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Thymflammation: The Role of a Constitutively Active Inflammatory Network and "Ectopic" Cell Types in the Thymus in the Induction of T Cell Tolerance and Beyond

Taras Kreslavsky[1,2]

[1]Division of Immunology and Respiratory Medicine, Department of Medicine Solna, Karolinska Institutet, Karolinska University Hospital, Stockholm, Sweden | [2]Center for Molecular Medicine, Karolinska University Hospital, Stockholm, Sweden

Correspondence: Taras Kreslavsky (taras.kreslavskiy@ki.se)

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ABSTRACT

The thymus exhibits constitutive activation of nearly all major inflammatory pathways, including sterile MyD88-dependent signaling and interferon production by mTECs, the presence of cellular and molecular components of type 1, type 2, and type 3 responses, as well as sustained B cell activation. The reasons for the existence of such a complex constitutively active inflammatory network at the site of T cell development—where the initial pathogen encounter is unlikely—have remained enigmatic. We propose that this inflammatory thymic 'ecosystem' has evolved to promote immunological tolerance to 'inflammatory self'—endogenous molecules absent from most peripheral tissues at steady state but upregulated during pathogen invasion. The spatial and temporal overlap with pathogen presence makes the discrimination of the inflammatory self from pathogen-derived molecules a unique challenge for the adaptive immune system. The frequent occurrence of diseases associated with autoantibodies against proinflammatory cytokines underscores the persistent risk of these molecules being misidentified as foreign. Their abundant representation in the thymus, therefore, is likely to be critical for maintaining tolerance. This review explores current insights into the thymic inflammatory network, its cellular and molecular constituents, and their role in the induction of T cell tolerance.

1 | Introduction

The generation of a highly diverse T cell receptor (TCR) repertoire by V(D)J recombination results in the production of self-reactive receptors by a fraction of developing T cells. To prevent autoimmunity, these cells must be functionally or physically inactivated in the thymus (central tolerance) or after their egress to the periphery (peripheral tolerance). Central tolerance relies on antigen availability at the site of lymphocyte development. In the thymus, several mechanisms promote the exposure of developing T cells to a diverse set of peripheral antigens. One such mechanism is the 'ectopic' expression of tissue-specific antigens by medullary thymic epithelial cells (mTECs) [1], in part driven by the specialized transcriptional regulator AIRE [2]. Moreover, minor subsets of mTECs can induce large parts of molecular programs of various peripheral cell types. These include thymus equivalents of tuft cells, microfold cells, keratinocytes, myocytes, ciliated epithelial cells, neuroendocrine cells, as well as cells combining some parts of both enterocyte and hepatocyte molecular programs [3–6]. These populations, collectively called thymic mimetic cells, are thought to further broaden the spectrum of tissue antigens available in the thymus. Finally, the

thymus harbors all major dendritic cell (DC) subsets, some of which migrate to the thymus from the periphery, resulting in the import of peripherally acquired self-antigens [7, 8].

Thymocytes that produce TCRs strongly recognizing self-antigens present in the thymus can be inactivated via two main mechanisms. Many autoreactive cells are eliminated by negative selection—apoptosis induced by strong TCR signaling [9]. However, some autoreactive thymocytes can instead be diverted into lineages with regulatory or innate-like properties [10]. The differentiation process of these subsets driven by strong signaling from an autoreactive antigen receptor is called agonist selection [10]. Agonist selection of MHC class II-restricted autoreactive thymocytes can result in the differentiation of regulatory T (Treg) cells through a multistep process that leads to upregulation of the transcription factor Foxp3, which orchestrates a large part of the regulatory T cell molecular program [11]. The peripheral suppressive functions of Tregs are also at least in part dependent on their recognition of self-antigens [12].

In contrast, agonist selection to innate-like T cell lineages, which in most cases takes place on MHC I or MHC I-like molecules, results in the differentiation of cells that can mount rapid effector responses reminiscent of those of innate immune cells. In this case, strong signaling from an autoreactive antigen receptor results in the execution of one of several specialized, restricted effector programs (e.g., T helper (Th)1-, Th2-, or Th17-like [13, 14]). Agonist selection on classical and non-classical MHC I molecules can result in differentiation into innate-like cells with a $CD4^{-}CD8\beta^{-}$ phenotype that constitute a large fraction of the intestinal intraepithelial lymphocytes [15–17]. Some innate-like T cell lineages, rather than being selected on MHC–peptide complexes, undergo agonist selection on MHC I-like molecules presenting non-peptide antigens. These include invariant natural killer T (iNKT) cells that recognize lipid antigens presented by CD1d and mucosal-associated invariant T (MAIT) cells that recognize vitamin B metabolites presented by MR1. Finally, while lineage diversion to innate-like subsets occurs only for a small fraction of αβ T cells, this pathway of differentiation is very prominent in the case of γδ T cells [18–20]. Interestingly, recent studies have demonstrated that many thymic γδ T cells and iNKT cells are not newly developing immature cells but rather mature tissue-resident lymphocytes [21, 22]. Possible reasons for this accumulation of effector T cells at the site of T cell development, where the initial pathogen encounter is unlikely, are discussed below.

2 | The Problem of 'Inflammatory Self'

Self/non-self discrimination by the adaptive immune system relies mainly on the context of antigen encounter. In the absence of pathogen-induced inflammation, autoreactive T cells are either physically eliminated or inactivated functionally [23]. Pathogen recognition by the innate immune system triggers inflammation (for the purpose of this review, broadly defined as any immune reaction to a pathogen or a sterile process involving similar components). This inflammatory response involves, among many other changes, the upregulation of co-stimulatory molecules required to 'license' a naïve T cell to initiate clonal expansion and execution of an effector molecular program. Inflammation also causes significant changes in the spectrum of presented self-epitopes, which occur through at least two mechanisms. First, the upregulation of inflammation-associated molecules directly changes the spectrum of expressed self-antigens [24]. Second, changes in the expression of antigen processing and presentation machinery components, such as the induction of immunoproteasome subunits, are likely to alter the repertoire of epitopes derived from non-inflammation-associated self-antigens [25, 26]. The resulting drastic change in the spectrum of presented self-epitopes [24] coincides in time and space with the emergence of pathogen-derived foreign antigens. The discrimination between self-epitopes induced by inflammation and foreign antigens, therefore, represents a unique challenge for the adaptive immune system.

The high risk of the inflammatory self being mistakenly recognized as foreign antigens is underscored by the frequent breakdown of tolerance to proinflammatory cytokines in various human diseases. Tolerance to various cytokines, including type I interferons (IFNs), IL-17, TNF, IL-6, and many others, is breached in numerous autoimmune disorders [27]. These include immunodysregulation polyendocrinopathy enteropathy X-linked (IPEX) syndrome (Foxp3 deficiency), autoimmune polyendocrine syndrome type 1 (APS-1) (AIRE deficiency), Sjögren's syndrome, rheumatoid arthritis, systemic lupus erythematosus (SLE), and many others (reviewed in [27]). Most strikingly, a significant number of otherwise healthy individuals produce anti-IFN autoantibodies with age, with approximately 4% of people over 70 exhibiting these antibodies [28]. Their presence is linked to poor outcomes in various viral infections, including infections with the influenza virus, SARS-CoV-2, West Nile virus, and herpesviruses, among others [28–32]. Of note, as these antibodies are often class-switched and hypermutated, they are likely to reflect a breach in both B and T cell tolerance.

Nevertheless, in most cases, the adaptive immune system remains tolerant to inflammation-associated self-antigens. The mechanisms of induction of this tolerance have remained unexplored until recently. Recent studies reviewed here reveal the surprising role of the complex inflammatory network persistently active in the thymus in promoting tolerance to this inflammatory facet of self. Here, we review current insights into this thymic inflammatory 'ecosystem' and its role in the induction of tolerance to inflammatory self. We discuss distinct inflammatory circuits that operate in the thymus under homeostatic conditions, including: (a) the interferon-driven inflammatory module, (b) a complex network of cell types associated with type 2 inflammation, (c) thymic sources of type 3 cytokines, (d) other inflammatory pathways activated in the thymus, including those that lead to activation of MyD88-dependent signaling, (e) signals orchestrating thymic B cell activation.

3 | The Interferon Inflammatory Module

3.1 | Constitutive Sterile Type I/III IFN Production and Signaling in the Thymus

IFNs are a group of cytokines that can be produced in response to viral infections and trigger antiviral defenses in various cell types. IFNs are classified into three distinct families: type I,

which includes IFNs β, ε, κ, ζ (mice), ω (humans), and multiple IFNα isoforms; type II, represented by IFNγ; and type III, comprising several isoforms of IFNλ [33]. This section discusses thymic expression and functions of type I and type III IFNs that share similar induction mechanisms, antiviral functions, and induce highly overlapping sets of IFNs-stimulated genes (ISGs) [33]. Thymic expression and function of the type II IFN, IFNγ, are discussed in the section 'IFNγ Production and Type 1 Inflammatory Pathways in the Thymus' below.

Signaling through type I and type III IFN receptors (composed of IFNAR1/IFNAR2 and IFNLR1/IL-10RB chains, respectively) results in the formation of a transcription factor complex consisting of STAT1, STAT2, and IRF9, which in turn induces a broad antiviral molecular program. Many ISGs directly or indirectly induced by these transcription factors encode antiviral effector proteins that interfere with various stages of the viral life cycle (such as viral entry, nuclear import of viral genomes, and replication) [33]. Of note, ISGs also encompass genes that encode components of the antigen processing and presentation machinery, including immunoproteasome subunits, with the latter induced by both IFNγ and type I/III IFNs [25]. IFN signaling induces a very large transcriptional program with several hundreds of ISGs strongly upregulated in response to type I/III IFNs [34–38]. Moreover, some estimates suggest that the expression of up to 10% of genes in the human genome can be modulated by IFN signaling [33]. The scale of these transcriptional changes is highlighted by the fact that, similar to proliferating cells, cells undergoing IFN signaling often form distinct clusters in scRNA-seq datasets [39–41]. Notably, while a large part of the IFN-induced program is stereotypical, many ISGs show various degrees of cell type specificity [34–38].

From the perspective of immunological tolerance, ISGs encode a broad array of self-antigens that are not expressed in most tissues at steady state but are rapidly upregulated in a variety of cell types upon virus recognition by the innate immune system. Moreover, through effects on the expression of the components of the antigen processing and presentation machinery, IFN signaling is likely to alter the spectrum of epitopes that are generated and presented for non-ISG self-antigens. These prominent changes in the self-immunopeptidome overlap in time and space with inflammation and the presence of viral antigens. Thus, in addition to the induction of tolerance to IFNs themselves, immunological tolerance has to be induced to hundreds, if not thousands, of ISGs, some of which are cell type-specific, as well as to IFN-induced epitopes from non-ISG-encoded self-antigens. This tolerance is of crucial importance, as illustrated by the fact that autoantibodies to type I IFNs are associated with poor prognosis in many viral infections [28–32].

How can this tolerance be achieved? Multiple lines of evidence indicate that type I and type III IFNs are constitutively produced and sensed in the thymus [38, 42–46]. An early study with a luciferase reporter under the control of IFNβ regulatory elements revealed that the spontaneous production of luciferase was by far the highest in the thymus, far above the levels observed in the secondary lymphoid organs, gut, or lung [42]. Analysis of cell types that expressed the reporter revealed that *Ifnb1* was predominantly expressed by mTECs, leading to the conclusion that, under non-inflammatory conditions, these cells may be the main source of IFNβ in the whole body [42]. Analysis of expression datasets indicated that not all type I/III IFNs are equally well represented in the thymus: while mTECs express high levels of *Ifnb1*, *Ifnl2*, and *Ifnl3*, genes encoding type I IFNs other than IFNβ are expressed at much lower levels [38, 47, 48]. Likewise, human mTECs predominantly express *IFNB1*, *IFNL1, IFNL2*, and *IFNL3* [38, 49]. The contribution of plasmacytoid DCs (pDCs) to the production of type I IFNs in the thymus remains incompletely understood. While some studies suggest the expression of α IFNs by these cells in human and mouse thymus [50, 51], side-by-side RNA-seq comparison of mouse mTECs and pDCs suggests that thymic pDCs express little, if any, type I/III IFNs at the steady state, indicating that mTECs may be the main source of these cytokines in the thymus [38, 47].

The expression of type I and type III IFNs by mTEC is not affected in germ-free mice and is independent of MyD88/TRIF signaling or sensing nucleic acids through MAVS/STING-dependent pathways [38]. Therefore, thymic IFN production is not induced by microbiota and is unlikely to reflect a response to endogenous retroviruses. Instead, their expression is dependent on AIRE [38, 42, 52], with the expression of *Ifnb1* being dramatically decreased in AIRE-deficient mTECs [38, 42], while *Ifnl2* and *Ifnl3* exhibit a milder downregulation [38, 52]. Analysis of fluorescent reporters driven by *Ifnl2* and *Ifnb1* regulatory elements demonstrated that, similar to many other AIRE-dependent genes, these cytokines are expressed by small and largely non-overlapping subsets of mTECs [38].

This sterile production of type I/III IFNs in the thymus, in turn, induces IFN signaling in a wide variety of cell types. Early evidence for this was obtained in studies with a Cre line driven by the regulatory elements of a canonical ISG—*Mx1*. Although *Mx1*-Cre is widely used as a poly(I:C)-inducible Cre line [53], in the thymus it exhibits spontaneous *Ifnar1*-dependent activity. This activity is largely restricted to the thymic medulla, resulting in a spontaneous 'fate mapping' of a large fraction of mTECs when *Mx1*-Cre is combined with a fluorescent reporter for Cre activity [43]. Interestingly, a 'real-time' *Mx1* reporter, *Mx1*-GFP, is expressed only in a small subset of mTEC cells [38]. Taken together, these results suggest a possibility that the majority of mTECs undergo transient IFN signaling rather than continuously respond to these cytokines. However, constitutive IFN signaling seems to occur in many other subsets of thymic antigen presenting cells (APCs), as *Mx1*-GFP is expressed by the majority of thymic DCs (including conventional DC1 (cDC1), cDC2, and pDC s) and thymic monocytes, as well as by sizable populations of thymic B cells and macrophages [38, 45]. Single positive (SP) thymocytes also undergo IFN signaling, as judged by their *Ifnar1*-dependent expression of the MHC class Ib molecule Qa2, as well as a number of canonical ISGs [46, 51]. Taken together, these results indicate that a variety of cell types undergo IFN signaling in the thymus. Moreover, RNA-seq experiments have demonstrated that this signaling induces a broad spectrum of ISGs in thymic APCs [38]. Comparison of *Ifnar1*$^{-/-}$, *Ifnlr1*$^{-/-}$, and *Ifnar1*$^{-/-}$*Ifnlr1*$^{-/-}$ mice showed that the expression of most, although not all, ISGs relied on a redundant function of type I and type III IFN signaling [38]. Of note, genes encoding several components of antigen processing and presentation machinery, including the immunoproteasome subunit-encoding genes *Psmb8* and *Psmb9*, were upregulated by type I/III IFN signaling

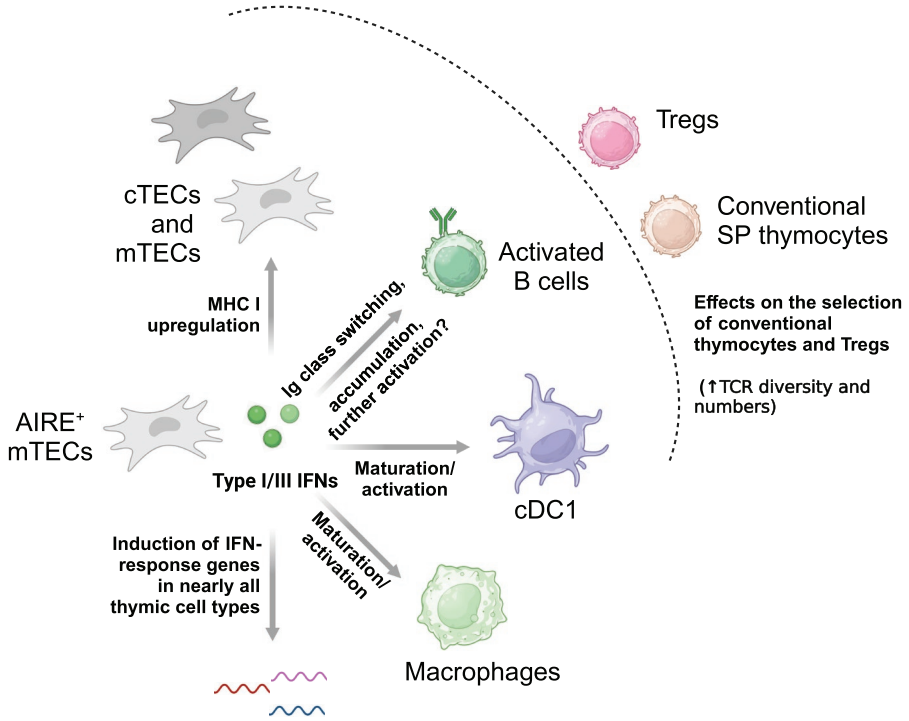

FIGURE 1 | Effects of type I/III IFNs on the components of the thymic microenvironment. Type I (predominantly IFNβ) and type III IFNs are produced by mTECs in an AIRE-dependent manner. IFN signaling results in the upregulation of a broad spectrum of ISGs in most thymic populations, induces maturation of thymic cDC1 cells and macrophages, regulates class switching, phenotype, and abundance of thymic B cells, and enhances MHC I expression by TECs. IFN signaling, most likely through these effects on thymic APCs, results in higher TCR diversity of both conventional CD4SP thymocytes and Treg cells.

in thymic DCs [38]. These results suggest that IFN signaling can also modulate the spectrum of self-epitopes generated and presented by thymic APCs from non-ISG-encoded self-antigens.

3.2 | Effects of Type I/III IFN Production on the Other Components of the Thymic Microenvironment

The constitutive IFN signaling, in addition to the induction of the canonical IFN response program, results in significant alterations in the composition and state of many thymic populations (Figure 1). Type I/III IFN signaling induces the activation and maturation of thymic cDC1 cells and macrophages [38]. Type III IFN signaling also positively regulates the levels of MHC I expression on mTECs and cTECs [52] and regulates the activation status and class switching in thymic B cells in a cell-intrinsic fashion [45] (see section 'Similarities and Differences in Thymic and Peripheral B cell Activation' below). Moreover, the overall abundance of thymic B cells is decreased in *Ifnar1*$^{-/-}$, *Ifnlr1*$^{-/-}$, and particularly—in *Ifnar1*$^{-/-}$*Ifnlr1*$^{-/-}$ mice [38]. Finally, *Ifnar1*$^{-/-}$*Ifnlr1*$^{-/-}$ mice also exhibit decreased TCR repertoire diversity and numbers of both mature conventional SP thymocytes and thymic Treg cells [38]. Some of these effects on thymic selection likely reflect changes in the composition, maturation status, and MHC expression of thymic APCs discussed above. In addition, type I IFN signaling may also play a cell-intrinsic role in Treg generation, as *Foxp3*-Cre-mediated deletion of *Ifnar1* results in a strong competitive disadvantage for Treg cells that is evident already in the thymus [54]. However, it is tempting to speculate that some of the Treg cells lost in *Ifnar1*$^{-/-}$*Ifnlr1*$^{-/-}$ mice could have recognized IFN-induced epitopes and therefore could regulate immune responses involving IFN signaling at the periphery.

Does IFN production in the thymus induce tolerance to inflammation-associated self-antigens upregulated in response to IFN signaling? Transfer of CD4 T cells from IFNβ-deficient mice into poly(I:C)-treated, but not PBS-treated, recipients results in enhanced proliferation of these cells compared to their WT counterparts [38], suggesting a partial loss of CD4 T cell tolerance to ISGs in the IFNβ-deficient mice.

Despite this representation of type I/III IFNs in the thymus, cytokines of this family are often targeted by autoantibodies, suggesting a breach in CD4 T cell tolerance. Anti-IFN autoantibodies arise in individuals with autoimmune diseases—for example, in SLE, Sjögren's syndrome, rheumatoid arthritis, IPEX, and APS-1 patients (the latter in line with the control of thymic expression of these IFNs by AIRE) [27]. Moreover, a substantial fraction of otherwise healthy individuals develops anti-IFN autoantibodies with age [28] and such autoantibodies are associated with poor prognosis in many viral infections [28–32]. While the exact mechanisms of this tolerance breach are unclear, it is interesting to note that such autoantibodies often target α IFNs as well as IFNω [30], which, in contrast to λ IFNs and IFNβ are only lowly expressed by human mTECs [38, 49]. This suggests that poor representation of type I IFNs other than IFNβ in the thymus may increase the risk of tolerance breach to these cytokines. Interestingly,

preferential targeting of α and ω IFNs holds true in APS-1 patients [30, 55, 56]. This may be consistent with the observation that, while the expression of genes encoding λ and β IFNs is strongly reduced in mTECs from AIRE-deficient mice, they remain detectable [38, 48]. In contrast, the expression of *Ifna* genes, already very low in wild-type mTECs, appears to be almost entirely abolished in *Aire*$^{-/-}$ thymus [38, 48], suggesting one possible explanation for the breach of tolerance to α IFNs in APS-1 patients.

Taken together, these findings, along with numerous other examples of autoantibodies targeting proinflammatory cytokines [27], support the notion that such molecules are at a constant risk of being misidentified by the immune system as foreign. Their abundant representation in the thymus may thus be essential for the preservation of tolerance to these inflammation-associated self-antigens.

3.3 | IFNγ Production and Type 1 Inflammatory Pathways in the Thymus

In addition to type I/III IFNs, IFNγ is also constitutively produced in the thymus. Cells expressing the IFNγ-YFP reporter are readily detectable in the thymus at steady state [57], and IFNγ is secreted by 0.3% of total thymocytes upon PMA/Ionomycin stimulation—a frequency much higher than that of IL-4 or IL-17A producers [58]. Almost two-thirds of these cells are T cells (predominantly iNKT cells with some contribution of other αβ subsets as well as γδ T cells), and about one-third—TCR-negative cells expressing Thy1 and/or NK1.1 [58], likely representing ILCs and/or NK cells [59, 60]. Analysis of scRNA-seq datasets indicates that some mTECs also express *Ifng* [58]. Despite this abundance of IFNγ producers, in contrast to *Ifnar1*$^{-/-}$*Ifnlr1*$^{-/-}$ animals incapable of type I/III IFN signaling, IFNγ receptor-deficient (*Ifngr1*$^{-/-}$) mice exhibit normal maturation of thymic DC1 and macrophages [38]. Furthermore, since the alterations in the thymic myeloid compartment observed in *Stat1*$^{-/-}$ mice (incapable of any canonical IFN signaling) closely mirror those in *Ifnar1*$^{-/-}$*Ifnlr1*$^{-/-}$ mice (which retain responsiveness to IFNγ), these defects are likely attributable entirely to the disruption in type I/III IFN signaling [38]. IFNγ receptor deficiency results in modestly decreased CXCR3 and Ly6C expression on thymic B cells, suggesting that these cells do undergo IFNγ signaling [45]. However, in contrast to mice incapable of type III IFN signaling, exhibiting a three-fold decrease in IgM^{-}IgD^{-} class-switched cells among thymic B cells (see below), the frequency of total class-switched cells in the thymus is unaltered in *Ifngr1*$^{-/-}$ mice [45]. It remains, however, to be analyzed if switching to specific Ig subclasses may be selectively altered in these animals. Finally, IFNγ signaling has a modest but detectable contribution to cytokine-driven stages of CD8SP differentiation [61]. Thus, despite the abundance of IFNγ-producing cells in the thymus, only relatively subtle effects of IFNγ signaling on the thymic microenvironment have been reported to date, and its possible broader impact on thymus physiology remains to be explored. For example, as IFNγ is a potent inducer of immunoproteasome subunits [25], it would be of interest to test if thymic IFNγ signaling results in changes in the antigen presentation machinery of thymic APCs. Likewise, little is known about the stromal populations that could constitute the niche for IFNγ-producing innate and innate-like lymphocytes in the thymus. Nonetheless, transpresentation of IL-15 by mTECs was shown to regulate the accumulation of thymic IFNγ-producing iNKT cells and γδ T cells [62–64].

In addition to IFNγ, other type 1 inflammation-associated cytokines are also represented in the thymus. For example, IL-15 is expressed by various thymic stromal populations, with the highest levels of expression detected in MHC II^{hi} mTECs [65]. Genes encoding both subunits of IL-12 are also expressed in the thymus [66, 67]; their expression is detectable in thymic DCs [68], and analysis of a thymic scRNA-seq dataset [69] indicates that mTECs also likely produce this cytokine. Likewise, TNF is constitutively produced in the thymus, including its expression by developing thymocytes [70].

Indirect evidence suggests that cells producing type 1 cytokines in the thymus can be involved in the induction of tolerance to these inflammation-associated self-antigens. Peptide immunization experiments using an MHC class I epitope in IFNγ demonstrate that a strong CD8 T cell tolerance is induced to this cytokine [58]. Bone marrow chimera experiments reveal a redundant role of hematopoietic and radioresistant populations in the induction of this tolerance, in line with its expression by both hematopoietic and stromal populations in the thymus [58]. While the exact contribution of central and peripheral tolerance to this process remains to be assessed, the presence of innate and innate-like IFNγ-producing cells in the thymus [57, 58] at frequencies sufficient to induce tolerance to other antigens [58] suggests that the thymic representation of IFNγ ensures tolerance to this cytokine.

4 | The Type 2 Cytokine Inflammatory Module

4.1 | Thymus-Resident Type 2 Immune Cells

The type 2 inflammatory network is one of the best-characterized components of the thymic inflammatory environment. A number of cell types associated with type 2 immune responses—such as thymic tuft cells [4, 5], eosinophils [71, 72], ILC2 cells [60, 73, 74], and a variety of Th2-like innate-like T cells [14, 75, 76] – are present in the thymus and form a complex network (Figure 2) discussed below. In line with the notion that type 2 inflammation is closely linked to tissue repair responses [77, 78], nearly all components of the thymic type 2 inflammatory ecosystem, including thymic tuft cells, iNKT cells, ILC2 cells, eosinophils, and IL-33-producing mesenchymal cells, have been implicated in thymus regeneration after damage [79–81]. These effects of the type 2 inflammatory network on thymus regeneration have recently been reviewed elsewhere [82] and are not further discussed here.

The stromal niche for type 2 inflammatory processes in the thymus appears to be at least in part provided by thymic tuft cells and thymic macrophages (Figure 2). Thymic tuft cells are an mTEC-derived population of mimetic cells that induce a molecular program, have transcription factor dependencies, and morphology reminiscent of those of tuft cells found in other locations [4, 5]. Tuft cells are chemosensory epithelial cells found in various organs, including the gastrointestinal tract and

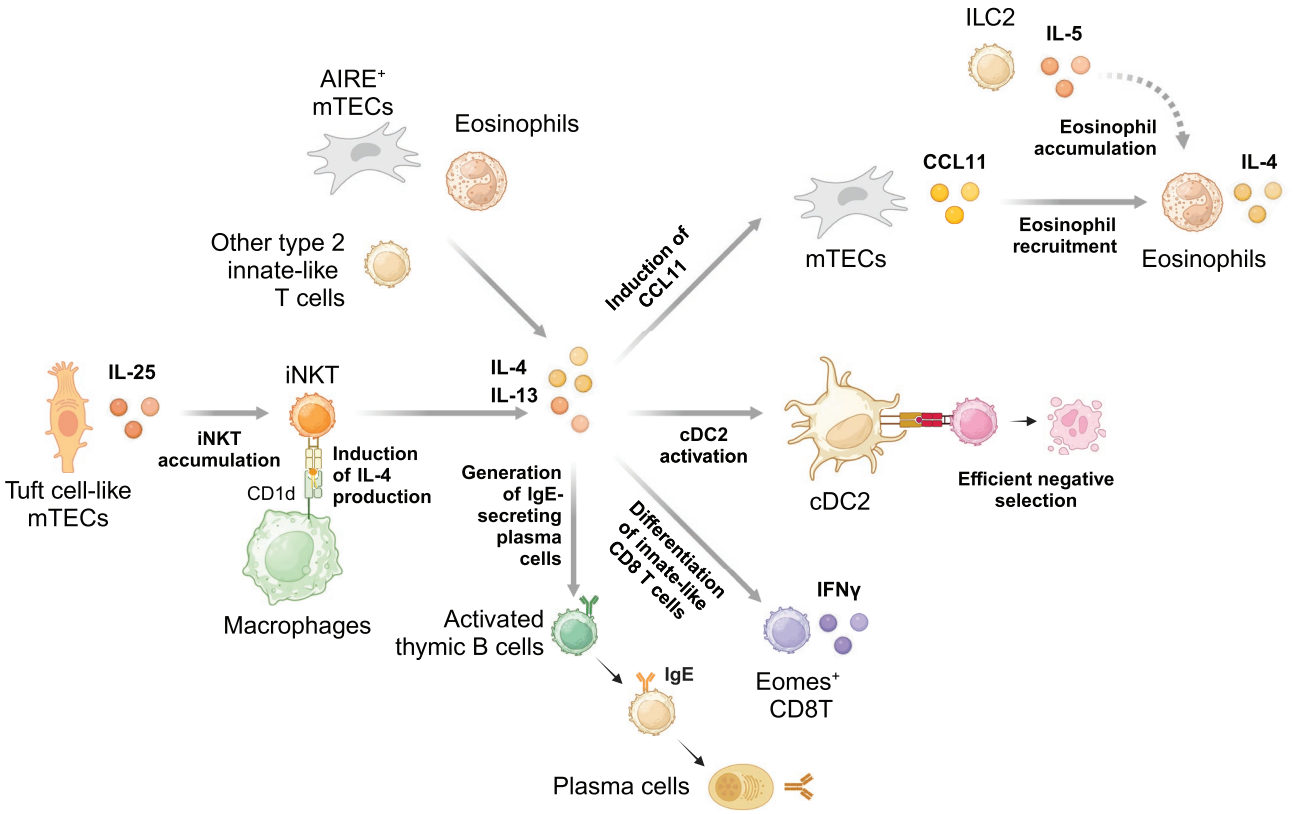

FIGURE 2 | Type 2 inflammatory network in the thymus. Type 2 cytokine-secreting iNKT cells (NKT2 cells) represent a central node of the type 2 inflammatory network in the thymus. The niche for these cells is at least in part provided by thymic tuft cells (through secretion of IL-25) and a myeloid population most likely corresponding to thymic macrophages (that regulate cytokine production by NKT2 cells through presentation of self-lipids on CD1d molecules). NKT2 cells are the main source of IL-4 and IL-13 in the thymus. Several additional populations contribute to the thymic production of these cytokines. IL-4 and IL-13 play a key role in thymus physiology by promoting the generation of IgE class-switched B lineage cells, driving the differentiation of Eomes$^+$ IFNγ-secreting innate-like CD8 T cells, and inducing cDC2 cell activation, thereby enhancing their capacity for efficient negative selection. In addition, these cytokines regulate the abundance of thymic eosinophils—possibly through the induction of CCL11 expression in mTECs. Thymic ILC2 cells also positively regulate the abundance of thymic eosinophils. While the underlying mechanism remains unclear, it was speculated that IL-5 secretion by ILC2 cells might mediate this effect.

respiratory system, that are crucial for sensing environmental cues and initiating type 2 immune responses [83, 84]. In line with the role of peripheral tuft cells in the orchestration of type 2 inflammation that occurs in part through their production of IL-25 [83, 84], thymic tuft cells also express IL-25, which in turn regulates the accumulation of NKT2 cells (iNKT cells that produce type 2 cytokines) [4]. Mice deficient for a key regulator of tuft cell differentiation, the transcription factor Pouf3f2, exhibit a marked reduction in thymic NKT2 cells [4, 63] (but some increase in thymic ILC2 cells [5]), and this defect is mirrored in IL-25-deficient mice [63].

NKT2 cells represent the central node of the type 2 inflammatory network in the mouse thymus. They are the most abundant population of cells that produce IL-4 and IL-13, key type 2 cytokines, in the thymus [80, 85]. While the selection of iNKT cells depends on the expression of CD1d by other thymocytes [86], the expression of CD1d by thymic myeloid cells is required to ensure the constitutive production of IL-4 by NKT2 cells [87]. Indeed, IL-4 expression by iNKT cells is largely abrogated upon deletion of *Cd1d* with *CD11c*-Cre but not *Zbtb46*-Cre, suggesting that it depends on CD11c-expressing cells other than the conventional DC subsets [87]. As thymic macrophages express CD11c [88] and as their depletion in *LysM*-Cre *Csf1r*$^{LSL-DTR}$ mice also strongly reduces the IL-4 expression by thymic iNKT cells, it is plausible that thymic macrophages are the key myeloid population required to sustain IL-4 production by iNKT cells [87]. Thus, endogenous lipid antigen presentation by thymic myeloid cells to NKT2 cells ensures constitutive IL-4 production by the latter population. This, in turn, has profound effects on many thymic populations, including DCs, B cells, TECs, and developing T cells, as discussed in detail below.

While NKT2 cells represent more than half of thymic IL-4-producing cells (as judged by analysis of IL-4-GFP reporter mice), other thymic innate-like populations, including both αβ and γδ T cell subsets, also produce IL-4 [58]. In the γδ T cell compartment, IL-4 is predominantly produced by Vγ1Vδ6.3 'γδ NKT cells', a subset of unknown antigen specificity that in terms of functional properties and transcriptional program are highly reminiscent of iNKT cells [89–92]. In the αβ T cell compartment, in addition to iNKT cells, thymic MAIT cells were recently reported to be capable of IL-4 production [75]. T cells are not the only source of IL-4 in the thymus, and eosinophils represent the second-largest subset of thymic IL-4 producers after iNKT cells [58, 71]. Finally, a small fraction of mTECs expresses *Il4*, and

this expression is largely restricted to the AIRE$^+$ compartment [58, 93].

This constitutive production of IL-4 and IL-13 has pleiotropic effects on the inflammatory network in the thymus, including impact on several thymic APC subsets. In the thymic DC compartment, these cytokines have recently been shown to activate cDC2 cells [94]. Remarkably, selective depletion of these activated CD301b$^+$ cDC2 cells impairs the negative selection of CD4 thymocytes, as demonstrated by TCR repertoire sequencing experiments, highlighting a non-redundant role of this small APC subset in establishing tolerance [94]. Furthermore, GFP expression under the control of *CD301b* regulatory elements is sufficient to induce the complete elimination of GFP-specific CD4 T cells [94]. These activated cDC2 cells are strongly reduced in IL-4R-deficient mice (incapable of both IL-4 and IL-13 signaling) and show a milder reduction in IL-4-, IL-13-, and CD1d-deficient mice [94]. This suggests that type 2 cytokines, partially derived from iNKT cells, drive their generation. Additionally, innate-like T cell-derived type 2 cytokines regulate thymic B lineage cells, as IgE-switched plasma cells, abundant in the thymi of wild-type Balb/c mice, are nearly absent in IL-4R-deficient animals and markedly reduced in IL-4- and CD1d-deficient mice [95].

Moreover, these cytokines are also essential for the accumulation of thymic eosinophils, another key component of the type 2 inflammatory network in the thymus, as evidenced by the marked reduction of these cells in IL-4R-deficient mice [80]. Although the exact mechanism underlying this phenotype remains to be fully characterized, recruitment of eosinophils to the thymus depends on their expression of chemokine receptor CCR3, with its primary thymic ligand, CCL11, being expressed by mTECs in an IL-4R-dependent manner [80]. Moreover, thymic eosinophils are strongly decreased in iNKT cell-deficient mice (but, somewhat unexpectedly, not in tuft cell-deficient animals) [80]. These findings support the notion that IL-4 and/or IL-13 production by iNKT cells regulates eosinophil accumulation in the thymus, potentially promoting their recruitment through CCL11 expression induced in mTECs. In addition, thymic eosinophil numbers are strongly reduced in ILC2-deficient *Il7r*-Cre *Rora*$^{fl/fl}$ mice, and it was suggested that ILC2 cells may regulate thymic eosinophils through IL-5 production [80]. Finally, the effects of type 2 cytokines on thymic stromal populations extend beyond the induction of CCL11 in mTECs, as IL-4 and/or IL-13 signaling in the thymic stroma also plays a role in regulating the egress of mature thymocytes through a yet-to-be-characterized mechanism [96].

In addition to its effects on other components of the type 2 inflammatory ecosystem in the thymus, IL-4 signaling also affects the developing T cells. Genetic alterations that lead to an expansion of IL-4-producing innate-like T cell subsets in the thymus are associated with a substantial increase in the generation of Eomes$^+$ CD8SP thymocytes [97–99]. Strikingly, the latter phenotype is not intrinsic to CD8 T cells; rather, it is induced by excess IL-4 production in these models [97–99]. This IL-4-driven developmental pathway is not a peculiarity of genetically modified strains, as wild-type Balb/c mice, which naturally have higher numbers of NKT2 cells compared to C57BL/6 mice, also exhibit an accumulation of Eomes$^+$ innate-like CD8 T cells [98]. This accumulation is abolished in IL-4R-deficient and CD1d-deficient Balb/c animals, suggesting the critical role of iNKT cell-derived IL-4 in this process [98]. Thus, somewhat paradoxically, type 2 cytokine production by thymic innate-like T cells also broadens the representation of type 1 inflammatory pathways in the thymus by inducing a subset of IFNγ-producing innate-like T cells. Finally, in addition to its role in the generation of innate-like CD8 T cells, IL-4 was recently suggested to positively regulate the intrathymic maturation of Treg cells [93].

4.2 | The Role of the Type 2 Inflammatory Network in the Induction of Tolerance

Multiple components of the type 2 inflammatory network described above have been directly implicated in the induction of T cell tolerance. First, thymic tuft cells are capable of inducing tolerance to the antigens they express, including the inflammation-associated self-antigens. This is evidenced by experiments in which transplantation of WT thymus, but not *Pouf3*$^{-/-}$ tuft cell-deficient thymus, into nude mice conferred tolerance to IL-25 immunization, indicating that thymic tuft cells induce tolerance to this alarmin [4]. Second, IL-4R-signaling activated CD301b$^+$ cDC2 cells, as noted earlier, play a non-redundant role in CD4 T cell negative selection [94]. Finally, it was demonstrated that both NKT2 cells and thymic eosinophils mediate tolerance to self-antigens that they express. Using IL-4-GFP as a model inflammation-associated self-antigen, it was shown that highly efficient central CD8 T cell tolerance to GFP in this system can be induced by both hematopoietic and stromal cells [58]. This finding aligns with the expression of IL-4-GFP by thymic innate-like T cells, eosinophils, and a rare subset of mTECs. Reaggregated thymus organ culture (RTOC) experiments demonstrated that IL-4-GFP-expressing thymic eosinophils are sufficient to induce the negative selection of GFP-specific CD8 thymocytes. Similarly, intrathymic transfer and RTOC experiments revealed that IL-4-GFP expression solely by iNKT cells was also sufficient to eliminate autoreactive CD8 thymocytes [58]. Remarkably, this elimination occurred without any involvement of professional thymic APCs, through direct T cell-to-T cell antigen presentation [58].

Thus, various cellular components of the type 2 inflammatory network constitutively operating in the thymus, including tuft cells, activated cDC2 cells, NKT2 cells, and eosinophils, can play a direct role in inducing tolerance to self-antigens associated with type 2 inflammation.

5 | The Type 3 Inflammatory Module

Thymic type 3 inflammation represents arguably the least characterized inflammatory module in the thymus. Nevertheless, it is clear that the key molecular and cellular components of type 3 inflammation are represented at the site of T cell development. For example, a key mediator of type 3 inflammatory responses, IL-23, is expressed by mTECs and thymic myeloid cells [50, 100] and steady-state *Il23a* expression in the thymus is higher than in the spleen, lymph nodes, liver, or colon [100]. This IL-23 production was suggested to positively regulate the clonal deletion of autoreactive thymocytes [100]. In turn, IL-23 regulates the production of IL-22 by thymic ILC3 cells, and further activation of

this pathway is crucial for thymic regeneration [101]. Moreover, neutrophils, a key effector population of type 3 inflammation, are present in the thymus [50, 102] and also contribute to local IL-23 production [50]. Finally, *Il17a* and *Il17f* are expressed both in mouse and human thymus [58]. However, cells expressing the IL-17 family cytokines seem to be less abundant than cells expressing IL-4 or IFNγ [58]. Analysis of reporter-positive thymic cells in IL-17A-GFP mice demonstrated that, similar to IL-4 and IFNγ, IL-17A is predominantly expressed by iNKT cells with smaller contributions of other αβ T cell subsets, γδ T cells, ILCs, and mTECs [58]. To our knowledge, the possible downstream effects of IL-17 signaling in the thymus remain uncharacterized. Likewise, it remains largely unclear what stromal populations could form the niche for the cell types associated with type 3 inflammation in the thymus. Nevertheless, some evidence suggests that mTECs can regulate the abundance of thymic IL-17-expressing γδ T cells through IL-7 production [103, 104]. In addition, keratinocyte-like mimetic mTECs were shown to form close contacts with thymic neutrophils [50] suggesting that they might provide a cellular niche for this granulocyte subset.

Evidence for the involvement of IL-17-expressing cells in the induction of tolerance comes from peptide immunization experiments demonstrating that expression of IL-17A-GFP by either hematopoietic or radioresistant cells is sufficient to induce CD8 T cell tolerance to GFP [58]. Moreover, RTOC experiments with sorted IL-17A-GFP-expressing iNKT cells provided direct evidence for the contribution of central tolerance to this process [58]. Finally, shifting the balance between the thymic representation of IL-17A-GFP-expressing cells and the developing GFP-specific CD8 T cells towards the latter population results in a fratricide killing of the peripheral Th17 cells [58]. These experiments directly demonstrate that decreased thymic abundance of an inflammation-associated self-antigen can result in the unprovoked elimination of an entire class of effector T cells at the periphery. It is conceivable that a similar balance shift occurs in AIRE deficiency. Indeed, while IL-17-producing γδ T cells are increased in the thymi of *Aire*$^{-/-}$ mice [103], analysis of expression datasets indicates that expression of *Il17a* and *Il17f* is nearly lost in AIRE-deficient mTECs ([105] and Jakub Abramson, personal communication), suggesting decreased thymic representation of these inflammation-associated self-antigens. At the same time, peripheral AIRE expression is required for efficient Th17 response to *Candida albicans* [106]. It is, therefore, conceivable that the decreased thymic abundance of type 3 cytokines, combined with their increased peripheral representation (e.g., due to inefficient but chronic responses to *Candida*), could underlie a breach of tolerance to these cytokines in APS-1 patients [107, 108]. More generally speaking, as IL-17-producing cells seem to be significantly less abundant in the thymus than their IL-4- and IFNγ-secreting counterparts [58], it is tempting to speculate that this poor thymic representation could contribute to the frequent targeting of IL-17 family cytokines by autoantibodies in patients with a variety of autoimmune diseases [27].

6 | Other Inflammatory Pathways in the Thymus

Type I/III IFN production and signaling is not the only innate inflammatory pathway constitutively active in the thymus. mTECs also express many toll-like receptors (TLRs) as well as MyD88, an adaptor protein involved in TLR signaling [109]. TEC-specific deletion of *Myd88* results in altered mTEC gene expression profiles, including decreased expression of several cytokine- and chemokine-encoding genes, indicating that mTECs undergo MyD88-dependent signaling at steady state [109]. Moreover, expression of MyD88-dependent genes is not altered in mTECs from germ-free mice, suggesting that MyD88 signaling in the thymus is not activated by microbiota [109]. MyD88-dependent signaling in TECs affects other components of the thymic microenvironment, as TEC-specific deletion of *Myd88* results in decreased frequencies of cDC2, monocyte-derived DCs, and Treg cells in the thymus as well as a reduced functionality of the remaining Treg cells [109]. It remains to be tested if this sterile MyD88-dependent signaling reflects the activation of TLRs (and if so—which ones) or signaling through the MyD88-dependent cytokine receptors.

In line with the latter possibility, keratinocyte-like mimetic cells were reported to express genes encoding IL-1 family cytokines, including IL-36α, IL-36β, and IL-36γ [50]. More generally speaking, Hassall's corpuscles formed by this mimetic population were suggested to represent important inflammatory hubs in the thymus [50, 110]. Indeed, these cells produce a number of antimicrobial factors, cytokines, and chemokines [50, 110, 111], form close contacts with thymic neutrophils [50] and were suggested to regulate thymic pDC function [50] and Treg generation [111].

In addition to IL-1 family cytokines, human Hassall's corpuscles were suggested to express S100A8/A9 [110]—molecules that might function both as antimicrobial agents and as DAMPs (danger-associated molecular patterns) that induce TLR4 signaling [112]. S100A8/A9 are not the only antimicrobial proteins produced by TECs, as both human and mouse mTECs also express a range of defensins [113]. This expression is controlled by AIRE, and tolerance to defensins is breached both in APS-1 patients and AIRE-deficient mice [113].

7 | The B Cell Immunity-Associated Module

Although IFN signaling and type 1, type 2, and type 3 inflammatory responses are activated at the periphery in response to specific pathogen types, all pathogen groups do elicit antibody-mediated responses. The B cell activation program in response to different pathogens involves both general components and pathogen type-specific aspects–such as switching to antibody isotypes most appropriate for the given threat. From the tolerance perspective, all these components represent inflammation-associated self-antigens and therefore must be represented in the thymus. Indeed, as discussed below, a complex network of cellular and molecular interactions ensures the constitutive activation of thymic B cells in modes that resemble peripheral responses to all main pathogen classes (Figure 3).

It is long acknowledged that both in humans and mice the thymus harbors small populations of B cells and plasma cells [114–117]. The origin of these B cells remains somewhat controversial. Early studies suggested that some B-lymphopoiesis can be occurring intrathymically [116, 118, 119]. In line with this notion, the presence of surface B cell receptor (BCR)-negative,

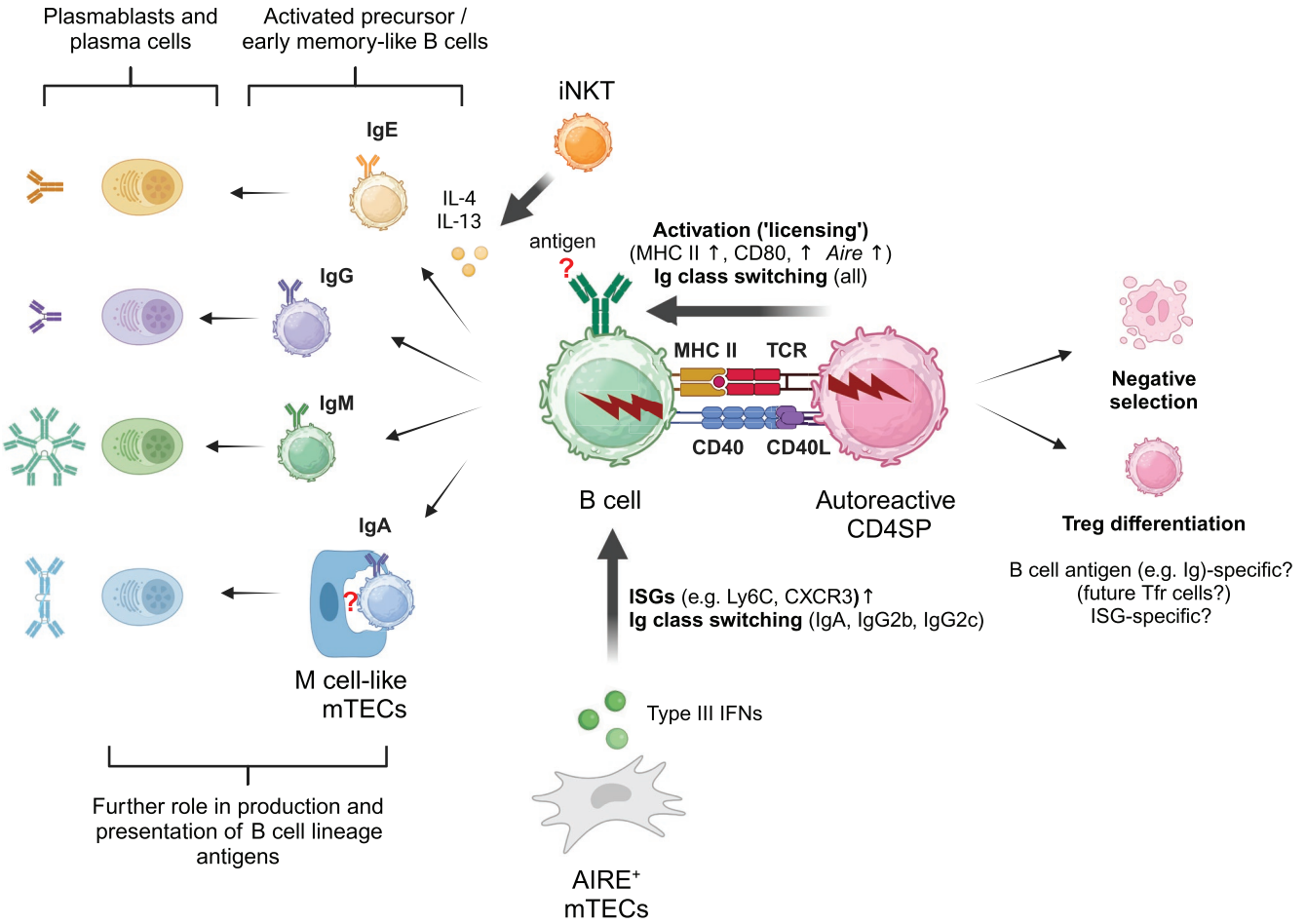

FIGURE 3 | Interactions and fates of thymic B lineage cells. Thymic B cells are activated ('licensed') by T cell 'help' provided by autoreactive CD4SP thymocytes that recognize self-epitopes presented by the B cells. This results in negative selection or Treg lineage diversion of the autoreactive thymocyte and CD40-mediated B cell activation. In contrast to the peripheral B cell activation, BCR-mediated antigen recognition is not strictly required for this process (but its possible involvement under physiological conditions remains to be assessed). Activated thymic B cells undergo class switching to nearly all Ig isotypes. Many thymic B cells seem to remain in an activated precursor/early memory-like state, while some differentiate into plasma cells. λ IFNs secreted by AIRE$^+$ mTECs positively regulate switching to most Ig isotypes, induce phenotypic changes in thymic B cells, and possibly provide an additional activation signal. IL-4 and IL-13 production by thymic iNKT cells induces the generation of IgE-switched B lineage cells, while interactions with microfold mimetic cells positively regulate the abundance of IgA-switched B cells through mechanisms that remain to be characterized.

CD19$^+$B220$^+$ cells expressing *Rag2*-GFP was reported in the thymus [120]. However, some of the latter studies involving detailed phenotypic [121] and transcriptional [95] characterization of thymic B lineage cells failed to identify cells corresponding to the early stages of B cell development in the thymus. Moreover, cell transfer experiments demonstrate that mature B cells readily home to the thymus [121]. The most immature B cells that can be confidently identified in these studies were transitional B cells [95]—a late stage of B cell development with fully rearranged BCR. In addition, some interspecies differences may exist, as the human thymus does contain a population of cells corresponding to early stages of B cell development (as judged by their co-expression of B cell signature genes and *RAG1*/*RAG2*) [69].

Thymic B cells are predominantly located in the medulla [45, 95, 122] and represent thymus-resident cells as evidenced by the results of parabiosis experiments [95, 120]. The majority of B cells and plasma cells have long dwelling times in the thymus, with only about 20% of thymic B cells replaced after a 2-week parabiosis [95] and about 40% replaced over a 6–8-week period [120]. These results align with a scenario in which the thymus is seeded by mature B cells from the periphery, which can then reside there for a long time. Thymic plasma cells in mice are also found in the medulla [95], while in humans they accumulate in the thymic perivascular space [123, 124].

In stark contrast to secondary lymphoid organs, where the majority of B cells remain in a naïve state, a very large fraction of thymic B cells are activated as judged by their up-regulation of activation markers (GL7, MHC II, CD80, AID [45, 120, 121, 125], as well as CD5 and CD11b [115]). Moreover, in line with AID expression [125], a very large proportion (~10%–20% in C57BL/6 mice) of thymic B cells are class-switched [45, 125, 126]. In C57BL/6 mice, this switched subset is dominated by IgA, IgG2b, and IgG2c isotypes (although IgG1 and IgE isotypes can also be detected) [45, 125], while in Balb/c mice, thymic B lineage cells also contain a sizable fraction of IgE-switched cells [95]. B cells with an acutely

activated phenotype ($GL7^+CD38^+$) are more abundant in neonatal mice, and neonatal but not adult thymic B cells are highly proliferative [45]. In line with this seemingly restricted expansion and constant seeding of the thymus by peripheral B cells, repertoire sequencing of thymic B cells and plasma cells demonstrated that they are highly polyclonal [95].

7.1 | Similarities and Differences Between Thymic and Peripheral B Cell Activation

Activation of B cells in secondary lymphoid organs results in the generation of tripotent 'activated precursor' B cells that can then give rise to plasmablasts, germinal center B cells, and non-germinal center-derived early memory B cells [127, 128]. While differentiation into both plasmablasts and germinal center B cells results in dramatic changes in B cell's transcriptome, early memory B cells remain transcriptionally very similar to the tripotent activated precursors [129]. Activated thymic B cells are phenotypically and transcriptionally reminiscent of these peripheral activated precursor B cells and early memory B cells [45]. Co-existence of these activated B cells and plasmablasts in the thymus suggests that at least some of the plasma cells are generated intrathymically and fetal thymus organ culture (FTOC) experiments provide evidence of the precursor-product relationships between the two populations [95]. Thus, two out of three main effector B cell subsets—memory B cells and plasma cells—are represented in the thymus. Interestingly, the thymus seems to be lacking any equivalent of germinal center B cells—a population that transcriptionally is very different from the early activated precursors [129]. Indeed, activated B cells in the thymus fail to upregulate the germinal center B cell signature (*Bcl6*, *S1pr2*, *Gcsam*) and are not affected by genetic manipulations that result in the loss of peripheral germinal center B cells (such as ICOS deficiency or ablation of Bcl6 in T cells) [45]. In line with the lack of germinal center B cells in the thymus, thymic B cells exhibit low levels of somatic hypermutation [95, 120].

Peripheral B cell activation is driven by three key signals—antigen recognition, T cell help, and cytokine signaling. It is initiated by the recognition of antigen by the BCR of a naïve B cell, which, in the case of protein antigens, leads to the presentation of the antigen epitopes on MHC class II molecules to activated CD4 T cells. These antigen-specific CD4 T cells in turn provide crucial co-stimulation to activated B cells, which in part occurs through the interaction between the co-stimulatory receptor CD40 on B cells and its ligand CD40L on T cells [130–132]. Finally, cytokines present in the milieu further modulate B cell activation and determine the Ig isotypes to which B cells undergo class switching [132].

In stark contrast to peripheral B cell responses, thymic B cell activation can be fully uncoupled from the antigen specificity of these cells. Indeed, BCR-transgenic model antigen-specific B cells readily undergo activation and class switching in the thymus in the absence of cognate antigen [121]. Nevertheless, activation of thymic B cells remains strictly dependent on CD4 T cell help, as it is completely abrogated in mice lacking CD40, CD40L, or CD4SP thymocytes [45, 121, 125]. Moreover, this activation requires MHC class II expression by thymic B cells and is abrogated when the TCR repertoire of CD4SP thymocytes is fixed in TCR-transgenic mice in the absence of cognate antigen [121, 125]. Finally, pulsing B cells with a CD4 T cell epitope is required and sufficient to induce the activation of B cells co-cultured with TCR transgenic CD4SP thymocytes specific to this epitope [121]. Taken together, these results are consistent with a model in which recognition of a self-epitope presented by a thymic B cell to autoreactive CD4SP thymocytes leads to B cell activation through CD40-CD40L stimulation (Figure 3). Strikingly, in contrast to B cell activation at the periphery, this thymic activation can take place without BCR-mediated recognition of cognate antigen. Moreover, instead of recognition of a foreign antigen by activated CD4 T cells, it seems to rely on the recognition of self-epitopes by autoreactive CD4SP thymocytes. Finally, instead of T cell activation, this interaction leads to induction of tolerance through negative selection or diversion to the Treg lineage (see below).

It is, however, important to note that while experiments with mice expressing a model antigen-specific BCR transgene clearly demonstrate that thymic B cell activation does not have to rely on B cell recognition of cognate antigen [121], it does not exclude the role of such recognition in a polyclonal setting. In line with a possible role of BCR specificity in thymic B cells, analysis of mice transgenic for the heavy chain from an anti-DNA antibody demonstrated that switched thymic B cells have a distinct light chain repertoire and are enriched for autoreactive specificities [125]. Moreover, thymic plasma cells are strongly decreased in a model antigen-specific BCR transgenic mice [95]. Thus, while antigen recognition does not seem to be strictly required for thymic B cell activation, BCR can have an impact on the fate of thymic B cells. Therefore, antigen specificities of polyclonal thymic B lineage cells remain to be defined, and, as discussed below, the answer to this question could have profound implications for our understanding of mechanisms of B cell-mediated central tolerance.

In addition, cytokine signaling and interplay with several thymic cell types is crucial for the regulation of Ig class switched subsets of B cells in the thymus. Production of type III IFNs (predominantly expressed in the thymus by mTECs, as discussed above) is required for the accumulation of thymic switched B cells [45]. B cell-specific deletion of IFNλ receptor results in decreased numbers of cells switched to IgA, IgG2b, and IgG2c, while switching to IgE is slightly increased [45]. Moreover, IgE-switched plasma cells that are relatively abundant in the thymus of Balb/c but not C57BL/6 mice are dependent on IL-4/IL-13 signaling and the presence of iNKT cells, a cell type that is the main source of IL-4 in the thymus [95]. Interestingly, cells producing another key regulator of B cell expansion, class switching, and plasma cell differentiation, IL-21, constitute about 0.2%–0.3% of CD4 thymocytes [57]. These cells express high levels of Nur77-GFP reporter, and their numbers are strongly decreased in AIRE-deficient mice, suggesting that they may be enriched for autoreactive specificities [57]. The effects of this IL-21 production on thymic B cells and other components of the thymic microenvironment remain to be assessed. Finally, the presence of IgA-switched B cells and plasma cells in the thymus is partially dependent on the presence of microfold mimetic cells [6]. Thymic microfold cells (M cells) are a mimetic population for a specialized epithelial subset usually located

above mucosa-associated lymphoid tissues, where these cells sample and transport antigens from the lumen to underlying immune cells. While the exact mechanisms by which microfold mimetic cells regulate IgA-switched plasma cells remain unclear, several observations suggest a complex interplay between thymic B cells and this mimetic population. Some thymic class-switched B cells colocalize with the microfold mimetic cells in a CCR6-dependent fashion, suggesting their physical interactions [6]. Furthermore, B cell-deficient and CCR6-deficient mice show a significant reduction in microfold mimetic cells [6], whereas *SpiB*$^{-/-}$ and *Sox8*$^{-/-}$ mice, which lack microfold cells, exhibit increased numbers of thymic B cells [3]. Collectively, these findings point to an intricate relationship between thymic B cells and microfold mimetic cells that remains to be further characterized.

7.2 | The Role of Thymic B Lineage Cells in the Induction of Tolerance

Several lines of direct and indirect evidence indicate that a crucial function of thymic B cells is the induction of tolerance to antigens that they express. Early studies demonstrated that the exclusive expression of MHC class II on B cells was sufficient to induce partial elimination of superantigen-reactive thymocytes [133]. In addition, in several MHC class II-restricted TCR-transgenic systems, the expression of antigen predominantly or exclusively by B cells was sufficient to induce efficient deletion of autoreactive CD4SP thymocytes [121, 134]. Moreover, total B cell- or activated B cell-restricted expression of Cre was sufficient to induce polyclonal CD4 T cell tolerance to an MHC II epitope of Cre, and the residual Cre-specific CD4 T cells in this setting were enriched for Treg cells [45]. Strikingly, even total thymic Treg numbers were reduced in B cell-deficient mice [45, 122, 135] and increased in BAFF-transgenic mice with an expanded thymic B cell compartment [122]. Of note, Treg expansion in the latter setting was dependent on MHC II expression by B cells, suggesting that the antigen-presenting function of B cells was crucial in this process of Treg induction or expansion [122].

Even more unexpectedly, much more subtle alterations in the B cell compartment, such as conditional ablation of type III IFN signaling in B cells and AID deficiency, resulted in decreased numbers of thymic Treg cells and altered Treg TCR repertoire, respectively [45]. These results suggest that a significant fraction of Treg cells could be selected on thymic B cells and some of them might recognize epitopes from different Ig isotypes. It is tempting to speculate that Treg cells recognizing activated B cells' antigens could participate in the regulation of humoral immune responses, for example, through the contribution to the T follicular regulatory (Tfr) cell pool [136].

Finally, a recent study identified a non-redundant role of thymic B cells in the induction of CD4 T cell tolerance to aquaporin-4 (AQP4), a self-antigen targeted in an autoimmune disease, neuromyelitis optica [137]. While AQP4 was expressed at low levels both by thymic B cells and mTECs, its expression by B cells was required and sufficient to eliminate AQP4-specific CD4 thymocytes [137]. Interestingly, AQP4 was induced in B cells by CD40 signaling, and AQP4 tolerance was lost in CD40-deficient mice [137].

The results described above clearly indicate the prominent role of thymic B cells in the induction of T cell tolerance both through negative selection and Treg diversion of CD4 thymocytes. It seems likely that thymic B cells could also contribute to the induction of CD8 T cell tolerance, although this remains to be formally demonstrated. What unique components of immunological self do B cells represent in the thymus? One obvious non-redundant function of thymic B cells could be the induction of tolerance to B cell lineage antigens, including those upregulated in the course of B cell activation and differentiation to effector subsets. Thymic B cell activation is often referred to as 'licensing' as it involves upregulation of MHC II, co-stimulatory molecules, and *Aire* [45, 121]. While this licensing is required for the induction of tolerance to antigens upregulated in activated B cells, such as AQP4 [137], it seems probable that even 'unlicensed' B cells would be sufficient to induce tolerance to the antigens that constitute the core B cell molecular program.

One obvious class of self-antigens that is highly expressed by B cells and plasma cells is antibodies. The striking loss of certain thymic Treg clonotypes in AID-deficient mice [45] suggests that B cells could make a major contribution to the induction of tolerance to different isotypes of immunoglobulins. It should be noted, however, that the loss of AID can have additional indirect effects on T cell tolerance, for example through possible changes in antigen uptake by switched B cells. In line with such indirect effects of AID deficiency on T cell tolerance unrelated to the elimination of Ig isotype-specific thymocytes, AID deficiency partially rescues the negative selection of TCR transgenic thymocytes specific to a model antigen [126]. The mechanisms underlying this phenotype remain to be characterized.

Another class of antigens that could be presented by thymic B cells is those encoded by ISGs. Type III IFN signaling induces a broad spectrum of both stereotypical (e.g., Mx1) and more cell type-restricted (Ly6D, Ly6C, CXCR3) ISGs in thymic B cells [38, 45]. Strikingly, B cell-specific ablation of type III IFN signaling results in a gross decrease in thymic Treg cells comparable to that observed in B cell-deficient mice [45]. Although many explanations for these results are possible, it is tempting to speculate that thymic B cells might be involved in the selection of ISG-specific Treg cells.

Licensed thymic B cells upregulate *Aire* [121], encoding for the transcriptional regulator responsible for the 'ectopic' expression of tissue-specific antigens in mTECs [2]. However, it remains to be tested if the expression of *Aire* by thymic B cells has functional consequences for their tolerogenic properties. It is important to note that, while transcription of *Aire* is clearly induced in licensed thymic B cells, AIRE protein is expressed in these cells at very low levels [121]. Moreover, while transcriptomics analysis did reveal gene expression changes in thymic B cells from AIRE-deficient mice [121], it remains to be identified which of these differences reflect B cell-intrinsic effects of AIRE deficiency.

It also remains to be tested if thymic B lineage cells solely mediate tolerance to antigens they express, or if antibody-mediated antigen uptake contributes to this tolerance induction. Some evidence for the latter notion was obtained in a TCR/BCR dual transgenic system, in which negative selection of TCR-transgenic

CD4 thymocytes recognizing a natural self-antigen was enhanced in the presence of B cells specific to the same antigen [120]. However, this setting in which nearly all thymic B cells recognize the same antigen contrasts with the physiological situation in which thymic B cells are highly polyclonal [95]. Given an overall low abundance of B lymphocytes in the thymus (~0.2% of total thymic cells [121]), it seems unlikely that individual autoreactive B cells targeting diverse soluble antigens could significantly contribute to the induction of tolerance to these antigens. In such a scenario, a negligibly small number of B cells would recognize each individual antigen. The situation could, however, be different if thymic B cells were enriched for polyreactive cells or cells recognizing epitopes exposed on apoptotic cells and cell debris. Indeed, in this setting, uptake of apoptotic cell fragments would result in the presentation of numerous exogenous antigens, some of which would likely be shared by many B cells, making their ability to mediate tolerance through antigen uptake more plausible. In line with this possibility, human thymic B cells may exhibit increased polyreactivity [138]. Innate-like B cell populations, such as B-1a cells [139] and marginal zone B cells [140] are thought to be enriched for such specificities. While no B cells with $CD21^{hi}CD23^{lo}$ marginal zone phenotype are detectable in the thymus [120, 122], a sizable minority of thymic B cells seems to have the canonical $CD19^{hi}B220^{lo}CD5^{+}$ B-1a cell phenotype [115, 116]. The relatedness of this subset to the B-1a lineage and their functional role in the thymus remain to be characterized. Finally, it is also conceivable that antibodies secreted by thymic plasma cells, if they recognize self-antigens present in the thymus, could contribute to the induction of tolerance through facilitation of antigen uptake and presentation by other thymic APCs. Indeed, in such a theoretical scenario even a single plasma cell of a rare specificity could facilitate antigen presentation by a significant number of the surrounding APCs.

8 | Other 'Ectopic' Cell Types in the Thymus

B cells, plasma cells, eosinophils, neutrophils, as well as a variety of innate-like T cell and NK/ILC subsets discussed above are not the only 'unexpected' hematopoietic cell types that can be found in the thymus. Many studies, including recent scRNA-seq atlases [38, 69, 94], suggest that the human and/or mouse thymus can also harbor small numbers of recirculating memory T cells, monocytes, mast cells, megakaryocytes as well as erythroid lineage cells (Table 1), although blood contamination remains to be fully excluded for some of these populations.

While the investigation of central tolerance often focuses on professional thymic APCs (such as TECs, DCs, and B cells), recent studies demonstrate that direct presentation of antigens by small numbers of thymic innate-like T cells and thymic eosinophils can mediate negative selection of MHC class I-restricted thymocytes [58] and show the contribution of thymic fibroblasts to central tolerance [47]. These results suggest that potentially any MHC class I-expressing cell type present in the thymus could participate in the induction of CD8 T cell tolerance. Moreover, it seems likely that at least some of the antigens expressed by such non-professional APCs can be picked up and presented on MHC II molecules by thymic DCs to induce CD4 T cell tolerance. Thus, in addition to ectopic antigen expression by mTECs, the recruitment or retention of these 'ectopic cell types' is likely to serve as a strategy to ensure the presence of self-antigens derived from the hematopoietic system within the thymus. In addition, thymic non-hematopoietic cell types other than TECs, for example endothelial cells and neurons [154, 155], should also be considered as possible sources of self-antigens for tolerance induction.

Nearly 90% of all protein-coding genes are represented in the thymus through ectopic antigen expression by mTECs [156]. Moreover, many inflammation-associated self-antigens are expressed both by mTECs and the 'ectopic' cell types discussed above [58]. What could be the evolutionary benefits of this seeming redundancy? One obvious reason for the physical presence of specialized peripheral cell types in the thymus could be the necessity to reach relatively high levels of antigen expression. Indeed, many tissue-specific antigens in mTECs are expressed at low levels, whereas some of them can be upregulated to very high levels in the specialized peripheral cell types. Some of these differences in the expression level, for example between mTECs and peripheral cell types specializing in the secretion of a particular protein (e.g., plasma cells), are likely to be very dramatic. Thus, it seems probable that while expression in mTECs can be sufficient to eliminate high-affinity clones specific to such antigens, expression by specialized cell types can be required to eliminate thymocytes that recognize these self-antigens with lower affinity. In addition, it is also conceivable that the antigen processing and presentation machinery can be somewhat different in different cell types and therefore the spectrum of epitopes produced by mTECs and other cell types may not be identical. From that perspective, it is interesting to note that although, as discussed above, AQP4 is expressed by mTECs and thymic B cells at comparable levels, B cells are required and sufficient to eliminate CD4 thymocytes specific to a particular AQP4 epitope, while mTECs contribute to this tolerance induction only marginally [137]. It is tempting to speculate that mTECs may not generate this epitope efficiently.

Of note, the exact same reasons, in particular the necessity to reach antigen expression levels characteristic of specialized peripheral cell types, may explain the evolutionary advantage of the differentiation of the mimetic cells in the thymus.

9 | Possible Origins of the Inflammatory Network in the Thymus

Although in jawed vertebrates the thymus is located far away from the body boundaries, it originates in embryogenesis from the third pharyngeal pouch [157]. In line with this origin of the thymus, it was suggested that in jawless vertebrates structures evolutionary and functionally related to thymus tissue are located in the tips of the gill filaments [158]. It is therefore conceivable that some of the thymic inflammatory pathways may have been inherited from mucosa-associated lymphoid tissues located in the gill region of early vertebrates.

It is also interesting to note that several peripheral cell types related to thymic mimetic cells, including ionocytes, tuft cells, as well as a population possibly corresponding to microfold cells, are present in fish gills [159, 160]. Therefore, it is conceivable

TABLE 1 | Rare hematopoietic cell types in the thymus[a].

Cell type	Reported in mouse thymus	Reported in human thymus	Formal demonstration of intrathymic localization and/or residency	Documented direct role in tolerance induction	Some of the other known functions in the thymus
B cells	Yes [115]	Yes [117]	Yes, parabiosis [95, 120], microscopy [45, 122, 137]	Yes, for CD4 tolerance, in vivo [45, 121, 133, 134, 137]	Regulation of microfold mimetic cell abundance [6]
Plasma cells	Yes [95]	Yes [114]	Yes, parabiosis [95], microscopy [95]	—	Secretion of natural antibodies and virus-specific antibodies [123, 124]
Memory T cells	Yes (reviewed in [141])	Yes [69]	Yes, microscopy [142]	Yes, in vivo [143, 144]	Local immunity [142] Repository of T cell memory [141]
Innate-like T cells	Yes (see text)	Yes [69]	Yes, parabiosis, i.t. transfer [21, 22], microscopy [85, 87]	Yes, for CD8 tolerance, in i.t. transfers and RTOCs [58]	Thymus regeneration [80] Production of IL-4 and IL-13 with effects on many cell types in the thymus (see text)
ILC1 and NK cells	Yes [59, 145]	Yes [146, 147]	—	—	—
ILC2	Yes [60]	Yes [73, 147]	Yes, microscopy [60]	—	Thymus regeneration [80, 81] Regulation of eosinophil abundance [80]
ILC3/LTi	Yes [60]	Yes [69, 147]	Yes, microscopy [148]	—	mTEC maturation [148] Thymus regeneration [101]
Eosinophils	Yes [71, 72]	Yes [149]	Yes, i.v. antibody injections [150], microscopy [72]	Yes, for CD8 tolerance, in RTOCs [58]	Thymus regeneration [80]
Neutrophils	Yes [50, 102]	Yes [69]	Yes, microscopy [50]	—	Constitutive IL-23 production, with a possible role in pDC activation [50] Clearance of apoptotic cells after irradiation [151]
Monocytes	Yes [38]	Yes [69]	—	—	—
Mast cells	—	Yes [69, 152, 153]	Yes, microscopy [152, 153]	—	—
Erythroid lineage cells	—	Yes [69]	—	—	—
Megakaryocytes	—	Yes [69]	—	—	—

[a]Cells corresponding to stages of T cell development, thymic DC subsets, and thymic macrophages are not included.

that the propensity of mTECs to give rise to some mimetic populations, including the ones with immune functions (tuft cells and microfold cells), may also reflect the evolutionary origins of TECs.

10 | Similarities and Differences Between Thymic and Peripheral Inflammatory Circuits

Although thymic inflammatory circuits in many ways resemble those utilized in the peripheral tissues in the course of immune responses, their mechanisms of initiation are fundamentally different: While peripheral inflammatory processes are usually triggered by pathogen encounter, most of the immune pathways in the thymus are triggered in a sterile manner. For example, while type I/III IFN signaling in the thymus results in the upregulation of a molecular program that includes many stereotypic antiviral components, the upstream signals are obviously different from those that are triggered in the course of viral infection. While at the periphery these IFNs are induced by activation of pattern recognition receptors, for example, by viral nucleic acids, in the thymus type I and III IFNs are induced in a microbiota- and endogenous retrovirus-independent, AIRE-dependent fashion [38, 42, 52]. Initiation of B cell activation in the thymus also has unique features. While peripheral B cell activation is initiated by BCR-mediated recognition of foreign antigen, thymic B cell licensing can be uncoupled from this BCR-mediated activation [121].

One noticeable exception from this sterile activation of inflammatory pathways in the thymus is the agonist selection of MAIT cells that is in part dependent on trafficking and presentation of microbiota-derived vitamin B2 metabolites in the thymus [161, 162]. The numbers of both Th1- and Th17-like MAIT cells are strongly reduced in the thymi and peripheral organs of germ-free mice [162]. Interestingly, the numbers of thymic eosinophils are also decreased in antibiotic-treated mice, suggesting that their recruitment or retention in the thymus is at least in part microbiota-dependent [150]. Despite these intriguing exceptions, thymic inflammatory circuits, while sharing many similarities with their peripheral counterparts, largely appear to rely on unique sterile mechanisms of activation.

11 | Concluding Remarks and Open Questions

As discussed in detail in this review, the thymus harbors a complex inflammatory network that functions largely in a sterile fashion and that ensures the representation of all major inflammatory/immune response-related pathways at the site of T cell development. We speculate that this inflammatory ecosystem has evolved (or, alternatively, had been maintained in evolution from some gill-associated lymphoid tissue) to enforce tolerance to inflammatory self.

Many questions about this inflammatory network remain to be answered. For example, the activation of multiple inflammatory pathways, some of which are thought to be mutually antagonistic, in a confined space of thymus medulla raises questions about their possible compartmentalization and cross-regulation. It would be of interest to assess the localization of various cellular and molecular constituents of this network and identify any possible spatial segregation of its different modules. Along the same lines, it seems likely that a lot remains to be learned about the thymic niches for various immune populations and the interaction of these populations with different components of the thymic stroma, including the diverse mimetic subsets. It is plausible that the interplay between microfold cells and thymic B cells, as well as tuft cells and NKT2 cells, are just the first examples of such interactions. Moreover, as the constitutive activation of many inflammatory pathways in the thymus does not result in full-blown inflammation, it is conceivable that 'thymflammation' may be kept in check by active regulatory mechanisms. If this is the case, such regulatory mechanisms are yet to be identified. Furthermore, while thymic B lineage cells represent one of the most studied 'ectopic' cell types in the thymus, their antigen specificity and its possible relevance to tolerance induction remain uninvestigated. Likewise, it remains unclear what triggers MyD88-dependent signaling in mTECs, whether TLRs are involved, and if yes—what ligands activate them. It also remains unclear if there are upstream signals resulting in the activation of type 2 inflammatory pathways in the thymus, for example, through the induction of IL-25 production by thymic tuft cells. Similarly, both the regulation and downstream consequences of the activation of type 1 and type 3 inflammatory circuits in the thymus remain to be defined. Finally, in addition to negative selection, at least some thymic inflammatory pathways contribute to Treg generation [38, 45]. It would be, therefore, of great interest to identify the exact antigens recognized by these Treg cells and assess their potential role in the negative regulation of specific branches of the inflammatory response in the periphery.

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Conflicts of Interest

The author declares no conflicts of interest.

Data Availability Statement

This is literature review; no primary data is included.

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