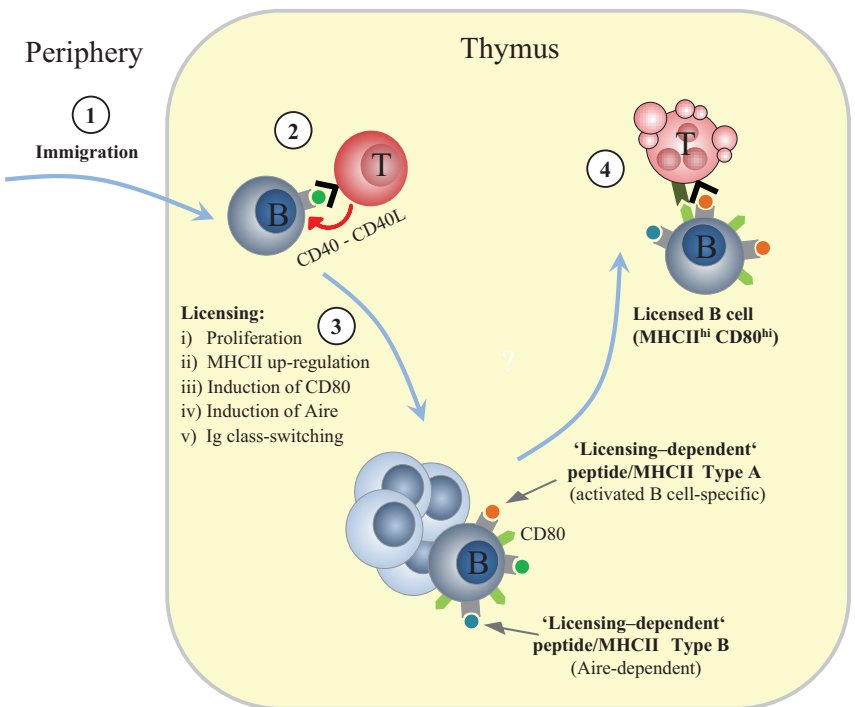

FIGURE 1 | Sequential phases of thymic B cell licensing. (1) Recirculating peripheral B cells can enter the thymus. Their peptide (p)MHCII ligandome is expected to mostly contain endogenously expressed "resting B cell autoantigens." Whereas the peripheral T cell repertoire is likely to be robustly tolerant toward these self-antigens, the nascent CD4 SP compartment is not (yet) fully purged of the respective specificities. (2) An unknown fraction of "B cell-reactive" CD4 SP cells within the diverse CD4 SP repertoire recognize "resting B cell autoantigens" and provide CD40 signals. CD40L is constitutively expressed by CD4 SP cells regardless of autoreactivity, presumably as a consequence of preceeding positively selecting interactions with cTECs. (3) Cross-talk with CD4 single-positive thymocytes induces proliferation, up-regulation of MHC class II, induction of CD80, induction of Aire, and Ig class-switching. (4) Licensed B cells delete autoreactive CD4 T cells, including TCR specificities that recognize "licensing-dependent" self-antigens; these are expected to include, but are not restricted to, self-antigens that are also up-regulated in activated B cells in the periphery (Type A) and Aire-dependent self-antigens (TRAs) (Type B).

cells led to deletion of superantigen reactive T cells (19). Myelin oligodendrocyte glycoprotein (MOG)-specific $CD4^+$ thymocytes were negatively selected when an epitope of MOG was exclusively presented by B cells (20). Perera et al. used autoreactive B cell receptor (BCR) transgenic mice to show that cognate T cells of the "same specificity" were negatively selected in the thymus (10). We showed that a "licensing-dependent" neo-antigen selectively up-regulated in immigrating B cells mediated negative selection through direct presentation (9).

What is known about the overall contribution of thymic B cells to central T cell tolerance? In mice lacking B cells, the size of the CD4SP cell compartment is significantly increased (9, 21). This resembles previous observations in mice that either lacked DCs or had a diminution of MHCII on mTECs, suggesting a non-redundant contribution of thymic B cells to negative selection of CD4 T cells (22, 23). Other recent reports showed that thymic Treg cells are decreased in B cell-deficient mice, and increased Treg numbers were observed in the thymus of Baff-transgenic mice harboring elevated B cell numbers (21, 24). Although these findings support a role of thymic B cell for central tolerance induction under physiological conditions, the exact spectrum of self-antigens that may require such a contribution remains to be characterized.

Do Thymic B Cells Pre-Empt the Self-Antigen Signature of Germinal Center B Cells?

The role of CD40 and the cognate interactions between B cells and CD4 T cells during thymic B cell licensing are reminiscent of the germinal center (GC) reaction (25). Indeed, a substantial fraction of thymic B cells display a Fas^+GL7^+ phenotype that is otherwise characteristic for GC B cells (9). It is therefore tempting to speculate that the tolerogenic potential of licensed thymic B cells might in particular comprise "activated-B-cell" autoantigens. Consistent with that idea, CD4 SP thymoctes from B cell-deficient mice are hyper-responsive to CD40-activated B cells (9). A central assumption of the GC paradigm is that CD4 T cell help needs to be tightly focused on epitopes of the foreign antigen that has been internalized via the BCR (26). In order to control BCR–hypermutation-related neo-autoreactivity among GC B cells, self-reactive B cells need to be deprived from cognate help. This not only requires robust CD4 T cell tolerance toward exogenously derived self-determinants that have been captured via the hypermutated BCR but also that the CD4 T cell repertoire is efficiently purged of reactivity toward any endogenously derived B cell autoantigen that is concurrently presented. Because thymic B cells may emulate the peptide/MHC composition of GC B cells in a tolerogenic setting, thymic B cell licensing may pre-empt T cell recognition of "activated B cell autoantigens" in an inflammatory context in secondary lymphoid tissues.

A Role for the BCR?

B cells efficiently present antigens that have been captured via the BCR (27). So, do BCR-specificity and/or -autoreactivity play a role in thymic B cell licensing and B cell-mediated central tolerance? Using BCR knock-in and T cell receptor transgenic mice specific for the same antigen, Perera et al. elegantly demonstrated that autoreactive B cells are particularly efficient APCs to induce negative selection of T cells with identical specificity (10). However, we found no evidence that BCR autoreactivity may favor B cell entry into the thymus or is a prerequisite to subsequently undergo licensing (9). Specifically, we employed SW_{HEL} mice to address these issues. In these mice, around two-thirds of peripheral B cells express a transgenic "anti-foreign BCR" specific for hen egg lysozyme (HEL), whereas the remainder of B cells carry endogenously rearranged BCRs, some of which may harbor autoreactivity. The relative abundance of HEL^+ B cells in the thymus exactly reflected their peripheral frequency, and both HEL^+ and HEL^- B cells underwent intrathymic licensing. Future work is needed to more conclusively address the composition of the thymic B cell repertoire, for instance, through BCR sequencing.

Open Questions

Is BCR Class-Switching in the Thymus Physiologically Relevant?

As a consequence of licensing, thymic B cells class-switch to IgG or IgA. It is therefore conceivable that thymic B cells present B lineage-specific "neo-epitopes" generated through isotype-class-switching or possibly also somatic hypermutation. In all likelihood, and in distinction from the GC reaction, class-switching in the thymus may occur "spontaneously," i.e., independent of cognate help downstream of BCR-mediated antigen capture. This has obvious implications for the emergence of "natural" Igs. Some thymic B cells display a memory B cell phenotype ($CD38^+Fas^{lo}$), and it is possible that IgG- or IgA-positive thymic B cells re-enter the blood stream. In fact, we observed a minute population of class-switched donor cell in the periphery after intrathymic injection of naive B cells (Tomoyoshi Yamano and Ludger Klein unpublished). More work is needed to clarify the role of the thymus as a potential source of natural Igs.

Do Thymic B Cells Shape the Thymic Microenvironment?

It was shown that thymic B cells express Ltα and Ltβ, and thereby regulate mTEC cellularity (28). Thymic B cells also express a variety of cytokines and chemokines, e.g., IL-10, IL-12, IL-16, and CCL22 (Tomoyoshi Yamano and Ludger Klein unpublished). These data suggest additional layers of cross-talk in the thymus. However, more work is needed to better understand whether and how thymic B cells may organize the thymic microenvironment.

Do Thymic B Cells Play a Role in Human Disease?

The presence of B cells with an "activated" phenotype in the healthy human thymus has long been recognized (29), but their origin and specificity has remained elusive. Ectopic GC-like structures are present in thymi of early-onset myasthenia gravis (MG) patients, and it was hypothesized that they are the site of auto-sensitization against the acetylcholine receptor (AChR) (30).

However, a recent characterization of antibody repertoires in MG thymi revealed expansion of a polyclonal repertoire unrelated to AChR specificities (31). Thus, it remains to be shown whether the accumulation of B cells in MG thymi is a cause or a consequence of disease.

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Regulatory T-cell development in the human thymus

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The thymus generates a lineage-committed subset of regulatory T-cells (Tregs), best identified by the expression of the transcription factor FOXP3. The development of thymus-derived Tregs is known to require high-avidity interaction with MHC-self peptides leading to the generation of self-reactive Tregs fundamental for the maintenance of self-tolerance. Notwithstanding their crucial role in the control of immune responses, human thymic Treg differentiation remains poorly understood. In this mini-review, we will focus on the developmental stages at which Treg lineage commitment occurs, and their spatial localization in the human thymus, reviewing the molecular requirements, including T-cell receptor and cytokine signaling, as well as the cellular interactions involved. An overview of the impact of described thymic defects on the Treg compartment will be provided, illustrating the importance of these *in vivo* models to investigate human Treg development.

Keywords: human thymus, regulatory T-cells, FOXP3, regulatory T-cell development, human thymic defects, primary immunodeficiency

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Introduction

Regulatory T-cells (Tregs) play a major role in immune homeostasis by preventing or limiting T-cell activation, particularly in the context of auto-antigens. Expression of the transcription factor forkhead box P3 (FOXP3), considered a master regulator of Treg development and function, is essential for their role in the maintenance of dominant tolerance [reviewed in Ref. (1)]. Conditions where FOXP3 is defective or absent, such as the recessive disorder immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome, are characterized by aggressive autoimmune manifestations that are usually fatal within the first 2 years of life, unless corrected via hematopoietic stem-cell transplantation (2–4).

Regulatory T-cells develop primarily in the thymus (thymus-derived Tregs, tTregs), although they can also be differentiated in the periphery (peripherally-induced Tregs). The delineation of these two populations in the peripheral Treg compartment is difficult due to the lack of specific markers. Nevertheless, tTregs are thought to be enriched in self-reactive T-cell receptors (TCRs) and to be critical for the maintenance of self-tolerance [reviewed in Ref. (1)]. Despite extensive research on tTreg development using murine models in the past 20 years, many questions remain unanswered regarding the mechanisms involved in the establishment and maintenance of the tTreg lineage [reviewed in Ref. (5, 6)]. Additionally, human tTreg studies are constrained by the limited number of tools available. Data have been mainly generated by *in vitro* manipulation of human thymic tissue or *in vivo* models using mouse/human chimeras [reviewed in Ref. (7)].

In this regard, the information provided by genetic human diseases characterized by thymus-related disturbances, such as the DiGeorge, the Omenn, and the autoimmune polyendocrinopathy candidiasis ectodermal dystrophy (APECED) syndromes, have been instrumental [reviewed in

Ref. (8, 9)]. DiGeorge syndrome features athymia in <1% of the patients, but in the majority of them the prevailing thymic hypoplasia is associated with mild to moderate T-cell lymphopenia, and increased incidence of infection and autoimmune diseases [reviewed in Ref. (10)]. Alterations in the circulating Treg compartment have been consistently reported in DiGeorge syndrome (11–13). Notably, a decreased proportion and number of $FOXP3^+$ cells in pediatric patients older than 2 years was observed when compared to age-matched healthy controls, in direct correlation with thymic output, as estimated by the numbers of $CD4^+$ recent thymic emigrants (RTEs, defined by the expression of CD31 and CD45RA) (12). Omenn syndrome results from hypormorphic mutations in severe combined immunodeficiency (SCID)-causing genes and is associated with the generation of a limited pool of T lymphocytes with a restricted repertoire and activated phenotype [reviewed in Ref. (8)]. Loss of corticomedullary junction and Hassall's bodies with depletion of autoimmune regulator gene (AIRE)-expressing medullary thymic epithelial cells (mTECs) and thymic dendritic cells (DCs) were described in two Omenn patients, in parallel with a dramatic decrease of Tregs in the thymus (14), supporting a role of AIRE-expressing mTEC and/or thymic DCs in their differentiation. In agreement, loss-of-function mutations in the *AIRE* gene (APECED syndrome) have been linked to a defective circulating Treg compartment (15–17). Not only is the frequency of naïve/resting Tregs (defined as $CD4^+FOXP3^+CD45RO^{neg}CD31^+$) decreased in APECED patients but also their levels of FOXP3 expression, function, and repertoire are altered, further supporting an abnormal tTreg development in the absence of AIRE (16).

A recent study that involved a paired analysis of thymic and blood samples in young children (newborns to 1-year-old) showed a direct correlation between the size of the two Treg compartments, further supporting the importance of the thymus for the establishment of the peripheral Treg pool early in life (18). Of note, both human and murine RTEs are endowed with enhanced potential to convert into peripherally-induced Tregs, when compared to their more mature counterparts, implying an additional role of the thymus for the setting of the peripheral Treg compartment (19). Furthermore, patients with athymia due to complete DiGeorge or FOXN1 deficiency have been shown to recover the peripheral Treg compartment upon allogeneic thymus transplantation, irrespective of the degree of HLA mismatching (20–22).

It is vitally important to understand human tTreg development in order to devise strategies to manipulate their generation as well as their repertoire (23). This mini-review will provide an overview of the current knowledge regarding human tTreg development, as well as the fundamental questions that remain to be addressed.

Commitment to the Treg Lineage: When?

The human thymic primordium is colonized by T-cell progenitors during the 8th week of gestation, but mature T-cells are only observed in the thymus at the 12th to 13th gestational weeks (24–26). At this stage, human tTregs can already be found in the thymus (27–29). The frequency of fetal human tTregs, identified by their elevated expression of the high-affinity IL-2 receptor alpha chain (IL-2Rα/CD25), was found to be stable throughout gestation (representing 6–7% of total thymocytes) and similar to the proportion observed in infant thymuses (28).

Fetal human tTregs already express FOXP3, as assessed at the gene expression level, as well as other markers related to their suppressive phenotype, such as cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) and glucocorticoid-induced TNFR-related protein (GITR) (27, 28). Moreover, these fetal human tTregs have the ability to suppress T-cell proliferation (27, 28).

One question that has been extensively addressed in mice, but due to technical limitations only in a few human studies, is when and how a thymocyte becomes committed to the Treg lineage. The discrete populations that express Treg markers, such as CD25 and FOXP3, in the human post-natal thymus mainly comprise mature CD4 single-positive (CD4SP, $CD4^+CD8^{neg}$) thymocytes, but also include CD8 single-positive (CD8SP, $CD4^{neg}CD8^+$) and double-positive (DP, $CD4^+CD8^+$) thymocytes, as well as cells in early pre-DP stages (27, 28, 30–36). In agreement, $FOXP3^+$/$CD25^+$ thymocytes can be found mostly in the medullary region of the human thymus, where mature thymocytes localize, with rare cells scattered in the cortex (30, 31, 35, 37, 38). The mechanisms that allow for human tTreg commitment to occur are still ill-defined.

We and others have reported pre-DP expression of FOXP3, namely, at the triple-negative and CD4 immature single-positive stages (33, 35) (**Table 1**). However, the contribution of this population to the human tTreg pool remains to be addressed.

Double-positive thymocytes expressing FOXP3 and/or CD25 are clearly identified in the human thymus. They additionally express other Treg function-associated markers, such as CTLA-4, CD39, and GITR (27, 28, 36), and exhibit suppressive function (32, 36) (**Table 1**). DP tTregs feature some degree of immaturity, as evidenced by the expression of recombination-activating gene 2 mRNA (34). Moreover, upon stripping of surface molecules using pronase, DP tTregs re-acquire both CD4 and CD8 at the surface, confirming their bona-fide DP status (36). Nevertheless, the majority of human DP tTregs express high levels of CD3 and CD27, which are associated with positive selection and maturity (27, 36) (**Table 1**). Importantly, DP tTregs are thought to significantly contribute to the CD4SP tTreg pool in humans, as predicted by linear regression models (36), and formally demonstrated by co-cultures of DP thymocytes with either TEC (36) or mature plasmacytoid (p)DCs (38). This observation contrasts with what has been described in murine models, where Foxp3 induction, although possible at the DP stage (45–47), mostly occurs at the CD4SP stage (48). Of note, human CD4SP $CD25^{neg}$ thymocytes are also permissive to FOXP3 acquisition (37, 39, 49).

CD4SP tTreg represent the major population of $FOXP3^+$ human thymocytes. They phenotypically mirror peripheral Tregs and exhibit efficient regulatory function (27, 28, 30, 36, 41, 43) (**Table 1**). The contribution of recirculating peripheral Tregs to this tTreg compartment is still debatable. It has long been proposed that activated T-cells may recirculate back to the thymus (50, 51), although this issue is particularly difficult to assess in humans. Recently, it was reported that a considerable proportion of human CD4SP tTregs may consist of recirculating

TABLE 1 | Characterization of human post-natal thymic Tregs.

Markers	Pre-DP	DP	CD4SP	CD8SP	Reference
FOXP3	+	+++	+++	++	(31, 33, 35, 36, 39, 40)
CD25	–	+++	+++	++	(30, 31, 33, 35, 36, 39, 41)
CTLA-4	+	+++	+++	++	(30, 31, 33, 36, 39, 41, 42)
CD127	–/+	+	–	–	(33–36)
HLA-DR	ND	++	+	+	(36)
CD39	ND	++	++	+	(36)
CD73	ND	–	–	+	(36)
CD103	ND	+	–	++	(36)
ICOS	ND	++	++	+	(39, 43, 44)
CD69	ND	++	++	+	(34, 36, 42)
CD27	ND	++	++	++	(36, 42)
Ki67	+	+	–/+	–/+	(36)
Suppressive capacity	ND	Yes	Yes	Yes	(30–32, 36, 39, 41)

ND, not determined.

cells (44). This was based on the observation that approximately one-fourth of these cells had lost CD31 and acquired ICOS and Tbet expression. Although it is not possible to exclude that a fraction of human tTregs may actually represent mature recirculating cells, there are some caveats to this interpretation. For instance, ICOS is already expressed by tTreg at the DP stage (39). Also, the high FOXP3 expression levels found within CD4SP ICOS^{+}CD31^{neg} tTregs (44) may reflect their interaction with ICOSL expressed on mTECs, as previously described (52). Moreover, TREC levels are reportedly comparable between the CD4SP CD25^{+} and CD25^{neg} thymocyte populations, and several logs higher that those found in circulating Tregs, supporting that the majority of CD4SP tTregs are at the final stage of T-cell development (53). Of note, Vbeta usage and spectratyping analyses supported that CD4SP tTregs and CD4SP FOXP3^{neg}/CD25^{neg} thymocytes have a similarly diverse repertoire (36, 53). To our knowledge, the direct comparison of the thymic CD4SP FOXP3^{+} and FOXP3^{neg} repertoires has not been reported. Its assessment will be important to clarify this issue since in peripheral cells the Treg repertoire has only 24% overlap with conventional CD4 T-cells (54). Additional studies will be instrumental in determining the magnitude and role of mature Treg recirculation in the human thymus.

Thus, tTreg lineage commitment may occur at various stages of human T-cell development.

Commitment to the Treg Lineage: How?

Studies in mice have clearly established the requirement for TCR stimulation in Treg lineage commitment [reviewed in Ref. (5, 6)]. In humans, technical limitations preclude a direct assessment of the role of TCR signaling in tTreg development. We and others have shown that human tTreg differentiation is associated with markers of positive selection, such as CD69 and CD27 (27, 28, 34, 36, 42) (**Table 1**). Moreover, binding sites for the TCR downstream targets NFAT and AP1 are present within the human *FOXP3* promoter that are directly activated by TCR stimulation (55). Notably, both DP and CD4SP tTreg express CTLA-4 (30, 36), a molecule that in mice was shown to be downstream of Nur77, an immediate early gene upregulated by TCR stimulation (56). Indirect evidence that enhanced TCR signaling strength may dictate thymocyte commitment into the Treg lineage can be further inferred by the increase in CD4SP CD25^{+}FOXP3^{+} thymocyte number in humanized mice treated with a superagonist anti-CD28 mAb (57). Moreover, human ZAP70-deficient patients present a dramatic decrease in the frequency and number of tTregs (58). Interestingly, CD4SP CD25^{+} tTregs were shown to frequently express two functional TCRs, in association with enhanced FOXP3 expression, suggesting that dual TCR expression may favor tTreg lineage commitment in humans (40). Overall, available evidence support that TCR signaling strength guides thymocyte commitment into the Treg lineage in humans, with a predicted impact on their self-reactivity.

Several additional signaling pathways and molecular factors have been implicated in human tTreg differentiation and/or proliferation, namely JAK3/STAT-5, Notch, CD80/CD86, ICOS/ICOSL, CD40/CD40L, thymic stromal lymphopoietin (TSLP), as well as the common-gamma chain (γC) cytokines interleukin (IL)-2 and -15 (37–39, 49, 52, 59). Watanabe et al. demonstrated that TSLP from Hassall's bodies activate myeloid (m)DCs, enabling them to induce tTreg differentiation from CD4SP CD25^{neg} thymocytes (37). pDCs are also capable of driving CD4SP non-regulatory thymocytes into the human Treg lineage, upon response to TSLP (49). Cognate interactions between mDCs or pDCs and CD4SP non-regulatory thymocytes are required, as differentiation was impaired by HLA-DR blockade (37, 49). Activation of pDCs with anti-CD40L and IL-3 also confers on them the capacity to differentiate post-selection DP CD69^{hi}TCR^{hi} thymocytes into human tTregs (38). Additionally, mTECs were shown to promote the survival and proliferation of human tTregs in an ICOSL-dependent mechanism that required the presence of conventional CD4SP cells as source of IL-2 (52). We have recently investigated the requirement of γC cytokines in human tTreg development and established a critical role for both IL-2 and IL-15 in their lineage commitment, as well as in tTreg proliferation and survival post-selection (39). This study also allowed the identification of macrophages and B lymphocytes as main IL-15 producers that likely represent two additional thymic antigen-presenting cell populations involved in human tTreg differentiation (39). In agreement, we found FOXP3^{+} cells in close vicinity of both macrophages and B cells in the human thymus (39). Accordingly, B lymphocytes were recently shown to be capable of selecting tTreg in mice (60, 61), and the majority of human CD4SP tTregs were shown to express CCR8 endowing them with the capacity to migrate in response to chemokines produced by macrophages (30).

As illustrated in **Figure 1**, the most immature thymocyte population that clearly expresses FOXP3, in addition to other Treg function-associated markers, such as CD25, CTLA-4, and CD39, and displays regulatory function is the cortical positively selected DP population (27, 28, 36). The thymic cellular populations and signals mediating their positive selection and concomitant recruitment to the Treg lineage may include cortical TECs and macrophages, as well as IL-2/IL-15, shown by immunohistochemistry to be expressed in the human thymic

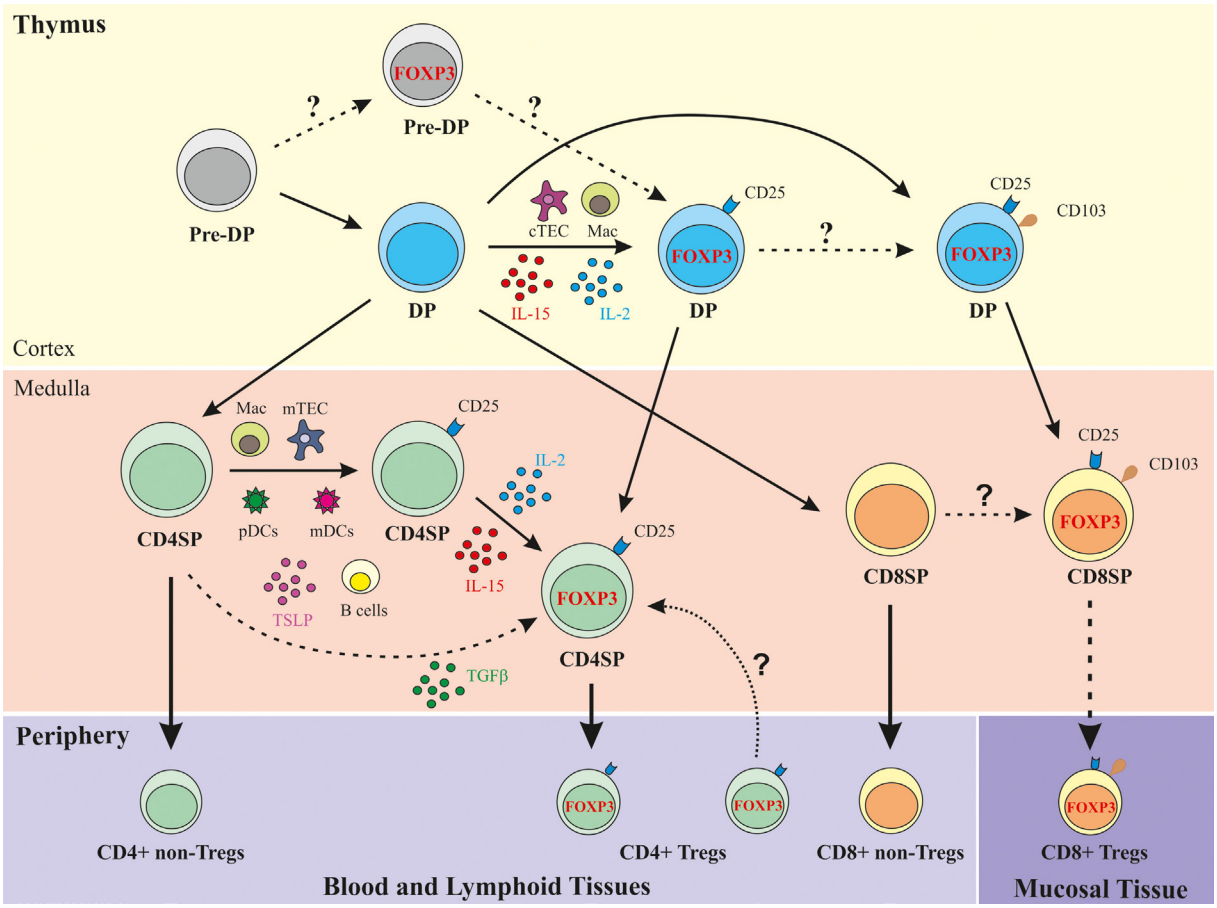

FIGURE 1 | Schematic representation of human Treg development in the human thymus. DP, double-positive ($CD4^+CD8^+$); CD4SP, CD4 single-positive ($CD4^+CD8^{neg}$); CD8SP, CD8 single-positive ($CD8^+CD4^{neg}$); cTEC, cortical thymic epithelial cell; mTEC, medullary TEC; Mac, macrophage; FOXP3, Forkhead box P3; TSLP, thymic stromal lymphopoietin.

cortex (39). The DP $FOXP3^+$ thymocyte compartment directly correlates with the CD4SP $FOXP3^+$ subset, denoting a precursor–product relationship (36). Upon interaction with activated pDCs, post-selection DP $FOXP3^{neg}$ cells may also differentiate into Tregs, in a costimulation-dependent manner (38). Medullary CD4SP $FOXP3^{neg}$ thymocytes can also acquire FOXP3 expression upon cognate interaction with activated pDCs or mDCs, in a costimulation- and IL-2-dependent fashion (37, 49). In addition, CD4SP $FOXP3^{neg}$ thymocytes may receive appropriate TCR and costimulation signals leading to CD25 acquisition and differentiation into tTreg precursors (CD4SP $CD25^+FOXP3^{neg}$ cells) (39). These precursors can differentiate into tTregs upon exposure to IL-2, produced mostly by cycling mature CD4SP thymocytes, or IL-15 secreted by macrophages, B lymphocytes, or mTECs (39). Whether concomitant TCR, costimulation, and γC cytokine signaling also commits medullary CD4SP $FOXP3^{neg}$ thymocytes into tTregs, is a possibility remaining to be addressed. Finally, CD4SP $CD25^{neg}FOXP3^{neg}$ thymocytes TCR-stimulated in the presence of costimulation, TGF-β and IL-2/IL-15 signaling can also acquire FOXP3 expression and differentiate into CD4SP tTreg (19, 39).

The Unique Properties of Human CD8SP tTregs

In addition to the CD4SP human tTregs, a CD8SP population with phenotypic and functional Treg characteristics is present in the human thymus. CD8SP human tTregs express several Treg-associated markers, such as FOXP3, CD25, CTLA-4, and GITR, although at lower levels than CD4SP tTregs (31, 36) (**Table 1**). Similarly to CD4SP tTregs, they express very low levels of the IL-7 Rα/CD127 molecule (36), and are able to suppress CD4SP $CD25^{neg}$ cells in a contact-dependent manner (31) (**Table 1**). A microRNA "signature" of CD8 Tregs has been recently defined in cord blood CD8 Treg (62).

Importantly, CD8SP human tTregs were found to express the α_E chain (CD103) of the $\alpha_E\beta_7$ integrin (36), a marker associated with CD4 Tregs in the periphery (63). CD103 was also expressed at the DP stage but was very low within CD4SP thymocytes, supporting a precursor–product relationship between $CD103^+$ DP and CD8SP human tTregs (36) (**Table 1**; **Figure 1**).

The role of CD103 expression on CD8SP $FOXP3^+$ thymocytes remains to be addressed. High expression of E-cadherin, the

main ligand of CD103, on mTEC (64) may promote retention of CD8 tTregs in the thymic medulla. Furthermore, in an *in vitro* cell adhesion assay, TEC-induced proliferation of CD8SP human thymocytes was inhibited by antibodies against E-cadherin or CD103, supporting a role for CD103 in CD8SP proliferation (64). Although the proliferation of CD8SP CD25$^+$ thymocytes was not addressed in this setting, the higher levels of the active cell-cycle marker Ki67 found within CD8SP FOXP3$^+$ as compared to CD8SP FOXP3^{neg} thymocytes (36) also support this hypothesis.

Despite the low frequency of CD8$^+$ Treg in the steady state, they may be of special relevance in pathologic conditions [reviewed in Ref. (65, 66)]. CD103 has been associated with T-cell migration to mucosal sites (67, 68). In agreement, in the setting of colorectal cancer, CD8$^+$FOXP3$^+$CD25$^+$ T-cells were significantly increased in peripheral blood and in colorectal cancer tissue, indicating that these cells may contribute to tumor immune escape and disease progression (69). In addition, studies in macaques have described a rapid expansion of CD8$^+$FOXP3$^+$CD25$^+$ Tregs in the blood and colorectal mucosa following pathogenic SIV infection (70). An increase in the frequency of CD8$^+$ Tregs has also been reported in HIV-1-infected patients (70).

In conclusion, a population of CD8$^+$ Tregs is generated in the human thymus. A subset of post-selection DP FOXP3$^+$ thymocytes expresses the tissue homing-associated molecule CD103, likely giving rise to the CD8SP FOXP3$^+$CD103$^+$ cells found in the medulla (**Figure 1**) (36). This finding supports the possibility that CD8$^+$ Tregs egress the thymus expressing markers associated with mucosal homing, which may explain their very low frequency in the blood.

Concluding Remarks

Cumulative evidence supports the existence of different pathways of Treg commitment in the human thymus that may occur at different stages of thymocyte differentiation. Their physiological contribution and the possible implications for the tTreg repertoire diversity remain unclear.

Thymus-derived Treg development does not seem to require a dedicated antigen-presenting cell population, as studies indicate that TECs, mDC, and pDC, as well as macrophages and B cells may be involved in tTreg selection. Finally, despite the proposed role of TCR signaling strength in human tTreg commitment, it has become increasingly clear that γC cytokines, particularly IL-2 and IL-15, are important mediators of lineage stabilization.

The thymus and specifically tTregs represent important therapeutic targets to manipulate tolerance in many clinical settings, namely autoimmune diseases, tumor immunity, and transplantation. Furthermore, their targeting is also critical to achieve full immunological reconstitution in primary and secondary immunodeficiencies and to decrease the morbidity associated with hematopoietic stem-cell transplantation. It is therefore of utmost importance to further investigate human tTreg development, in order to take full-advantage of the current development of immune-based therapies.

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The contribution of chemokines and migration to the induction of central tolerance in the thymus

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As T cells develop, they migrate throughout the thymus where they undergo essential bi-directional signaling with stromal cells in distinct thymic microenvironments. Immature thymocyte progenitors are located in the thymic cortex. Following T cell receptor expression and positive selection, thymocytes undergo a dramatic transition: they become rapidly motile and relocate to the thymic medulla. Antigen-presenting cells (APCs) within the cortex and medulla display peptides derived from a wide array of self-proteins, which promote thymocyte self-tolerance. If a thymocyte is auto-reactive against such antigens, it undergoes either negative selection, via apoptosis, or differentiation into the regulatory T cell lineage. This induction of central tolerance is critical for prevention of autoimmunity. Chemokines and adhesion molecules play an essential role in tolerance induction, as they promote migration of developing thymocytes through the different thymic microenvironments and enhance interactions with APCs displaying self-antigens. Herein, we review the contribution of chemokines and other regulators of thymocyte localization and motility to T cell development, with a focus on their contribution to the induction of central tolerance.

Keywords: thymus, negative selection, central tolerance, chemokine receptors, thymocyte migration

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Introduction: Coordination of T Cell Development with Intrathymic Localization

Thymocytes migrate through distinct thymic microenvironments at discrete stages of differentiation in order to receive essential signals from surrounding stromal cells that govern further differentiation and selection (1, 2) (**Figure 1**). Early thymocyte progenitors (ETP) localize to the cortical side of the cortico-medullary junction (CMJ). As they commit to the T-lineage, thymocytes migrate into the mid-cortex, where they rearrange T cell receptor (TCR) β chain genes (3). Cells that successfully express TCRβ pass the β-selection checkpoint, and undergo proliferation and differentiation near the sub-capsule. Subsequent double positive (DP) thymocytes are localized throughout the cortex, where they rearrange TCRα chain genes. DP cells that receive weak TCR signals in the cortex undergo positive selection, promoting survival and differentiation of self-MHC-restricted single positive (SP) cells. SP thymocytes migrate into the medulla, where auto-reactive cells receiving strong TCR signals are culled from the repertoire or diverted into the regulatory T cell (Treg) lineage. We will review migratory and adhesion cues governing localization and cellular interactions of differentiating thymocytes and stromal cell subsets, with an emphasis on signals that promote central tolerance. Recent advances and open questions will be highlighted.

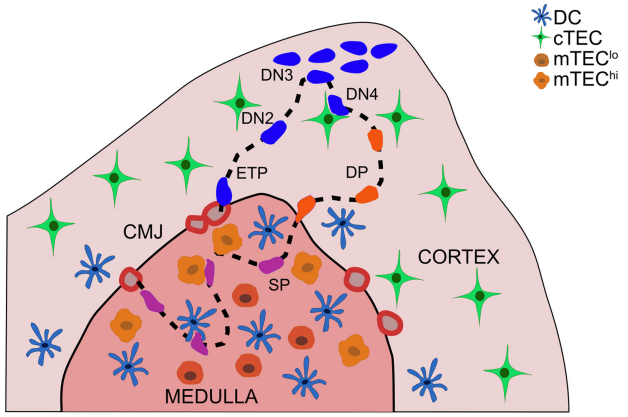

FIGURE 1 | Thymocyte migration through distinct thymic microenvironments occurs in an ordered fashion, enabling appropriate interactions with stromal cells. Thymocyte progenitors enter the thymus through vessels at the cortico-medullary junction (CMJ). ETPs ($CD3^-CD4^-CD8^-c\text{-}Kit^+CD44^+CD25^-$) integrate cTEC-derived signals in the cortex near the CMJ, which promote survival and T-lineage-commitment. DN2 ($CD3^-CD4^-CD8^-c\text{-}Kit^+CD44^+CD25^+$) thymocytes migrate into the mid-cortex, as they rearrange TCRβ chain genes. Subsequent DN3 ($CD3^-CD4^-CD8^-c\text{-}Kit^-CD44^-CD25^+$) thymocytes that pass the β-selection checkpoint proliferate at the sub-capsule, and differentiate through a DN4 ($CD3^-CD4^-CD8^-c\text{-}Kit^-CD44^-CD25^-$) stage to become DP ($CD4^+CD8^+$) thymocytes. DP cells, which rearrange TCRα chain genes, are localized throughout the cortex, with a bias toward the medulla. Interactions with cTECs induce positive selection of DP cells expressing TCRs with low avidity for self-peptide:MHCs. Auto-reactive DP thymocytes can be negatively selected in the cortex. Positively selected DP cells begin to migrate rapidly and enter the thymic medulla, guided by chemokine gradients, as they differentiate into SP thymocytes. SP cells rapidly scan mTECs and DCs during their 4–5-day residence time in the medulla to encounter a wide array of self-peptides, which induce auto-reactive cells to undergo apoptosis or diversion into the Treg lineage. Mature SP thymocytes egress from the thymus through blood vessels in the CMJ.

Migration and Stromal Interactions During Early Stages of Thymocyte Differentiation

Common lymphoid progenitors or their immediate progeny enter the thymus through vasculature at the CMJ (4), and subsequently give rise to developing T cells (5–7). Transmigration through the endothelium is initiated by selectin-mediated rolling (P-selectin), followed by firm adhesion via integrins (α4β1 and αLβ2) in concert with chemokine receptor signaling (CCR9, CCR7) (8–12). Within the thymus, cortical thymic epithelial cells (cTECs) provide IL7, SCF, and DLL4, which are indispensable for survival, differentiation, and T-lineage-commitment of thymocyte progenitors (13–15). ETP and double negative 2 (DN2) cells express CXCR4, which promotes chemotaxis toward cTEC-derived CXCL12 (16–20). Cortical thymocytes also express integrin α4β1, which binds VCAM-1 on cTECs. CXCR4 deficiency or impaired VCAM-1 adhesion inhibits thymocyte differentiation and migration from the CMJ to the mid-cortex (20–23). It remains to be determined how CCR7 promotes both thymic entry of progenitors into the cortex, and medullary accumulation of SP thymocytes (see below). As ETP do not express CCR7, rapid downregulation of CCR7 following thymic entry may enable cortical progenitor localization.

Migration and Stromal Interactions of Thymocytes Undergoing β-Selection

DN3 cells completing TCRβ rearrangements localize to the outer capsule (4). In addition to pre-TCR signals, activation of CXCR4 (24), NOTCH-1 (13, 25), and IL7R via cTEC ligands (1, 26) are required for differentiation and expansion at the β-selection checkpoint. The consequences of or signals governing sub-capsular localization of proliferating post-β selection cells remain to be elucidated (3). CCR9 is first expressed at the DN3 stage, and DN3 through DP thymocytes migrate toward CCL25, expressed by cTECs (17, 18, 27). Deficiency or overexpression of CCR9 prevents DN3 accumulation at the sub-capsule (12, 28, 29). However, a role for CCR9 in sub-capsular localization is hard to reconcile with the distribution of CCL25 throughout the cortex (30) or the CCR9-responsiveness of DP cells, which are also present throughout the cortex (17, 18). Moreover, we have shown that pre-positive selection DP thymocytes, which are CCR9 responsive, accumulate near the medulla, not the sub-capsule (31). Thus, signals governing DN3 accumulation at the sub-capsule remain to be identified.

Migratory Cues Governing Localization and Stromal Interactions of DP Thymocytes

We speculate that plexinD1 may promote rapid motility and peri-medullary accumulation of pre-positive selection DP cells (31). Sema3e, a soluble plexinD1 ligand produced in the medulla, inhibits CCR9-mediated chemotaxis, releases integrin α4β1 catch bonds, and is required for medullary localization of post-positive selection thymocytes (32–34). However, pre-positive selection DP cells also express plexinD1; thus, DP cells that reach the peri-medullary cortex, perhaps through random migration (35), would encounter Sema3e, potentially diminishing CCR9-mediated migration back into the cortex, and relaxing adhesion to VCAM-1 on cTECs, thus increasing motility. Recent studies demonstrate that GIT2, which modulates actin reorganization during cellular migration, also promotes rapid migration of cortical thymocytes (36). GIT2 and plexinD1 may coordinately enhance the ability of DP cells to efficiently scan cTECs for positively selecting ligands, which is consistent with the impaired positive selection in *Git2*$^{-/-}$ mice (36). Future studies may resolve the roles of plexinD1 and GIT2 in localization, migration, and positive selection of pre-positive selection DP thymocytes.

Migratory Cues and APCs Governing Cortical Negative Selection

Although the medulla is a critical environment for negative selection, there is mounting evidence that the cortex promotes deletion of a significant number of auto-reactive thymocytes. Thymocytes undergoing negative selection were recently quantified using *Bim*$^{-/-}$; Nur77^{GFP} mice (37), in which apoptotic cells survive due to deficiency in the Bcl2 family

member Bim, and GFP levels reflect TCR signal strength, enabling quantification of cells that should have been deleted due to strong TCR signaling. In the absence of Bim, GFP^+ DP and GFP^+ SP cellularity was increased, demonstrating that negative selection occurs in both compartments. Interestingly, the increase in GFP^+ DP cells was up to threefold higher than GFP^+ SPs (37), suggesting that over 90% of positively selected DP thymocytes are fated for cortical deletion (38). Another study analyzed Helios levels in *Bim*$^{-/-}$ mice to estimate that 55% of TCR-signaled thymocytes are deleted at the DP stage (39). Together, these studies indicate that the majority of negative selection occurs in DP cells, raising the question of which cortical antigen-presenting cells (APCs) promote central tolerance.

Cortical thymic epithelial cells are uniquely responsible for inducing positive selection (40); however, their role in negative selection remains ambiguous. Early studies established that thymic grafts transplanted into allogeneic athymic hosts were tolerated by host-derived T cells (41–45). Developing T cells are likely tolerized to graft-derived peptide:MHCs expressed by medullary TECs (mTECs) or DCs, which does not clarify whether cTECs induce negative selection. To address this, transgenic mice were developed in which MHC-I (46) or MHC-II (47) was expressed exclusively on cTECs. cTECs in these mice induced positive selection of CD8SP or CD4SP thymocytes, respectively, but could not tolerize polyclonal thymocytes. In light of the essential contribution of mTECs to negative selection against diverse self-antigens (see below), these findings do not resolve whether cTECs induce deletion of some auto-reactive clones. Expression of model antigens uniquely in cTECs resulted in deletion of TCR transgenic thymocytes, indicating that cTECs can mediate negative selection (48). However, when a TCR transgene was more faithfully expressed at the later DP stage in the HY^{CD4} model, cTECs expressing the cognate antigen induced TCR activation, but not apoptosis of auto-reactive DP cells (49). Thus, cTECs can clearly activate auto-reactive TCRs, but their ability to mediate deletion remains uncertain.

DCs have emerged as likely mediators of cortical negative selection. DCs express high levels of costimulatory and MHC molecules, enabling strong TCR activation (50). Strikingly, in a model of cortical negative selection, thymocytes undergoing apoptosis were localized adjacent to cortical DCs, and negative selection was impaired when DCs were conditionally ablated (49). The migratory cues that promote thymocyte:DC interactions during cortical negative selection have yet to be elucidated. Cortical DCs accumulate near vasculature, where CCR7 ligands are presented (30, 51, 52). Thymic DCs undergo CCR7-mediated chemotaxis (53), suggesting CCR7 may position DCs near cortical blood vessels. CCR7 was also postulated to induce cortical thymocytes to associate with DCs under positively selecting conditions (52). However, CCR7 is not up-regulated until the SP stage (54), when thymocytes home to the medulla, and CCR7 was dispensable for cortical deletion in the HY^{cd4} model (49). Thus, CCR7 signaling may position cortical DCs near vasculature, but is unlikely to promote thymocyte:DC interactions during cortical negative selection. CCR2 also contributes to cortical DC positioning, as it recruits migratory DCs to perivascular spaces in the cortex to induce deletion against blood-borne antigens (55, 56). CCX-CKR1 (CCRL1) regulates bioavailability of CCL19, CCL21, and CCL25, but its expression by cTECs and impact on tolerance are currently controversial (57, 58). Further investigation is needed to elucidate the contributions of APCs and migratory cues governing cortical negative selection.

Migration of Post-Positive Selection Thymocytes into the Medulla

The migration of post-positive selection thymocytes into the medulla is critical for the induction of central tolerance. If the medulla does not develop, or thymocytes cannot accumulate therein, negative selection is impaired, and autoimmunity arises (59–64). Only positively selected thymocytes gain access to the medulla (31); recent evidence suggests CXCR4 is responsible for cortical retention of DP cells (65). Following positive selection, thymocytes migrate much more rapidly (12–16 μm/min post-selection versus 6–8 μm/min pre-selection) and undergo chemotaxis toward the medulla (31, 66, 67). It is commonly assumed that thymocytes enter the medulla at the SP stage. However, plexinD1 deficiency results in relocalization of $CD69^+$ cells from the medulla into the cortex, suggesting post-positive selection $CD69^+$ DP cells may enter the medulla (32, 33). Furthermore, the kinetics of medullary entry after positive selection, compared to the timing of differentiation from the DP to SP stage indicates that $CD69^+$ DP cells enter the medulla (68). Thus, positive selection likely induces rapid thymocyte medullary entry; further studies are required to determine if and how $CD69^+$ DP cells overcome cortical retention to enter the medulla.

The chemokine receptor CCR7 is critical for thymocyte localization in the medulla (31, 51). CCR7 is expressed by SP thymocytes (51, 54, 69, 70), while the ligands CCL19 and CCL21 are expressed by mTECs (71). In mice deficient for CCR7 or its ligands, medullary accumulation of SP cells is diminished, negative selection is impaired, and autoimmunity ensues (59, 60). Although CCR7 is required for SP chemotaxis toward the medulla and accumulation therein, *Ccr7*$^{-/-}$ SP cells enter and migrate within the medulla (31). In contrast, SP medullary entry is abrogated by pertussis toxin (31, 69), which blocks signaling through $G\alpha_i$-associated G protein coupled receptors (GPCRs), including chemokine receptors. Thus, other GPCRs must contribute to thymocyte medullary localization. We speculate that CCR4 may contribute to medullary entry. CD69 + DP and CD69 + CD4SP thymocytes express CCR4 (54, 69) and undergo chemotaxis toward the ligands CCL17 and CCL22 (17), which are expressed in the medulla (18, 72). CCR4 is up-regulated early after positive selection, while CCR7 is expressed on more mature SP cells (54), suggesting differential roles in guiding thymocytes into the medulla. CCR4 may be responsible for initial medullary entry of post-positive selection cells, while CCR7 may promote retention of maturing SPs (**Figure 2**). Future studies are required to address the relative contributions of CCR4 and other GPCRs to medullary entry and central tolerance, though a recent study did not identify a role for CCR4 in these processes (54).

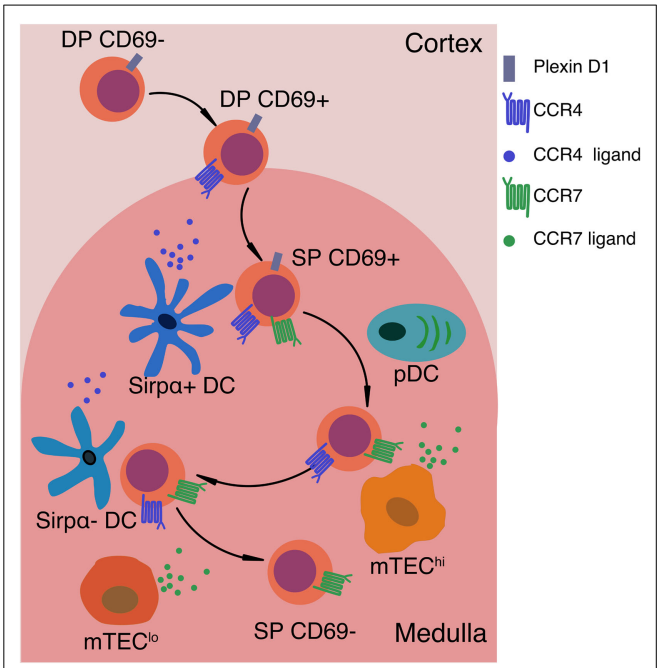

FIGURE 2 | Signals that impact motility and localization of positively selected thymocytes. After positive selection, thymocytes up-regulate CD69 and the chemokine receptor CCR4. CCR4 ligands are expressed by medullary DCs, thus creating a chemotactic gradient that may promote medullary entry of post-positive selection thymocytes. PlexinD1 is expressed on DP and immature SP thymocytes, and may also promote medullary entry by inhibiting responses to cortical chemokines and releasing thymocytes from tight interactions with cTECs. As SP cells mature, they up-regulate CCR7, promoting chemotaxis toward the gradient of CCR7 ligands produced by mTEC^{lo} (CD80^{lo}MHC-II^{lo}) and mTEC^{hi} (CD80^{+}MHC-II^{hi}) cells. CCR7 signaling is critical for maintaining SP thymocytes within the medulla. In the absence of CCR7, SP cells do not undergo efficient negative selection against TRAs. Expression of CCR7 and CCR4 on SP thymocytes may also promote chemokinesis, or rapid motility of SP thymocytes, as well as efficient interactions with the two main subsets of medullary APCs, mTEC^{hi} cells and DCs, respectively. Thus, chemokine-guided migration likely impacts multiple aspects of SP motility and cellular interactions that are required to ensure SP thymocytes efficiently scan numerous medullary APCs to encounter the full array of self-antigens that induce central tolerance.

APCs Governing Medullary Negative Selection and Treg Generation

Once SP thymocytes migrate into the medulla, they encounter heterogeneous APCs that enforce self-tolerance (**Figure 2**). Medullary APCs display peptides derived from a wide array of tissue-restricted antigens (TRAs), proteins otherwise expressed by peripheral tissues. mTEC^{hi} cells express high levels of CD80 and MHC-II, as well as the transcriptional regulator AIRE, which induces mTEC differentiation and expression of diverse TRAs that were previously epigenetically silenced (73–81). AIRE-dependent expression of such TRAs is essential for the induction of central tolerance in mice and humans (82–87). Medullary DCs also contribute to negative selection; they can be divided into intrathymically derived Sirpα^{-} conventional DC (cDC), migratory Sirpα^{+} cDC, and plasmacytoid DCs (pDC) (88, 89). Other APCs, such as B cells, may also contribute to negative selection (90–92), but are not discussed here.

Several experimental models indicate that mTECs can directly present peptide:MHCs to mediate negative selection and Treg induction. Negative selection against model self-antigens was intact following ablation of DCs or MHC-II expression on hematopoietic cells, demonstrating that mTECs can be sufficient to mediate negative selection (93, 94). Furthermore, miRNA-mediated reduction of MHC-II expression in mTECs resulted in diminished negative selection of TCR transgenic thymocytes to a model TRA, demonstrating that direct antigen presentation by mTECs is required for deletion in some cases (95). Direct presentation of TRAs by mTECs can also induce Treg differentiation (96). While endogenous proteins in mTECs will naturally access the MHC-I processing and presentation pathway, presentation on MHC-II is facilitated by macroautophagy, which is required for central tolerance (97). Thus, mTECs have an intrinsic capacity to present diverse self-antigens to mediate central tolerance of CD4SP and CD8SP cells.

DCs are also critical for thymic central tolerance. DC ablation in a CD11c-DTA model resulted in impaired negative selection and fatal autoimmunity (98). MHC-II ablation on hematopoietic cells impaired both Treg induction and negative selection against serum-borne and soluble TRAs (99–101). Sirpα^{+} cDC and pDC can acquire peripheral antigens and traffic them to the thymus to induce negative selection (102, 103). Also, in some models of mTEC-expressed TRAs, DCs isolated from the thymus stimulate TRA-specific T cells specific more efficiently than mTECs themselves, indicating that antigens are transferred efficiently from mTECs to DCs to mediate deletion (104). Transfer of model TRAs from mTECs to DCs can be AIRE-dependent and required for negative selection (99, 104). The mechanisms of antigen transfer between mTECs and DCs remain to be elucidated. mTECs may secrete or release antigen in vesicles; DCs may acquire antigen by endocytosis of apoptotic mTECs (105); or peptide:MHC complexes may be acquired by DCs from mTEC cell membranes (104, 106). Thus, the heterogeneous thymic DC compartment promotes central tolerance against peripheral, blood-borne, and mTEC-derived self-antigens.

While both mTECs and DCs induce tolerance to some antigens, their relative contributions to central tolerance of polyclonal thymocytes have been difficult to ascertain. Using TCR repertoire analysis of Treg and naïve T cells, Perry et al. recently compared the impact of restricting antigen presentation to DCs versus mTECs (107). mTECs and DCs mediated negative selection of non-overlapping TCRs, and DCs deleted about threefold more TCRs. These findings are in keeping with studies showing that both subsets are important for negative selection. Furthermore, both mTECs and DCs induced Treg differentiation. AIRE was critical for negative selection and Treg induction of lower frequency TCRs, and the Sirpα^{-} subset of cDC was required for AIRE-dependent Treg generation (107). Importantly, this study compared the effects of diminished MHC-II expression on mTECs with ablated MHC-II expression on DCs, and may thus underestimate the relative contribution of mTECs to central tolerance. Nonetheless, it is clear that complete central tolerance will require efficient thymocyte interactions with both mTECs and DCs.

Migratory Cues Promoting Medullary Central Tolerance

Given that DCs acquire TRAs from mTECs, it is likely DCs must localize near mTECs to mediate efficient central tolerance. Consistent with this, XCR1, which is expressed on Sirpα$^-$cDC, was required for localization of cDC to the center of the medulla (53). In *Xcl1*$^{-/-}$ mice, Treg cellularity was diminished, the TCR repertoire was altered, and autoimmune manifestations occurred, indicating that medullary localization of Sirpα$^-$cDC is required for central tolerance (53). This suggests a model in which XCR1 promotes direct apposition of Sirpα$^-$DCs with mTECs for TRA acquisition. Sirpα$^+$ cDC and pDC, which carry peripheral antigens into the medulla, migrate into the thymus through vasculature in a P-selectin, VLA4, and GPCR-dependent manner (103). CCR9 is required for thymic entry of pDC, but the corresponding GPCR for Sirpα$^+$ cDC has not been identified (102). Although thymic DCs express CCR7 and migrate toward CCR7 ligands, *Ccr7*$^{-/-}$ DCs localize properly within the medulla (53). Thus, signals required for medullary localization of Sirpα$^+$ DCs and pDCs remain to be identified.

SP thymocytes were recently estimated to have a medullary residence time of ~4–5 days (108), shorter than the previous estimate of ~12 days (109), and each AIRE-dependent TRA is expressed on only 1–3% of AIRE$^+$mTEC^{hi} cells (74, 110). Thus, thymocytes must rapidly scan multiple mTECs and DCs to encounter the full spectrum of medullary self-antigens that promote central tolerance. Chemokines can promote lymphocyte chemokinesis (111), and CCR7 has been shown to promote rapid motility of SP thymocytes (31). Fast SP migration is also dependent on MST1, which promotes integrin-mediated binding of SP thymocytes to ICAM1 in the context of CCL21 (112). It remains to be determined whether other chemokine signals are required for rapid motility of SP thymocytes.

It remains to be established whether interactions between thymocytes and medullary APCs are driven by chemotaxis toward APCs or random encounters due to fast SP motility. Several studies suggest chemokines may facilitate T cell:APC interactions in secondary lymphoid organs. Using microspheres releasing CCL19 and CCL21, a recent study demonstrated that when sources of CCR7 ligands were interspersed, T cells hopped between microspheres, potentially facilitating antigen sampling (113). Both CCR4 and CCR7 have been implicated in promoting T cell:APC interactions that drive naïve T cell activation (114, 115). Thus, CCR4 and CCR7 may also promote cellular interactions between SP cells and DCs and mTECs, respectively. Indeed, Mst1 was required for efficient interactions between SP cells undergoing negative selection and Aire$^+$mTECs expressing a model TRA (112), suggesting that CCR7 may enhance adhesion between SP cells and mTECs via intergrin:ICAM1 interactions. Furthermore, CCR7 deficiency was recently shown to result in increased Treg cellularity (54), which may also reflect the contribution of CCR7 to avid APC interactions. Although the basis for the decision to undergo apoptosis versus Treg specification is not resolved, current models favor an avidity model in which the highest avidity TCR signals promote negative selection, while a range of slightly lower avidity signals promote Treg induction as well as negative selection (116, 117). Thus, if CCR7 promotes T cell:APC interactions, CCR7 deficiency might result in lower avidity interactions that favor Treg induction. The fact that CCR7 ligands are expressed by mTECs, while CCR4 ligands are expressed by DCs also raises the possibility that CCR7 and CCR4 promote interactions with mTECs and DCs, respectively (**Figure 2**). Further investigation will be required to elucidate the contribution of chemokines or other adhesion molecules to interactions with medullary APCs driving central tolerance.

Areas for Future Investigation

Chemokine receptors and integrins promote migration and adhesion required for thymocyte:stromal interactions that drive T cell differentiation and selection. However, multiple localization and migration cues remain to be elucidated. We have not identified signals driving localization of DN3 thymocytes to the sub-capsule, accumulation of pre-selection DP cells near the medulla, or thymocyte:APC interactions during cortical negative selection. The identities of GPCRs other than CCR7 that promote medullary entry and APC interactions remain to be determined. We are just beginning to appreciate that localization of stromal cells is critical for thymocyte differentiation, and future studies will likely identify factors driving proper stromal organization. Thus, many open questions remain regarding the localization and adhesion cues that promote differentiation of a fully functional and self-tolerant T cell compartment.

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Differential expression of microRNAs in thymic epithelial cells from *Trypanosoma cruzi* acutely infected mice: putative role in thymic atrophy

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A common feature seen in acute infections is a severe atrophy of the thymus. This occurs in the murine model of acute Chagas disease. Moreover, in thymuses from *Trypanosoma cruzi* acutely infected mice, thymocytes exhibit an increase in the density of fibronectin and laminin integrin-type receptors, with an increase in migratory response *ex vivo*. Thymic epithelial cells (TEC) play a major role in the intrathymic T cell differentiation. To date, the consequences of molecular changes promoted by parasite infection upon thymus have not been elucidated. Considering the importance of microRNA for gene expression regulation, 85 microRNAs (mRNAs) were analyzed in TEC from *T. cruzi* acutely infected mice. The infection significantly modulated 29 miRNAs and modulation of 9 was also dependent whether TEC sorted out from the thymus exhibited cortical or medullary phenotype. *In silico* analysis revealed that these miRNAs may control target mRNAs known to be responsible for chemotaxis, cell adhesion, and cell death. Considering that we sorted TEC in the initial phase of thymocyte loss, it is conceivable that changes in TEC miRNA expression profile are functionally related to thymic atrophy, providing new clues to better understanding the mechanisms of the thymic involution seen in experimental Chagas disease.

Keywords: Chagas disease, thymus atrophy, thymic epithelial cell, microRNA, thymocyte migration

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Introduction

The thymus is a common target organ in infectious diseases (1). This primary lymphoid organ is responsible for bone marrow-derived T cell precursors differentiation from the most immature $CD4^-CD8^-$ phenotype to $CD4^+CD8^+$ and finally in $CD4^+CD8^-$ or $CD4^-CD8^+$ T cells that will colonize secondary lymphoid organs (2). These maturation steps occur while these cells migrate through the thymic lobules and interact with microenvironmental cells, particularly

Abbreviations: AIRE, autoimmune regulator; ECM, extracellular matrix; pGE, promiscuous gene expression; TEC, thymic epithelial cells.

thymic epithelial cells (TEC) (3). TEC guide the T cell maturation by production of cytokines, chemokines, hormones, adhesion molecules, extracellular matrix (ECM) proteins, and by expression of different ligands, like Notch, as well as self-peptides in the context of major histocompatibility complex (MHC). The self-peptide presentation determines T cell fate through positive and negative selection events, where immature lymphocytes expressing randomly rearranged T-cell receptor will be selected based on their differential ability to recognize the complex peptide/MHC (4–7). All intrathymic T cell maturation steps generate lineage committed and self-tolerant T cells capable to perform immunological functions in the periphery. However, the intrathymic homeostasis is disrupted in numerous acute infectious diseases leading to thymus atrophy (1). The transient thymic involution can be caused not only by infection but also due to other forms of stress and also occurs progressively with aging in a permanent way, as reviewed elsewhere (8). The biological advantages of thymic involution are currently uncertain, although there is evidence that thymic alterations triggered by *Trypanosoma cruzi* infection explain part of the clinical outcomes observed in chagasic patients (8, 9).

Chagas disease acute phase is characterized by apparent circulating parasites and tissue parasitism with intense production of reactive nitrogen intermediates, such as nitric oxide (NO) and cytokine release: interleukin (IL)-12, interferon (IFN) γ, tumor necrosis factor (TNF) α by macrophages, natural killer (NK) and T cells, with an activation pattern characterized by a polarized type-I response (10). However, there is also the production of anti-inflammatory cytokines, such as IL-4, IL-10, and transforming growth factor (TGF) β, that together with glucocorticoids (GC) control the immune response (11). Such response plays a role in containing parasite replication in acute phase and influences disease severity during the chronic phase of the infection (12).

Trypanosoma cruzi acute infection in mice causes a severe thymic atrophy, which becomes noticeable during early infection and increases progressively in parallel with parasitemia and pro-inflammatory cytokine levels (10). Additionally, even though *T. cruzi* infected cells can be found in the thymus (13, 14), current evidence demonstrates that the organ is mostly affected by systemic effects of the infection (15, 16). Actually, the parasite-associated response goes beyond the immune system with the activation of hypothalamus–pituitary–adrenal axis, resulting in hormonal imbalance that affects intrathymic homeostasis (9, 17). The neuroendocrineimmnune imbalance promotes a massive depletion of immature $CD4^+CD8^+$ T cells, which together with the export of these thymocytes to periphery, trigger thymic atrophy (16). Those intrathymic migratory abnormalities somehow benefit the immature thymocytes to bypass negative selection events, which reinforces the role of TEC in thymic atrophy, since in acutely *T. cruzi* infection, TEC enhanced the deposition of ECM, such as laminin and fibronectin, as well as chemokines, favoring developing T-cell migration (18–21). Nevertheless, the mechanism by which TEC mediate thymic involution remains poorly understood. microRNAs (miRNAs) can be envisioned as one group of candidates. miRNAs are small non-coding RNA molecules that suppress gene expression at the post-transcriptional level, and are fine-tuning regulators of diverse biological processes (22, 23).

In these respect, it has been shown that induction of thymic involution through poli(I:C) treatment is under tight control of miRNA-29a, which regulates interferon-α receptor (IFNαR1) in TEC, resulting in a very sensitive mechanism of thymic atrophy (24). In fact, TEC are programed to reduce functionality and suspend thymopoiesis in response to IFN-α (8). Recent studies suggest that miRNAs are important factors in the maintenance of tissue-restricted antigens expression in medullary TEC (7). Taken together, a molecular regulation of infection-associated thymic involution prompted us to analyze the expression of miRNAs in cortical and medullary TEC from *T. cruzi* acutely infected mice.

Materials and Methods

Experimental Acute *Trypanosoma cruzi* Infection

Male C57BL/6 mice were provided by the Oswaldo Cruz Foundation animal facilities (Rio de Janeiro, Brazil). Five weeks old mice were infected by intraperitoneal injection of 1×10^3 *T. cruzi* (Y strain) trypomastigotes. The parasites were maintained by serial passages in male mice from the same strain, harvested after 7 days post-infection (dpi) through cardiac puncture. The collected blood was harvested in vials containing 200 µl of sodium citrate, centrifuged (1,200 rpm) for 10 min, later the plasma was collected after incubating for 30 min in 37°C and centrifuged (3,000 rpm) during 10 min. The pellet containing parasites was resuspended and the trypomastigote concentration was estimated using Neubauer chamber in order to prepare a solution with 5,000 parasites/ml was prepared. Each mouse was infected with 200 µl of this solution. The uninfected (control) mice were kept under the same conditions through the infection progress.

Parasitemia was estimated for all infected animals by direct microscopic observation of 5 ml blood obtained from the tip of the tail. Initially, 10 mice were infected and the parasitemia was done on the following 6–18 days, once the parasitemia pick was determined, the estimation of circulating trypomastigotes was done solely 8 dpi to confirm that the infection was well succeeded.

All experiments and animal handling were conducted according to the rules prescribed by the official ethics committee for animal research of the Oswaldo Cruz Foundation.

Analysis of Thymocyte Subpopulations

Sixteen mice were infected as described above and their thymuses were harvested between 9 and 12 dpi, 4 thymuses plus 1 from control mouse per day (total of 16 acutely Chagas infected mice and 4 controls). The organs were individually squeezed in PBS containing fetal calf serum 10% (Gibco). For analysis of thymocyte subsets, cells were resuspended in mouse serum during 15 min and incubated with specific monoclonal antibodies for 30 min at 4°C in the dark (anti-CD4/APC, anti-CD8/FITC, from BD Pharmingen), followed by washing and analysis on flow cytometer FACS Canto II (BD Biosciences) and using the FACS Diva v6.1.3 software. In order to determine specific fluorescence intensity, the background staining values obtained with fluorochrome-matched

IgG isotype controls were subtracted. Thymocytes from 12 to 14 dpi also underwent these procedures, four infected and two control mice each time, in order to confirm that the infection led to thymic atrophy.

The variation of $CD4^+CD8^+$ cells due to infection progression was tested by one-way ANOVA, followed by the Tukey's honestly significant difference (HSD) *post hoc* test.

Thymic Epithelial Cell Sorting

Five thymuses from 12 days post-infection mice or control mice were used for TEC isolation procedure, which was performed as described (25) with some modifications. Briefly, thymuses were minced and transferred to round-bottom tubes and agitated in 50 ml of RPMI-1640 for 30 min for initial thymocytes release, after which the remaining tissue was digested with two sequential changes of collagenase/DNAse I solution [50 mg/ml collagenase D (Roche), 1 mg/ml DNAse I (Roche) in RPMI medium] at 37°C for 15 min each, followed by one collagenase/dispase/DNAse I [50 mg/ml collagenase/dispase (Roche), 1 mg/ml DNAse I in RPMI medium] at 37°C for 30 min under continuous stirring. Cells were then centrifuged, pooled, and resuspended in cold EDTA/FACS buffer (5 mM EDTA in PBS with 2% FCS and 0.02% NaN_3), filtered through 100 µm mesh and counted in Neubauer chamber. Then, anti-CD4 and anti-CD8 Dynabeads (Invitrogen) were added at 500 µl/10^8 cells according to the manufacturer's protocols. Remaining cells were then pooled and recovered by centrifugation, washed in EDTA/FACS buffer and 5×10^6 cells dispensed into the wells of a 96-well round-bottomed plate for staining. We incubated the biotinylated anti-mouse I-A[b] primary antibody (BD Pharmingen) for 30 min at 4°C, followed by a wash in 100 µl of EDTA/FACS buffer, after we added the secondary APC-Cy7-conjugated streptavidin and the conjugates: FITC-conjugated UEA-1 lectin (Vector), PerCP conjugated anti-CD45 antibody (clone 30-F11), PE-conjugated anti-Ly51 antibody (clone 6C3), APC-conjugated CD326 antibody (EpCAM, clone D8.8), all from BD Pharmingen. Cells isolated and stained as outlined above were resuspended in EDTA/FACS buffer at 1×10^6 cells/ml. Sorting was performed in a FACS Aria II cell sorter (BD Biosciences). Samples were collected in 50% (v/v) fetal calf serum in RPMI, recovered by centrifugation, counted and analyzed for purity.

RNA Extraction

The sorted population was submitted to RNA extraction using miRNEasy (Qiagen), which allows the isolation of small RNA (with miRNAs) and messenger RNA (mRNA) separately. To allow normalization of sample-to-sample variation in miRNA isolation, cDNA synthesis and real-time PCR, synthetic *Caenorhabditis elegans* miRNA cel-miR-39 (Qiagen) was added as 5 µl of 25 pmol solution to each denatured sample (i.e., after combining the sample with Qiazol) and quantified in all samples with an average recovery ranging from 26 to 36 in crossing point (CP) (Figure S1 in Supplementary Material). After this, we proceeded with other extraction steps following the manufacturer's instructions. The quantity and quality of RNA were assessed on NanoDrop ND-1000 Spectrophotometer (Thermo Scientific) and 2100 Bioanalyzer (Agilent Technologies) using the small RNA LabChip kit and RNA 6000 nano kit (Agilent Technologies).

AIRE Gene Expression by Quantitative Polymerase Chain Reaction

Gene expression for *AIRE* (Autoimmune regulator gene) and reference genes were carried out using 30 ng of total RNA with SuperScript III kit (Invitrogen) for reverse transcription reaction and FAST SYBR Green Master Mix (Applied Biosystems) and the following primers: *AIRE* (F-GGCAGGTGGGGATGGAATGC and R-TTCAGACGGAGCGTCTCCTGG), *HPRT* (F-TCCCAGC GTCGTGATTAGCGATG and R-GGCCACAATGTGATGGCC TCCC) and *RPL13* (F-CCAAGCAGGTACTTCTGGGCCGGAA and R-CAGTGCGCCAGAAAATGCGGC) for quantitative polymerase chain reaction (qPCR) on Step ONE Plus Fast Real Time PCR System (Applied Biosystems).

miRNA Expression Profiling

microRNA (30 ng) was submitted to reverse transcription by poly-A-tailing using RT^2 miRNA First Strand Kit (Qiagen) as described in the manufacturer's protocol. We then performed miRNA expression profiling using a custom PCR array plate with 85 miRNA (Qiagen) and RT^2 SYBR Green qPCR Mastermix (Qiagen) on Step ONE Plus Fast Real Time PCR System (Applied Biosystems). For normalization, we used all five references cel-miR-39, snoRNA142, snoRNA251, Rnu6, and snoRNA20 (Figure S1 in Supplementary Material) after gene expression stability analysis (26).

Quantitative PCR Analysis

The fluorescence accumulation data of real-time RT-PCR reactions of each sample were used to fit four parameters sigmoid curves to represent each amplification curve using the library qPCR (27) for the R statistical package version 3.1.2 (28). The cycle of quantification was determined for each amplification, by the maximum of the second derivative of the fitted sigmoid curve. The efficiency of each amplification reaction was calculated as the ratio between the fluorescence of the cycle of quantification and the fluorescence of the cycle immediately preceding that. The estimated efficiency of each miRNA or gene was obtained by the mean of the efficiencies calculated for each amplification reaction of that precise miRNA or gene. microRNA normalization among the different amplified samples was achieved by the calculation of normalization factors given by the geometric mean of the expression value of all expressed miRNAs in a given sample (26). *AIRE* normalization was done by the geometric mean of the expression value of *HPRT* and *RPL13* reference genes. The comparisons of means of normalized miRNA or AIRE expression values between groups were performed by a non-parametric one-way ANOVA with 1,000 unrestricted permutations, followed by *post hoc* pair-wise comparisons with Bonferroni adjustment by a non-parametric *t*-test also with 1,000 permutations (29). Additionally, false-positive ratios (FDR) were estimated to adjust for multiple comparisons (30). Results were represented in graphs displaying the expression levels mean ± SE. Two-tailed levels of significance $\leq$0.01, 0.05, and 0.1 were considered as "highly significant," "significant," and "suggestive," respectively.

Bioinformatics-Based Enrichment Analysis of miRNA Targets

To predict miRNA targets, we identified putative target genes based on predictions from five online softwares: *miRanda*[1], *Microcosm Target*[2], *miRNAMap*[3], *miRTarBase*[4], and *Target Scan*[5]. Any gene was considered a putative target if it was predicted in at least three out of the five predicting software. We then performed a gene set enrichment analysis (GSEA) with putative target genes. A gene set was defined as all putative target genes that share the same ontology based on the gene ontology (GO) database (31). The over representation was assessed with a statistical score based on a hypergeometric test with p-values $\leq$0.001. The Rgraphviz package[6] was used to illustrate the relationship between putative targets, miRNAs and biological processes, and in calculations of the k-core structures of the input networks using the degree as centrality measure. Graphs follow virtual physical models with low energy configuration, and only vectors containing the maximum core membership for each vertex, equal to 11 or greater, were displayed.

Results

Thymic Atrophy in *Trypanosoma cruzi* Acute Infection

Since the interaction between thymocytes and TEC play a major role in T-cell development, variations in TEC gene expression may alter the thymic environment with consequences on thymocyte fate (4). Accordingly, we analyzed miRNA profiles variation due to infection in the initial point of thymic atrophy to avoid secondary effects caused by thymocyte loss or consequent microenvironmental modifications. We used the decay of $CD4^+CD8^+$ thymocytes number to define when the thymus should be harvested. Intraperitoneal acute infection led to a parasite load picking at 8 dpi, and characterized by the high number of metacyclic trypomastigotes found circulating in the peripheral blood (**Figure 1A**). During the following days a decrease of $CD4^+CD8^+$ thymocytes was observed (**Figure 1B**) and later, a severe thymic atrophy with an average loss of 80% of $CD4^+CD8^+$ thymocytes was seen on the 14th dpi (**Figure 1C**). On the 12th dpi, this cell subpopulation was significantly reduced when compared with cell counting from control and 9–10 dpi mice (**Figure 1B**), preceding the thymic atrophy, thus we perform the following experiments using samples at this time point of infection.

Cortical and Medullary TEC Sorting

In order to prepare pure populations of primary (freshly harvested) cortical and medullary TEC (respectively cTEC and mTEC), thymuses from control and infected mice were harvested at 12 dpi and disaggregated by enzymatic digestion, where most thymocytes were eliminated and TEC were enriched, allowing cell sorting. Sorted population was then stained

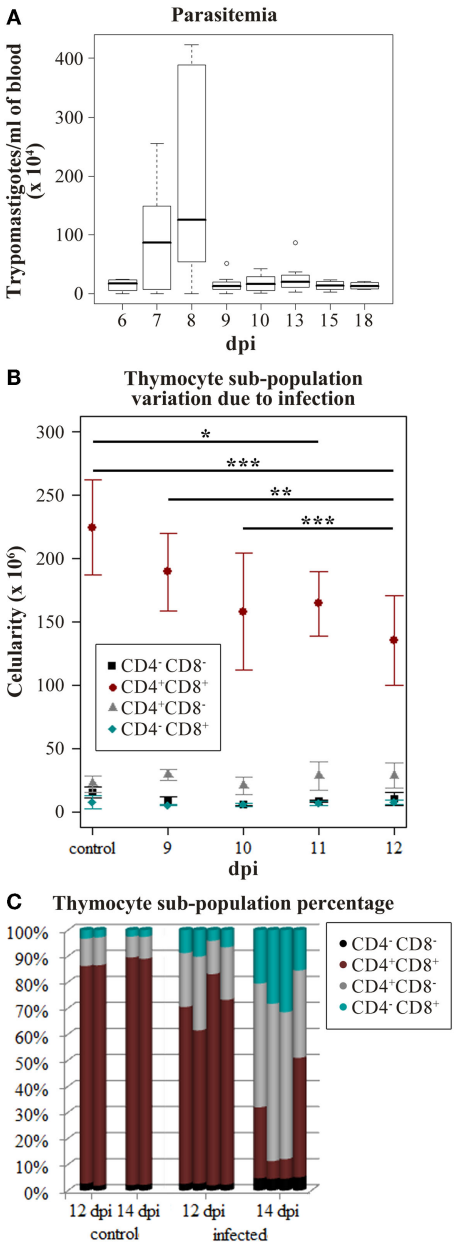

FIGURE 1 | *Trypanosoma cruzi* acute infection induces progressive thymic atrophy and $CD4^+CD8^+$ thymocyte loss. Nine C57BL/6 mice were infected with 1,000 metacyclic trypomastigotes and in the following 6–18 days the parasitemia was accessed to verify the infection progression. **(A)** The box plot shows the median amount of parasites per blood milliliter, upper and lower quartiles, maximum and minimum values (whiskers) excluding outliers (circles). Once the infection peak was identified (8 dpi), the thymocyte subpopulation variation in the following days was studied in order to identify when the $CD4^+CD8^+$ thymocyte loss starts. **(B)** The cytofluorometric profiles of thymocytes were obtained staining with anti-CD4 and anti-CD8. Thymocytes from three or four mice were submitted to FACS analysis on days 9–12 post-infection (dpi). The plots represent the mean number of cells ±SD in each day. The described decrease on $CD4^+CD8^+$ thymocyte subpopulation due to infection progression was confirmed (ANOVA p-value = 0.0002) and the Tukey's honestly significant difference (HSD) post-test revealed significant differences in each dpi. (adjusted p-value $\leq$*0.05, **0.01, and ***0.001) **(C)** Thymocyte subpopulation percentages data derived from the cytofluorometric profiles show that by 14 dpi the thymic atrophy is reached. Each bar represents thymocytes from a single mouse.

[1]http://www.mirbase.org/ftp.shtml
[2]http://www.microrna.org/microrna/getDownloads.do
[3]ftp://mirnamap.mbc.nctu.edu.tw/miRNAMap2/miRNA_Targets/Mus_musculus/
[4]http://mirtarbase.mbc.nctu.edu.tw/php/download.php
[5]http://www.targetscan.org/cgi-bin/targetscan/data_download.cgi?db=mmu_61
[6]http://www.bioconductor.org/packages/release/bioc/html/Rgraphviz.html

with Ulex europaeus Lectin 1 (UEA1) and antibodies against CD45, MHC-II, EpCAM, and Ly51 and sorted (**Figures 2A–D**). Post-sort analysis revealed more than 98% purity for cortical TEC ($CD45^-MHCII^+EpCAM^+Ly51^+UEA1^-$) and 95% for medullary TEC ($CD45^-MHCII^+EpCAM^+Ly51^-UEA1^+$) populations (**Figures 2E–F**, respectively).

To further validate the purity of sorted cell populations, we analyzed *AIRE* gene expression (**Figure 2G**), typical of medullary TEC. As expected, the *AIRE* expression was higher in medullary TEC populations, with the subpopulation classified as cTEC exhibiting average expression close to zero. In fact, the data from three independent sorting pointed out that, on average, *AIRE* relative expression on mTEC is 29.45 times higher than in cTEC. There was no significant difference between samples from control and infected condition ($p = 0.31$), indicating that the infection by itself does not affect *AIRE* levels, although we have detected a significant interaction effect. This result suggests that the difference on *AIRE* expression due to TEC phenotype varies if there is infection ($p = 0.04$). Actually, the detected levels in TEC from infected animals where 13.45 times lower than samples from control mice.

Changes in TEC-Derived microRNA Profiling in Response to Experimental Chagas Disease

We analyzed herein 85 miRNAs in order to approach putative molecular alterations in TEC following response to *T. cruzi* acute infection, and that might be related to the previously reported thymic atrophy and abnormal scape of immature thymocyte (20, 21, 32, 33). We found that 29 out of the 85 miRNAs were significantly differently expressed between TEC from infected and normal mice (adjusted $p \leq 0.05$), all were up-regulated (**Figure 3**) whereas differences in further 13 miRNAs were suggestive (Figure S3 in Supplementary Material).

We also detected significant interaction effect for 9 miRNAs (adjusted p-value ≤ 0.05 FDR corrected), where the response to the infection differed according to the TEC phenotype (**Figure 3**; Figure S2 in Supplementary Material), indicating that the increase rate of miRNA expression is higher in cortical TEC.

Additionally, seven miRNAs exhibited a consistent pattern of no amplification in TEC from infected animals (miR-144, miR-208b, miR-291b-3p, miR-295, miR-302a, miR-488, and miR-654-3p, Figure S4 in Supplementary Material). These miRNA can target genes involved with TGF-β signaling pathway (Palu et al., unpublished data).

Trypanosoma cruzi Acute Infection Increases Expression of miRNA known to Modulate Important Biological Processes

More than 60% of mammalian mRNAs are regulated by miRNA, whereas many can be targeted by more than one miRNA. Conversely, a single miRNA can have more than one target (34). Here, we identified miRNAs modulated in TEC due to *T. cruzi* infection, based on differential expression between infected and control mice.

To approach the putative roles of these miRNAs, we identified potential targets using available algorithms. Yet, these algorithms usually predict hundreds of potential target genes for a single miRNA and often generate false-positive candidates. In order to reduce such a high number of theoretical targets, and to make a more reliable prediction, we applied five different algorithms, and considered as potential targets only those genes predicted by at least three of these algorithms. The results from miRNAs predicted targets analysis were then combined with GO-term enrichment analysis to identified biological processes over represented among the list of target genes, so that to identify miRNA associated biological functions. Significant enrichment of predicted targets revealed cell adhesion, cell migration, and cell death among others biological processes (**Figure 4**).

Potential Network among miRNAs and Corresponding Predicted Targets

Given the lack of data regarding TEC molecular pathways during infection, we evaluated *in silico* potential interaction network between 29 differentially expressed miRNAs and the predicted targets related to cell death, cell migration, and cell adhesion (Table S1 in Supplementary Material). The complexity of the relationships is shown in **Figure 5**, where the elements shown were selected based on having the minimum of 11 relations. All 17 miRNAs have at least one putative target related to the negative regulation of extrinsic apoptotic signaling (GO:2001237), a process that was also related to 12 out of the 58 illustrated genes. Nevertheless, among the genes involved in cell death, only *Bcl2l11* was exclusively related to positive regulation of cell death. These miRNAs could be targeting *Serpine1*, *Tgfbr1*, *Vegfa*, *Igf1*, *Hgf*, *Snai2*, *Rffl*, *Map2k5*, *Itgav*, and *Sgms1* mRNAs, which are related to inhibition of apoptotic externals signals.

Many putative targets (32 out of 56) were related to cell migration, whereas 18 were associated to chemotaxis. More interesting, the presence of 21 targets associated with positive regulation of cell migration suggests that miRNAs could be inhibiting molecules that promote migration. Among the 17 targets associated to adhesion, 8 are known to be involved in positive regulation (GO:0045785) and cell-cell adhesion (GO:0016337). The increase in miRNA targeting genes that favor adhesion may explain some of the described alterations in *T. cruzi* acutely infected mice.

Discussion

The mechanisms by which TEC regulate thymic atrophy appear to be under the tight control of miRNAs (24). Here, we analyzed the miRNA expression in cortical and medullary TEC from *T. cruzi* acutely infected mice. Our results provide novel insights into the molecular regulation of TEC-associated thymic involution secondary to infection, using the experimental model of acute Chagas disease.

It has been previously shown that thymic involution, reduction on T cell output, increased susceptibility to autoimmune disease and loss of TEC numbers are associated with ablation of mature miRNAs (7). Although those are events similar to the ones observed in infected mice, it is noteworthy that the infection induced an upregulation of differentially expressed miRNAs in both cTEC and mTEC subsets, whereas in some cases the increase in expression was significantly higher only in cTEC, suggesting that the *T. cruzi* infection triggers different responses according to TEC phenotype.

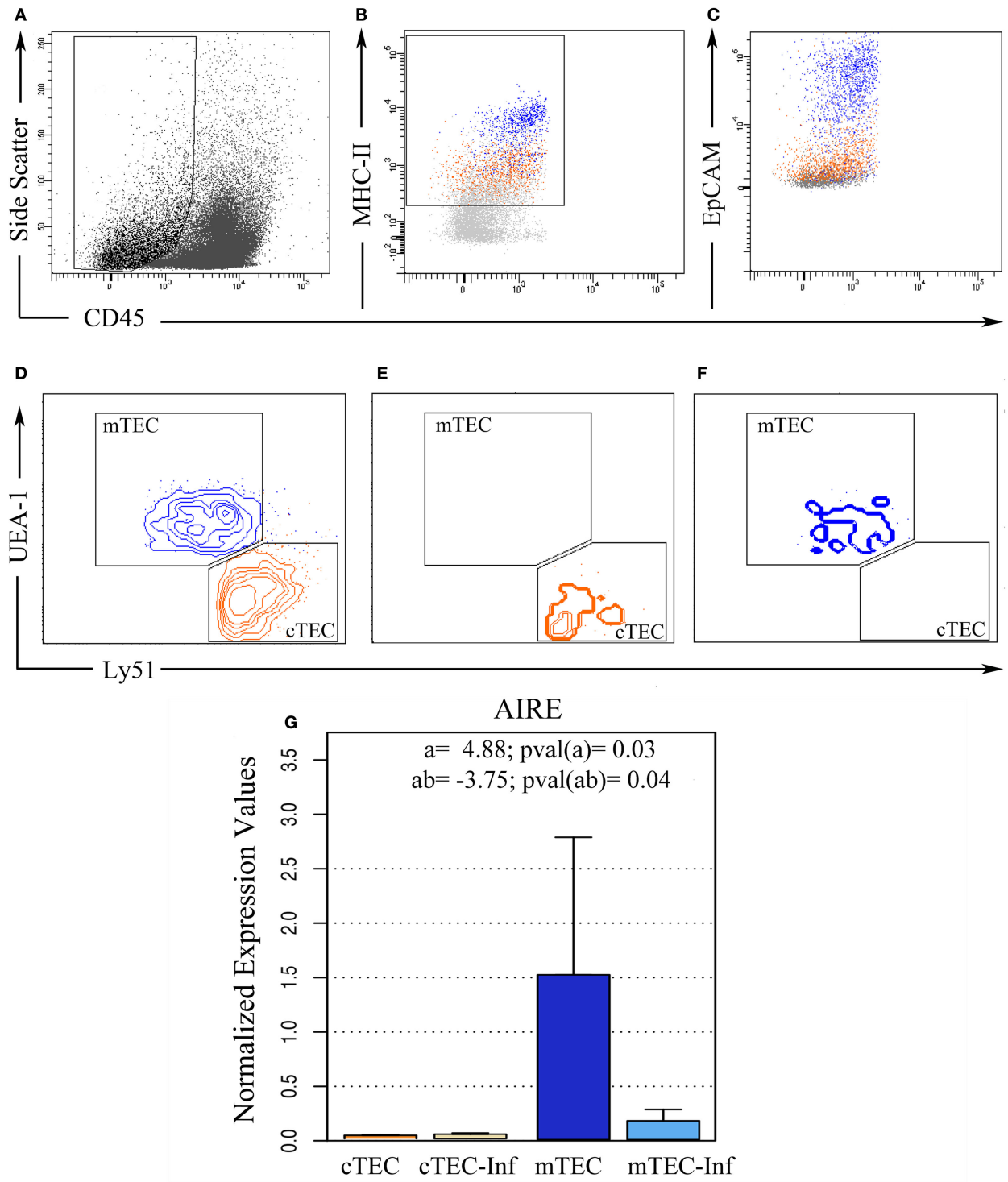

FIGURE 2 | *Ex vivo* thymic epithelial cell sorting. Five replicates of thymic cell pools from control and infected mice were sorted using flow cytometry in order to isolate TEC. **(A)** Initially, $CD45^-$ cells were selected based on size, **(B)** from this population, MHC-II positive cells were isolated after EpCAM confirmation **(C)**. **(D)** Then, according to UEA-1 and Ly51 surface markers, these cells were distinguished between cortical TEC (cTEC, $Ly51^+$, and UEA^-, orange) and medullary TEC (mTEC, $Ly51^-$, and UEA^+, blue) phenotypes. Post-sorting analysis revealed 98% purity in cTEC **(E)** while 95% purity in mTEC **(F)**. **(G)** After miRNA isolation, the remaining mRNA from three experimental pools allowed us to analyze *AIRE* gene expression, confirming if the sorted cells matched the correct expected TEC profile. The bar plot represents the average expression in each condition. "a" indicates the magnitude of the expression ratio ($\log^{-2}$) due to TEC phenotype, whereas a positive value shows a higher expression in mTEC. "ab" indicates the expression ratio magnitude (in $\log^{-2}$) as consequence of the combination between infection and cell type, whereas a negative value shows that the *AIRE* expression in infected mTEC is lower than in control mTEC.

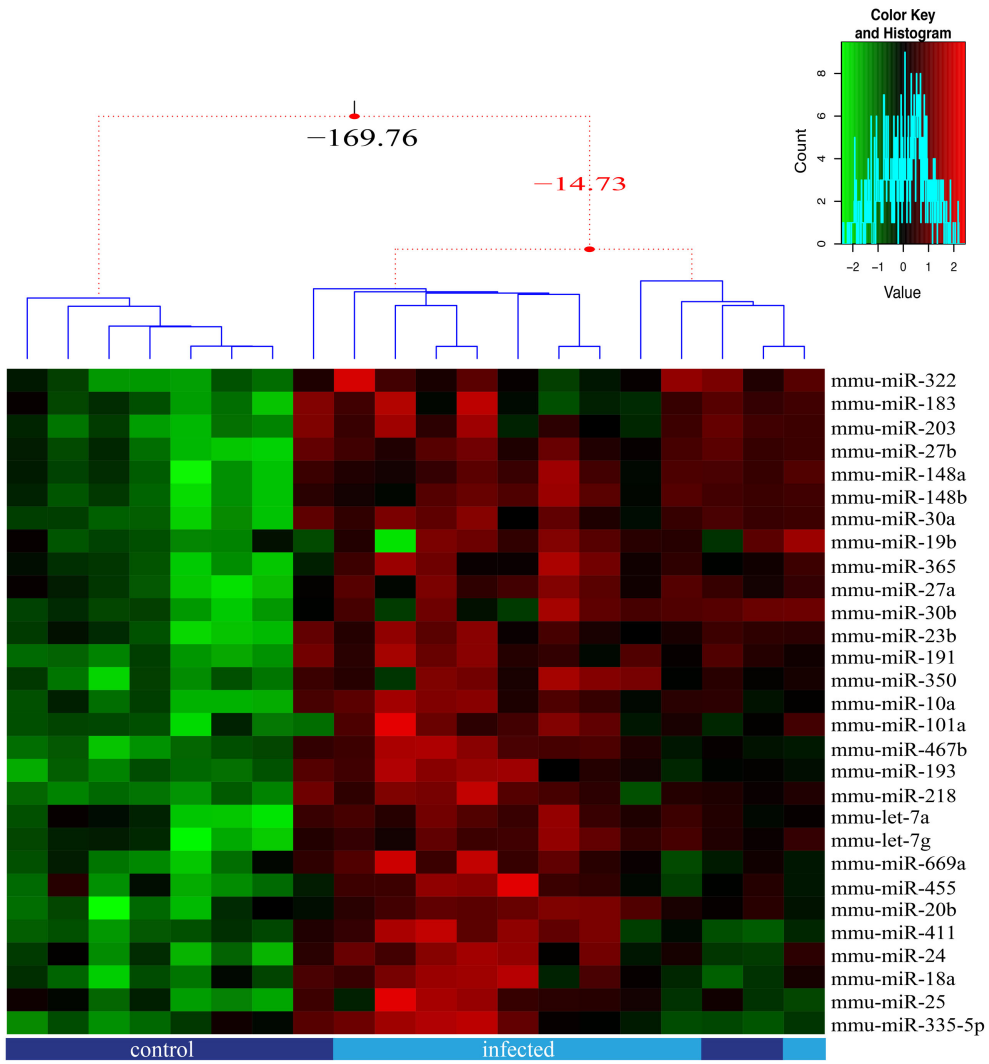

FIGURE 3 | miRNA relative expression in thymic epithelial cells from control and infected mice. The miRNA expression of 85 miRNAs was analyzed for each sorted TEC population, in five replicates, with samples from control (dark blue) and infected (light blue) mice being paired. We present the 29 miRNAs that were significant (adjusted *p*-value $\leq$0.05) differentially expressed due to infection and the samples clustered according to expression profile similarity. Three groups of samples were identified based on their expression profile, as illustrated by the dendrogram.

Among miRNAs significantly modulated due infection, miR-27a and miR-27b, also exhibited dependence whether TEC sorted out from the thymus exhibited cortical or medullary phenotype. Mature miR-27a and miR-27b differ from each other by just one nucleotide and are transcribed from paralog clusters, the intergenic miR-23a~27a~24 cluster (localized in chromosome 9q22) and the intronic miR-23b~27b~24 cluster (localized in chromosome 19p13) (35, 36). Yet, there is limited information regarding the functions of this cluster in infectious diseases. Herein, we found an upregulation of the miR-23b~27b~24 cluster, thus at variance with the findings observed in primary macrophages, which exhibit rapid decrease miR-27a and miR-27b expression upon murine cytomegalovirus infection (37). Nevertheless, the *Cryptosporidium parvum* infection, a protozoan parasite that infects the gastrointestinal epithelium, causes miR-27b upregulation that suppresses KH-type splicing regulatory protein and contributed to epithelial production of NO, helping the epithelial antimicrobial defense (38). In *T. cruzi* infection, the serum levels of NO increase both in mice and humans (9, 39), and high intracardiac contents of NO synthase and NO metabolites have been detected (40). Interestingly, *T. cruzi* can infect TEC and, although only small fractions of TEC are invaded (13, 14), the presence of the parasite may trigger NO production.

Regarding the 23b~27b~24 cluster upregulation, miR-24 can be highlighted. It is known that mRNA-target for a particular miRNA depends of cell context and this is the case of miR-24, which has been described in apoptosis and cell survival (41–44). In cardiomyocytes, miR-24 directly targets the proapoptotic protein Bim and inhibits apoptosis. Moreover, *in vivo* delivery after myocardial infarction suppressed cardiac cell death and rescued cardiac dysfunction (42). Yet, miR-24 function is complex since it enhanced survival in myeloid and B cell lines, as well

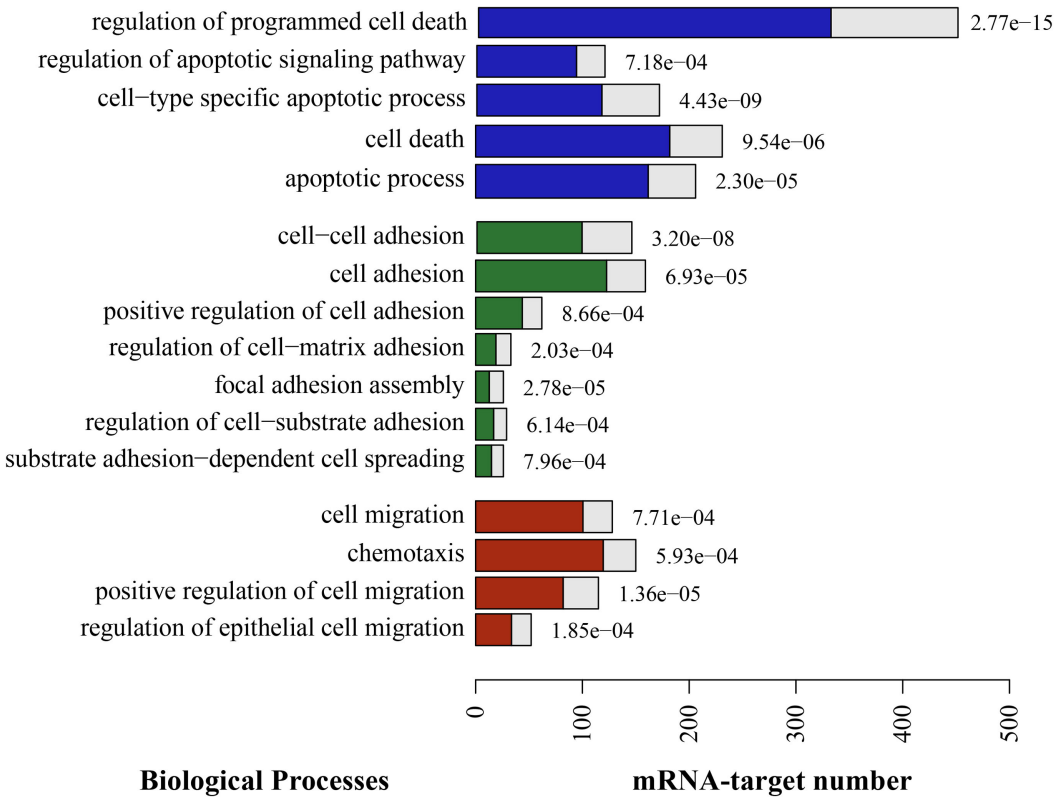

FIGURE 4 | Putative enriched biological processes modulated by TEC miRNA following *Trypanosoma cruzi* infection. The putative mRNA targets for the 29 miRNAs that were differentially expressed due to infection are consistently involved in a long list of biological processes. Here, we show the most relevant processes in the context of cell death (blue), adhesion (green), and migration (red). The full length of the bars represent the total putative target mRNAs known to be involved in this process, in opposition to the colored bar inside, that represents the expected mRNA number. On the right side of each bar, there is the adjusted *p*-value indicating that the enrichment of those events is not by chance.

as primary hematopoietic cells (44). Importantly, it has been shown that miR-24 expression differed in age-related thymic involution. When comparing young *versus* aged TEC (a mix of cTEC and mTEC) a decrease in miR-148b, miR-19b, miR-24, and miR-322 expression was seen in aging (45). Herein, using *T. cruzi*-induced thymic atrophy, we showed an upregulation of these miRNAs in TEC. Both infection and aging-induced thymic involution are due to multifactorial events; in the aging case, we can highlight the sex hormone dependence and the increase in adipose tissue, whereas in infection, the immune inflammatory response and stress-related hormones are undoubtedly relevant (16, 46, 47). Nevertheless, it has yet to be established the tuned regulation of this miRNA in the various thymic atrophy induced situations.

It is important to take into account that changes in miRNA profile seen here are in consequence of the thymic stress brought out by the infection with activation of the immune system and the hypothalamus–pituitary–adrenal axis. This stress results in hormonal imbalance (high levels of GC) that affects intrathymic homeostasis promoting thymic atrophy by a massive depletion of immature $CD4^+CD8^+$ T cells and the export of immature thymocytes to periphery (9, 16, 17). Under stress conditions, miRNA can act as restorer to homeostasis or as an enforcer of new gene expression program so that to adapt to the new condition (34). An example is the regulatory action exerted by miR-10a and miR-182 upon Th1- or Th2-associated T regulatory cells, respectively, where $CD4^+Foxp3^+$ cells orchestrate distinct miRNA pathways in response to local environmental factors (48). Furthermore, miR-10a expression is stimulated by TGF-β, making it a good example of how environmental factors coordinate distinct miRNA pathways and regulates cell fate. In fact, TGF-β seems to be a molecular node of the infection since the gene encoding its receptor appears in the middle of our microRNA network (**Figure 5**), where the gene for TGF-β2 is also present. TGF-β is able to regulate $CD4^-CD8^-$ development through direct interaction with thymocytes but also by binding to TEC surface (49). Although, it is unclear if the increased miR-10a in TEC from infected mice is part of a host response due TGF-β enhancement or if miR-10a is a fine-tuning factor in TEC, our results suggest that TGF-β signaling is a key pathway in the thymic involution process. More studies will be necessary to define TGF-β role in TEC, but it has been already shown that the inhibition of this pathway decelerates the process of age-related thymic involution (50), therefore suggesting a common pathway between thymic involution due to senescence and infection.

Interestingly, GC also regulated miRNAs (51, 52). The systemic stress induced by dexamethasone intraperitoneal injection, a synthetic GC causes a significant loss of the $CD4^+CD8^+$

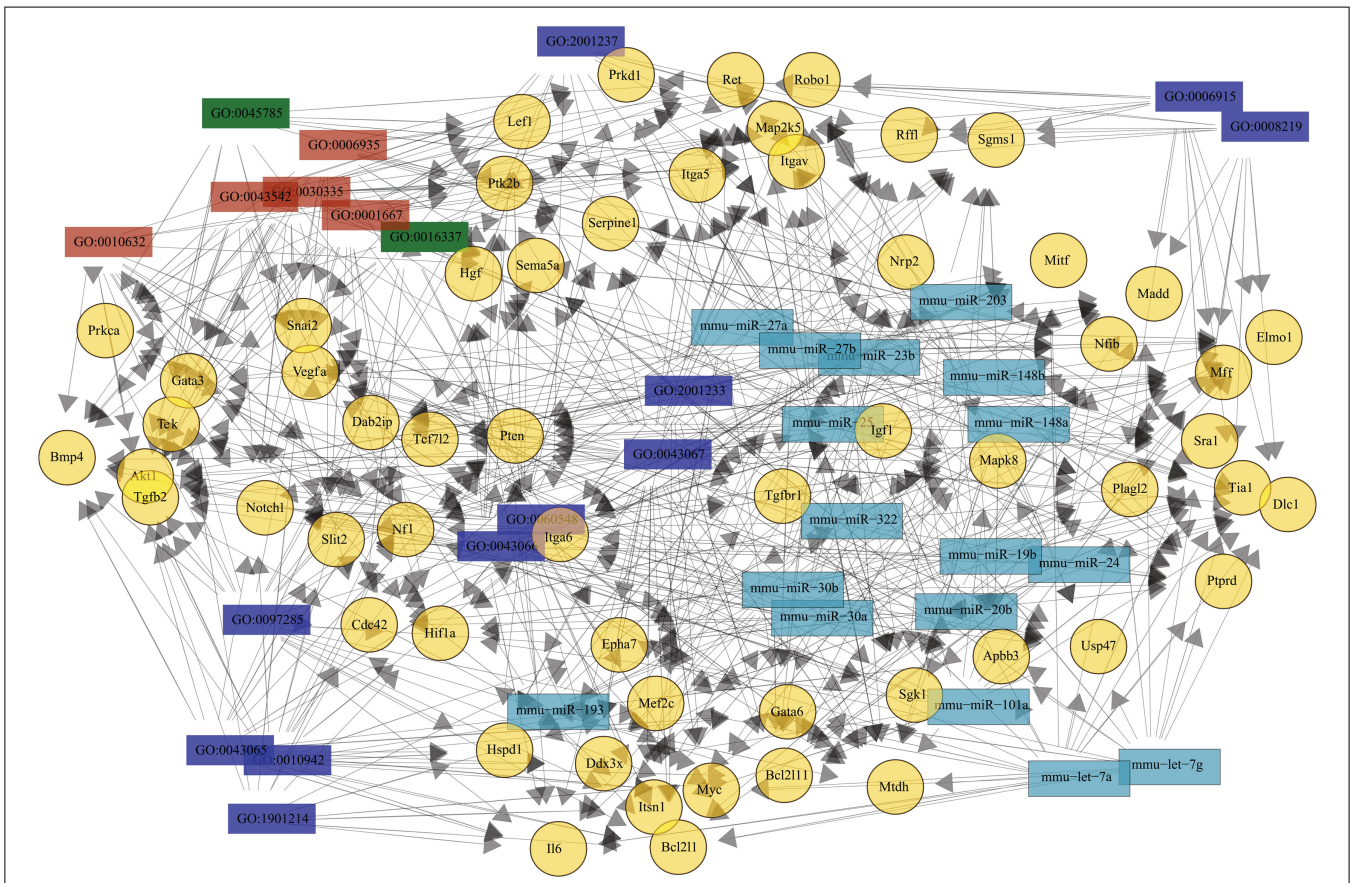

FIGURE 5 | Theoretical relationship among the most recurrent miRNA, mRNA, and biological processes. The network of the 29 differentially expressed miRNAs and their targets is very complex. To illustrate this network, only molecules or processes related with at least 11 elements are shown. There are 17 miR differentially expressed due to infection (cyan) and their 58 putative targets (yellow), plus 18 biological processes related to it. Biological processes are identified by gene ontology (GO) tags, where blue represents cell death, red are cell adhesion, and green cell migration. The relationships are illustrated by the arrows.

thymocytes within 24 h and a reduction in miR-17-92 cluster (miR-17, miR-20a, miR-20b, and miR-106a) in whole thymus samples (52). Although we were also studying miRNA expression in a condition where there is $CD4^+CD8^+$ cell loss, in TEC we observed an upregulation of miR-20b and a suggestive increase in miR-20a expression, suggesting that intrathymic regulation of miR-20b is cell type specific.

Glucocorticoids can also reduced the protein expression of Drosha's co-factor DGCR8/Pasha and Dicer, two indispensable enzymes for miRNA bioprocessing pathway in thymocytes (51). Moreover, Dicer- and DGCR8-deficient mice are incapable to sustain proper thymic architecture and promote thymocyte development, with a severe loss of TEC, demonstrating the miRNA role in TEC maintenance and function (32, 33). In fact, in mice where TEC do not produce miRNA due conditionally inactivate *Dgcr8* gene, there is a specific loss of mature $mTEC^{hi}$ and $AIRE^+$ subsets that induce a breakdown in thymic central tolerance with the presence of autoantibodies or development of spontaneous autoimmunity (33). On the other hand, *AIRE* knockdown results in modulation of different miRNAs (53, 54), with upregulation of miR-20b, miR-191, and miR-411 (54), which is consistent with our observation. In our study, there was donwregulation of *AIRE* expression due infection, concomitant with upregulation of those miRNAs.

Abnormally release of potential autoreactive T cells from the thymus occurs in patients with severe clinical form of Chronic Chagas disease and also in mouse experimental model (19). Intriguingly, despite the thymic escape of T cells bearing "forbidden" T cell receptor that should be deleted by negative selection (21), some evidence points to normal promiscuous gene expression in infected thymuses suggesting that negative selection can induce tolerance. Indeed, the escape of $CD4^+CD8^+$ T cells to the periphery seems to be more related with a higher fibronectin-driven migration than defects in negative selection (18, 20, 21). Considering that TEC play a role in thymocyte migration and that we defined enhanced intrathymic fibronectin and laminin deposition in *T. cruzi* acutely infected mice (1), we performed biological processes GO-term enrichment analysis and evaluated *in silico* potential interaction network among the 29 differentially expressed miRNAs and their predicted targets. This network analysis

predicted cell adhesion, regulation of cell–matrix adhesion, cell migration, chemotaxis, regulation of programed cell death, and apoptotic processes to be altered as a consequence of *T. cruzi* acute infection. Taking together, these data point out miRNA as candidates to orchestrate thymic atrophy from the TEC perspective, since the alteration herein studied precedes the involution.

The intrathymic T cell migration is a multivectorial process where each individual vector represents a given molecular interaction, as, for example, those interactions mediated by ECM. Accordingly, changes in the ECM contents should result in modulation of thymocyte migration (4, 20). Although there are studies in cancer (55), there is a lack of information concerning the role miRNAs in regulating ECM molecules in the thymus, and more particularly in TEC. We found correlations indicating putative intrathymic functions for some miRNAs, such as miR-183 that direct regulates integrin β1 expression (56), miR-143, suppressing fibronectin directly (57), miR-218 controlling focal adhesion kinase (58), and miR-203 increasing metalloproteinase-1 expression (59).

Overall, in this study, we show differentially expressed miRNAs in TEC from *T. cruzi* acutely infected mice, highlighting miRNAs as possible mediators of thymic atrophy. To our knowledge, this is the first study to show miRNA expression in TEC from infected mice. Further studies are needed to define the targets and dissect the role of TEC miRNAs in the context of infection.

Author Contributions

LL-L and CP designed and performed the experiments, analyzed and interpreted the data, and wrote the manuscript; MR-A performed quantitative polymerase chain reaction analysis and the bioinformatics analyses; BP performed the cell sorting; AM contributed to cell sorting standardization and to writing the manuscript; MG-S and AC contributed to the microRNA assays; and WS conceived the project, provided the budget, and participated in writing the manuscript.

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Supplementary Material

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fimmu.2015.00428

Figure S1 | Selection of multiple internal control reference for normalization. **(A)** Calculation of average expression stability values of five different internal control reference, including one spike-in (cel-miR-39), using geNorm (23). The geometric mean of five small RNAs (Rnu6, cel-miR-39, snoRNA251, snoRNA 142, and snoRNA 202) was used for normalization. Crossing point mean of the samples shows the level of expression for **(B)** cel-miR-39 **(C)**, snoRNA 142, and **(D)** snoRNA202.

Figure S2 | Significant differentially expressed miRNA in thymic epithelial cells from control and infected mice separated by TEC phenotype. The miRNA expression of 85 miRNAs were analyzed for each sorted TEC subpopulation, in five replicates, where samples from control, cortical TEC (orange) and medullary TEC (dark blue) and infected, cortical infected TEC (yellow) and medullary infected TEC (light blue). We present the 29 miRNAs that were significantly differentially expressed (adjusted p-value $\leq$0.05) due to infection.

Figure S3 | Suggestive differentially expressed miRNA in thymic epithelial cells from control and infected mice separated by TEC phenotype. The expression of 85 miRNAs was analyzed for each sorted TEC subpopulation, in five replicates, where samples from control, cortical TEC (orange) and medullary TEC (dark blue) and infected, cortical (yellow) and medullary TEC (light blue) are showed. We present the 13 miRNAs that were suggestive significance (adjusted p-value $\leq$0.1) differentially expressed due to infection.

Figure S4 | miRNAs with a consistent pattern of no amplification in TEC from infected animals. The miRNA expression of 85 miRNAs was analyzed for each sorted TEC subpopulation, in five replicates, where samples from control, cortical TEC (orange) and medullary TEC (dark blue) and infected, cortical (yellow) and medullary TEC (light blue) are showed. We present the seven miRNAs that exhibited a consistent pattern of no amplification in TEC from infected animals, being clearly detected in control samples.

Table S1 | List of microRNAs and the respective putative mRNA targets.

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Positive and negative regulatory mechanisms for fine-tuning cellularity and functions of medullary thymic epithelial cells

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Self-tolerant T cells and regulatory T cells develop in the thymus. A wide variety of cell–cell interactions in the thymus is required for the differentiation, proliferation, and repertoire selection of T cells. Various secreted and cell surface molecules expressed in thymic epithelial cells (TECs) mediate these processes. Moreover, cytokines expressed by cells of hematopoietic origin regulate the cellularity of TECs. Tumor necrosis factor (TNF) family RANK ligand, lymphotoxin, and CD40 ligand, expressed in T cells and innate lymphoid cells (ILCs), promote the differentiation and proliferation of medullary TECs (mTECs) that play critical roles in the induction of immune tolerance. A recent study suggests that interleukin-22 (IL-22) produced by ILCs promotes regeneration of TECs after irradiation. Intriguingly, tumor growth factor-β and osteoprotegerin limit cellularity of mTECs, thereby attenuating regulatory T cell generation. We will review recent insights into the molecular basis for cell–cell interactions regulating differentiation and proliferation of mTECs and also discuss about a perspective on use of mathematical models for understanding this complicated system.

Keywords: medullary thymic epithelial cells, autoimmune disease, negative feedback, mathematical modeling, T cells, thymus

Introduction

Thymic epithelial cells (TECs) are essential for T cell development and self-tolerance induction in the thymus (1). TECs are classified into medullary TECs (mTECs) and cortical TECs (cTECs) according to their localizations in the thymus. While cTECs mainly support the early differentiation and proliferation of T cells and positive selection of self-MHC-restricted T cells, several lines of evidence indicate critical roles of mTECs in preventing the onset of autoimmune diseases in human and mice (2). mTECs uniquely express many kinds of tissue-specific self-antigens (TSAs) (2–4). mTECs expressing high levels of MHC class II and co-stimulatory molecules, such as CD80, would be capable of directly presenting these TSAs to medullary T cells (5). Alternatively, TSAs in mTECs are transferred to thymic dendritic cells (DCs) and indirectly presented to T cells (6, 7). When T cells recognize these presented TSAs through high avidity interactions, they undergo apoptosis or are converted into regulatory T cells (Tregs) (2). Expression of TSAs is, in part, regulated by nuclear protein autoimmune regulator (AIRE), and dysfunctional mutations in AIRE provoke autoimmune diseases in humans (3, 8, 9). In addition to such roles in preventing autoimmunity, recent studies suggest that immune tolerance to some tumors might be under the control of mTECs (10–12). Therefore, understanding

cellular and molecular mechanisms to regulate mTEC differentiation, proliferation, and apoptosis is an important issue.

Recent studies have revealed new aspects of mTEC differentiation and proliferation (13–15). One recent study indicated a new mechanism that promotes recovery of TECs following thymic injury induced by γ-irradiation (13). In addition, molecular mechanisms of negative regulation in mTEC differentiation have been reported (14, 15). In this review, we will summarize these new findings. Moreover, these new findings together with previous studies imply the existence of considerably more complicated cellular and molecular mechanisms regulating mTEC cellularity than was previously recognized. Such a sophisticated system can ensure precise regulation of TEC functions in T cell differentiation, selection, and tolerance induction. Therefore, we also present our perspectives on how mathematical modeling might contribute to understanding regulation of TEC cellularity and functions.

Positive Regulatory Mechanisms for mTEC Differentiation and Regeneration

Tumor Necrosis Factor Receptor Family Signaling and NF-κB Pathways in mTEC Differentiation

Medullary TECs constitute a heterogeneous cell population under constant differentiation (1). The roles of tumor necrosis factor (TNF) receptor family signaling on mTEC differentiation were previously summarized (16). Therefore, we briefly mention this topic. Receptor activator of NF-κB (RANK), CD40, and lymphotoxin β-receptor (LtβR), and all members of the TNF receptor family, have been reported to promote mTEC differentiation. RANK signaling appears to play a dominant role in the differentiation of mTECs expressing Aire (16–19). The role of CD40 may be similar to that of RANK in the postnatal period because deletion of both RANK and CD40 signaling resulted in almost complete loss of Aire-expressing mTECs, as compared to a partial reduction of mature mTECs by the absence of either RANK or CD40 signaling (16). In the embryonic thymus, only RANK signaling is active (18) because thymic expression of CD40 ligand starts in the perinatal period (20). LtβR might control several distinct steps in mTEC differentiation (16, 21–23). An early study suggests that LtβR signaling induces Aire expression (24). However, later studies did not support the direct connection between LtβR signaling and Aire expression (25, 26). This apparent discrepancy remains to be solved. Moreover, some studies revealed that the absence of LtβR signaling causes a disturbance in three-dimensional organization of mTECs (27, 28), suggesting a distinct role of LtβR signaling from other TNF receptor signaling.

Ligands of these TNF receptor family members are expressed mainly in cells of hematopoietic origin. Previous studies revealed that RANK ligand (RANKL) is expressed in innate lymphoid cells (ILCs) and positively selected $CD4^+CD8^-$ T cells (CD4SP) (16, 19). Moreover, RANKL expression was detected in fractions of $CD4^-CD8^-$ thymocytes (double negative, DN) and γδT cells (29, 30). Conditional deletion of RANKL from each cell type would be needed to identify what types of cells are major sources of the RANKL for mTEC differentiation unambiguously. CD40 ligand is most highly expressed in CD4SP (19, 31). The ligand of LtβR is a heterotrimer consisting of secreted lymphotoxin α and membrane bound lymphotoxin β. Expression of lymphotoxin β appears to be high in CD4SP, CD8SP, and ILCs (19, 25, 32). Consequently, interactions with these cells might be required for differentiation of mTECs.

Signaling by RANK, CD40, and LtβR activates the transcription factor NF-κB. The NF-κB family consisting of five members (i.e., RelA, RelB, c-Rel, p105/p50, and p100/p52) that form hetero- and homodimers and are sequestered in the cytosol typically by binding to their inhibitor protein IκBs in unstimulated cells. Signal-dependent degradation of IκBs by the ubiquitin–proteasome pathway results in the translocation of NF-κB into the nucleus, which in turn promotes the expression of genes controlling various cellular responses (33). RANK, CD40, and LtβR signaling are capable of activating two distinct NF-κB pathways: the canonical NF-κB pathway and the non-canonical NF-κB pathway (16, 33). Briefly, activation of the canonical NF-κB pathway leads to nuclear translocation of RelA or c-Rel bound to p50. On the other hand, the non-canonical NF-κB pathway results in nuclear translocation of RelB bound to p52.

RelB deficiency and a dysfunctional mutation of NF-κB inducing kinase (NIK), a signal transducing molecule of the non-canonical NF-κB pathway, cause a severe reduction in the number of mature mTECs (34–37), suggesting an essential role for the non-canonical NF-κB pathway in mTEC differentiation. TNF receptor-associated factor 6 is a signal transducer of the canonical NF-κB pathway and is also essential for the mTEC differentiation (38). These data imply that roles of the canonical and non-canonical NF-κB pathways are not redundant, but that both are essential for mTEC differentiation. Elucidation of the functional differences between these two NF-κB pathways in mTEC differentiation remains to be determined.

Role of IL-22 Signaling in the Regeneration of TECs

Many stressors, such as psychological stress, virus infection, chemotherapy, and irradiation in bone marrow transplantation therapy, provoke acute thymic involution in which TECs and thymocytes rapidly decrease (39, 40). Although recovery from these acute thymic injuries usually occurs, incomplete recovery of thymic cells can increase the risk of immunodeficiency and autoimmunity (39, 40). Therefore, understanding the molecular and cellular mechanisms of acute thymic involution and its recovery is necessary. A recent study revealed a critical role for IL-22 in the repair of TECs after thymic involution induced by radiation (13).

IL-22 is reportedly produced by T helper 1 (Th1) cells, Th17 cells, and Th22 cells (41, 42). In addition, group 3 ILCs secret high amounts of IL-22 (41, 42). The IL-22 signal is transmitted through the IL-22 receptor (IL-22R), consisting of IL-22R1 and IL-10R2 subunits, and activates various downstream signaling (41). The IL-22 signal promotes the regeneration of epithelial cells in the liver, airway, and intestine after injury (42).

Dudakov et al. (13) reported that expression of IL-22 is upregulated in the thymus after total body irradiation in mice, which may mimic thymic injury by radiation therapy for malignant leukemia. IL-22 expression showed an inverse correlation with changes in thymic cell numbers after the irradiation. They further demonstrated a delay of TEC recovery in IL-22-deficient mice

(*Il22*$^{-/-}$) after the irradiation. Because thymic cell numbers were not reduced in the thymus of untreated *Il22*$^{-/-}$ mice, IL-22 appears to function in the regeneration of TECs specifically. They also determined that lymphoid tissue inducer (Lti), which belongs to group 3 ILCs, was the producer of IL-22 in this context. IL-22 expression in the Lti was induced by signaling of IL-23 secreted from thymic DCs. The reduction of CD4^{+}CD8^{+} thymocytes (double positive, DP) by irradiation triggered the secretion of IL-23 from DCs, although the molecular mechanism remains unclear. IL-22 appears to enhance proliferation of mTECs as well as cTECs. Because Aire-positive mTECs are reportedly post-mitotic (43), it should be determined in the future whether IL-22 signaling alone is capable of inducing the proliferation of Aire-positive mTECs in this situation or whether other signals are necessary for the recovery of Aire-positive mTECs.

Negative Regulatory Mechanisms for Fine-Tuning the Cellularity of mTECs

Negative Regulation of mTEC Cellularity by TGF-β Signaling

Tumor growth factor (TGF)-β has diverse functions during the development and homeostasis of various tissues (44). Binding of TGF-β to its cell surface receptor complex, consisting of type II receptors (TGF-β RII) and the type I receptors (TGF-β RI), induces activity in its cytoplasmic serine/threonine kinases, thereby activating Smad protein complex. The activated Smad complex is subsequently translocated into the nucleus and promotes gene expression. As a result, TGF-β signaling induces anti-proliferative and pro-apoptotic effects in many types of cells.

In the thymus, TGF-β is expressed by cTECs and immature thymocytes (45, 46). On the other hand, the TGF-β receptor complex is expressed in both cTECs and mTECs (47). Hauri-Hohl et al. have recently reported a role for TGF-β signaling in regulation of mTEC number (14). They prepared mice lacking TGF-β RII expression specifically in TECs. Interestingly, the cellularity of only mTECs was increased by the lack of TGF-β signaling in TECs. Consistently, administration of a TGF-β RI inhibitor also increased the mTEC number. Thus, TGF-β signaling limits mTEC cellularity selectively, although both mTEC and cTECs should receive the signals.

The limitation of mTEC number by TGF-β signaling is less likely due to its anti-proliferative and pro-apoptotic effects. Instead, *in vitro* data have suggested that TGF-β signaling interferes with the activation of non-canonical NF-κB signaling; the mechanism, however, remains unclear. Given that RANK, CD40, and LtβR signaling all activate non-canonical NF-κB signaling, it is possible that TGF-β inhibits non-canonical NF-κB signaling triggered by these receptors, thereby limiting the mTEC cellularity. This idea might explain the mTEC-selective inhibition by TGF-β.

Regulation of Gene Expression and Differentiation of mTECs by the ETS Family Member Spi-B

The Ets family transcription factor Spi-B has been recently identified as a regulator of mTEC differentiation (15). RANKL signaling rapidly upregulates Spi-B expression in *in vitro* thymic stromal culture via the NIK-dependent NF-κB pathway. Lack of Spi-B caused an increase in the number of mTECs expressing high levels of MHC II. On the other hand, expression of co-stimulatory molecule CD80, CD86, and some of TSAs in mTECs were strikingly reduced in Spi-B-deficient (*Spib*$^{-/-}$) mTECs. Thus, Spi-B apparently has dual functions in mTEC differentiation: Spi-B limits the number of mature mTECs and promotes some mTEC-functional genes. In addition, expression of osteoprotegerin (OPG), a decoy receptor of RANKL (48), was significantly reduced in *Spib*$^{-/-}$ mTECs. OPG was previously reported to be a negative regulator of mTEC differentiation by inhibiting RANKL signaling (19). Moreover, the Spi-B-mediated limitation of mTEC cellularity was not detected in the absence of OPG. These facts suggest that Spi-B induced by RANK signaling upregulates OPG expression in mTECs, thereby competitively inhibiting the RANKL signal-inducing mTEC differentiation (**Figure 1**). Thus, this negative feedback regulation finely tunes the cellularity of mTECs. Noticeably, negative regulation of mTEC differentiation by the Spi-B–OPG axis starts in the peri- to neonatal period, during which Aire mediates long-lived tolerance (49, 50).

Biological and Physiological Significance of Negative Regulation of mTEC Cellularity

The effect of TGF-β signaling in mTECs on thymic T cell differentiation was investigated (14). The number of SP thymocytes and the frequency of CD4SP were mildly increased by the absence of TGF-β signaling. Moreover, export of thymic T cells to the periphery was delayed in the postnatal period of these mice. These data suggest that the increase in mTEC number prolongs the dwelling time of mature T cells in the thymic medulla. The absence of TGF-β signaling in TECs resulted in an increase in thymic Tregs and their precursors and a reduction in the frequency of thymic and peripheral Th17 cells.

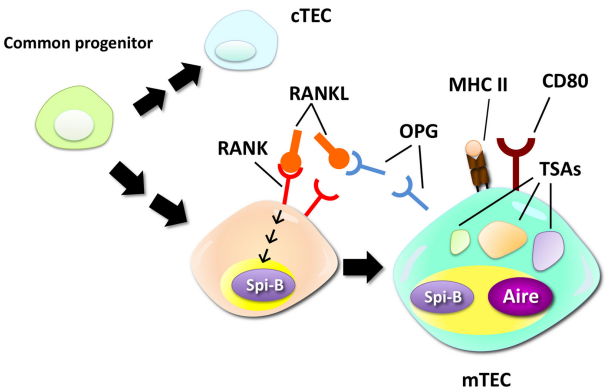

FIGURE 1 | Negative regulation of mTEC differentiation by the RANK–Spi-B–OPG–RANKL feedback loop. mTECs are derived from a common progenitor that can give rise to both mTECs and cTECs. RANK signaling promotes differentiation of relatively immature mTECs into mTECs expressing high levels of MHC class II (MHC II), CD80, and Aire. A recent study suggested that RANK signaling upregulates expression of Spi-B. Spi-B promotes expression of some TSAs, CD80, and osteoprotegerin (OPG), a secreted decoy receptor for RANK, in mTECs. OPG, in turn, competitively inhibits RANKL–RANK interactions, thereby inhibiting the RANKL-dependent process of mTEC differentiation.

Osteoprotegerin-deficient ($Opg^{-/-}$) mice were used to investigate the role of the negative feedback circuit consisting of RANKL–Spi-B–OPG in thymic T cell selection (15). The OPG deletion in the thymic stroma led to an increase in the number and frequency of Tregs and Treg precursors in the thymus. Together with the findings on TGF-β-mediated TEC regulation, this suggested that negative regulation of mTECs attenuates the generation of thymic Tregs. Importantly, the increase in Treg generation by the deletion of OPG initiates in the perinatal period. A recent study revealed that Tregs generated during this period are functionally distinct from those produced in the adult thymus and that these Tregs play a critical role in long-lived tolerance induction (49, 50). Therefore, fine-tuning in the generation of Tregs during this period by this negative feedback loop could have an impact on T cell tolerance in adults.

What is the physiological impact of these negative regulations? Suppose that mTECs simply played a role in preventing the onset of autoimmune disease by negative selection and conversion of Tregs. In this case, the inhibitory regulations of mTEC differentiation might be harmful to the body. Indeed, the absence of TGF-β signaling in TECs attenuates autoimmunity caused by a chronic ablation of Tregs (14), suggesting a reduction of self-tissue reactive T cells by abolishing TGF-β-mediated negative regulation. Thus, this finding supports the idea that negative regulation of mTECs would increase the risk of autoimmunity. Besides the critical role of mTECs in inducing tolerance toward various self-tissues, recent studies have shown that mTECs promote T cell tolerance to tumors (10–12). The roles of RANKL–Spi-B–OPG negative feedback regulation in tumor immunity were tested. When this negative feedback loop was abolished by OPG depletion in the thymic stroma, tumor growth, and incidence of carcinogenesis were increased (14). These findings suggest that this negative feedback regulation might promote tumor immunity and optimize the trade-off between prevention of autoimmunity and induction of tumor immunity. Thus, negative regulation of mTEC number may contribute to immune responses toward self-antigens in tumors that are originally derived from self-tissues.

Perspective on Mathematical Modeling for TEC Cellularity

As described above, many cell types and molecules are involved in the regulation of mTEC cellularity. Consequently, T cell selection and tolerance induction supported by mTECs could be finely tuned by a combination of various mechanisms under steady state and pathological condition. Mathematical modeling would help us to understand this complicated situation. However, mathematical modeling on dynamics of TECs, including mTECs, has not been reported yet. On the other hand, there are several studies on mathematical modeling of thymocyte development. The similar mathematical approach as that used for investigations into thymocyte development can be employed for TEC development. Moreover, because interactions with thymocytes are critical for differentiation, proliferation, and survival of TECs, dynamics of thymocytes should be included in the mathematical modeling of TECs. In this section, we briefly discuss about a perspective on use of mathematical models to understand dynamics of TEC population by referring to previous mathematical modeling studies on thymocytes.

Generally, tracking cell fates over time at the single-cell level is experimentally demanding and almost impossible *in vivo*. Therefore, mathematical models are indispensable to extract biologically relevant information on cellular dynamics and differentiation from population-level measurements. As more detailed information is obtained by new experimental methods, the mathematical models have also evolved from simple ordinary differential equations (ODEs) to cellular automata, compartment models, and stochastic models in order to account for different subtypes of lymphocytes, their cellular heterogeneity, and spatial niches that they reside.

Although dynamics of thymocyte populations were modeled by their types, i.e., DN, DP, and SPs, the interactions of thymocytes with TECs were not explicitly incorporated in many models (51–55). In order to investigate the contributions of cortical and medullary selection, the influence of the TECs and the thymic environment were incorporated more explicitly into models in other studies. Fano et al. modeled the interaction of the thymocytes with the cortical and medullary APCs to estimate the fractions of the positively and negatively selected thymocytes in the cortex and the medulla in relation to the diversity of presenting ligands (56). In other studies (57, 58), the anatomical structure of the thymus, together with cell types, was incorporated explicitly into an investigation of the interrelation between thymocyte migration and selection. In these studies, however, the TECs were considered to be in a static thymic environment. In reality, the TECs also differentiate and proliferate homeostatically in the thymus.

Influence of the thymocyte dynamics should be incorporated into the mathematical modeling of TEC development because not only intra- but also inter-regulation of thymocytes and TECs is quite important when we consider the differentiation process of TECs and recovery of TECs from damage and its involution by aging, in which both thymocytes and TECs change their population dynamically (59, 60). Thus, we think that mathematical models will be crucial for understanding the joint dynamics of thymocytes and TECs by disentangling their complicated cell–cell interactions.

Concluding Remarks

Several types of cells and various positive and negative signaling pathways appear to control the cellularity of mTECs under physiological and pathological condition. Because cellular development and recovery from injuries are time-dependent processes, these mechanisms should be regulated in a precise and timely manner. Employment of mathematical modeling is a promising approach to understand these temporally regulated processes in the future.

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REVIEW
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A tale from TGF-β superfamily for thymus ontogeny and function

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Multiple signaling pathways control every aspect of cell behavior, organ formation, and tissue homeostasis throughout the lifespan of any individual. This review takes an ontogenetic view focused on the large superfamily of TGF-β/bone morphogenetic protein ligands to address thymus morphogenesis and function in T cell differentiation. Recent findings on a role of GDF11 for reversing aging-related phenotypes are also discussed.

Keywords: TGF-β, BMP, thymus, thymopoiesis, T cell development

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Introduction

The adaptive immune system evolved as a complex set of defense mechanisms amplified by the specificity properties of antigen receptor-bearing B and T lymphocytes (1). Following blood trafficking into the thymus, bone marrow-derived lymphoid progenitors become committed to T cell lineage development. Within this organ, cell specialization occurs gradually in a manner that T cell development results in the generation of conventional CD4 and CD8 αβ T cells along with natural killer T cell (NKT; an innate-like T cell subpopulation), regulatory T cell (Treg), and γδ T cell subsets (2). Classically, commitment to T cell lineage was found to rely on the Delta-class Notch ligand Delta-like 4 (DLL4) and the interleukin-7 (IL-7) along with kit and flt3 ligands at stages usually prior to TCRβ chain assembling (3–6). Branching into distinct paths can be observed throughout the mainstream developmental pathway, from the double-negative (DN; $CD4^-CD8^-$) T cell precursors to the highly expanded double-positive (DP; $CD4^+CD8^+$) cells, and the resulting mature single-positive (SP; $CD4^+CD8^-$ or $CD4^-CD8^+$) stages. Thus, at specific niches, the thymus provides to developing T cells signals that trigger a series of ordered events leading to cell proliferation, TCR gene rearrangements, and selective checkpoints along with massive cell death (7). Altogether, these events culminate in a proper repertoire of distinct and specialized mature thymocyte subpopulations able to emigrate to the periphery. In this review paper, we highlight the role of members of the large transforming growth factor-β (TGF-β) superfamily (**Box 1**) during thymic ontogeny, thymic epithelial cell (TEC) differentiation and function, as well as T cell maturation. Lastly, we discuss recent information on a possible regenerative potential of TGF-β ligands to rescue aging-related thymus atrophy.

TGF-β Signaling and Thymus Formation

Organogenesis relies on well-organized interactions between distinct germ layers and differentiating cell types controlled by intricate molecular hierarchies. Thymus development occurs from common parathyroid bilateral rudiments in the epithelial endodermal lining of the third pharyngeal pouch around embryonic days (E) 9.0–9.5 in mice and early week 5 in humans (**Figure 2A**) (41–44). As growth continues through E10.5 in mice and early week 6 in humans, the contact between the third pharyngeal pouch and the third pharyngeal cleft ectoderm determines paired organ primordia with

BOX 1 | Multiple roads for signaling by TGF-β superfamily members.
The TGF-β superfamily comprises TGF-β1–3, bone morphogenetic proteins (BMPs), growth and differentiation factors (GDFs), Nodal, activins/inhibins, Müllerian inhibiting substance (MIS)/anti-Müllerian hormone (AMH), and Lefty. These ligands were initially grouped accordingly to the functional roles observed following their original identification (8–11). As it became clear that most ligands play multiple functions depending on cell type, developmental stage, or tissue conditions, they are now classified by sequence similarity and the downstream pathway they activate (12). Each family member has an overall basic structure, in which inactive forms are produced with an N-terminal secretion peptide and a large propeptide domain known as latency-associated peptide (LAP). Cleavage of the propeptide domain by proprotein convertases releases a mature domain at the C-terminus, which eventually dimerizes (13). The propeptide domain has major regulatory roles. It influences protein stability and functions as chaperone during secretion, also mediating diffusion through interactions with the extracellular matrix and inhibiting the active peptide form even after cleavage (14–16).

Signaling by TGF-β superfamily members occurs through a similar mechanism, but operates with distinct components. Ligands bind single-pass transmembrane receptor serine/threonine kinases, which relay the signal for intracellular effectors capable of translocating into the nucleus to modulate gene transcription (**Figure 1**). More specifically, these receptors are classified into two structurally similar types. Ligand binding occurs only through type II receptors, which then recruit and phosphorylate type I receptors [e.g., Ref. (17, 18)]. Type II receptors, such as ActRII (*Acvr2a*) or ActRIIB (*Acvr2b*), may take part in many distinct pathways or may be specific for a given group of ligands, such as AMHR2 (*Amhr2*) for MIS/AMH, BMPRII (*Bmpr2*) for most BMPs and Gdf9, and TβRII (*Tgfbr2*) for TGF-βs (19, 20). Type I receptors are also known as activin receptor-like kinases (ALKs) due to their sequence similarity to activin receptors (21). These receptors are usually specific to a more restricted set of ligands. For instance, Nodal, Gdf1, Gdf11, activins, and inhibins bind ActRII to recruit Alk4 (*Acvr1b*) and Alk7 (*Acvr1c*) or they bind ActRIIB to recruit either Alk4, Alk7, or Alk5 (*Tgfbr1*) (19). Together, type II and type I receptors form a heterotetrameric complex, in which the type I receptor further phosphorylates intracellular effectors of the Smad family (22). Depending on the ligand/receptor complex they are responding to, receptor-activated Smads (R-Smads) can be subdivided into two groups: a BMP-related set gathers Smad1, Smad5, and Smad9 (formerly Smad8), whereas Smad2 and Smad3 are responsive to TGF-β-related signals (**Figure 1**). An N-terminal MH1 domain negatively regulates the MH2 domain, being indispensable for Smad translocation into the nucleus and DNA binding (23–25). However, these functional properties do not hold true for all R-Smads. In particular, Smad2 seems to interact to DNA only indirectly (24).

A common mediator Smad (co-Smad), or Smad4, integrates signals from both branches by associating with the R-Smads (**Figure 1**). They form transcriptional complexes able to translocate into the nucleus (26–28). Nuclear transportation of Smads depends on accessory proteins, particularly importins, exportins, and nucleoporins (29, 30). The presence of DNA molecules harboring Smad-binding elements favors heterodimerization between R-Smads and co-Smad (28). They ultimately associate with cell-type-specific transcription factors and co-activators to regulate a plethora of target genes (31).

Regulation of Smad activity occurs through multiple mechanisms (32). Two inhibitory Smads (I-Smads) impair signaling by competing with R-Smads for receptors or by co-Smad interaction (33). For instance, Smad6 forms stable interactions with type I receptors, blocking phosphorylation of Smad2 and Smad1, but not Smad3 (34, 35). Similarly, Smad7, the other I-Smad member, also binds type I receptors and suppresses further phosphorylation by targeting them for proteasome-dependent degradation (35, 36). The available literature on the molecular interactions of TGF-β superfamily members is vast, but not in the scope of this review. Further information can be found elsewhere (33, 37–40).

stratified epithelium and a central lumen lined by precursors of medullary thymic epithelial cells (mTECs). These cells are characterized by the expression of both claudin-3/4 and cytokeratin-5 (K5) (46, 47). Further development of thymic medulla also depends on the successful establishment of the cortical region, as observed in mice with arrested T cell development (48). Within each primordium, a dorso-rostralmost domain expressing *Gcm2* gives rise to a parathyroid gland from E9.5 in mice or as early as the onset of week 6 in humans, whereas a ventro-caudalmost domain identified by *Foxn1* expression produces a thymic lobe from E11.25 in mice or mid-week 6 in humans (**Figure 2B**) (44, 49–52). Epithelial cell proliferation fills the pharyngeal pouch lumen by forming cord-like structures with smaller lumina, similar to branching morphogenetic events in other organs (**Figure 2C**) (47). In this context, activation of Foxn1 blocks the respiratory development (53) and, along with subsequent colonization by lymphocyte precursors, seems to be responsible to produce a concentric medulla less densely cellular than the surrounding cortex (47). Fetal liver-derived lymphocyte progenitors colonize the embryonic thymus from E11.5 in mice and week 8 in humans (54, 55), whereas short-term apoptotic events around E12.0 disconnect the developing anlagen from the embryonic pharynx (41). The rudiments migrate downwards at different paces, gradually resolving the *Gcm2*- and *Foxn1*-restricted domains into two morphologically distinct structures enclosed by neural crest-derived mesenchyme (**Figures 2C–F**) (51, 56). Parathyroid primordia usually lag behind and move toward the tracheal region dorsally to the thyroid gland, whereas thymic rudiments move ventrally and more caudally into the thoracic cavity (**Figures 2D,E**). The thymic primordia ultimately fuse at the midline to produce a bi-lobed organ above the developing heart (**Figure 2F**). Unlike mice, humans exhibit superior parathyroid glands derived from the fourth pharyngeal pouch (**Figure 2**) (43), whereas organogenesis of the human thymus is essentially similar to mice both morphologically and molecularly (44). Each of these morphogenetic events during thymus organogenesis is controlled by a multitude of signals, including members of the TGF-β superfamily.

Thymus Specification and Thymic Epithelial Cell Differentiation

Early production of Bmp4 by the endoderm, the surrounding neural crest-derived mesenchyme, and the overlying ectoderm of the third pharyngeal arch and cleft raised the possibility that bone morphogenetic protein (BMP) signals may trigger thymus and parathyroid formation (57). However, conditional inactivation of *Bmp4* in both pharyngeal endoderm and mesenchyme using a *Foxg1–Cre* line had no effect in organ induction, but resulted in abnormal morphogenesis (see below) (58). This could be the result of a short-time window of 24 h necessary to establish the prospective thymic and parathyroid domains as observed in chicken embryos (59). Indeed, Patel et al. have observed using a $Bmp4^{lacZ}$-reporter line that the onset of Bmp4 production occurred at E9.5 in the ventral pharynx close to the third pouch entrance, but not in the pouch endoderm or mesenchyme proper (57). Expression in these tissues was later achieved and expanded to the overlying ectoderm (57). The realization that endoderm patterning occurs before primitive gut and pharyngeal pouch formation still hampers the identification of signals responsible for thymus specification *in vivo* and other members of the TGF-β superfamily may also be at play (60). Particularly, activin A is

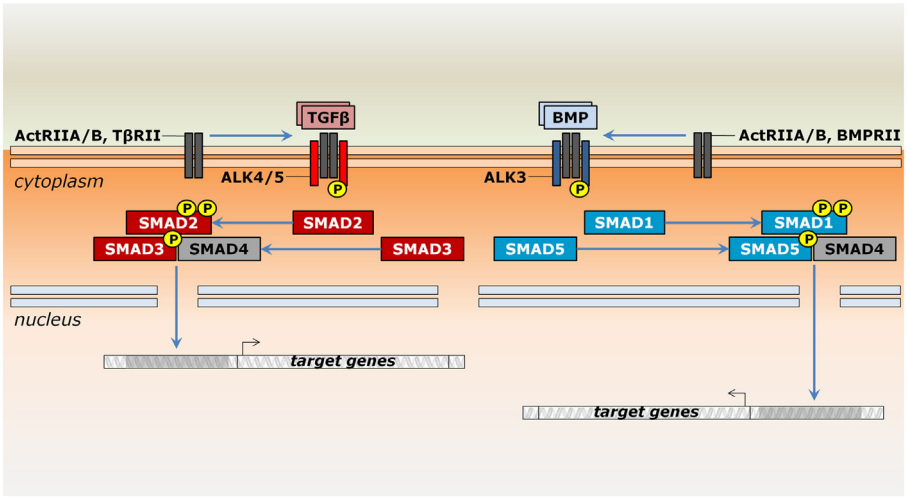

FIGURE 1 | Signaling by ligands of the TGF-β superfamily in the thymus. Members of the TGF-β superfamily may signal by either the TGF-β (reddish) or the BMP branch (bluish). Upon binding to type II serine/threonine receptors occurs the recruitment of type I receptors, which further phosphorylate Smad proteins. Whereas ActRIIA and ActRIIB may be shared between both pathways, TβRII and BMPRII are specific to TGF-β and BMP signaling, respectively. In general, Smad2 and Smad3 relay signals from the Alk4, Alk5, and Alk7 receptors, while Smad1, Smad5, and Smad8/9 are phosphorylated by Alk2, Alk3, and Alk6 receptors. However, Alk2, Alk6, and Alk7 are not expressed during thymocyte maturation. Modulation of gene expression occurs after Smad complex translocates into the nucleus and depends on the interaction with additional protein complexes (not shown).

required to induce definitive endoderm prior to the differentiation of third pharyngeal pouch endoderm *in vitro* (61). Since gene targeting of some superfamily ligands or their receptors results in embryonic lethality (62–64), new conditional mutants should be produced taking into consideration that gene deletion may have to occur earlier and at different embryonic compartments than previously thought.

The possibility that thymus induction depends on synergistic effects of TGF-β superfamily ligands with non-superfamily signals is a likely case (59). Endoderm-derived undifferentiated epithelial cells comprise a homogeneous population phenotypically defined as cytokeratin (K)5^{+}K8^{+}EpCAM^{+}MTS24^{+} in the thymic primordium of mouse embryos at E12.0 (65). When a single progenitor cell labeled with enhanced yellow fluorescent protein (eYFP) was microinjected into an unlabeled syngeneic thymus rudiment with the same age, and transplanted under the kidney capsule, both cortical and medullary portions showed scattered eYFP^{+} TECs also positive for region-specific markers after 4 weeks, revealing that common bipotent progenitors are able to produce both epithelial lineages during embryogenesis (65). Recently, thymic epithelial progenitor cells (TEPCs) bearing stem-cell features were also identified in the thymus of adult mice as a MHCII^{low}α6 integrin^{high}Sca-1^{high} subset (66). They mature in a highly complex stepwise process not fully understood, ultimately producing cortical TECs (cTECs) or mTECs (67).

Cortical TECs are sparsely distributed and may be identified as CD45^{-}EpCAM^{+}Ly51(CD249)$^{+}$*Ulex europaeus* lectin 1 (UEA-1)$^{-}$K5^{-}K8^{+} cells with high levels of both MHC II and the proteasome subunit β5t (68–71). Considering the TGF-β-related pathways, cells from neonatal mice express both the *Acvr2a* (ActRII) and *Acvr2b* (ActRIIB) genes for the common receptors, in addition to *Acvr1* (Alk2), *Bmpr1a* (Alk3), and *Bmpr2* (BMPRII) for the BMP-specific receptors, and the TGF-β-specific type I receptors, Alk4 (*Acvr1b*) and Alk5 (*Tgfbr1*), and type II receptor TβRII (*Tgfbr2*) (71, 72). This set of receptor genes allows cTEC to respond to both signaling branches of the TGF-β superfamily, even though the BMP receptor, *Bmpr1b* (Alk6), and the TGF-β receptor, *Acvr1c* (Alk7), are not present. Yet, expression of subunit genes *Inha* and *Inhbb* for inhibins and activins, *Bmp2* and *Bmp4*, and *Tgfb1* and *Tgfb3* makes possible the existence of an autocrine circuitry for thymic homeostasis, and indicate that these factors might influence early thymopoiesis (71, 72).

In the thymic medulla, mTECs are characterized by a CD45^{-}EpCAM^{+}Ly51^{-}K5^{+}K8^{-} phenotype with variable levels of UEA-1, MHCII, CD80, and Aire (67). These distinct expression profiles seem to take part in the differentiation program in which MHCII^{high}CD80^{high} mature mTECs expressing Aire are responsible for the production of numerous peripheral self-antigens in the thymus, a critical event for central tolerance (67, 73–77). Hence, SP cells that strongly interact with self peptides through MHC molecules (pMHC) arrest migration, exhibit sustained TCR activation, persistent high levels of cytosolic Ca^{2+}, and early caspase activation, leading to macrophage-dependent phagocytosis (78, 79). Surprisingly, thymocyte apoptosis triggers the production of all three TGF-β ligands by dendritic cells (DC), macrophages, and TECs in the medullary region of neonate or adult thymuses, a phenotype that was partially impaired in *Bim* mutants (80). In addition, apoptosis-driven production of TGF-β signals resulted in an increased generation of thymic regulatory T (tTreg) cells (see below) (80). Interestingly, mTECs are the cell type in the thymus that express most ligand genes of the TGF-β superfamily and their cognate receptors – *Inha* and *Inhbb*, *Bmp2*, *Bmp3*, *Bmp4*, *Bmp5*, *Bmp6*, and *Gdf6*/*Bmp13*, *Gdf3*, *Gdf6*/*Bmp13*, *Gdf8*/*myostatin*, *Gdf10*, *Gdf11*, and *Gdf15*, *Lefty1* and *Lefty2*,

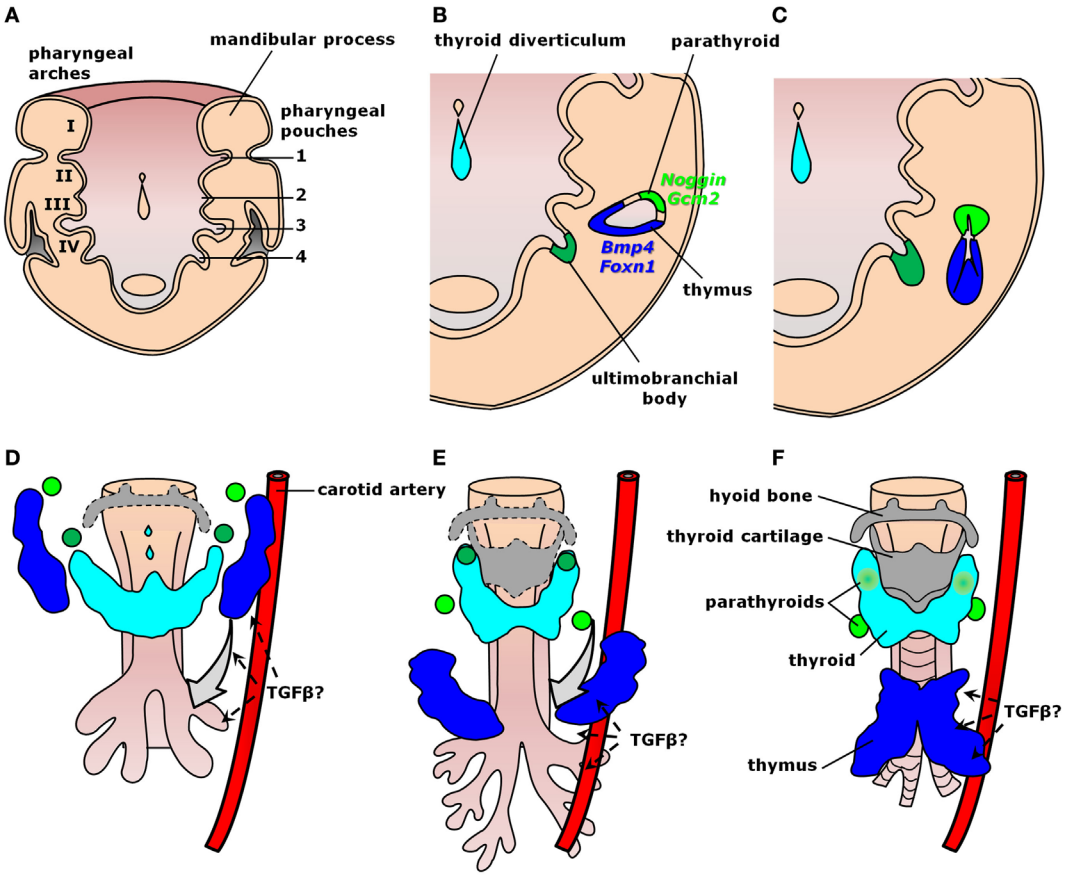

FIGURE 2 | Signaling by TGF-β superfamily members during thymus organogenesis. Schematic representation of thymus formation at different stages of development. **(A–C)** Thymus specification viewed dorsally at the ventral half of the pharyngeal region. **(A)** The common parathyroid–thymus primordium arises from the third pharyngeal pouch endoderm. **(B)** Within each anlage, mTEC precursors line a central lumen surrounded by a dorso-rostralmost domain expressing the BMP-antagonist *Noggin* and the parathyroid specific gene *Gcm2* (light green), whereas the ventro-caudalmost domain expresses *Bmp4* and the thymus-specific gene *Foxn1* (blue). **(C)** Each primordium grows in size while proliferating cells fill the rudiment lumen, later colonized by lymphocyte precursors to produce an inner medulla. **(D–F)** Thymus migration toward the heart. The inferior parathyroid (light green) and the thymus (blue) primordia are gradually resolved as they migrate downwards. **(D)** TGF-β cues from the endothelium of pharyngeal blood vessels (e.g., carotid arteries) seem to orient thymic and parathyroid migration toward their final location. **(E)** The third pharyngeal pouch-derived thymic and the inferior parathyroid rudiments pass by the primordia of the superior parathyroid (dark green), which migrate only a short distance downward the tracheal region. **(F)** Fusion of the thymic primordia occurs at the midline just above the developing heart (not shown) [modified from Ref. (45)].

Tgfb1, *Tgfb2*, and *Tgfb3* along with *Acvr2a* (ActRII), and *Acvr2b* (ActRIIB), *Acvr1* (Alk2), *Bmpr1a* (Alk3), and *Bmpr2* (BMPRII) for BMP/growth and differentiation factor (GDF) signaling, and *Acvr1b* (Alk4) and *Tgfbr1* (Alk5) for the TGF-β/Activin/Nodal pathway, in addition to the type III receptor gene *Tdgf1* (Cripto) (71, 72, 81, 82).

The possibility that members of the TGF-β superfamily produced by mTECs may influence T cell differentiation or impact thymus physiology cannot be ruled out and remains to be thoroughly investigated. For instance, despite the previously identified BMP ligands in mTECs – Bmp3/osteogenin, Bmp5, Bmp6, and Bmp13 – there is no available functional information regarding their activities in the thymus to our knowledge. It is known, on the other hand, that Bmp6 exerts an antiproliferative effect in peripheral CD19$^+$ B cells and induces apoptosis in CD27$^+$ memory B cells (83). By contrast, *Tgfbr2* deficiency in differentiating T cells increased apoptosis of TCRβ^{high}CD4$^+$ and TCRβ^{high}CD8$^+$ mature SP cells after anti-CD3 treatment or of TCRβ^{high}OT-II T cells after antigen-dependent stimulation, thus revealing that TGF-β signals might be involved in thymocyte-negative selection (84). Interestingly, loss of *Tgfbr2* in TECs using a *Foxn1–Cre* mouse line resulted in an expansion of the mTEC compartment – especially MHCII^{high} cells – without affecting cTEC cellularity and the morphology of the corticomedullary junction (85). Indeed, other lymphocyte-derived signals than TGF-β ligands are known to influence mTEC maturation, a phenomenon that is largely known as "thymic cross-talk" (86).

Signaling by TGF-β superfamily members appears to play a secondary role in regulating a master regulator of thymus development and function. Inactivation of the transcription factor Foxn1 results in an athymic phenotype despite the formation of an epithelial anlagen during embryogenesis (49, 87). Expression

of *Foxn1* in thymic primordia is anticipated by the production of Bmp4 and Wnt4 in the epithelium and the adjacent mesenchyme of the third pharyngeal pouch from E10.5 in mice and from mid-week 6 in human embryos (23, 57, 88). Accordingly, *in vitro* treatment of fetal thymic organ culture (FTOC) with BMP4 or overexpression of *Wnt4* in a TEC cell line upregulated the expression of *Foxn1* (88, 89). However, conditional inactivation of *Bmp4* in the pharyngeal endoderm and mesenchyme did not affect *Foxn1* expression (58), similarly to transgenic embryos expressing the BMP-antagonist *Noggin* in TECs (90). In turn, information on blockage of Wnt4 and its effect over the expression of *Foxn1* is limited. In particular, Talaber et al. have shown that a single administration of dexamethasone caused the reduction of both Wnt4 and Foxn1 levels (91). Interestingly, conditional deletion of β-catenin in mTECs using a *BK5–CreER*^{T} line resulted in *Foxn1* downregulation (92). Altogether, the available evidence suggests that induction or maintenance of such an essential transcription factor in the thymic epithelia relies on an intricate molecular hierarchy with a key participation for BMP and WNT signals, which may provide some kind of redundancy for TEC differentiation and function.

With a great potential for translational medicine, differentiation of TEPCs from mouse or human embryonic stem cells (ESCs) can be achieved under culture conditions by the addition of selected growth factors, including TGF-β superfamily ligands. For instance, Lai and Jin have initially reported that incubation with Fgf7, Bmp4, Egf, and Fgf10 produced K5^{+}K8^{+}EpCAM^{+} cells from mouse ESCs (93). These cells were able to further differentiate into medullary K5^{+}K8^{-} and cortical K5^{-}K8^{+} TECs when transplanted with CD4^{-}CD8^{-}CD45^{+} thymocytes under the kidney capsule and sustain normal T cell maturation (93). In humans, an Activin A-dependent inductive stepwise process first differentiate ESCs into definitive endoderm (94), and later into *SOX2*$^{+}$*FOXA2*$^{+}$*CDX2*$^{-}$ anterior foregut endodermal cells by the concurrent inhibition of BMP and Activin/TGF-β signaling using Noggin and the type I receptor-specific inhibitor SB-431542, respectively (95). Further development into TEPC may be achieved by relatively similar approaches, generally modulating retinoic acid, canonical Wnt, and BMP level (61, 96).

Thymus Colonization by Lymphoid Precursors

Colonization of the thymic primordia occurs through intermittent cell flow based on chemokine-dependent mechanisms (55, 97–100). It begins discretely prior to organ vascularization with T cell-restricted progenitors that are unable to definitely populate the thymus (55, 97). Cell influx is transiently interrupted during thymus migration to the thoracic cavity (42). Then, a second wave of cell colonization brings multipotent T cell- and NK-cell progenitors before birth (55). The most significant chemokines currently identified for attracting early T lineage progenitors (ETPs) to the developing avascularized thymus are CCL25 and CCL21 (98). Curiously, whereas CCL25 is produced by both *Foxn1*-positive TECs and the adjacent parathyroid primordium, CCL21 is expressed only by *Gcm2*-positive cells (99, 101). These ligands signal, respectively, through the CCR9 and CCR7 receptors present in CD45^{+} ETPs (102–105). However, it is still poor defined whether members of the TGF-β superfamily directly or indirectly influence or are modulated by these chemokines during thymus colonization. In particular, Gordon et al. observed delayed ETP homing into *Bmp4*-deficient thymic primordia at E11.5, but no significant differences in CCL25 expression in relation to wild-type thymus (58). The relationship with CCL21, other chemokines and their cognate receptors in the embryonic thymus, if present, remains to be determined. Of note, many pathological conditions and morphogenetic events show participation of TGF-βs, BMPs/GDFs, and activins/inhibins in the modulation of chemokine production and *vice versa* (106–113).

Interaction of immigrating lymphocyte progenitors with the thymic stroma is critical for adult thymus organization, but not for TEC differentiation during embryonic development. Using CD3ε transgenic mouse embryos, known to exhibit arrested T cell maturation at the triple negative (TN) CD3^{-}CD4^{-}CD8^{-}CD44^{+}CD25^{-} ETP stage (114, 115), Jenkinson et al. have shown that K5^{+}K8^{+} bipotent TEPCs normally differentiate into functional K5^{+}K8^{-} medullary and K5^{-}K8^{+} cortical TECs, although adult thymus in these transgenic animals exhibit persistent flat organization with morphologically abnormal cortex (115, 116). In particular, transfer of normal bone marrow cells into RAG2$^{-/-}$; tgε26 chimeric mice, in which bone marrow cells from mice mutant for the recombination activating gene 2 (RAG2) were previously transplanted into newborn tgε26 mice, rescued thymic organization and cellularity in the adult (48).

Thymus Migration

The subsequent migration of the thymus into the thoracic cavity also relies on signaling by members of the TGF-β superfamily and depends on neural crest cells. Despite a minor contribution in thymus cellularity, forced production of the BMP-antagonist Noggin in the caudal hindbrain prior to neural crest migration using *B2-NC:Noggin* transgenic mice culminated in thymic hypoplasia or aplasia later in development (117). Indeed, Bmp2 induces Cdc42-dependent actin cytoskeleton reorganization and filopodia formation in neural crest cells, consequently affecting their subsequent migration (118). Moreover, conditional loss of *Bmp4* in mice expressing *Foxg1–Cre* impaired the separation between correctly patterned parathyroid and thymus, which also exhibited a partially compromised capsule (58). Yet, based on observations performed for thyroid migration (119), Gordon and Manley have proposed that the downward migration of the thymus may be driven by signals from the pharyngeal blood vessels, more specifically the carotid arteries (**Figures 2D–F**) (42). Remarkably, mouse embryos with cardiac neural crest cells deficient for the type I receptor Alk5 (*Tgfbr1*) show defective cardiac outflow development, with atypical branching of carotid arteries and failed migration of still connected parathyroid and capsule-encased thymus (120). This raises the possibility that the directional cue for thymus migration might be Alk5 ligand (e.g., TGF-β1–3 or Gdf11), possibly secreted or released through the endothelium (**Figures 2D–F**). By contrast, conditional inactivation of *Tgfbr2* in TECs by a *Foxn1–Cre* mouse line does not affect thymus final positioning (121). Although producing distinct phenotypes, each signaling branch by members of the TGF-β superfamily is involved in the downward migration of thymic

primordia and reveals a critical, but still poorly understood role for the neural crest-derived capsule during thymus organogenesis. Neural crest cells may also differentiate into endothelial cells, pericytes, and smooth muscle cells, and were found to persist in adult mice up to the onset of thymus involution (122).

Thymus Organization and Maturation of T Cells Under TGF-β Superfamily Signals

The adult thymus exhibits two gross anatomical regions easily identified by their histological staining patterns. The peripheral cortex harbors more immature and mostly small thymocytes, and is darker-stained due to a higher cell density. A corticomedullary junction supplied by numerous septal blood vessels makes the transition between the cortex and the central medulla. This latter region is paler due to cell size and a lower T cell density (123–125). A capsule of connective tissue encases the organ. It consists of an outer layer rich in type I collagen and an inner layer of reticular fibers containing type III collagen, and projects type I collagen-containing septa into the parenchyma, partially subdividing the thymus into smaller lobules (126).

Signals from members of the TGF-β superfamily have a major influence on T cell differentiation and thymus homeostasis. As secreted molecules, they may be locally produced by thymic stromal cells and act over developing T cells as paracrine factors or be produced by the thymocytes themselves and work autocrinely. Alternatively, factors from the developing T cells may similarly operate over stromal cells to support thymus homeostasis. However, thymocytes do not express most members of the TGF-β superfamily and the ones present vary in expression as cells differentiate (**Figure 3**). Similar changes are also found for receptor genes (127). Such differences in gene expression occur during T cell maturation, but also when comparing the same stage from fetal and adult thymuses (127–131). Nevertheless, provision of soluble growth factors seems to rely mostly to stromal cells, particularly TECs (71, 127). It is still possible that members of the TGF-β superfamily also act over large distances, being produced by other organs and reaching the thymus through the circulatory system (132). The importance of endocrine stimuli for intrathymic T cell maturation has been largely investigated (133), but whether a given TGF-β ligand exerts long-range effects over thymopoiesis remains to be properly addressed.

Changes in phosphorylation levels of Smad2/3 (pSmad2/3) and Smad1/5/8 (pSmad1/5/8), respectively, used as read-outs for the activities of TGF-β/Activin/Nodal and BMP/GDF signaling, follow differences in the expression of respective cognate receptors as thymocytes mature (134, 135). Thymocytes differentiate in a stepwise process that involves the somatic rearrangement

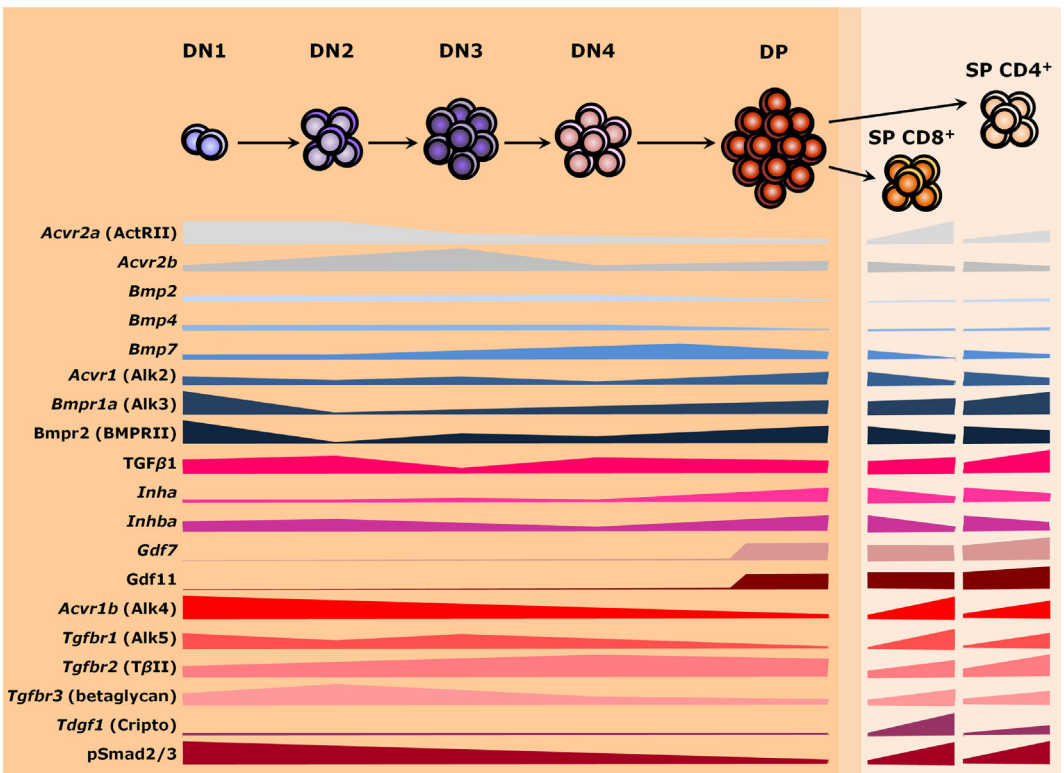

FIGURE 3 | TGF-β superfamily during thymopoiesis. Levels of selected ligands, receptors, and Smad intracellular effectors during the differentiation of αβ T lymphocytes. Common receptors between the TGF-β and the BMP branches are colored in shades of gray, whereas components of the BMP and the TGF-β pathways are colored in shades of blue and red, respectively. The darker orange region of the scheme represents the thymic cortex, whereas light orange represents the thymic medulla. A thin corticomedullary region is represented in between the cortex and medulla. Omitted components are either not present during thymocyte maturation or no information is available at present. DN, double-negative; DP, double-positive; SP, single-positive.

of T cell receptor (TCR) genes while migrating in close contact with stromal cells and the extracellular matrix (ECM) throughout thymic compartments (136, 137). In this process, a major group of αβ TCR-bearing T cells are produced, which ultimately function by recognizing peptide antigens presented by class I or class II major histocompatibility complexes (MHC I or MHC II, respectively) on the surface of host cells (138). Alternatively, a distinct lineage of T cells bearing γδ TCR chains develop, which recognize a quite unique group of molecules (139). Noteworthy, intrathymic lineage restriction and cell fate are determined not only by the type of TCR and its avidity for self-antigens but also by the acquisition of co-receptors that relay signals to intracellular effectors during T cell activation. Hence, generation of distinct cell types are tightly controlled as thymocyte progresses through thymic niches (7). Herein, we will point out some key aspects of the expression and influence of TGF-β superfamily signaling molecules on the distinct paths of thymocyte development: from $CD4^-CD8^-$ DN T cell precursors (further subdivided in DN1 to DN4 stages based on the surface expression of CD44 and CD25) to the highly expanded immature $CD4^+CD8^+$ DP cells, and upon the mature $CD4^+CD8^-$ or $CD4^-CD8^+$ SP cells.

DN1 to DN2 Cells

Entry of bone marrow-derived $Lin^-cKit^{high}CD44^+CD25^-$ cells, or ETPs, into the thymus occurs through the corticomedullary junction. In this intermediate region, these immature cells with T cell–B cell–myeloid potential come into contact with $K5^+K8^+$ bipotent TEPCs and mature T cells (67, 124, 140). They subsequently move into the thymus cortex toward the subcapsular zone as DN cells, as defined by the lack of CD4 and CD8 co-receptors (7, 141). In the cortex, developing thymocytes then upregulate CD25 – the α chain of the IL-2 receptor – to become $CD44^+CD25^+$ DN2 cells, which undergo D_β to J_β recombination of the β-chain locus (142, 143). This DN1 to DN2 transition is accompanied by a strong downregulation of *Bmpr1a* (Alk3) and *Bmpr2* (BMPRII) expression (127, 129). Most cells at this stage present high levels of pSmad2 along with Alk4 (*Acvr1b*) and ActRII (*Acvr2a*) on their cell surface, although a few cells also exhibit Alk5 (*Tgfbr1*) and TβRII (*Tgfbr2*) receptors (134). DN cells also express the type III co-receptor betaglycan/TβRIII (*Tgfbr3*), with highest levels at DN3 cells (144). Betaglycan seems to increase the binding strength of some ligands with their cognate receptors, therefore potentializing their effects (145–147). Thymocytes express no *Bmpr1b* (Alk6), *Acvr1* (Alk2), and *Acvr1c* (Alk7) during thymopoiesis (127, 129). Yet, high levels of inhibin βA subunit (*Inhba*) and TGF-β1 (*Tgfb1*) contrast with reduced levels of the inhibin α subunit (*Inha*), Bmp2, Bmp4, and Bmp7 at the DN2 stage (81, 82, 127, 130). When *Inha* mutants were used for E14.0 FTOC, a partial arrest at the DN2 stage impaired further T cell maturation (148). Likewise, antibody-dependent blocking of betaglycan in E14.0 FTOC resulted in a reduction of both DN2 and DP cells (144). By contrast, addition of TGF-β1 or TGF-β2 in E14.0 FTOC strongly inhibited T cell development by mainly impairing the differentiation of DN1 cells into DN2 (149). A slightly less strong impact after BMP4 treatment of E15.0–E15.5 FTOC or suspension cultures of fetal thymocytes resulted in cell cycle arrest at the DN1 stage without induction of apoptosis (89, 150). The use of BMP4-treated chimeric human–mouse FTOC produced similar findings (81), revealing a conserved role for Bmp4 during evolution. Besides, partial redundancy between BMP ligands also seems to occur in the thymus, since treatment of FTOC with BMP2, but not with BMP7, similarly affected the production of DP cells (150).

DN2 to DN3 Cells

Following T cell differentiation into $CD44^{-/low}CD25^+$ DN3 cells, V_β to DJ_β recombination gives rise to the β chain of the pre-TCR (143). At this stage, the levels of *Inha*, *Bmp2*, and *Bmp4* remain relatively low, *Bmp7* becomes upregulated up to the $CD3^-CD8^+$ intermediate single-positive (ISP) stage, and expression of *Inhba* and *Tgfb1* declines (82, 127, 130). Levels of Alk4 (*Acvr1c*), Alk5 (*Tgfbr1*), and ActRII (*Acvr2a*) gradually reduce as thymocytes mature, in contrast to TβRII, which is slowly upregulated – at this stage, Alk4 and Alk5 are co-expressed (134). Expression of *Bmpr1a* (Alk3) and *Bmpr2* (BMPRII) presents a small recovery at the DN3 and DN4 stages (127, 129). Nevertheless, conditional inactivation of *Bmp7* in the hematopoietic lineage using a *vav-iCre* line had no significant impact on T cell differentiation and total cell numbers, likely because endoderm-derived cTECs and mTECs may supply enough Bmp7 or other redundant factor for the mutant thymocytes (71, 82, 150). In particular, subcapsular cTECs, cortical DCs, and mTECs express *Bmp2* and *Bmp4* (71, 81, 82). Activation of the Bmp4 pathway in stromal cells indirectly impacts the DN to DP transition, as revealed by reconstitution experiments with thymocyte-depleted stroma treated with BMP4 or untreated stroma with BMP4-treated DN cells (89). Of note, although highly expressed up to the transition from DN2 to DN3, being downregulated up to the DP stage, and sustained at low levels at SP subsets (127), the gene referred as *Bmp1* is a procollagen C-proteinase involved in ventral body wall closure during embryogenesis. To our knowledge, there is no available functional information regarding its role during thymopoiesis, except that it was also found in cTECs and mTECs (71, 151).

DN3 to DN4 Cells

Should rearrangements result in unproductive β chains, DN3 cells undergo apoptosis and are phagocytized by cortical macrophages or DCs in a process termed β-selection (143, 152). Otherwise, successful recombination leads to a reduction in CD25 expression and the expansion of $CD44^-CD25^-$ DN4 thymocytes (153, 154). Both activin A and inhibin A similarly stimulate the DN3 to DN4 transition, as revealed in FTOC from wild-type fetuses at E14.0. However, treatment with activin A led to higher numbers of mature $CD24^{low}CD8^+TCR\beta^{high}$ T cells at the expense of $CD4^+$ cells, in contrast to inhibin A treatment, which stimulated the transition from DN4 to DP cells (148).

DP Cells

Rearrangement of the TCRα chain occurs at the DP stage and cells move from the cortical zone toward the thymic medulla (143, 155). During this migration, cTECs present self peptides through MHC molecules (pMHC) to the TCR of intermingling DP thymocytes in a process known as positive selection, in which interactions of low-avidity drive clones to survive and continue

maturation (156). At the DP stage, Alk4 (*Acvr1b*), Alk5 (Tgfbr1), and ActRII (*Acvr2a*) reach their lowest levels, but the number of cells concomitantly presenting Alk5 and pSmad2 increases in relation to Alk4-positive cells (134). By contrast, *Bmpr1a* (Alk3) and *Bmpr2* (BMPRII) are highly expressed (127, 129). Two members of the GDF subgroup, Gdf7 and Gdf11, seem to be induced in DP cells and sustained at SP stages, with $CD4^+$ T cells presenting relatively higher levels than $CD8^+$ T cells (127). Gdf7 signals through BMP-specific receptors as Alk3 and BMPRII, whereas Gdf11 binds TGF-β-related receptors, as Alk4 and Alk5 (157–159). Their roles on T cell function are largely obscure, if any. Mouse mutants for *Gdf7* exhibit variable hydrocephalus and fail to produce a class of commissural neurons (160). Male mutants are sterile due to impaired differentiation and branching morphogenesis of the seminal vesicle, with no other affected reproductive structure (161). In turn, mutants for *Gdf11* show homeotic transformations due to a delayed trunk to tail transition (162, 163). They die after birth because of renal defects, which may vary from hypoplasia to complete bilateral agenesis (164). Curiously, oral infection with Gram-negative bacteria, *Aggregatibacter actinomycetemcomitans*, in rats led to a chronic upregulation of *Gdf11* expression among other cytokines in both peripheral $CD45RA^+CD4^+$ T cells and B cells (165). At present, however, little is known on the effects of GDFs over thymopoiesis.

SP Cells

Still in the cortex, differentiating thymocytes start to lose the expression of either CD4 or CD8 and migrate toward the medulla. The choice for either CD4 or CD8 SP lineage seems to occur at a transitional step defined as $CD4^+CD8^{low}$ and depends on TCR interaction with the MHC class II or class I, respectively (166, 167). Additionally, it also relies on the triggering of a transcriptional machinery that operates distinctly for final differentiation (165, 166). Noteworthy, the SP cells sustain *Bmpr1a* (Alk3) and *Bmpr2* (BMPRII) expression, and upregulate Alk5 (*Tgfbr1*) and TβRII (*Tgfbr2*), which lead to increased levels of pSmad2 (84, 127, 129, 134). At this stage, fine-tuning of TGF-β signaling may occur by type III co-receptors – $CD4^+CD8^-$ cells upregulate *Tgfbr3* (betaglycan), whereas $CD4^-CD8^+$ cells exhibit higher levels of Cripto (*Tdgf1*) (127, 144). Genetic loss of *Tgfbr3* in FTOC resulted in decreased numbers of both DP and SP cells, probably related to the high rates of apoptosis in DN, DP, and $CD4^+$ SP subsets (144). An apoptotic phenotype was also observed in the liver of *Tgfbr3* mutants (168). However, a functional role for Cripto during thymopoiesis is currently unresolved, despite its importance for TGF-β binding and inhibition (169). Mutants for this gene present a strong deleterious phenotype during gastrulation and die shortly afterward (170, 171). Modulation of TGF-β family members, their receptors, and co-receptors at the DP stage is therefore associated with the terminal differentiation of thymocytes.

Impact of TGF-β Signals on the Differentiation of Thymic Regulatory T Cells

Regulatory T (Treg) cells have the ability to suppress autoreactive T cells, and they can originate from the thymus or be induced in the periphery (172). Thymus-derived Treg (tTreg) arise in the thymus from SP $CD4^+$ T cells that escape negative selection during maturation by presenting TCR signals of variable affinities (80, 172–174). More specifically, TCRs with high avidity for self-antigens trigger a new upregulation of CD25 (IL-2 receptor α chain) and therefore exhibit an increased responsiveness to IL-2, ultimately inducing the expression of the transcription factor forkhead box P3 (*Foxp3*) through a STAT5-dependent mechanism (175–177). Foxp3 is the critical transcription factor for Treg cell lineage, as its loss abolishes tTreg cells and lead to systemic autoimmunity and death (178, 179). Conversely, forced expression of *Foxp3* in $CD25^-CD45RB^{high}CD4^+$ SP cells transferred into severe combined immunodeficiency (SCID) hosts suppressed exacerbated inflammation (180). Unlike previously thought (181), however, expression of *Foxp3* in developing tTreg cells induced apoptosis instead of cell survival. Cell death is prevented by limiting concentrations of γc-mediated survival signals enough to sustain only fewer than one million $Foxp3^+$ cells (182).

Signals from members of TGF-β superfamily also play important roles over the differentiation and survival of tTreg cells. In particular, conditional loss of *Tgfbr1* (Alk5) in thymocytes seems to be involved in tTreg specification, since a *Lck–Cre* mouse line completely blocked differentiation of tTreg cells in neonatal mice, whereas later inactivation of *Tgfbr1* by a *Foxp3–Cre* line produced no differences in tTreg numbers as compared to wild-type mice (80, 183). In addition, the intrathymic injection of an anti-TGF-β antibody suppressed *Foxp3* expression in a TCR transgenic $CD4^+CD25^-$ SP cells (80). Of note, impaired Alk5 signaling induced by the *Lck–Cre* line caused no significant impact on $CD4^+$ and $CD8^+$ SP cell numbers (183). A later increase in Treg cells induced in the periphery (pTreg) in these mutant mice relied on IL-2 signaling, since ablation of this cytokine produced no detectable cells in organs, such as the spleen and liver (183). Similarly, thymocyte deficiency of *Tgfbr2* from a *CD4–Cre* mouse line resulted in reduced numbers of tTreg cells due to Bim-dependent apoptosis likely independent of γc-signaling, without affecting TCR-$\beta^{high}CD4^+Foxp3^-$ mature T cells in neonatal mice (84). Unlike *Tfgbr1*-mutant thymocytes, conditional deletion of *Tgfbr2* also resulted in low numbers of pTreg cells (84). Induction of pTreg cells relies on the Smad3-dependent upregulation of *Foxp3* triggered by activation of both TCR and TGF-β signaling and facilitated by retinoic acid, which increased pSmad3 accessibility to regulatory sequences of the Foxp3 promoter and concurrently counteracted the suppressing effects of a c-Jun N-terminal Kinase (JNK) inhibitor (184, 185). Genetic analyses of the regulatory CNS1 region of *Foxp3*, which contains binding sites for NFAT, Smad3, and RAR/RXR, revealed that tTreg cell development occurs independently of its activation, whereas its chromosomal deletion largely impaired the production of pTreg cells in secondary lymphoid organs (184–186). In accordance to the different requirements revealed for tTreg in comparison to pTreg populations, TGF-β1 is essential for the peripheral differentiation and maintenance of pTreg cells, but seems to be dispensable for tTreg maturation (187).

Taking into consideration the upregulation of all three TGF-β ligands by stromal cells upon thymocyte apoptosis in the thymus, along with recent findings regarding mutants for distinct

TGF-β-specific receptors (80, 84, 183), it is possible that TGF-β ligands may play a redundant yet underestimated role in the immune system. Noteworthy, mutants for TGF-β2 and TGF-β3 also exhibit perinatal mortality, a characteristic that complicates the examination of their role in adults (188–190). Although at first sight, the phenotypes observed in these mutants were generally non-overlapping, some particular structures showed similar defects between single mutants (e.g., cleft palate in either TGF-β2 and TGF-β3 mutants) or exclusive abnormalities in compound mutants, such as abnormal brain vascular morphogenesis and impaired midline fusion along with earlier embryonic lethality in *Tgfb1*^{RGE}*;Tgfb3* and *Tgfb2;Tgfb3* compound mutants, respectively (191, 192). However, development of tTreg was never evaluated in these compound mutants. An alternative explanation may consider the participation of a previously unappreciated ligand of the TGF-β superfamily in the differentiation of tTreg cells. Whether this is indeed the case, this candidate ligand should probably signal through Alk5 and TβRII receptors to phosphorylate Smad2 and Smad3 intracellular effectors. Thereby, likely ligands to be thoroughly evaluated due to their expression pattern and receptor affinity are Gdf11 and Gdf8/myostatin – curiously two members that showed redundancy in patterning the axial skeleton as revealed by *Gdf11;Mstn* double mutants. Unfortunately, examination of fetal thymus morphology and T cell differentiation using FTOC was not performed in these mutants (193).

Noteworthy, TGF-β signals also regulate the thymic development of IL-17-producing cells. A subset of γδ T cells acquire the capacity to produce IL-17 inside the thymus via a TGF-β1-dependent machinery, and both Tgfβ1$^{-/-}$ and Smad3$^{-/-}$ mice were shown to be completely devoid of IL-17-producing γδ T cells (194). Additionally, NKT17 cells comprise a thymic-derived IL-17-producing, CD1d-restricted, and glycolipid antigen-reactive T cell subset (195, 196). These cells express high levels of TβRII and depend on TGF-β signals for differentiation and survival within the thymus and in the periphery (197, 198).

A TGF-β Member for Thymus Rejuvenation?

Aging is an inherent process of living beings, normally associated with gradual loss of function and structure over time – accumulation of reactive species, DNA damage, abnormally folded proteins, and telomere shortening are just some of the molecular changes that may be followed by increased apoptosis, cell transformation, or other cellular event that will ultimately lead to death (199). Although this negative scenario was initially thought to be irreversible, numerous evidences point out that at least in part it is possible to slow down or eventually reverses some specific aging phenotypes. Taking the thymus as example, aging is easily recognizable by a sharp decrease in cellularity of both lymphoid and stromal compartments, whereas the number of thymic adipocytes inversely increases (200, 201). Ultimately, these thymic changes lead to a reduction of naïve T cells in the periphery along with an increase of memory T cells, which reflects in the organism ability to respond to both infection and tumorigenesis (202).

Multiple factors may trigger thymic involution, including the production of sex steroid hormones from puberty, increased calorie intake, or diminished levels of some growth factors and cytokines, such as fibroblast growth factor 7 (FGF7)/keratinocyte growth factor (KGF), insulin-like growth factor (IGF-1), growth hormone (GH), interleukin-7 (IL-7), and IL-22 (203). Modulation of each of them is able to rescue the aged thymic phenotype and restore the immune function at some level (204–210). However, some of these strategies may be inefficient, invasive, non-specific, or produce undesirable side effects to be used in humans (211). A quest for thymic rejuvenation therapies therefore faces daunting challenges in the clinic. Of particular interest, forced expression of *Foxn1* was shown to effectively reprogram fibroblasts into TECs or regenerate fully involuted thymuses at many different experimental setups, both *in vitro* or *in vivo* (212–214). In this context, signals that control *Foxn1* expression might be used to restore the integrity of the thymic epithelial niche and subsequently flourish thymopoisesis in the elderly. In this scenario, administration of soluble factors, such as ligands of the TGF-β superfamily, may be used as regenerative drugs.

Recent findings have revealed that levels of some circulating factors vary with age and that heterochronic parabiosis, i.e., a surgical procedure that connects the circulatory systems of animals with different ages, was able to reverse age-related phenotypes as cardiac hypertrophy (132). These authors further identified the TGF-β member Gdf11 as responsible for restoring cardiac function in old mice, a finding that was further expanded to other systems. In particular, daily treatment of old mice with recombinant GDF11 improved skeletal muscle mass and strength, as well as the integrity of brain vasculature and cognitive function (215, 216). In culture, Gdf11 promoted osteoblastogenesis while inhibiting adipogenesis in bone marrow-derived cells (217). Administration of GDF11 in endothelial progenitor cells triggered cell sprouting and migration, also revealing a role in the formation of blood vessels (218).

Whether Gdf11 or other circulating factor can be used as a rejuvenating cytokine for the thymus remains to be thoroughly assessed. Indeed, *Gdf11* is expressed in the thymus of young mice (132), whereas the levels of its non-exclusive receptors, Alk4 and Alk5, vary in thymocytes and TECs, as previously discussed. Of note, however, therapy with Gdf11 produced some side effects in mice (219), and a recent study by Egerman et al. has recently questioned the aforementioned observations (220). Whereas these controversial data on Gdf11 await further investigation, it is noteworthy that heterochronic parabiosis did not reverse thymic involution, but caused atrophy with mild effects on T cell subpopulations of young mice and a reduction in the number of $CD4^+CD25^+Foxp3^+$ regulatory T cells in old partners to the level of the young pair (221). Although a putative rejuvenating factor for the thymus still awaits to be determined, this controversial matter helps to bring the debate on the role of TGF-β superfamily members for the thymus function.

Concluding Remarks

Although the differentiation of T cells is mainly driven by the rearrangement of TCR genes, many members of the TGF-β superfamily exert critical roles in their stepwise progression during thymic migration. Historically, special attention had been

given to the activity of TGF-β ligands in the induction of Treg cells and tolerance to self-antigens, as well as to BMP signaling on thymus organogenesis. However, other members are also produced by developing thymocytes, thymic stromal cells, or may circulate throughout the body by the blood stream and reach the thymus. These ligands signal through the same limited sets of type I and type II receptors to produce dissimilar outcomes either by affecting distinct stages or cell types (e.g., thymocytes versus TECs). How such TGF-β superfamily ligands affect T cell maturation, thymus proper physiology, or its involution remain poorly understood and should be the focus of future research. In addition, a scenario in which a TGF-β superfamily member or its inhibitor acts to rejuvenate the aged thymus may be a likely case for future research.

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