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Intrathymic Regulation of Dendritic Cell Subsets and Their Contributions to Central Tolerance

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ABSTRACT

Thymic dendritic cells (DCs) are critical mediators of central tolerance, cooperating with medullary thymic epithelial cells (mTECs) and B cells to establish T-cell self-tolerance to the proteome. The DC compartment is highly heterogeneous and is comprised of three major subsets, plasmacytoid dendritic cells (pDCs) and two conventional dendritic cell (cDC) subsets, cDC1 and cDC2. Thymic cDC1 and cDC2 arise from distinct progenitors and access the thymus at different stages of their differentiation, but both become activated by cellular and secreted cues received within the sterile thymus environment. Activated cDC1s and cDC2s have been implicated in presenting distinct types of self-antigens to induce central tolerance. Thus, understanding how the distinct cDC subsets are regulated within the thymus environment will provide important insights into mechanisms governing self-tolerance. Furthermore, the thymic DC compartment undergoes age-associated compositional and transcriptional changes that likely impact the efficiency and quality of central tolerance established over the lifespan. Here, we review recent findings from our lab and others on mechanisms regulating thymic DC activation, the distinct roles of thymic DC subsets in central tolerance, and age-associated changes in thymic DCs that could impact T-cell selection.

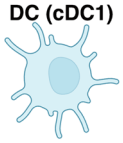
1 | Introduction

Dendritic cells (DCs) are a subset of leukocytes, derived from hematopoietic stem cells (HSCs) in the bone marrow, that play essential roles in bridging innate and adaptive immune responses [1]. DCs are sentinel cells present in diverse tissues, where they sample the extracellular space. Upon encountering pathogens, DCs become activated via signaling through pattern recognition receptors (PRRs) and cytokine receptors, resulting in a plethora of transcriptional and associated functional changes that enable them to activate an appropriate T cell response. Activated DCs stabilize presentation of peptides derived from proteins acquired at the site of infection on major histocompatibility complexes (MHC); they increase expression of cytokines and costimulatory molecules, and they alter expression of adhesion molecules and chemokine receptors to promote migration to draining lymph nodes. Within the lymph nodes, DCs colocalize with and activate naïve T cells that subsequently expand and differentiate into effector and/or memory T cells that mount protective immune responses to clear pathogens [1–3]. Through interactions with activated T cells, DCs receive reciprocal signals, further licensing them to become even more effective antigen presenting cells (APCs) [4–6]. In addition to this canonical role for DCs in activating protective T cell responses, during periods of homeostasis, DCs in secondary lymphoid tissues promote T-cell tolerance to self-antigens, with a notable role in inducing regulatory T cell responses to commensal microbiota in the intestine [7–9]. Moreover, DCs are present in the thymus, where they play an essential role in establishing central tolerance to self-antigens by inducing autoreactive thymocytes to undergo either negative selection or differentiation into the regulatory T

cell (Treg) lineage [10–14]. DCs make up only 0.5% of total thymus cellularity [15]; nonetheless, their importance in establishing and/or maintaining self-tolerance is highlighted by the impaired central tolerance and spontaneous fatal autoimmunity that occurs in a genetic mouse model of DC ablation [16].

Thymic DCs are heterogeneous cells characterized by expression of CD11c and MHCII in the absence of macrophage markers, such as CD64 [15, 17] (Figure 1). They can be subdivided into three main subsets: conventional DC1 (cDC1), which are phenotypically SIRPα^{-}CD8α^{+}XCR1^{+}, conventional DC2 (cDC2), which are phenotypically SIRPα^{+}CD8α^{-}XCR1^{-}, and plasmacytoid DCs (pDC), which can be identified as PDCA-1^{+} B220^{+} CD11c^{low} cells [17, 18] (Figure 1). cDC subsets reside primarily in the thymic medulla, where they have the opportunity to present antigens to post-positive selection thymocytes to induce central tolerance. While both cDC1 and cDC2 express high levels of MHCI, MHCII, and costimulatory molecules, multiple lines of evidence indicate that these two DC subsets present a nonoverlapping repertoire of self-antigens to thymocytes, suggesting they make distinct contributions to central tolerance [11, 12]. cDC1s are highly efficient at acquiring and cross-presenting medullary thymic epithelial cell (mTEC)- derived antigens to developing thymocytes, allowing them to contribute to central tolerance against tissue-restricted antigens (TRAs), which are proteins expressed by mTECs that are otherwise expressed in only a few differentiated tissues [19–22]. In contrast, cDC2s differentiate extrathymically and migrate into the thymus, where they can present self-antigens acquired in peripheral tissues or acquired from circulation to developing thymocytes [17, 23, 24]. pDCs are a distinct cell type known for their ability to produce Type I interferons in response to viral infections, but not for their ability to present antigens to induce T-cell responses [25–27]. A role for pDCs in central tolerance has been reported, but not extensively documented [28]. Thymic pDCs are not as efficient at acquiring mTEC derived antigens [10, 29, 30] and also do not efficiently induce Treg differentiation from CD4SP thymocytes [10, 19, 31, 32]. Thus, the role of pDCs in the thymus is yet to be resolved.

In this review, we will focus on the regulation and function of conventional DC subsets, cDC1s and cDC2s, in the thymus. We will first examine the origin of thymic cDC subsets and the intrathymic regulation of their activation. We will also discuss key mediators by which cDCs induce central tolerance and highlight the differential roles of cDC1s and cDC2s in establishing tolerance to different classes of self-antigens. Lastly, we will examine age-associated changes in the thymic DC compartment that could modulate T cell selection throughout the lifespan.

Thymic Dendritic cell subsets:	Conventional Type 1 DC (cDC1) 	Conventional Type 2 DC (cDC2) 
Markers:	CD11c, MHCII, CD8α, XCR1, CCR7†	CD11c, MHCII, SIRPα, CD14 †, CX3CR1†, CCR7 †, CD301†, CCR2†
Transcription factors§:	Irf8, Batf3, Id2	Irf4, Klf4, Zeb2, Irf2, Relb, Notch2

† Indicates markers expressed only by a subset of cDC1 or cDC2

§ Transcription factors involved in the differentiation and/or homeostasis of indicated subset

FIGURE 1 | Distinct characteristics of major conventional dendritic cell subsets in the thymus. Select cell-surface markers identifying cDC1 and cDC2 are shown. Major transcription factors implicated in the differentiation of these distinct cDC subsets are also indicated.

2 | Regulation of DCs in Thymus

2.1 | Intrathymic Differentiation of cDC1s

cDC1s develop intrathymically from bone marrow-derived hematopoietic progenitors [17, 33]. Early studies suggested that cDC1s and T-lineage committed thymocytes arise intrathymically from a shared progenitor [34–36]. However, it is now clear that thymic cDCs and thymocytes are derived from distinct progenitors. Bone-marrow-derived multipotent progenitors (MPP) differentiate into thymic cDCs after injection into the circulation of irradiated congenic recipient mice, while common lymphoid progenitors (CLP) do not, despite the fact that both progenitors give rise to T-lineage restricted thymocytes [37]. Furthermore, CLPs enter the thymus from the circulation and rapidly generate thymocytes, while MPPs first seed the bone marrow and differentiate into CLP prior to giving rise to thymocytes with a 1-week delay relative to CLPs [38]. Together, these findings indicate that cDC1s differentiate from a hematopoietic precursor downstream of MPPs that is distinct from CLPs, while thymocytes are immediate progeny of CLPs. The distinct origin of cDC1s and thymocytes is further supported by lineage-tracing experiments in which the vast majority of thymic cDC1s were not derived from IL-7R^{+} hematopoietic progenitors, which include CLP and lymphoid-primed MPP (LMPP), the precursors of thymocytes [39]. Additionally, retroviral barcoding combined with next-generation sequencing (NGS) revealed that a large majority of thymic DCs did not originate from the same intrathymic progenitor as T cells [40]. Importantly, the DN1c subset of thymic DN1 cells was identified as the precursor of thymic cDC1s, while thymocytes arise from DN1a and DN1b subsets that comprise early thymocyte progenitors (ETP), the most immature intrathymic precursors of T-lineage cells [33, 41, 42]. Furthermore, macrophage-DC progenitors (MDP), common DC precursors (CDP), and pre-DCs, which are sequential DC-lineage precursors, isolated from the bone marrow all give rise to thymic cDC1s when injected into the blood of nonconditioned host mice [33], indicating that thymic cDC1 likely arise from the same DC progenitors that produce cDC1 in the periphery [43].

Studies highlighting differential roles of transcription factors provide further insight into the hematopoietic hierarchy giving rise to thymic cDC1s. Flt3 and Flt3 ligand (Flt3L) are required for the general differentiation and homeostasis of DCs, including those within the thymus [44, 45]. Flt3L deficient mice have a severe reduction in CDPs and pre-cDCs in the bone marrow, as well as a significant reduction of cDC1 and cDC2 in the thymus [46, 47]. Furthermore, *Irf8* is required for the generation of pre-cDC1s from CDPs. Global deletion of *Irf8* resulted in a marked reduction of pre-cDCs and cDC1s, suggesting that *Irf8* is the transcription factor responsible for specifying the cDC1 fate [32, 48]. Three different enhancers that regulate *Irf8* activity have been noted to be particularly important for either pre-DC, DC, or monocyte differentiation. The +32 kb enhancer

is essential for cDC1 development but not for cDC1 fate specification. Deletion of this enhancer region resulted in the complete absence of cDC1s in the skin-draining lymph nodes (sLN) and spleen. The +41 kb enhancer is crucial for the differentiation of pre-cDC1 from CDP. Excising the +41-kb *Irf8* enhancer led to impaired differentiation of pre-cDC1s from CDPs, ultimately resulting in the elimination of cDC1s [48]. Lastly, the −50 kb enhancer is not required for DC development but rather regulates *Irf8* expression in monocytes and macrophages. Within the thymus, *Irf8* deficiency also results in a reduction of DN1c cells, giving further credence to their identity as intrathymic cDC1 precursors [33]. *Batf3*$^{-/-}$ mice almost entirely lack cDC1s, but not cDC2s (CD8α^{-}CD11c^{+}DCs) in the thymus, spleen, and lymph nodes [32, 49]. Interestingly, *Batf3* deficiency does not impact CDPs or pre-cDC1s [50]. Together these results indicate that *Batf3* plays an important role in the differentiation of cDC1s from pre-cDC1s, while *Irf8* is needed earlier in the differentiation of pre-cDC1s [51]. It remains to be determined if intrathymic DN1c cells are impacted by *Batf3* deficiency or if the differentiation block occurs after the DN1c stage. cDC1 differentiation from CDPs also depends on *Nfil3*-mediated downregulation of *Zeb2*, which suppresses cDC1 differentiation, and a concomitant increase in *Id2* [52]. In addition to its role in the differentiation of pre-cDC1s, *Irf8* is also critical for maintaining cDC1s, as conditional deletion of *Irf8* in cDC1s leads to the adoption of alternative cell fates, including cDC2s [53]. Thus, the transcriptional regulation of cDC1 lineage commitment that occurs in peripheral cDC1s is largely maintained for thymic cDC1s and implicates differentiation from MDP to CDP to pre-DC within the bone marrow, followed by egress of pre-DC into circulation and subsequent entry into the thymus where they differentiate into DN1c cells that finally give rise to cDC1s.

Homing of cDC1 precursors into the thymus was initially thought to be independent of CCR7, as the number of thymic cDCs is not reduced in mice deficient for CCR7 [54]. However, a recent study indicated that thymic homing of a Lin^{-}CD11c^{+}MHCII^{-}Flt3^{+}Sirpa^{low} pre-cDC1 precursor is dependent on the CCR7-CCL21 axis [55], such that CCR7 deficiency resulted in an intrathymic reduction in both pre-cDC1s and cDC1s. Notably, data from our lab and others suggest that global CCR7 deficiency does not impact the accumulation of thymic cDCs within the medulla, where CCR7 ligands are enriched [54, 56]. Instead, the XCR1-XCL1 axis has been implicated, as mature cDC1s express XCR1, while the ligand XCL1 is expressed by mTECs, and *Xcl1* deficiency was initially shown to diminish the medullary accumulation of thymic cDC1s [57]. However, a more recent study has challenged this observation: analysis with MiCasa, a computational tool that evaluates subtle changes in tissue organization, revealed that *Xcl1* deficiency may impact global medullary organization, rather than the localization of cDC1s within the medulla [58]. Thus, additional studies are needed to identify the migratory cues that recruit cDC1 progenitors into the thymus, yielding DN1c cells, and subsequently enforce the medullary localization of resultant cDC1s.

2.2 | Migration of cDC2s Into the Thymus

Unlike cDC1s, cDC2s do not differentiate from a DC precursor within the thymus, but instead migrate into the thymus from the periphery [17, 23, 31]. Nonetheless, similarly to cDC1s, cDC2s also arise from myeloid precursors in the bone marrow, including CDPs and pre-DCs, as indicated by the finding that thymic cDC2s are greatly reduced in mice lacking Flt3L [46, 47]. Recent studies indicate that pre-DCs within the bone marrow can be subdivided based on transcriptional and protein expression profiles into precommitted pre-DC1s (Lin^{-}CD11c^{+}MHCII^{-}CD24^{high}CD8a^{-}) and pre-DC2s (Lin^{-}CD11c^{+}MHCII^{-}Ly6C^{+}SiglecH^{-}) [59–61]. In addition, a lymphoid-derived pDC-like precursor was recently identified using a CD300c-lineage tracing approach, and this progenitor gives rise to some cDC2s that merge transcriptionally with cDC2s derived from the myeloid CDP lineage. These studies highlight the heterogeneity of bone marrow progenitors that give rise to the cDC2 lineage, perhaps reflecting the heterogeneity of cDC2 subsets identified in the thymus [61].

Several transcription factors, including *Irf4*, *Klf4*, *Zeb2*, *Irf2*, *Relb, and Notch2* in conjunction with *Rbpj*, are implicated in the differentiation of DC precursors to the cDC2 lineage in the periphery, but not all of them impact thymic cDC2 cellularity [62]. cDC2s express high levels of *Irf4*, and genetic ablation of *Irf4* expression leads to a partial loss of cDC2s in the periphery. The *Irf4*-deficient cDC2s that remain are impaired in their capacity to support IL-17-producing T cells in response to infection or under homeostatic conditions in mucosal tissues [63, 64]. The impact of *Irf4* deficiency on thymic cDC2s was not evaluated. *Klf4* deficiency impairs the differentiation of pre-cDCs into *Irf4*-expressing cDC2s, leading to a significant reduction in cDC2s in the spleen, lymph nodes (LNs), lungs, and small intestine; it remains unclear if *Klf4* directly regulates *Irf4* expression [65]. Deletion of *Klf4* also completely impairs the development of CD11b^{-}CD24^{-} cDC2s in skin-draining LNs, and this subset promotes Th2 responses in vitro [65, 66]. ZEB2 is another transcription factor critical for cDC2 differentiation. Conditional deletion of *Zeb2* in CD11c-expressing cells reduces the number of cDC2s in the spleen, although pDC differentiation is more severely hampered [67, 68]. A recent study revealed that triple mutations in the *Zeb2* enhancer region, at the three sites where NFIL3 and C/EBP compete for binding, lead to loss of *Zeb2* expression selectively in myeloid progenitors, blocked specification of pre-cDC2s, and ablated DC2s in the periphery in vivo [69]. *Irf2* is responsible for the development of the CD4^{+} subset of cDC2s [70, 71], as *Irf2*$^{-/-}$ mice have fewer splenic CD4^{+}CD11b^{+} DCs. Interestingly, *Relb* is required for the development of cDC2s, defined here as CD8a^{-} DEC205^{-} DCs, in the spleen, but not in the thymus [72]. While global *Relb* deficiency reduced cDC2s in both organs, bone marrow chimeras revealed a 10-fold reduction in splenic cDC2s derived from *Relb* deficient versus WT hematopoietic progenitors, but thymic cDC2s were not impacted. The authors suggest this discrepancy could reflect impaired *Relb*-mediated mTEC differentiation in the constitutive knockout, resulting in a secondary impact on thymic cDC2s, while mTECs were *Relb*-sufficient in bone marrow chimeras. In the spleen, another subset of cDC2s, characterized by the expression of the adhesion molecule ESAM [73], is ablated in mice with conditional *Notch2* deletion in DCs. Consistent with a role for NOTCH signaling in regulating cDC2s, conditional deletion of *Rbpj* in DCs leads to a three-fold decrease in the number of cDC2s in the spleen, but thymic cDC2s are not impacted [74]. The impact of *Irf4*, *Klf4*, and *Zeb2* deficiency on the homeostasis of cDC2s in the thymus remains to be determined, particularly as *Relb* and

NOTCH signaling deficiencies seem to reduce peripheral cDC2 populations without impacting thymic cDC2s.

A combination of adhesion molecules and chemokine receptors regulates the migration of cDC2s into the thymus. The chemokine receptor CCR2 is required for the homeostasis of cDC2s in the thymus [75, 76], as global *Ccr2* deletion leads to a reduction in the frequency of thymic cDC2s. Moreover, *Ccr2* deficiency increases the number of cDC2s in the BM and reduces their frequency in blood, suggesting that CCR2 is important for the egress of cDC2s from the BM into circulation, such that *Ccr2* deficiency may reduce the availability of cDC2s for thymic entry. CCL2, a CCR2 ligand that is expressed by endothelial cells, cortical thymic epithelial cells (cTECs), and mature mTECs, accumulates in perivascular regions, where thymic cDC2s are also found [75–77]. Consistent with their localization in perivascular spaces, cDC2s acquire antigen from circulation to induce negative selection and Treg induction [75, 76]. However, we and others find that most cDC2s localize within the parenchyma of the medulla, along with the majority of cDCs [19, 22, 78, 79]. Thus, CCR2 may play more of a role in regulating the cellularity of cDC2 in the blood than in regulating thymic entry or perivascular localization per se. Consistent with the role of other chemokine receptors in regulating perivascular localization, a recent study identified a $CX3CR1^+$ subset of cDC2s that localizes within perivascular spaces in a CX3CR1-dependent manner, where they acquire antigens from circulation to induce central tolerance [24]. While CX3CL1 deficiency reduced the accumulation of cDC2s within perivascular space, the homing of DCs to the thymus was not impaired. Thus, the chemokine receptor(s) responsible for the recruitment of cDC2s into the thymus remains to be identified. One recent study determined that $CX3CR1^+$cDC2s from the small intestine traffic microbial antigens into the thymus of young mice in a CX3CR1- and CCR5/CCR2-dependent manner, resulting in the expansion of thymocytes responsive to commensal-derived antigens [48]. It remains to be determined if these $CX3CR1^+$cDC2s are distinct from the $CX3CR1^+$ "trans-endothelial" DCs in the perivascular space [24, 80]. In addition to confirming a role for chemokine receptor signaling in regulating cDC2 thymic entry, adoptive transfer experiments in the presence of blocking antibodies revealed that p-selectin and the integrin VLA-4, which binds VCAM-1, are required for the recruitment of cDC2s into the thymus [23]. Recent work has also shown that stimulation of TLR9 in thymic mTECs leads to an influx of a $CD14^+$ subset of cDC2s into the medulla and to increased transfer of mTEC-derived antigens to these DCs. Although TLR signaling increased expression of chemokines by $mTEC^{hi}$ cells, including *Cxcl1*, *Cxcl2*, *Cxcl3*, and *Cxcl5*, deficiency in the corresponding chemokine receptor *Cxcr2* did not impair $CD14^+$cDC2 recruitment after TLR9 stimulation [81]. The authors suggest that CXCR2 may instead position $CD14^+$cDC2s near mTECs, but the chemokine receptors responsible for the recruitment of $CD14^+$cDC2s into the thymus remain to be identified. Because we find that many $CD14^+$cDC2s express CX3CR1, CX3CR1 and CCR5/CCR2 may regulate the entry of $CD14^+$cDC2, as indicated above [19]. However, it remains to be determined if the $CD14^+$cDC2s that accumulate with TLR stimulation and the intestinal-derived $CX3CR1^+$ cDC2s are the same or distinct subsets. Taken together, these studies indicate that thymic cDC2s are quite heterogeneous, and cDC2 subsets may enter the thymus using distinct homing molecules, localize to different thymic regions, and contribute differentially to central tolerance.

2.3 | Activation of DCs in the Thymus

A notable feature of the thymic DC compartment is that some cDC1s and cDC2s have activated phenotypes, including elevated expression of MHCII, CCR7, CD40, and the T-cell costimulatory molecules CD80 and CD86 [19, 21, 54, 82, 83]. Studies from our lab and others have shown that activated DCs (aDCs) localize within the medulla, and aDC1s efficiently present mTEC-derived antigens to developing thymocytes to establish central tolerance [19, 21, 22, 30], underscoring the importance of elucidating mechanisms regulating thymic DC activation. In the periphery, DCs are activated by pathogen-derived stimuli that signal through PRRs, such as toll-like receptors (TLRs), and by cytokines, including interferons, that augment and modulate DC activation and differentiation [84–88]. Strikingly, the transcriptome of thymic aDC1s is largely overlapping with that of PRR-triggered peripheral cDC1s [21], despite the sterile nature of the thymus environment. In fact, activation of thymic DCs occurs normally in germ-free mice, which are devoid of both pathogens and commensal bacteria, as well as in mice triply deficient for *Myd88*, *Mavs*, and *Trif*, the adaptors of PRR signaling, raising the question of how thymic DCs become activated [79, 89].

Crosstalk between post-positive selection thymocytes and DCs is an important driver of thymic DC activation. The number of aDC1s and aDC2s declines when thymocyte differentiation is blocked pre-positive selection, indicating that positively selected thymocytes likely provide signals that induce DC maturation [19, 82, 90]. Our lab and others have identified CD40-CD40L signaling as a critical mediator of thymocyte-DC crosstalk driving DC activation. CD40L is expressed primarily by CD4SP thymocytes, and cognate interactions between CD4SP and DCs result in CD40-dependent activation of both cDC1s and cDC2s [19, 82, 90]. The involvement of CD40 signaling in thymic DC activation mirrors DC licensing in secondary lymphoid organs, where CD40L-expressing CD4 T cells undergo cognate interactions with DCs, inducing CD40 signaling that drives increased expression of costimulatory molecules and enhanced DC survival [5, 91]. Our recent findings show that CD8SP thymocytes also play an important role in activating thymic DCs [19]. A role for CD8SP cells in thymic DC activation was largely dismissed because the number of aDCs is not reduced in $\beta 2m^{-/-}$ mice, in which CD8SP cells fail to differentiate [82]. Furthermore, in a mixed bone marrow chimera setting, $\beta 2m^{-/-}$ cDC1s and cDC2s become activated at comparable frequencies to WT DCs, indicating that cognate interactions with CD8SP thymocytes are not required for DC activation [82]. However, cognate interactions between CD8SP cells and DCs do promote DC activation, although in a CD40-independent manner [82, 90]. Furthermore, our recent single-cell transcriptional profiling data reveal a large transcriptional shift in cDC1s from $\beta 2m^{-/-}$ thymi, as well as a decline in $CD14^+$ $CX3CR1^+$ DC2s [19]. In the absence of CD8SP thymocytes, both cDC1s and cDC2s have reduced Type I interferon (IFN) gene signatures as they become activated, and IFN signaling has recently been identified as a key driver of cDC1 activation [19, 83]. Specifically, Type III interferon signaling, which shares many transcriptional targets with Type I IFN signaling, is required for activation of cDC1s [92]. $Ifnlr1^{-/-}$ and

Ifnar1$^{-/-}$ *Ifnlr1*$^{-/-}$ mice both show a significant reduction in the number of aDC1s, but not aDC2s [83]. Nonetheless, thymic cDC2s signal constitutively through IFNLR1 and IFNAR1, consistent with our finding that the transcriptional signature of type I IFN signaling is reduced in thymic cDC2s in the absence of CD8SP cells [19, 83]. mTECs are the major producers of Type I and Type III IFNs in the thymus, which they express in a partially *Aire*-dependent but PRR-signaling independent manner [92–94]. Thus, while it remains to be determined if CD8SP cells directly activate interferon receptor signaling in thymic DCs, the predominant thymic expression of interferons by mTECs suggests that CD8SP thymocytes may instead regulate mTEC IFN production. In addition to IFN signaling, IL-4 signaling is required for activation of CD301^{+}cDC2s, which are required for efficient negative selection [78]. IL-4 is expressed in the thymus mainly by thymic resident iNKT2 cells, indicating that in addition to crosstalk with conventional thymocytes, signals from innate lymphocytes also promote thymic DC activation [95, 96].

The chemokine receptor CCR7 also modulates homeostasis of the thymic DC compartment, with consequences for central tolerance. As discussed above, CCR7 promotes migration of pre-DC1s into the thymus. In the absence of CCR7, the number and frequency of cDC1s decline, possibly as a direct consequence of reduced recruitment of thymic pre-DC1s [55]. Our findings indicate a distinct, nonexclusive mechanism by which CCR7 alters thymic DCs. CCR7 is expressed by thymic aDC1 and aDC2 [21, 54, 82]. We find that CCR7 deficiency results in increased apoptosis of thymic aDC1s, likely contributing to the lower cDC1 to cDC2 ratio in *Ccr7*$^{-/-}$ thymi [54]. CCR7 deficiency also results in increased thymic Treg cellularity, which is unexpected given that selection of Treg occurs in the medulla, and CCR7 enforces medullary accumulation of CD4SP cells that are selected to become Treg [54, 97–99]. However, our findings indicate that *Ccr7* deficiency in thymic DCs, not in CD4SP thymocytes, is responsible for the increase in thymic Treg cellularity [54]. In adult *Ccr7*$^{-/-}$ mice, an expanded number of recirculating Treg account for the increase in Treg cellularity; however, increased Treg are already observed by postnatal day 4, when Treg are first selected in the thymus, indicating that CCR7 deficiency in DCs likely results in an increase in newly generated Treg at this age as well [54, 97]. The mechanisms by which CCR7 deficiency alters thymic DCs and the impact of such alterations on Treg selection and recirculation require further evaluation.

3 | Molecular Mediators of Tolerogenic Interactions Between Thymic DCs and Thymocytes

Presentation of sufficiently high-avidity self-antigens that activate TCR signaling in thymocytes is necessary, but not sufficient, for thymic APCs to induce negative selection and Treg induction. Here, we will review cell-surface and secreted proteins expressed by thymic DCs that contribute to central tolerance (Figure 2A). CD70, which is upregulated by CD40 signaling in DCs, binds to CD27 expressed by thymocytes [104, 106]. Global genetic deletion of either CD27 or CD70 reduces Treg cellularity in the thymus [104]. CD27 is not required for generation of CD4^{+} CD25^{+} Treg precursors (TregP), which are present at normal numbers in *Cd27*$^{-/-}$ mice; however, subsequent FOXP3^{+}TregP are reduced. CD27 signaling inhibits apoptosis of CD25^{+}TregP by reducing expression of pro-apoptotic Bcl2 family members. Thus, CD27 is required for survival of differentiating Treg, but not for initial TCR-mediated agonist selection. Other tumor-necrosis factor receptor superfamily (TNFRSF) members, whose ligands are expressed by thymic DCs as well as by mTECs, also play key roles in regulating Treg differentiation [105]. OX40, GITR, and TNFR2 are all expressed by Treg and CD25^{+}TregP at higher levels than on conventional CD4SP. Expression levels of these TNFRSF molecules correlate with TCR signal strength, and their expression is dependent on CD28 signaling during agonist selection. GITRL and TNF are expressed by both mTECs and DCs, whereas OX40L is expressed exclusively by mTECs. Binding to these TNFSF ligands increases TregP sensitivity to IL-2, promotes their conversion into mature Treg in vitro, and elevates thymic Treg cellularity in vivo. Conversely, blockade of all three TNFRSF members abrogated Treg differentiation in vivo. Together, these results indicate that DCs express TNFSF ligands that costimulate TregP via TNFRSF superfamily members that are induced after initial agonist selection, and TNFRSF signaling is required for T_{reg} development [105]. Several studies have also established a requirement for costimulation through CD28, which is expressed by CD4SP cells, in establishing central tolerance through both negative selection and Treg induction. CD28 on thymocytes binds to CD80 and CD86, which are expressed at high levels by aDCs and mature mTECs that have been signaled through CD40. CD28 costimulation is important not only for upregulation of antiapoptotic proteins, but also for *Foxp3* expression and upregulation of TNFRSFs by TregP, as mentioned above [10, 100–103, 107]. Furthermore, expression of CD80 by DCs, in the absence of expression by B cells or TECs, was sufficient to sustain normal thymic Treg numbers [101]. Notably, deletion of CD80 and CD86 on DCs, despite expression of CD80 by B cells and TECs, led to an increase in CD4 T cells due to impaired clonal deletion, demonstrating an essential role for DCs in providing CD80-mediated costimulation to autoreactive thymocytes to mediate central tolerance.

As discussed above, CD40 is a TNFRSF protein expressed by thymic DCs that is essential for their activation (Figure 2B). Following engagement with CD40L-expressing thymocytes, CD40 signaling drives expression of costimulatory molecules by DCs, including CD80, CD70, and 4-1BB [82, 91]. Thus, while CD40 does not costimulate thymocytes directly during central tolerance induction, CD40 expression by DCs is required for central tolerance. We find that conditional deletion of *Cd40* specifically in cDC1s reduces aDC1 cellularity, resulting in a significant decline in the frequency of polyclonal thymocytes undergoing negative selection [19]. Furthermore, we find that CD40 deficiency specifically in cDC1s diminishes the frequency of CD4SP selected into the Treg lineage, consistent with another study in which conditional deletion of CD40 in DCs reduced the frequency of polyclonal Treg in the thymus [108]. These results indicate that aDC1s are essential for both negative selection and Treg induction.

Previous work from our lab shows that cDC2s and aDCs express the chemokines CCL17 and CCL22, ligands for CCR4, a chemokine receptor that supports central tolerance (Figure 2C) [79, 98]. CCR4 signaling promotes the migration of early post-positive selection thymocytes into the medulla, where they encounter DCs, mTECs, and B cells that display a broad range of self-antigens to induce central tolerance. *Ccr4* deficiency impairs the accumulation of post-positive selection DP and semi-mature CD4SP

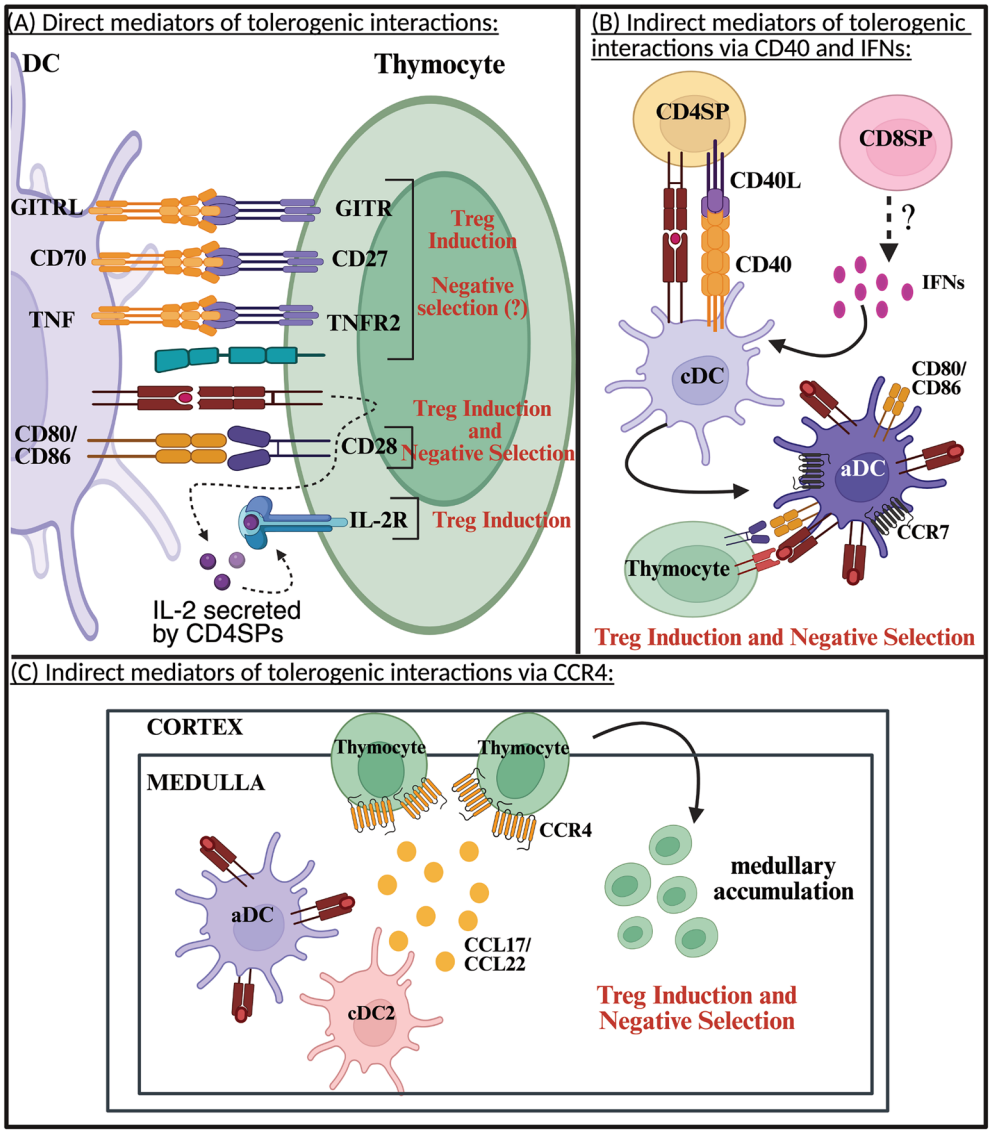


FIGURE 2 | Molecular mediators of DC interactions with thymocyte subsets that contribute to central tolerance through direct or indirect mechanisms. (A) DCs express CD80 and CD86, which bind to CD28 on thymocytes. Signaling though CD28 is essential for both Treg induction and negative selection of strongly self-reactive thymocytes [10, 100–103]. Thymic DCs also express the TNFSF members CD70, GITRL, and TNF which can bind to the corresponding receptors on CD4SP thymocytes [104, 105]. Activation of these TNFRSF members on thymocytes that have been activated by self-antigen and CD28 signaling, along with IL-2R signaling, is required for diversion to the Treg lineage [105]. (B) When post-positive selection CD4SP thymocytes, which express CD40L, undergo cognate interactions with DCs, they activate CD40 signaling in cDC1s and cDC2s, promoting DC activation that involves increased expression of MHC complexes, CCR7, and costimulatory molecules [19, 82, 90]. Through either direct or indirect mechanisms, CD8SP thymocytes induce a Type I IFN signature in DCs that promotes DC activation [19, 83]. Activated DCs, in turn, are responsible for efficient central tolerance, including negative selection and Treg induction. (C) The CCR4 ligands CCL17 and CCL22 are expressed by cDC2s and aDCs. These chemokines mediate accumulation of early postpositive selection thymocytes in the medulla and promote thymocyte-DC interactions, likely accounting for the requirement for CCR4 in the early stages of negative selection and Treg induction [79, 98].

cells within the medulla. Interestingly, live-cell imaging also revealed that *Ccr4*$^{-/-}$ CD4SP thymocytes spend significantly less time interacting with medullary DCs than *Ccr4*-sufficient cells, perhaps contributing to the reduced frequency of thymocytes undergoing negative selection in *Ccr4*$^{-/-}$ mice [79, 98]. Together, these results suggest that chemokines produced by DCs not only recruit thymocytes into the medulla but also directly promote interactions with DCs that induce central tolerance, consistent with the role of chemokine receptors in promoting T cell-DC interactions in secondary lymphoid organs [109, 110].

4 | The Role of Thymic DCs in Establishing Central Tolerance to Different Classes of Self-Antigens

4.1 | Tolerance to Tissue-Restricted Antigens

In order to induce central tolerance to the distinct transcriptomes expressed by differentiated cell types that comprise all organs, the repertoire of self-antigens displayed by thymic APCs must be highly diverse (Figure 3). To accomplish this,

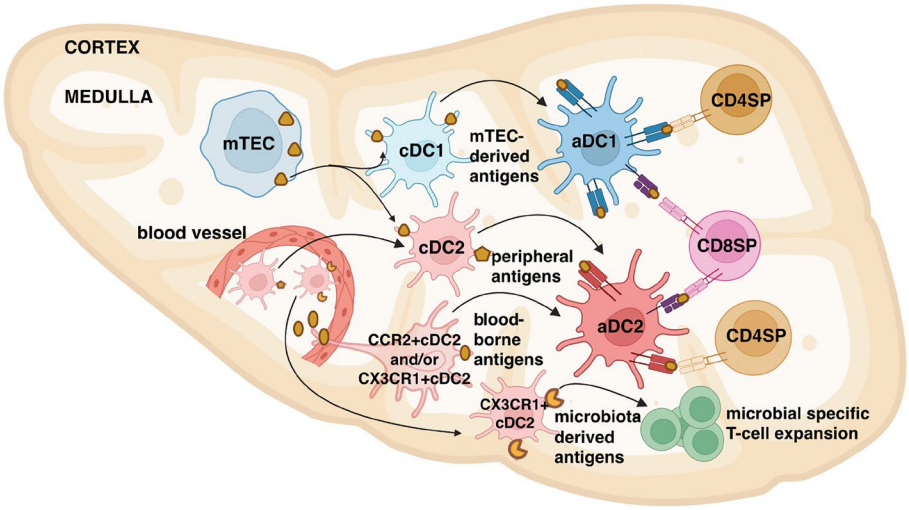


FIGURE 3 | Unique DC subsets present self-antigens from distinct sources to thymocytes to induce central tolerance. aDC1s and aDC2s efficiently present antigens acquired from mTECs to developing CD4SP and CD8SP thymocytes [19, 20, 22, 30, 89, 111]. cDC2s can also traffic peripheral antigens into the thymus to induce tolerance against self-antigens that are not expressed by mTECs. CCR2$^+$cDC2s and CX3CR1$^+$cDC2s are able to present circulating or blood borne antigens to thymocytes [24, 75]. Additionally, CX3CR1$^+$cDC2s carry microbiota induced antigens from the intestine into the perinatal thymus, where they induce proliferation of microbial specific T cells [80].

mTECs collectively express about 90% of the transcriptome, including TRAs. TRAs, such as insulin and MOG, are proteins expressed by mTECs that are otherwise expressed in only a few peripheral tissues. About 4000 TRAs are expressed under control of the transcriptional regulator *Aire*, which promotes TRA transcription as well as mTEC differentiation [112–116]. Other TRAs are expressed by the recently discovered diverse mimetic mTEC subsets, which express lineage-defining transcription factors and the genetic programs associated with differentiated cell types, like intestinal Tuft cells or M cells [117–121]. Differentiation of mimetic mTEC subsets is also variably dependent on *Aire* [120]. Consistent with a key role for *Aire* in driving TRA expression in conventional and mimetic mTECs, mice and people lacking *Aire* have reduced TRA expression and experience multiorgan autoimmunity due to defective central tolerance [122–127]. Of note, only 1%–3% of mTECs express any given TRA, creating a sparse mosaic of self-antigen expression by mTECs in the medulla; thus, if mTECs were solely responsible for presenting TRAs to thymocytes, central tolerance would be inefficient, likely enabling maturation and egress of autoreactive T cells [112–116].

Fortunately, while thymic DCs do not express *Aire*, they acquire TRAs from mTECs for presentation to thymocytes, increasing the efficiency of central tolerance to sparsely expressed self-antigens (Figure 3) [12, 13, 22, 111, 128]. For some Aire-dependent TRAs, presentation by mTECs is sufficient to induce central tolerance, but for others, presentation by DCs is required [10–12, 30, 129]. Studies using TCR transgenic (Tg) mice and Aire-dependent neo-self-antigens indicate that the nature of the self-antigen, such as whether it is secreted or membrane-tethered, as well as whether antigen presentation occurs on MHCI versus MHCII impacts whether DCs and/or mTECs play a greater role in driving thymocyte negative selection [11, 89]. 2-photon imaging studies from our lab indicate that both mTECs and DCs efficiently present model *Aire*-dependent TRAs, inducing activation of both CD4SP and CD8SP TCR Tg thymocytes [89]. Notably, when the model TRA was expressed in a secreted form, as opposed to membrane-tethered, DCs presented the antigen to CD4SP more efficiently than mTECs. In keeping with our results, a separate study demonstrated that while TCR Tg CD4SP thymocytes were deleted in an *Aire*-dependent manner to both the trans-membrane and secreted forms of the same model TRA used in our studies, DCs were required for negative selection only for the secreted form [11]. Moreover, we found that DCs presented peptides from the membrane-tethered form of the TRA to CD8SP thymocytes more efficiently than did mTECs, suggesting that thymic DCs are particularly efficient at acquiring and cross-presenting membrane-bound self-antigens expressed by mTECs on MHCI [89]. In a complementary approach, another study used TCR repertoire sequencing to identify TCR clones that required MHCII expression by *Aire*$^+$mTECs versus DCs for negative selection or Treg induction. The expected contributions of each APC type were validated by analysis of thymocytes expressing the candidate TCRs, via retroviral transduction, in mice in which either mTECs or DCs were ablated for MHCII [30]. Notably, this study indicated that both DCs and AIRE$^+$ mTECs contribute to negative selection and Treg induction of TCRs selected in an *Aire*-dependent manner. Additional studies have addressed the relative contributions of DCs versus mTECs to central tolerance of polyclonal thymocytes. Ablating MHCII on bone marrow-derived APCs, which include DCs, or reducing MHCII levels on mTECs leads to increased numbers of CD4SP thymocytes [129, 130]. Selective deficiency of MHCII on DCs also increases CD4SP numbers, demonstrating a nonredundant contribution of DCs to negative selection [131]. However, multiple studies have shown that negative selection is most severely impaired when both mTECs and DCs are unable to present self-antigens, indicating the importance of both APC types in driving central tolerance [32, 129].

A number of studies indicate that cDC1s are more efficient than cDC2s at acquiring and presenting mTEC-derived antigens to

induce central tolerance. Relative to cDC2s, cDC1s more efficiently acquire fluorescent proteins expressed by mTECs [20, 22, 30, 111]. Recent work from our lab shows that when specific DC subsets are sorted from the thymus of a mouse in which Ovalbumin (OVA) is expressed by mTECs as a model *Aire*-dependent TRA, aDC1s most efficiently present this TRA on MHCI, resulting in robust proliferation of OT-I TCR Tg $CD8^+$ T cells [19]. Notably, aDC1s are extremely efficient at cross-presenting this mTEC-derived TRA, inducing as much OT-I proliferation as positive control splenocytes incubated with the cognate OVA peptide. These findings are in keeping with our imaging studies, discussed above, showing that DCs can present TRAs to thymocytes more efficiently than the mTECs themselves that express the TRA [89]. We also found that out of seven purified thymic DC subsets, aDC1s most efficiently displayed antigens expressed by mTECs on MHCII, consistent with a previous study [19, 21]. Although aDC1s are particularly efficient at presenting mTEC-derived antigens on MHCI and MHCII, we found that aDC2s were the next most efficient DC subset, cross-presenting TRAs on MHCI to induce OT-I T cell proliferation and presenting mTEC-derived antigens on MHCII, while non-activated cDC1 and cDC2 subsets showed minimal activity in either of these assays [89].

Multiple mechanisms by which cDC1s acquire antigen from mTECs have been identified. $AIRE^+$ mTECs undergo rapid turnover in the homeostatic thymus [132], and apoptotic mTECs may be phagocytosed by thymic APCs, promoting presentation of mTEC-associated self-antigens. In keeping with this possibility, cDC1s express the scavenger receptor CD36, which has been implicated in binding phosphatidylserine (PS) present on the membrane of apoptotic bodies. CD36 facilitates transfer of cell surface, but not cytoplasmic antigens, from apoptotic mTECs to DCs, resulting in display of these antigens on MHCII molecules [20]. TCR repertoire analysis indicated substantial overlap between TCRs dependent for selection on *Cd36* versus *Batf3*, indicating that CD36-mediated antigen transfer is a major mechanism by which cDC1s acquire mTEC-derived self-antigens to promote central tolerance. In addition to processing and presenting phagocytosed self-antigens, cDCs can acquire and display intact peptide–MHC complexes from other cells, a process known as "cross-dressing". Thymic DCs can cross-dress with both MHCI and MHCII complexes acquired from TECs or other DCs, but not from B cells, and cross-dressing occurs more efficiently in thymic versus splenic DCs [29] Although both cDC1s and cDC2s acquired peptide–MHC complexes to a similar extent, cDC1s were more efficient than cDC2s in inducing T cell proliferation to antigen presented by cross-dressed complexes. These studies were carried out largely in vitro, and future studies should address the contributions of cross-dressing to central tolerance in vivo.

Despite the clear role for cDCs in mediating negative selection and Treg generation, and the superior capacity of cDC1s to acquire and present mTEC-derived self-antigens relative to cDC2s, there has been some debate about whether cDC1s play an essential role in establishing central tolerance in the thymus. TCR repertoire analysis followed by retrogenic validation studies of thymocytes from wild-type versus $Batf3^{-/-}$ mice, which are deficient for cDC1s, identified some TCRs that require cDC1s for negative selection, and others that require cDC1s for selection into the Treg lineage, indicating that cDC1s shape the TCR repertoire by contributing to both arms of central tolerance [20, 30]. However, another study that evaluated the TCR repertoire of thymic Treg from BATF3-deficient versus wild-type mice found no significant changes [10]. The discrepancy in TCR repertoire analyses may reflect a difference in analytical approaches, or possibly, the use of different TCRβ chains. Moreover, another study noted no abnormalities in thymic Treg numbers in cDC1-deficient $Batf3^{-/-}$ or $Irf8^{-/-}$ mice, while depletion of mTECs resulted in about 50% reduction in thymic Tregs [32]. Nonetheless, in further support of a role for cDC1s in central tolerance, thymocytes selected in the absence of cDC1s induced tissue-specific inflammation in multiple organs when transplanted into lymphopenic hosts, and CD36-dependent antigen transfer from mTECs to DCs suppressed allo-reactive T cell responses that resembled GVHD in the context of bone marrow transplantation [20]. Moreover, CD70 expression by cDC1s, not cDC2s, is essential for Treg generation in vitro [104]. Recent studies show that mice with a significant reduction in the frequency of aDC1s ($Ifnlr1^{-/-}$ and $Ifnar1^{-/-}/Ifnlr1^{-/-}$ mice) exhibited a decline in Treg progenitors and Treg cells, further suggesting that aDC1s are responsible for Treg induction [83]. Our recent study also indicates that cDC1-specific deletion of CD40 results in fewer aDC1s in the thymus, an increased number of CD4SP thymocytes, and a reduced frequency of Treg [19]. Together, these findings indicate that aDC1s play a nonredundant role in supporting negative selection and Treg induction, and while aDC1s are required for selection of some TCR specificities, they act largely in conjunction with mTECs to enforce broad central tolerance.

Several studies suggest that despite their somewhat inferior capacity to acquire TRAs, cDC2s also contribute to central tolerance to mTEC-derived antigens. Our 2-photon imaging studies indicate that compared to cDC1s, cDC2s activate a higher frequency of TCR Tg CD8SP and CD4SP thymocytes with specificity for an mTEC-expressed model-TRA in live thymic tissue [89]. In addition, aDC2s are a close second to aDC1s in their capacity to acquire and present mTEC-derived antigens, enabling them to induce fairly robust proliferation of naïve TRA-specific CD8 T cells, as discussed above [19]. Moreover, ablation of a large proportion of cDC2s resulted in a significant reduction in polyclonal negative selection, as revealed by TCR repertoire sequencing and evaluation of the frequency of thymocytes undergoing negative selection [78]. Multiple studies also implicate cDC2s in efficient Treg selection [10, 22, 31, 32, 54]. Research from our lab and others demonstrates that compared to the cDC1 lineage, cDC2s and aDC2s induce a higher frequency of Treg when cocultured with $CD25^-Foxp3^-CD4SP$ cells in vitro [10, 19, 31, 32, 54, 81, 133]. Furthermore, we find that thymic Tregs increase in both adult and perinatal CCR7-deficient mice as a consequence of CCR7 deficiency in the DC compartment [54]. CCR7 deficiency results in an increased proportion of cDC2s and apoptosis of aDC1s, as discussed above. Notably, DCs from $Ccr7^{-/-}$ thymuses induced more Treg in vitro, which reflected the shift in composition toward elevated frequencies of cDC2s [54]. In another study, selection of a naturally occurring Treg TCR with specificity for an Aire-dependent prostate-specific self-antigen

required cDCs for selection, but was not dependent on cDC1s, suggesting cDC2s are crucial for selection of this TRA-specific Treg clone [10]. Overall, these studies indicate that cDC2s acquire antigens from mTECs and play a critical role in mediating negative selection and Treg generation to TRAs.

4.2 | Tolerance to Peripheral Antigens

cDC2s migrate into the thymus, where they become activated, suggesting they have the potential to induce tolerance to antigens acquired in peripheral tissues [14, 17, 23, 134] (Figure 3). A seminal study showed that cDCs constitutively migrate from blood into the thymus, but migration is inhibited by inflammatory signaling in DCs, suggesting DCs may selectively traffic self-antigens from homeostatic tissues to promote thymic central tolerance while avoiding presentation of pathogen-derived antigens that could induce unwanted tolerance [23]. In keeping with this possibility, DCs presenting exogenously provided OVA in a noninflammatory setting induced negative selection of OVA-specific CD4SP thymocytes [89]. Furthermore, expression of OVA antigen under the control of a cardiac myocyte-specific promoter resulted in negative selection of OVA-specific CD4SP cells [23]. Negative selection in this model was VLA-4-dependent, suggesting that DCs were required to traffic OVA into the thymus, rather than OVA being ectopically expressed as a TRA by mTECs to promote deletion [23]. A recent study identified a subset of CX3CR1$^+$cDC2s that carry microbiota-derived antigens from the intestine into the perinatal thymus [80]. Interestingly, however, these DCs did not induce tolerance, but instead resulted in an increased number of microbial-specific T cells, although the mechanisms underlying this phenomenon remain to be clarified [80]. Together, these studies indicate that cDC2s traffic antigens into the thymus from peripheral tissue for presentation to developing thymocytes.

Thymic cDC2 subsets also promote tolerance to antigens acquired from circulation. Intravenous injection of hen egg white lysozyme (HEL) revealed greater antigen uptake by cDC2s, although cDC1s also acquired HEL [133]. When purified from the thymuses of these mice, cDC2s, along with cDC1s, induced negative selection and Treg induction of HEL-specific thymocytes in vitro. Another study showed that *Ccr2*$^{-/-}$ mice have a reduced number of cDC2s, resulting in impaired negative selection of OVA-specific CD4SP TCR Tg cells in the presence of circulating OVA. Interestingly, some cDC2s localize within perivascular spaces in the thymus in a CCR2-dependent manner, where they should have ready access to circulating antigens [75]. Consistent with this idea, a recent study identified CX3CR1$^+$cDC2s that are positioned around vascular walls in the thymus, where they project processes into the vascular lumen to capture circulating antigens [24]. Perivascular positioning of this DC subset is aided by expression of CX3CR1, which binds the chemokine CX3CL1 that is expressed by thymic capillaries; disrupting this chemokine axis resulted in impaired negative selection in response to circulating antigens. Collectively, these studies indicate that cDC2 subsets are specialized for acquisition and presentation of blood-borne antigens, but the relationship of these subsets to one another remains to be clarified.

5 | Age Associated Changes in Thymic DCs

5.1 | Thymic DCs in Perinates

The perinatal window of development is a crucial time in which the immune system both establishes homeostasis with the environment, including tolerance to peripheral self-antigens and commensal microbes that colonize barrier tissues, and first responds to newly encountered pathogens [135–139]. Conventional T cells in the perinatal period are more self-reactive than in adults, as denoted by higher expression of CD5, which is thought to reflect greater strength of TCR signaling during positive selection [140–144]. Alternatively, elevated CD5 levels could reflect less efficient negative selection in the perinatal period, consistent with the finding that diabetogenic T cells are produced by neonatal through postnatal day 7 (P7) thymuses, but are not produced in thymuses older than P10 [145]. Perinatal CD4$^+$ T cells are also prone to mounting T_H2, as opposed to T_H1 immune responses, which is at least in part due to insufficient IL-12 production by DCs in secondary lymphoid organs needed to promote T_H1 differentiation [135, 146–148]. In addition, perinatal CD8$^+$ T cells preferentially differentiate into virtual memory cells which rapidly secrete cytokines in response to pathogens prior to undergoing apoptosis, but do not efficiently generate memory T cell responses [138, 149–151]. Moreover, Aire-dependent Treg selected uniquely in the perinatal period have a more suppressive signature than those selected in the adult thymus and persist into adulthood where they are required for protection against tissue-specific autoimmunity [137, 152]. The profound differences in the function of T cells produced from perinatal versus adult thymi suggest that age-associated changes in the thymic environment could alter thymocyte differentiation and selection.

Age-associated changes in the thymic DC compartment over the neonatal to juvenile (P28) period are consistent with an altered capacity to induce central tolerance. Our recent single-cell transcriptional profiling studies demonstrate that the composition of thymic DCs changes with age, such that aDC1s decline in frequency over the neonatal to juvenile period, while cDC2s become more prevalent [153]. A previous study also indicated that cDC1s decline from neonates to adults, whereas cDC2s increase with age [154]. The age-associated increase in cDC2s was implicated in more efficient negative selection in adults, indicating that the lower frequency of cDC2s in neonatal mice leads to defective central tolerance. This finding is in keeping with the significant decline in negative selection observed after cDC2 ablation [78]. Moreover, given the different capacity of aDC1 versus cDC2 to support central tolerance to distinct classes of self-antigens, as discussed above, the change in DC subset composition in the perinatal period likely promotes age-associated changes in the repertoire of TCRs undergoing negative selection and Treg induction. In fact, T cells expressing TCRs specific for a peptide of *Padi4* switch from differentiating down the Treg lineage to undergoing predominantly negative selection between perinatal and adult periods [155]. Strikingly, we found that a gene expression signature associated with Type I IFN responses increases in cDC1s and cDC2s between P3 and P7, timing that is strikingly concordant with the decline in

differentiation of diabetogenic T cells [145, 153]. An early age-associated increase in the production of IFNβ1 and IFNλ2 in the thymus was recently demonstrated using reporter mouse strains [83]. As discussed above, Type III interferons, which activate expression of genes in the Type I IFN pathway, were recently identified as key signals driving activation of thymic aDC1s [83]. Notably, we find that the increased Type I IFN signaling signature in the perinatal period correlates with elevated expression of genes involved in antigen processing and presentation on MHCI [153]. Together, these studies strongly suggest that age-associated changes in the composition and transcriptional profiles of DCs over the neonatal to juvenile transition likely contribute to stage-specific thymocyte selection and downstream T-cell activity.

5.2 | Thymic DCs in Aged Mice

Following an expansion phase during the neonatal to juvenile transition, and a brief homeostatic phase in juvenile mice, the thymus begins to undergo involution strikingly early, between 1 and 2 months of age [156]. Age-associated thymic involution is characterized by reduced thymocyte cellularity and T cell output, as well as diminished numbers and altered differentiation of TECs [135, 157–159]. Moreover, aging is associated with a reduced frequency of naïve relative to memory T cell subsets, and an increase in T-cell self-reactivity, indicating that self-tolerance could be impaired [160–162]. In keeping with this possibility, we and others have shown that Aire^{+} mTEC^{hi} cells, which express TRAs, decline not only in number but also as a proportion of mTECs as early as 3 months of age [158, 163]. Moreover, expression of Aire-dependent TRAs, representing multiple tissues, declines by 1 year of age [164]. As TRA expression declines, DCs undergo age-associated changes. We find a dramatic drop in the number of DCs of all subsets starting around 6 months of age [163]. Notably, the composition of the DC compartment also changes with age; the frequency of aDC1s declines, accompanied by an increase in the frequency of cDC1s. Moreover, the frequency of cDC2s increases, with a modest reduction in aDC2 frequencies [163]. These findings are complementary to a previous study showing that the proportion of the overall DC1 lineage (cDC1s + aDC1s) declines gradually with age, relative to an increased proportion of the DC2 lineage [165]. As aDC1s are particularly efficient at acquiring and presenting TRAs from mTECs, the decline in both Aire^{+} mTECs and aDC1s suggests that central tolerance to mTEC-derived antigens may be impaired with age. Although we did not observe a decline in the frequency of polyclonal thymocytes undergoing negative selection, heterochronic live thymic slice assays revealed that by middle age (12 months), the thymus microenvironment is impaired in its ability to support negative selection and Treg induction to moderate avidity self-antigens, including model TRAs [163]. These findings are consistent with the impaired negative selection of polyclonal thymocytes to the TRA apolipoprotein B, an autoantigen involved in atherosclerosis, in the mouse thymus by 6 months of age [166]. Using transcriptional profiling data of sorted thymic cDC1+ aDC1 and cDC2+ aDC2 from mice at 1, 3, and 6 months of age, we also identified an increase in expression of genes induced by LPS, such as *Il1a, Il1b, Cxcl2, Il6, Il12b, Il18*, and *Tnf*, by 6 months of age, suggesting a pro-inflammatory environment generated by aging DCs could alter selection and/or contribute to thymic involution [167]. Together, these studies suggest that age-associated changes in thymic DCs may contribute to impaired central tolerance to TRAs by middle age, which could play a role in the increased incidence of autoimmunity by middle age, warranting further examination of the impact of aging on thymic DCs.

6 | Conclusions and Future Directions

The thymic DC compartment, which plays a critical role in central tolerance, is highly heterogeneous, as revealed by recent single-cell transcriptional profiling studies from our lab and others. Thymic DCs are shaped by signals from developing thymocytes and TECs, which change over the lifespan [19, 78, 83, 153]. cDC1s develop from intrathymic progenitors and subsequently become activated within the thymus in response to CD40 signaling driven by cognate interactions with CD4SP thymocytes, IFN signaling that depends on the presence of CD8SP thymocytes, and Type III IFNs produced in an Aire-dependent manner by mTECs [33, 34, 82, 83, 156]. aDC1s are strongly implicated in driving central tolerance to mTEC-acquired self-antigens [19, 21, 30, 111]. Future studies are needed to resolve the role of CD8SP cells in regulating cDC1 transcriptional profiles and functions. cDC2s differentiate extrathymically prior to migrating into the thymus, where they proliferate and become activated in response to signals from SP thymocytes, as well as other environmental cues such as IFN signaling and IL-4 signaling [17, 19, 23, 95, 96]. cDC2s are more heterogeneous than cDC1s and have been implicated in various aspects of central tolerance, from trafficking self-antigens acquired in peripheral tissues into the thymus to induce negative selection, to presenting self-antigens acquired from circulation for negative selection, to inducing Treg selection to mTEC-derived TRAs [10, 14, 23, 24, 75, 133]. Furthermore, a subset of cDC2s traffics microbial antigens into the thymus for presentation to thymocytes [80]. It will be important to clarify whether some of the distinct cDC2 activities can be attributed to different or overlapping cDC2 subsets and to investigate mechanisms by which cDC2s contribute to the expansion of microbial-specific thymocyte clones. Finally, the thymic DC compartment undergoes age-associated changes in transcription and composition both early after birth and during age-associated thymic involution [135, 154, 163, 165]. Future studies are needed to evaluate how thymic DCs shape the TCR repertoire and the function of T cells produced by the thymus at different ages.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

Data sharing not applicable to this article as no datasets were generated or analysed during the current study.

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