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Instructive Cues of Thymic T Cell Selection

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Keywords

thymus, clonal deletion, Foxp3$^+$ regulatory T cells, T cell receptor, cytokines, thymic antigen-presenting cells

Abstract

A high diversity of αβ T cell receptors (TCRs), capable of recognizing virtually any pathogen but also self-antigens, is generated during T cell development in the thymus. Nevertheless, a strict developmental program supports the selection of a self-tolerant T cell repertoire capable of responding to foreign antigens. The steps of T cell selection are controlled by cortical and medullary stromal niches, mainly composed of thymic epithelial cells and dendritic cells. The integration of important cues provided by these specialized niches, including (*a*) the TCR signal strength induced by the recognition of self-peptide-MHC complexes, (*b*) costimulatory signals, and (*c*) cytokine signals, critically controls T cell repertoire selection. This review discusses our current understanding of the signals that coordinate positive selection, negative selection, and agonist selection of Foxp3$^+$ regulatory T cells. It also highlights recent advances that have unraveled the functional diversity of thymic antigen-presenting cell subsets implicated in T cell selection.

INTRODUCTION

The thymus contains two distinct anatomical sites: the medulla and the surrounding cortex. These regions are composed of several antigen-presenting cells (APCs), mainly thymic epithelial cells (TECs) and dendritic cells (DCs), which create specialized microenvironments coordinating T cell selection events (**Figure 1**). T cell receptor (TCR) gene rearrangements allow the generation of a highly diverse αβ TCR repertoire composed of more than 10^{15} distinct TCRs (1, 2). Given that TCR gene rearrangements occur semirandomly, some TCRs will inevitably recognize self-antigens. Therefore, the TCR repertoire needs to be selected for its ability to recognize for- eign peptide–MHC complexes while being self-tolerant. Impairment of antigen presentation in the thymus can result in a breakdown in T cell tolerance, triggering autoimmune disorders such as type 1 diabetes and multiple sclerosis.

During their development, thymocytes sequentially migrate within cortical and medullary stromal niches. Their high motility allows interaction with a large array of self-peptide-MHC complexes presented by numerous APCs and responding to cytokine signals. They undergo several selection processes, including β-selection, positive selection, negative selection (also called clonal deletion), and commitment to the regulatory T cell (Treg) lineage. Their fate is determined by the strength of the

TCR signal, triggered by the recognition of peptide-MHC complexes and by the integration of costimulatory and cytokine signals provided by numerous thymic APCs. Only ~5% of developing thymocytes are selected, and ~1–4 × 10^6 mouse naive T cells are ex- ported daily to the periphery. In this review, I summarize our latest knowledge on intrathymic αβ T cell selection and discuss the instructive cues that drive the generation of a self-tolerant T cell repertoire. I give particular emphasis to signals derived from TCRs, costimulatory molecules, and cytokine receptors and the recently appreciated thymic APC heterogeneity.

T CELL DEVELOPMENT AND SELECTION

Early T Cell Development and Thymic Positive Selection

T cell development requires continuous homing of bone marrow–derived T cell progenitors via blood vessels at the corticomedullary junction (3). At this early stage, these cells lack the TCR coreceptors, CD4 and CD8, and are termed $CD4^-CD8^-$ double-negative (DN) cells. During their migration toward the subcapsular region, DN thymocytes can be further subclassified into distinct subsets based on expression of CD44 and CD25 (also known as IL2Rα chain) (4–6). DN1 ($CD44^+CD25^-$) cells include early T cell progenitors that express the receptor tyrosine kinase c-Kit (also known as CD117), which promotes their survival by interacting with its lig- and expressed by endothelial cells (7, 8). Early T cell progenitors develop into DN2a ($CD44^+CD25^+CD117^{hi}$) and then into DN2b ($CD44^+CD25^+CD117^{lo}$) cells. DN2b cells are more committed to the T cell lineage than DN2a cells, although they retain other lineage po- tentials (9). During DN2-to-DN3 ($CD44^-CD25^+$) transition, natural killer (NK) cell potential and DC potential are lost upon engagement of Notch-1 receptor by its ligand, Delta-like 4 (10, 11). Sequence-specific transcription factors involved in T cell lineage identity have been recently reviewed (12). At the DN3 stage, cells become fully committed to the αβ T cell lineage and ex- press a pre-TCR complex at the cell surface by association of the TCR β chain with an invariant pre–TCR α chain and CD3 molecules. Low-affinity interactions between the TCR β subunit of the pre-TCR and peptide-MHC complexes induce pre-TCR signaling that rescues cells from apoptosis, allowing their development into DN4 ($CD44^-CD25^-$) cells (13, 14). This first major checkpoint, called β-selection, enables amplification of cells with productive TCRβ rearrange- ments. These cells upregulate CD4 and CD8 coreceptors, leading to double-positive (DP) cells,

which progress through an immature $CD8^+CD4^-CD3^-$ intermediate single-positive (ISP) stage (15). DP thymocytes rearrange their TCR α chain to produce a cell surface αβ TCR and reside three to four days in the cortex.

DP thymocytes that received TCR signals upregulate CD4 and downregulate CD8 expression, becoming phenotypically $CD69^+CD4^{hi}CD8^{lo}$ intermediate thymocytes. Cells bearing αβ TCR that recognize with low to moderate affinity peptides presented by MHC-I or MHC-II are positively selected as $CD8^+$ or $CD4^+$ single-positive (SP) thymocytes, respectively (16). This TCR-dependent process that promotes thymocyte survival is termed positive selection (**Figure 1**).

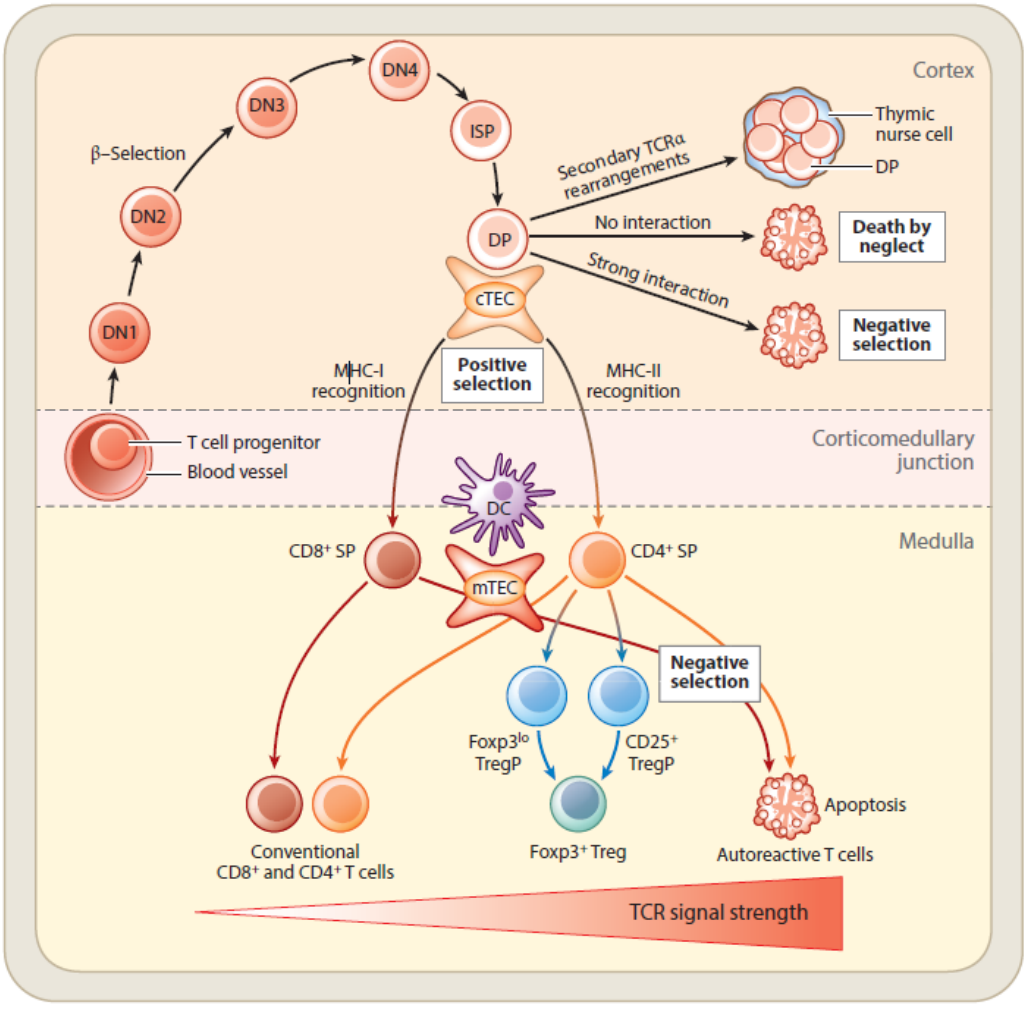


Figure 1

T cell selection in the thymus. T cell progenitors enter the thymus via blood vessels localized at the corticomedullary junction. The development of DN thymocytes into discrete subsets (DN1–DN4) occurs during their migration toward the subcapsular region. DP cells generated in the outer cortex, through an immature ISP stage, migrate back into the inner cortex. DP thymocytes can be engulfed by thymic nurse cells, supporting secondary TCRα rearrangements. DP thymocytes that have successfully rearranged their TCRs scan cTECs for positively selecting ligands. DP thymocytes not recognizing peptide-MHC complexes with sufficient affinity die by neglect, whereas those that strongly recognize these complexes die by negative selection. Only T cell clones recognizing peptide-MHC complexes with adequate affinity continue their development. Recognition of peptide-MHC-I and peptide-MHC-II complexes drives the differentiation of DP thymocytes into $CD8^+$ and $CD4^+$ SP thymocytes, respectively. After positive selection, $CD8^+$ and $CD4^+$ SP thymocytes migrate into the medulla. They scan via their TCRs the presence of self-peptides mainly presented by mTECs and DCs. The strength of TCR interactions dictates T cell fate. Weak interactions promote their survival, whereas strong interactions cause negative selection by apoptosis. Intermediate to high interactions divert $CD4^+$ SP thymocytes to the Treg lineage. Strong TCR signals generate $CD25^+$ Treg precursors, whereas weaker TCR signals generate Foxp3lo Treg precursors. Abbreviations: cTEC, cortical thymic epithelial cell; DC, dendritic cell; DN, double negative; DP, double positive; ISP, intermediate single positive; mTEC, medullary thymic epithelial cell; SP, single positive; TCR, T cell receptor; Treg, regulatory T cell; TregP, Treg precursor.

However, the majority of DP thymocytes (~85–90%) express TCRs that do not recognize peptide-MHC complexes and die by neglect in absence of TCR-mediated survival signals (17). In line with this, a study based on a high-throughput, unbiased clonal screen of random αβ TCR pairs has found that 85% of TCRs fail to induce selection. Only 15% of random TCRs induce signaling and lead to positive (7.5%) or negative (7.5%) selection (18). The development of $CD8^+$ and $CD4^+$ SP thymocytes takes two to four and one to two days, respectively (19). Unlike peripheral T cells, DP thymocytes respond efficiently to low TCR signals inducing the expression of the antiapoptotic molecule Bcl-2 (20–23). TCR signaling leads to a gradual increase in intra- cellular calcium, activating the calcium-dependent phosphatase calcineurin and the downstream transcription factor NFAT (nuclear factor of activated T cells), both required for positive selection (24–26). Transient calcium elevations are accompanied by migratory pauses during the first 24 h of positive selection, progressively becoming briefer (25, 27). The comparison of thymocytes bearing MHC-I-restricted TCRs with distinct self-reactivities using two-photon time-lapse microscopy showed that the positive selection of thymocytes exhibiting low self-reactivity occurs with transient calcium fluxes and brief migratory pauses (28). Positive selection can take over a week for $CD8^+$ thymocytes with low self-reactivity. Nevertheless, whether the positive selection of $CD4^+$ thymocytes with low self-reactivity is similar to that of $CD8^+$ thymocytes requires further investigation. Interestingly, the subunit CNOT3 of the CCR4-NOT deadenylase complex, implicated in poly(A) tail shortening, protects thymocytes from apoptosis during positive selection by inhibiting upregulation of the proapoptotic gene *Puma* (29). This indicates that posttranscriptional regulation likely influences thymocyte survival during positive selection. Although Erk-associated phosphorylation is essential for positive selection (30), dephosphorylation changes triggered by TCR activation regulate thymocyte survival (31). Indeed, T cell–specific deletion of protein phosphatase 2A (PP2A) deregulates dephosphorylation of apoptosis-related proteins in DP thymocytes, resulting in abnormal apoptosis that could be corrected by Bcl-2 transgene expression.

Thymic Negative Selection and TCR Signal Strength

A substantial proportion of DP thymocytes bearing TCRs specific for ubiquitous self-antigens die by negative selection in the cortex (32, 33) (**Figure 1**). Thymocyte apoptosis is difficult to visualize and quantify, because dying cells are rapidly engulfed by macrophages (17). Thymocyte deletion is mediated by the mitochondrial apoptotic pathway and requires the BH3-only protein family member Bim (34, 35). Using mice lacking *Bim* and expressing a *Nur77*-GFP (green fluorescent protein) transgene, which reflects the TCR signal strength perceived, Stritesky et al. (36) estimated that six times more cells undergo negative selection than positive selection. Furthermore, 75% of thymocytes were found to be deleted at the DP stage whereas only 25% were eliminated at the SP stage. The Ikaros family transcription factor Helios was found to discriminate $CD4^+$ thymocytes undergoing positive and negative selection (33). Using Helios, researchers identified two major waves of Bim-mediated negative selection in 55% of cortical $CCR7^-CD24^+CD4^{lo}CD8^{lo}$ DP thymocytes and in 20% of medullary $CCR7^+CD4^+$ SP thymocytes. These estimations indicate that the proportion of DP thymocytes that die by negative selection in the cortex is much higher than previously thought. The p53-upregulated modulator of apoptosis, Puma, also cooperates with Bim to delete autoreactive thymocytes (37). Indeed, mice deficient in both *Bim* and *Puma* have a higher accumulation of mature SP thymocytes and a broader autoimmunity than *Bim*-deficient mice. These observations provide compelling evidence that the apoptosis of autoreactive thymocytes prevents organ-specific autoimmunity. Since the estimation of the proportion of cortical and medullary apoptotic thymocytes was based exclusively on Bim expression (33, 36) and Puma cooperates with Bim to induce apoptosis, these proportions might be even higher.

TCR-mediated positive selection triggers CCR7 upregulation, driving SP thymocyte migration into the medulla (38, 39). At this stage, SP thymocytes, expressing the activation marker CD69 and the heat-stable antigen CD24, are defined as semimature. These cells are further screened for self-reactivities by being exposed to organ-specific antigens, called tissue-restricted self-antigens (TRAs). Mice with defective medulla development and function display signs of autoimmunity; thus, exposure to TRAs is necessary to achieve complete tolerance (40). Contrary to mature $CD69^{-}CD24^{-}$ SP thymocytes that proliferate upon TCR stimulation, semimature $CD69^{+}CD24^{+}$ SP thymocytes are susceptible to apoptosis upon high-affinity recognition of peptide-MHC ligands. TCR stimulation thus leads to a different outcome depending on the maturation state of SP thymocytes (41).

Since thymocytes with specificity to any single self- peptide-MHC complex are rare, their identification remains difficult. Most studies have used TCR transgenic mice also expressing the cognate antigen, either naturally or transgenically. These models revealed the importance of TCR reactivity in thymic selection and clonal deletion efficiency. For instance, a seminal study showed that transgenic thymocytes for the male H-Y antigen, presented by MHC-I molecules, are massively deleted in males but not females (42).

A subsequent study showed that the appropriate expression of the H-Y TCR α chain at the DP stage results in clonal deletion at the SP stage (43). Nevertheless, the abnormal high frequencies of monoclonal TCR-specific thymocytes in these transgenic models could fail to faithfully recapitulate T cell selection. Therefore, peptide-MHC tetramers were employed to quantify self-specific clones within an unmanipulated T cell repertoire (44). One-third of specific MHC-II-restricted thymocytes were detected in mice expressing a ubiquitous cognate antigen, in contrast to mice lacking the antigen, indicating that ∼70% of autoreactive cells are deleted. Another study, based on peptide-MHC-II tetramers, showed that the fate of $CD4^{+}$ thymocytes depends on the expression pattern of the self-antigen (45). $CD4^{+}$ thymocytes specific for easily accessed or highly expressed
antigens are massively deleted. In contrast, $CD4^{+}$ thymocytes specific for antigens with limited thymic expression are partially deleted whereas those specific for foreign antigens or self-antigens not encountered during their development are ignored. Intriguingly, $CD8^{+}$ thymocytes are more susceptible to apoptosis than $CD4^{+}$ thymocytes, providing an explanation for the CD8/CD4 imbalance (46).

Negative selection may not be fully efficient, and autoreactive T cell clones can reach the periphery, even in healthy individuals. Indeed, immunization with some self-antigens induces autoimmunity, indicating that the peripheral T cell repertoire contains self-specificities. For example, human circulating $CD8^{+}$ T lymphocytes specific for β cell antigens expressed by medullary TECs (mTECs) were found in healthy donors and patients with type 1 diabetes alike (47, 48). Furthermore, the frequency of human $CD8^{+}$ T cells specific for the SMCY self-peptide de- rived from the Y chromosome (the H-Y equivalent) is only approximately threefold lower in men than in women. These observations indicate that around one-third of SMCY-specific T cells es-
cape clonal deletion in males (49). Similarly, a pioneering study using tetramers found that one- third of male-specific Smcy3 $CD8^{+}$ thymocytes escapes clonal deletion in male mice (50). Since the efficiency of clonal deletion depends on the affinity and avidity of the studied TCR for its cognate antigen, its degree of global efficiency is certainly difficult to estimate in a normal poly- clonal T cell repertoire in the absence of TCR sequencing. Furthermore, the incomplete representation of splice isoforms could account for clonal deletion escape. For example, the absence of expression of a splice variant of the myelin antigen proteolipid protein (PLP) induces the spontaneous development of experimental autoimmune encephalomyelitis in SJL TCR transgenic mice expressing a TCR derived from a pathogenic T cell clone (51, 52). Even if clonal deletion is in- complete, tolerance mechanisms likely rely on thymus-derived, antigen-specific $Foxp3^{+}$ Tregs, at least for some TRAs (53).

Foxp3$^+$ Regulatory T Cell Development and TCR Signal Strength

SP thymocytes bearing strongly autoreactive TCRs do not necessarily undergo clonal deletion. A fraction of them are redirected into the Foxp3$^+$ Treg lineage, a process called agonist selection (54, 55) (**Figure 1**). Strong TCR signals can also induce the differentiation of other lineages, such as invariant NK T cells (iNKTs) or CD8αα$^+$ intraepithelial lymphocytes (IELs), a concept reviewed elsewhere (56). Strong TCR signals driving Foxp3$^+$ Treg development were initially observed in TCR transgenic mice expressing the agonist ligand. Treg selection was observed when TS1 mice with a transgenic TCR recognizing an epitope of PR8 influenza virus hemagglutinin (HA) were crossed with transgenic mice expressing the PR8 HA as a neo-self-antigen (57, 58). Similar observations were made with other TCR transgenic systems such as DO11.10 TCR transgenic mice crossed with mice expressing ovalbumin (OVA) (59–61). Strikingly, thymic Tregs are absent when TCR transgenic mice are bred on a *Rag* (recombination activating gene)-deficient background (54), revealing the importance of self-antigen recognition in Treg selection. Accordingly, the TCR repertoire of Foxp3$^+$ Tregs exhibits limited overlap with nonregulatory T cells and resembles autoreactive T cells of *Foxp3*-deficient mice (62). Furthermore, Treg selection for one given TRA can be critical to prevent organ T cell infiltration. Indeed, altered Treg selection for a single prostate-specific antigen renders mice susceptible to prostate-specific T cell infiltration (63).

Several studies concluded that Treg selection is induced by an intermediate TCR avidity be- tween those that drive positive and negative selection (55). Transgenic mice expressing natural Treg-derived TCRs, backcrossed on a *Rag*-deficient background, develop very few Foxp3$^+$ Tregs (64, 65). Efficient Treg selection occurs only at very low clonal precursor frequencies in the presence of a polyclonal population, suggesting that intraclonal competition is required for Treg generation. However, numerous TCR transgenic mice backcrossed on a *Rag*-deficient background, such as Marilyn-*Rag2*$^{-/-}$, 3BBM74-*Rag2*$^{-/-}$, B3K508-*Rag1*$^{-/-}$, and OTII-*Rag2*$^{-/-}$, show defective medulla formation with altered mTEC development (as discussed below) (66, 67). Therefore, the poor development of Tregs in transgenic mice expressing natural Treg-derived TCRs could be due to a low representation of its cognate self-antigen in the medulla and/or weak production of IL-2 in the absence of self-reactive CD4$^+$ thymocytes (68). Furthermore, mixed bone marrow chimeras and the intrathymic transfer of TCR transgenic Tregs into wild-type recipients lead to Foxp3$^+$ Treg numbers that reach a plateau, indicating that clonal Treg selection involves a saturable niche, probably due to restricted availability of the cognate antigen, even in a normal medullary microenvironment.

Treg selection relies on a two-step model (69, 70). The first step is driven by strong TCR/ CD28 stimulation that induces CD25 expression, thereby generating CD25$^+$Foxp3$^-$ cells, called CD25$^+$ Treg precursors. The second step depends on γ-chain cytokines, mainly IL-2 and IL-15, promoting Foxp3 upregulation and CD25$^+$Foxp3$^+$ Treg development. A second Treg precursor lacking CD25 expression and characterized by a low level of Foxp3, called Foxp3^{lo} precursor, was subsequently identified (71, 72). Intrathymic transfer of either CD25$^+$ or Foxp3^{lo} precursors leads to CD25$^+$Foxp3$^+$ Treg development (72, 73), indicating that Tregs arise from two developmental pathways. Whereas CD25$^+$ precursors appear at day 2 after birth, Foxp3^{lo} precursors are detectable only at day 4. Furthermore, the TCR repertoire of Foxp3^{lo} precursors shows little overlap with that of CD25$^+$ precursors and resembles that of mature CD25$^+$Foxp3$^+$ Tregs (73). Accordingly, Foxp3^{lo} precursors also show a more mature CD69$^-$MHC-I$^+$ phenotype.

These observations indicate that the mature Treg pool is heterogeneous, with cells derived from both precursors. In accordance with this, two mature thymic Treg subsets defined as Triple^{lo} (GITR^{lo}PD-1^{lo}CD25^{lo}) and Triple^{hi} (GITR^{hi}PD-1^{hi}CD25^{hi}) show a distinct TCR repertoire (74). Triple^{hi} Tregs express TCRs with stronger self-reactivity than Triple^{lo} Tregs. Although it is tempting to speculate that Triple^{hi} and Triple^{lo} Tregs would be derived from CD25^{+} and Foxp3^{lo} precursors, respectively, their developmental relationship remains to be established. Interestingly, CD25^{+}Foxp3^{+} Tregs have distinct functional activities, with Tregs derived from Foxp3^{lo} precursors protecting from T cell–induced colitis and those derived from CD25^{+} precursors protecting from experimental autoimmune encephalomyelitis (73, 75). Similarly to Tregs derived from Foxp3^{lo} precursors, Triple^{lo} Tregs limit T cell–induced colitis, whereas Triple^{hi} Tregs control lymphoproliferation in lymph nodes (74). Considering the therapeutic potential of Tregs for a variety of autoimmune disorders in clinical trials, further investigations are required to decipher the mechanisms driving these two Treg developmental pathways (76, 77). Improving our understanding could help in generating Tregs from thymuses discarded during cardiac surgery (an interesting source of sufficient quantities of stable Tregs) that will protect against specific pathologies (78).

ANTIGEN-PRESENTING CELLS IN T CELL SELECTION

Functional Properties of Cortical TECs

Cortical TECs (cTECs) constitute a three-dimensional reticular network facilitating interactions with cortical thymocytes. They express epithelial cell adhesion molecule (EpCAM) and the surface markers Ly51 (encoded by *Enpep*) and CD205 (encoded by *Ly75*) but lack reac- tivity to the lectin *Ulex europaeus* agglutinin 1 (UEA-1). cTECs are thus commonly defined as EpCAM1^{+}Ly51^{+}CD205^{+}UEA-1^{-}. Given their paucity, they remain difficult to study and their diversity is probably underestimated. Nevertheless, they are heterogeneous based on their levels of MHC-II, CD40, and the Notch ligand Delta-like 4, which likely reflect their maturation state (**Table 1**). Recent single-cell transcriptomic analyses also identified two main cTEC subsets in humans: cTEC^{lo} and cTEC^{hi}, characterized by increasing expression levels of HLA class II genes, *PSMB11*, *PRSS16*, and *CCL25* (79). Signals provided by thymocytes beyond the DN1 stage drive cTEC maturation. This is illustrated by the transplantation of bone marrow cells from *Rag2*$^{-/-}$ mice, which show a block at the DN3 stage, into human CD3ϵ transgenic recipients (Tgϵ26 mice), in which T cell development is arrested at the DN1 stage (80, 81). Development of DN3 thymocytes in mice after transplantation restores the cortical organization and the differentiation of CD40^{+}MHC-II^{hi} cTECs.

cTECs control (*a*) T cell progenitor recruitment by expressing CXCL12 and CCL25 chemokines, (*b*) T cell lineage engagement by providing the Notch ligand Delta-like 4, and (*c*) immature thymocyte survival and proliferation by secreting IL-7 and stem cell factor (SCF) cytokines (10, 11, 82–84). Although DP thymocytes recognizing ubiquitous self-antigens die by negative selection in the cortex (32), cTECs are not likely implicated in this process. Transgenic mice expressing MHC-II molecules only in cTECs generate autoreactive CD4^{+} T cells, arguing against their implication in clonal deletion (85). Cortical clonal deletion is likely mediated by rare DCs that are in contact with dying autoreactive thymocytes (32). Importantly, cTECs

Table 1 cTECs, mTECs, and DCs involved in thymocyte selection: non-exhaustive list of markers classically used to identify distinct subsets of cTECs, mTECs, and DCs

Cell type	Phenotype	Cell type	Phenotype
 cTEC^{lo}	EpCAM^{+}UEA-1^{-}Ly51^{+}CD205^{+}MHC-II^{lo}CD40^{lo}DLL4^{lo}	 Post-Aire mTEC	EpCAM^{+}UEA-1^{+}Ly51^{-}MHC-II^{lo}CD80^{lo}Aire^{-}KRT10^{+}Involucrin^{+}
 cTEC^{hi}	EpCAM^{+}UEA-1^{-}Ly51^{+}CD205^{+}MHC-II^{hi}CD40^{hi}DLL4^{hi}	 Tuft-like mTEC	EpCAM^{+}UEA-1^{+}Ly51^{-}CCL21^{-}MHC-II^{lo}DCLK1^{+}Pou2f3^{+}
 CCL21^{+} mTEC^{lo}	EpCAM^{+}UEA-1^{+}Ly51^{-}MHC-II^{lo}CD80^{lo}CCL21^{+}	 cDC1	CD11c^{int}MHC-II^{+}CD8α^{hi}Sirpα^{-}CD11b^{-}XCR1^{+}
 Aire^{-} mTEC^{hi}	EpCAM^{+}UEA-1^{+}Ly51^{-}MHC-II^{hi}CD80^{hi}Aire^{-}	 cDC2	CD11c^{int}MHC-II^{+}CD8α^{lo}Sirpα^{+}CD11b^{+}XCR1^{-}
 Aire^{+} mTEC^{hi}	EpCAM^{+}UEA-1^{+}Ly51^{-}MHC-II^{hi}CD80^{hi}Aire^{+}	 pDC	CD11c^{int}MHC-II^{lo}PDCA1^{+}B220^{+}

Abbreviations: Aire, Autoimmune regulator; cTEC, cortical TEC; cDC, conventional dendritic cell; DLL4, Delta-like ligand 4; EpCAM, epithelial cell adhesion molecule; mTEC, medullary TEC; pDC, plasmacytoid dendritic cell; TEC, thymic epithelial cell; UEA-1, *Ulex europaeus* agglutinin 1.

support positive selection of thymocytes through their unique expression of protein degradation machineries (**Figure 2*a***). They express the thymoproteasome subunit β5t (encoded by *Psmb11*), which is required for MHC-I presentation of low-affinity self-peptides specialized for $CD8^+$ thymocyte positive selection (86, 87). *Psmb11*$^{-/-}$ mice show defective positive selection of $CD8^+$ thymocytes, indicating a key role for this catalytic subunit in the generation of a diverse repertoire of MHC-I-restricted T cells. Similarly, cTECs express the lysosomal endopeptidase cathepsin L (encoded by *Ctsl*) and the thymus-specific serine protease TSSP (encoded by *Prss16*), which contribute to the generation of MHC-II-restricted self-peptides specialized for $CD4^+$ T cell positive selection (88, 89). Mice deficient for *Ctsl* have a drastic reduction in $CD4^+$ thymocyte positive selection, whereas mice deficient for *Prss16* have impaired positive selection for some TCR specificities. Furthermore, cTECs show a high constitutive macroautophagy that contributes to MHC-II presentation of cytosolic proteins (90). By encompassing viable DP thymocytes, a fraction of cTECs generate multicellular complexes, called thymic nurse cells (91). They are believed to participate in T cell selection by creating a microenvironment favorable to secondary TCRα rearrangements of long-lived DP thymocytes (92).
Thus, cTECs play multiple roles in early T cell development and selection, including pro- genitor cell homing, T cell lineage commitment, thymocyte proliferation, and positive selection.

Nevertheless, it remains unknown whether a single cTEC can simultaneously promote all these functions or whether discrete cTEC subsets are specialized for specific functions. Further inves- tigations are required to elucidate cTEC functional heterogeneity.

Functional Properties of Medullary TECs

mTECs are more abundant than cTECs and are classically defined as EpCAM1+Ly51−UEA-1+. They were initially described as two subsets: MHC-IIloCD80lo (mTEClo) and MHC-IIhiCD80hi (mTEChi), with mTEChi derived from progenitors included in the mTEClo subset (93, 94). mTECs play a unique role in T cell selection through their exclusive ability to express self-antigens that mirror peripheral tissues. Reports of pioneering studies in the 1990s described the expression of TRA genes in the thymus, such as pancreatic and ocular genes (95–97). Later in 2001, mTECs were identified as the unique cells that express a broad range of TRAs (98). RNA-sequencing analyses revealed that mTEChi express up to 85% of the genome, i.e., more than 18,000 genes
(99). This promiscuous gene expression is activated in part by the transcription factors Aire (autoimmune regulator) and Fezf2 (Fez family zinc finger 2) transcription factors, controlling the expression of ∼3,000–4,000 and ∼600–700 genes, respectively (99–102). Nevertheless, the molecular regulators of about half of TRAs remain to be identified. A given TRA is expressed by only ∼1–3% of mTECs, implying that the full repertoire of TRAs is covered by ∼200– 500 mTECs at a given time point (103). The high motility of SP thymocytes during their 4- to 5-day residency in the medulla probably facilitates efficient scanning of TRAs (19).

mTECs not only constitute an antigen reservoir but also act as bona fide APCs. They can efficiently induce clonal deletion of CD8+ thymocytes and present endogenously expressed TRAs via MHC-II molecules using macroautophagy (104, 105) (Figure 2b). Diminished MHC-II expression on mTECs results in the escape of autoreactive CD4+ thymocytes from clonal deletion with enhanced Treg selection, demonstrating the autonomous contribution of mTECs in CD4+ T cell tolerance (106). An increased mTEC compartment leads to higher Treg numbers (107, 108). Conversely, a decreased mTEC compartment results in reduced Treg numbers (109, 110), implicating

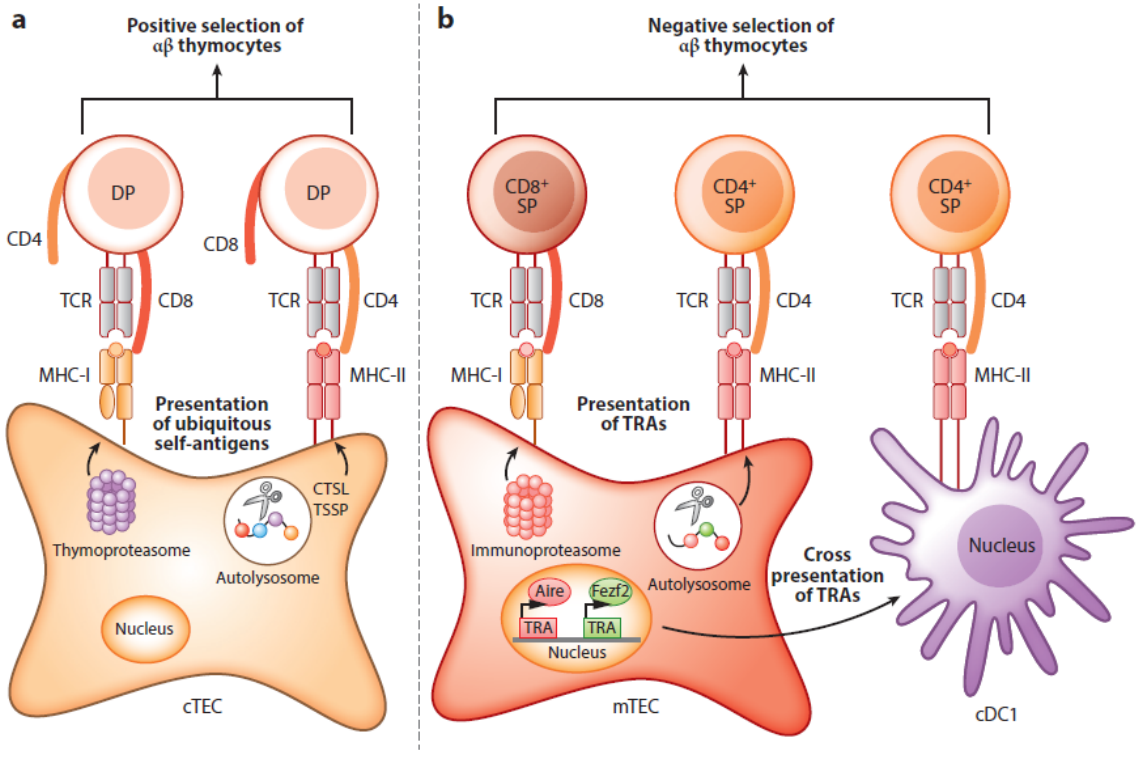


Figure 2

Functional properties of cTECs and mTECs in positive and negative selection of thymocytes. (*a*) cTECs induce the positive selection of DP thymocytes by expressing unique antigen-processing and -presentation machineries. They are equipped with a cortex-restricted thymoproteasome containing a β5t subunit that produces a unique set of MHC-I-associated peptides for the selection of $CD8^+$ SP thymocytes. By expressing CTSL and TSSP, they generate MHC-II-associated self-peptides required for the positive selection of $CD4^+$ SP thymocytes. Constitutive macroautophagy in cTECs favors the presentation of a broad spectrum of peptides via MHC-II molecules, enlarging the T cell repertoire of positively selected thymocytes. (*b*) By expressing TRAs, regulated by Aire and Fezf2, mTECs are crucial for the negative selection of $CD8^+$ SP and $CD4^+$ SP thymocytes. Macroautophagy favors the direct presentation of endogenous TRAs by mTECs, thus contributing to autoreactive thymocyte deletion. TRAs can also be transferred to and presented by cDC1s, which reinforces negative selection. Abbreviations: Aire, Autoimmune regulator; cDC1, type 1 conventional dendritic cell; cTEC, cortical thymic epithelial cell; CTSL, cathepsin L; DP, double positive; Fezf2, Fez family zinc finger 2; mTEC, medullary thymic epithelial cell; SP, single positive; TCR, T cell receptor; TRA, tissue-restricted self-antigen; TSSP, thymus specific serine protease.

mTECs in Treg selection. Furthermore, HA expression under the *Aire* promoter leads to HA- specific Treg induction, independently of antigen transfer to DCs, demonstrating that mTECs can autonomously induce antigen-specific Tregs (60). Accordingly, mTECs foster the generation of $CD25^+$ Treg precursors (111). Furthermore, the costimulatory signal CD70 provided by mTECs and DCs rescues Tregs from apoptosis (112). Lineage tracing and single-cell RNA sequencing recently uncovered that mTECs are highly heterogeneous (**Table 1**) (recently reviewed in 113, 114). In particular, $mTEC^{lo}$ include $mTEC^{hi}$ progenitors and a $CCL21^+$ subset, implicated in the medullary positioning of positively selected thymocytes. They also include post-Aire cells that have downregulated MHC-II expression and thymic tuft cells resembling gut chemosensory epithelial tuft cells. $mTEC^{hi}$ include two subsets, $Aire^-Fezf2^+$ and $Aire^+Fezf2^+$. Single-cell transcriptomic analyses revealed a largely conserved mTEC heterogeneity in humans (79, 115). Although mTECs are heterogeneous, the contribution of distinct subsets in T cell tolerance in- duction remains only partially understood.

Alike mTECs control T cell selection; conversely, thymocytes control mTEC differentiation, a process referred to as thymic cross talk (40, 116). $CD4^+$ thymocytes play a dominant role in this process, as illustrated by impaired differentiation of $Aire^+$ mTECs and an underdeveloped medulla in mice deficient in $CD4^+$ thymocytes (66, 67). By providing RANKL (encoded by *Tnfsf11*) and CD40L (encoded by *Cd40lg*), $CD4^+$ thymocytes activate RANK (encoded by *Tnfrsf11a*) and CD40 (encoded by *Cd40*) signaling, promoting mTEC differentiation (reviewed in 114, 117). RANKL and CD40L signals are delivered upon antigen-specific TCR/MHC-II interactions with mTECs. These findings are consistent with the fact that membrane-bound rather than soluble RANKL induces mTEC differentiation (118). Furthermore, autoreactive thymocytes remain viable for a while after encountering a negative-selection ligand, which would allow them to provide instructive signals to mTECs (119). Therefore, mTEC differentiation is probably cou- pled with T cell selection, even if further investigations would be worthwhile to determine the TCR signal threshold for this process.

Functional Properties of DCs

DCs contribute to T cell selection, since their constitutive ablation impairs clonal deletion of $CD4^+$ thymocytes and leads to spontaneous fatal autoimmunity (120). There are three main subsets of thymic DCs: two subsets of $CD11c^{hi}$ conventional DCs (cDCs) and $CD11c^{int}$ plasmacytoid DCs (pDCs) (40, 121) (**Table 1**). Approximately 70% of cDCs are resident cells of intrathymic origin (also called cDC1s; $CD11c^{hi}CD8\alpha^{hi}Sirp\alpha^-$), and ~30% are migratory cells of extrathymic origin (also called cDC2s; $CD11c^{hi}CD8\alpha^{lo}Sirp\alpha^+$). Three thymic DC counterparts have been identified in humans: DC1s ($XCR1^+CLEC9A^+CD11b^-$), DC2s ($SIRPA^+CLEC10A^+CD11b^+$), and pDCs ($IL3RA^+CLEC4C^+$) (115, 121). This heterogeneity suggests a distinct contribution of DC subsets in thymocyte selection.

cDC1s express the chemokine receptor XCR1, which contributes to their medullary localization in response to XCL1 produced by $Aire^+$ mTECs (122). They form a dense network with mTECs, which allows them to cross present TRAs (104, 123). This phenomenon contributes to the deletion of autoreactive $CD8^+$ and $CD4^+$ thymocytes (104, 124). Furthermore, the rapid turnover of mTECs (two to three weeks) is suspected to favor TRA cross presentation by cDCs (94, 125, 126). Several mechanisms of intercellular antigen transfer have been proposed, including membrane exchange, apoptotic body uptake, exosome transfer, and gap junctions (127). Interestingly, a recent study found that CD36, a scavenger receptor preferentially expressed by cDC1s, facilitates the transfer of cell surface antigens from mTECs to cDC1s (128). CD36-mediated cross presentation participates in TCR repertoire selection of conventional T cells and Tregs.

Nevertheless, other mechanisms remain to be identified, since CD36 is dispensable for the transfer of cytoplasmic antigens. Interestingly, the combined deletion of cDC1s (Batf3−/− mice) and mTECs (Traf61'TEC mice) leads to severe multiorgan autoimmunity, while mTEC deletion alone results in chronic liver inflammation (129). This indicates that cDC1s and mTECs cooperate to safeguard against autoimmunity. cDC1s and mTECs select distinct clones among Tregs and conventional T cells, and approximately half of Aire-dependent clonal deletion and Treg selection requires cDC1s (130). These findings indicate a division of labor between mTECs and cDC1s in shaping the TCR repertoire. Although mTECs express thousands of TRAs, they cannot cover the whole spectrum of peripheral self-antigens (99). However, cDC2s and pDCs can transport into the thymus inoffensive self-antigens captured in the periphery that otherwise would not be presented to thymocytes (40, 131–135). The notion that migratory DCs can delete autoreactive thymocytes is supported by the fact that the adoptive transfer of OVA-loaded DCs into OTII transgenic mice, expressing an MHC-II-restricted TCR specific for OVA, leads to the deletion of DP and SP thymocytes (131, 132, 136). This further reinforces the notion that cDCs are important mediators of cortical negative selection. cDC2s migrate to the thymus in a CCR2-dependent manner and are localized in the cortex and around blood vessels, allowing them to capture and present circulating antigens (134). Overall, cDC2s exhibit a more activated phenotype than cDC1s, characterized by high MHC- II, CD40, and CD80 expression (133). Furthermore, although cDC1s are particularly efficient in cross presenting self-antigens, cDC2s are also capable of capturing mTEC material (124, 137).
cDC2s participate in the negative selection of CD4+ thymocytes and Treg selection (133, 134, 138). However, their contribution in CD8+ T cell selection remains unclear.

pDCs continuously migrate via the bloodstream into the thymus, where they adopt a semimature phenotype by upregulating CD11c and MHC-II molecules (139). Their recruitment depends on the chemokine receptor CCR9 (132). However, CCR9 deficiency does not fully block their thymic entry, indicating that other chemokine receptors are involved in this process. In line with this, CCR2 is an appealing candidate since (a) pDCs express CCR2, (b) thymic CCL2 overexpression leads to massive pDC recruitment in the thymus, and (c) thymic pDCs are reduced in Ccr2−/− mice (140, 141). The recruitment of pDCs into the thymus likely relies on both CCR9 and CCR2, although the generation of Ccr9−/− × Ccr2−/− double-knockout mice would help to clarify this question. Similarly to their peripheral counterparts, thymic pDCs can function as bona fide APCs. Although pDCs participate in the deletion of autoreactive CD4+ thymocytes (132), their role in CD8+ T cell and Treg selection remains unclear. pDCs were recently found to be capable of mi- grating from the colon into the thymus in early life, suggesting that they might induce tolerance to commensal bacteria (142). In line with this hypothesis, TCR sequencing indicated that a substantial fraction of colon-associated Foxp3+ Tregs correspond to thymus-derived Tregs and that microbiota composition influences the repertoire of these cells (143).

Interestingly, whereas DCs control T cell selection, reciprocally, thymocytes regulate the functional properties of DCs. The maturation of cDC1s and cDC2s, characterized by increased MHC-II levels, is driven by autoreactive $CD4^+$ thymocytes via CD40-CD40L interactions (144). Nevertheless, it remains unknown whether SP thymocytes control pDC functional properties. Furthermore, antigen-specific interactions between mTECs and $CD4^+$ thymocytes regulate the recruitment of peripheral DCs into the thymus. Upon these interactions, lymphotoxin α (LTα), upregulated in $CD4^+$ thymocytes, negatively regulates the expression of CCR2 ligands, fine-tuning cDC2 and pDC entry and clonal deletion (66, 136). Consequently, $Lta^{-/-}$ mice show increased thymic recruitment of cDC2s and pDCs, resulting in enhanced clonal deletion of cortical and medullary thymocytes (136). Thus, interactions between mTECs and $CD4^+$ thymocytes regulate the pool of peripheral DCs in the thymus. The implication of RANKL, also involved in these interactions, in thymic DC biology remains to be determined. These findings illustrate the complex interplay between different cellular actors that

mediates efficient T cell selection.

SURFACE RECEPTORS DIRECTING THYMOCYTE SELECTION

Costimulatory Molecules

The CD28 costimulatory molecule and its two ligands, CD80 and CD86 (also known as B7-1 and B7-2), play a major role in thymocyte selection. *Cd28*$^{-/-}$ and *Cd80*$^{-/-}$ × *Cd86*$^{-/-}$ mice have impaired Treg development from the precursor stage (73, 145, 146). CD28 costimulation acts concomitantly with TCR stimulation to promote differentiation into the Treg lineage by inducing Foxp3 expression (147). As a costimulatory molecule, the CD28-CD80/CD86 axis likely favors Treg generation from CD4^{+} thymocytes bearing TCRs of low to intermediate affinity. Further- more, CD28 drives Treg generation by activating the NF-κB pathway, including the nonclassical member c-Rel, that critically promotes Treg precursor development (146, 148–151).

The role of the CD28-CD80/CD86 axis in clonal deletion has been debated (55). Pobezinsky et al. (152) reported that clonal deletion requires CD28-mediated costimulation at the CD4^{+}CD8^{lo} intermediate stage of thymocyte development. Moreover, in the absence of CD28, surviving thymocytes are diverted to anergic TCRαβ^{+} DN cells that then migrate to the intes- tine, where they become CD8αα^{+} IELs. Thus, CD28 costimulation is required for clonal dele- tion, and CD8αα^{+} IELs probably derive from autoreactive thymocytes that survive negative se- lection. A recent study revisited the role of CD28-CD80/CD86 costimulation in clonal deletion (153). *Cd80*$^{-/-}$ × *Cd86*$^{-/-}$ mice have increased numbers of CD4^{+} and CD8^{+} thymocytes, a phe- nomenon observed from the CD4^{+}CD8^{lo} intermediate stage. CD80/CD86 costimulation likely triggers clonal deletion rather than prolonging the survival of thymocytes undergoing deletion, since they remain decreased even upon Bcl-2 introduction in *Cd80*$^{-/-}$ × *Cd86*$^{-/-}$ mice. Peptide- MHC-II tetramers used to identify endogenous antigen-specific polyclonal CD4^{+} thymocytes showed that CD28-CD80/CD86 costimulation is important for clonal deletion and Treg selection of TRA-specific CD4^{+} thymocytes. However, it seems dispensable for the deletion of thymocytes specific for widely expressed antigens. Furthermore, CD28 costimulation was also shown to be essential to clonal deletion events in both the cortex and the medulla (154).

CD40-CD40L costimulation also participates in thymocyte selection. Contrary to CD28, largely expressed by thymocytes, CD40L is restricted to CD4^{+} thymocytes (67, 155). CD40- CD40L interactions, involving both DCs and mTECs, are required for normal numbers of thymic Tregs (144, 156, 157). Treg reduction in *Cd40*- or *Cd40lg*-deficient mice is likely due to a decreased homeostatic proliferation resulting from lower thymic IL-2. However, it cannot be excluded that thymic Treg reduction is due to reduced thymic B cells, as CD40$^{-/-}$ mice have few thymic B cells and B cell–deficient mice have reduced Tregs (158). In contrast to reports on CD28-CD80/CD86 costimulation, few studies have found a role for CD40-CD40L in clonal deletion. Early works indicated that CD40L is required for the deletion of thymocytes recognizing endogenous super- antigens (159). However, bone marrow chimera, containing both *Cd40lg*-expressing and -deficient thymocytes, indicated that CD40L likely acts in a non-cell-autonomous manner during negative selection (160). Accordingly, it was later shown that CD40-CD40L, together with CD28- CD80/CD86 control mTEC development (66, 67, 161, 162). CD40-CD40L interactions are also required for thymic DC maturation and a normal thymic B cell pool (163–165). Thus, CD40- CD40L and CD28-CD80/CD86 costimulatory axes control the functional properties of mTECs, DCs, and B cells, all implicated in tolerance induction. Studies focused on these costimulatory axes should consider the defects associated with medullary APCs in mice deficient for these receptors and ligands.

Tumor Necrosis Factor Receptors

TCR and CD28 stimulation induces the expression of tumor necrosis factor receptors (TNFRs) such as GITR (also known as Tnfrsf18), OX40 (also known as Tnfrsf4) and TNFR2 (also known as Tnfrsf1b) at higher levels in Treg precursors than in conventional $CD4^+$ thymocytes (166, 167). The expression level of these TNFRs correlates with TCR signal strength measured by Nur77. This upregulation depends on CD28 since *Cd28*-deficient Treg precursors lose GITR, OX40, and TNFR2 expression (166). Stimulation of Treg precursors with GITRL or OX40L promotes their conversion into $Foxp3^+CD25^+$ Tregs by increasing their responsiveness to IL-2. High expression of these TNFRs on Treg precursors thus likely confers a selective advantage in Treg differentiation. Conversely, GITRL and OX40L neutralization inhibits the generation of mature $Foxp3^+CD25^+$ Tregs. Furthermore, the single deletion of *Gitr* or *Ox40* leads to a modest reduction in mature Tregs, suggesting that these TNFRs collectively drive Treg development. Given the heterogeneity of mTECs and DCs (40, 113) (**Table 1**), it remains to be defined which subtype of medullary APCs expresses GITRL, OX40L, and TNF-α and whether these ligands are provided in a dedicated niche. It would be also interesting to investigate whether these ligands are delivered simultaneously or sequentially during their migration in the medulla and whether their delivery is coupled or not to antigen presentation.

CYTOKINES IN T CELL SELECTION IL-2 and IL-15

In addition to the TCR/CD28 signals triggered by the recognition of self-peptide-MHC com- plexes presented by thymic APCs, cytokines have emerged as important regulators of Treg se- lection (Figure 3a). They serve as molecular messengers between thymic APCs and developing thymocytes. In particular, common cytokine receptor gamma chain (γc)-dependent cytokines are critical for Treg development. These γc-dependent cytokines prevent the deletion of developing Tregs by providing survival signals that counterbalance the proapoptotic effects of Foxp3 (71, 168). Mice deficient for γc, which is shared by IL-2, -4, -7, -9, -15, and -21 receptors, fully lack Tregs

Thymic Foxp3+ Tregs express the IL-2Rβ, IL-4Rα, IL-7Rα, IL-15Rα, and IL-21Rα chains

The genetic deletion of specific receptor chains has revealed that IL-2 and, to a lesser degree, IL-15 play a dominant and nonredundant role in Treg development. Mice that are Il2−/−, Il15−/−, or Il2ra−/− display a modest reduction in Foxp3+ Tregs in their thymuses (169, 171, 172). By con- trast, mice deficient for both Il2 and Il15 or for Il2rb, which encodes a common chain for IL-2 and IL-15 receptors, have a profound reduction in thymic Foxp3+ Tregs (169, 171–173) (Figure 3b). In vitro stimulation of CD25+ Treg precursors with IL-2 or IL-15 induces their differentiation into Foxp3+CD25+ cells, although IL-2 is more efficient (69, 170). However, it cannot be excluded that IL-15 in vivo trans-presentation via high- affinity IL-15Rα expressed by mTECs enhances the efficiency of IL-15 to induce Foxp3 (Figure 3b). IL-2 stimulation of Foxp3lo Treg precursors induces their differentiation into Foxp3+CD25+ cells more efficiently than IL-2 stimulation of CD25+ precursors (71, 72). In humans, IL-2 and IL-15 also drive CD4+CD25+CD127+ Treg precursor differentiation into FOXP3+ cells and promote their proliferation and survival (174). Furthermore, in the absence of IL-15Rα but not IL-2, the generation of Foxp3lo Treg precursors is reduced (72). The stimulation of IL-2 and IL-15 receptors induces STAT5 signaling, which drives Treg development. Mice with a T cell–specific deletion of Stat5 show a severe defect in Tregs (171). Conversely, constitutive expression of active STAT5 rescues Treg development in

Il2rb-deficient mice. STAT5 directly regulates expression of Foxp3 by binding to its promoter and a distal enhancer called conserved noncoding sequence 0 (171, 175, 176).

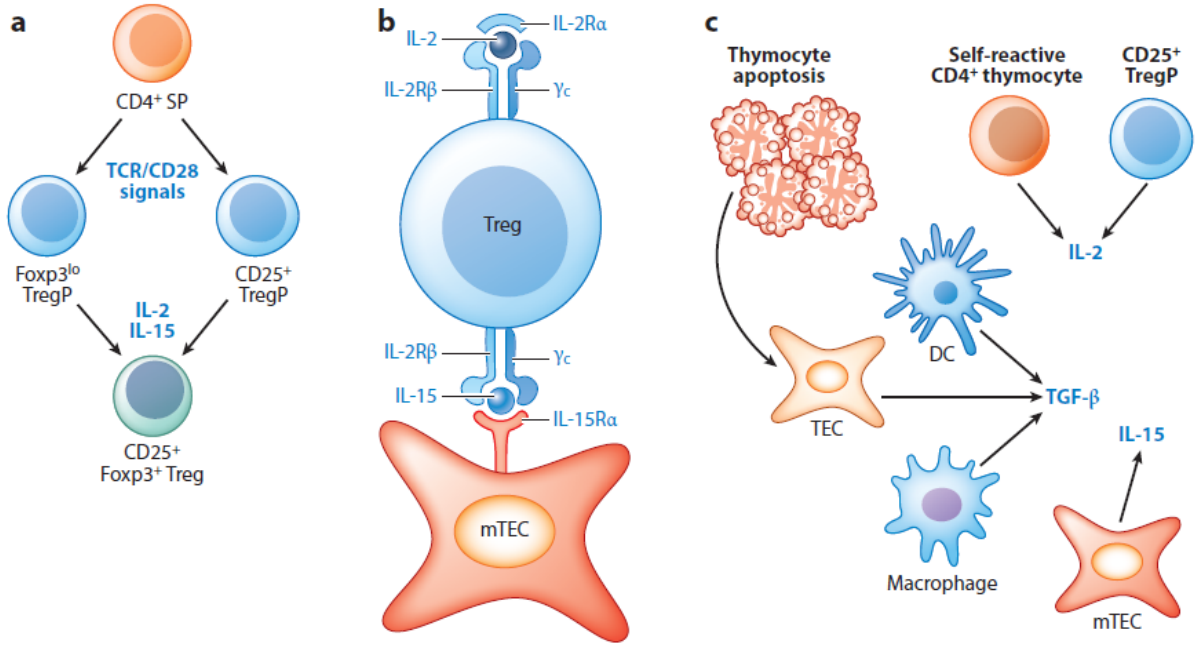


Figure 3

Cytokines involved in $CD25^+Foxp3^+$ Treg cell development. (*a*) TCR/CD28 signals drive generation of $Foxp3^{lo}$ and $CD25^+$ TregPs, while IL-2 and IL-15 cytokine signal integration promotes their development into mature $CD25^+Foxp3^+$ Tregs. (*b*) The heterotrimeric IL-2 and IL-15 receptors share two subunits: IL-2Rβ and the common cytokine receptor gamma chain (γ_c). They have distinct α subunits: IL-2Rα and IL-15Rα, respectively. IL-2 is a secreted cytokine binding the heterotrimeric IL-2 receptor on the Treg cell surface, whereas IL-15 is trans-presented by the membrane-bound IL-15Rα of mTECs. (*c*) Several cells secrete cytokines implicated in $CD25^+Foxp3^+$ Treg development. IL-2 is mainly provided by self-reactive $CD4^+$ thymocytes and also likely by $CD25^+$ TregPs, whereas IL-15 is mainly produced by mTECs. The production of TGF-β by DCs, TECs, and macrophages would be regulated by thymocyte apoptosis. Abbreviations: DC, dendritic cell; mTEC, medullary thymic epithelial cell; SP, single positive; TCR, T cell receptor; TEC, thymic epithelial cell; Treg, regulatory T cell; TregP, Treg precursor.

Recent efforts were made to identify the cellular sources of IL-2. DCs bearing the cognate self-antigen have been initially proposed to provide locally IL-2 (177). However, transgenic mice with a conditional deletion of the Il2 gene revealed that self-reactive CD4+ thymocytes, includ- ing Treg precursors, are the critical source of IL-2 for Treg development (68, 178) (Figure 3c). Since thymocyte apoptosis has been proposed to drive Treg generation (179), this raises the ques- tion of whether IL-2 production by self-reactive CD4+ thymocytes is coupled to their apoptosis. Moreover, it remains to be seen whether IL-2 is produced by recirculating T cells exhibiting an activated phenotype (180). Hematopoietic chimeras showed that in contrast to IL-2, IL-15 pro- vided by stromal cells supports the development of Foxp3lo precursors and CD25+Foxp3+ Tregs
(72). IL-15 reporter mice were used to show that mTECs are the main producers of IL-15 (181) (Figure 3c). Since mTECs express self-antigens involved in Treg selection, it remains unclear whether TCR and IL-15 receptor engagements occur simultaneously.

Transforming Growth Factor β

Similar to findings for Tgfb1−/− mice, T cell conditional deletion of Tgfbr1 or Tgfbr2 results in an early fatal inflammatory syndrome (182–184), demonstrating that TGF-β signaling crucially controls T cell tolerance. The level of active TGF-β1 increases during the neonatal period, concomitantly with medulla development, and TGF-β receptor II (TGF-βRII) is upregulated during the transition from DP to SP thymocytes. These observations suggest that TGF-β signaling might regulate T cell selection (179, 185).
Although it is widely accepted that TGF-β controls Foxp3 induction in naive CD4+CD25−
T cells in the periphery (186), its role in thymic Treg development has been debated. Initial analyses of mice with a T cell–specific deletion of Tgfbr2 (Cd4Cre × Tgfbr2fl/fl mice) suggested that TGF-β signaling is dispensable for Treg development in 12- to 16-day-old mice (182, 183). However, a subsequent study showed that mice deficient for Tgfbr1 specifically in T cells (LckproximalCre × Tgfbr1fl/fl mice) lack CD25+Foxp3+ Tregs at postnatal days 3–5, showing that TGF-β signaling in thymocytes controls the emergence of thymic Tregs (187). Notably, although at this early stage CD25+ Treg precursors are unaffected, both Foxp3+ Treg precursors and mature CD25+Foxp3+ Tregs are almost absent, which could be in line with a role in Foxp3 in- duction. The proportion of thymic CD25+Foxp3+ Tregs with an activated phenotype drastically increases when these mice develop a systemic inflammatory syndrome by three to four weeks of age. This increase is due to IL-2 overproduction and heightened responsiveness, since genetic ablation of Il2 in these mice leads to the complete absence of Foxp3+ Tregs. Thymic Tregs from Cd4Cre × Tgfbr2fl/fl mice highly express Bim, Bax, and Bak proapoptotic proteins and weakly express the antiapoptotic protein Bcl-2 (185). Remarkably, deletion of the proapoptotic gene Bim in these mice restores Foxp3+ Treg numbers in four-day-old thymuses, indicating that TGF-β protects Tregs from Bim-induced cell death.
Interestingly, the production of TGF-β by DCs, macrophages, and TECs is stimulated by thy- mocyte apoptosis (179) (Figure 3c). Thymocyte apoptosis enhanced by treatment with dexam- ethasone, gamma irradiation, or anti-CD3 antibody increases TGF-β production. Furthermore, gamma irradiation after birth increases thymocyte apoptosis, TGF-β levels, and Treg frequencies. Conversely, decreased thymocyte apoptosis in gamma-irradiated Bim−/− mice leads to reduced
TGF-β production and Treg frequencies. Since thymocyte apoptosis increases after birth (17), this finding likely explains the delay of Treg emergence by day 3 as compared to conventional thymo- cytes. Thus, the negative selection of autoreactive thymocytes seems to control Treg production

efficiency. Although further studies are required to clarify the role of TGF-β in Treg selection, it is likely that TGF-β signaling acts as both a differentiation and a survival factor for thymic Tregs. Few studies have attempted to investigate the role of TGF-β signaling in the negative selec- tion of thymocytes. To address this question, OTII transgenic mice were crossed with RIP-mOVA mice, in which the rat insulin promoter (RIP) drives the synthesis of a membrane-bound form of ovalbumin (mOVA), specifically in mTECs. The deletion of TCRβ^{hi} OTII thymocytes was en- hanced on a *Tgfbr2*$^{-/-}$ background in eight-day-old mice (185). This phenotype was accompanied by reduced survival of these cells, suggesting that TGF-β signaling protects thymocytes from negative selection. The identification of Helios as a marker of autoreactive conventional CD4^{+} thymocytes has helped in evaluating the proportion of thymocytes undergoing negative selection in an unskewed polyclonal repertoire (33). *Cd4*^{Cre} × *Tgfbr2*$^{fl/fl}$ mice show an increased proportion of Helios^{+} cortical and medullary CD4^{+} thymocytes at seven to ten days of age, a phenotype also observed in fetal thymic organ culture (188). A reduced proportion of these autoreactive CD4^{+} thymocytes express the proapoptotic factor Bim and the active caspase 3, indicating that TGF-β signaling controls the survival of highly autoreactive thymocytes. In line with defective negative selection, these cells preferentially accumulate at the corticomedullary junction and escape from negative selection in the medulla. Interestingly, TGF-β signaling upregulates RANKL expression in autoreactive CD4^{+} thymocytes, known to trigger Aire^{+} mTEC differentiation (114). TGF-β by itself is inefficient at inducing RANKL expression and requires synergy with TCR stimulation (189). Accordingly, RANKL levels in Helios^{+} autoreactive CD4^{+} thymocytes are reduced in *Cd4*^{Cre} × *Tgfbr2*$^{fl/fl}$ mice (188). Thus, highly autoreactive thymocytes also escape from negative selection by failing to provide RANKL signals to mTECs, resulting in altered Aire^{+} mTEC differentiation and self-antigen expression. Although TGF-β signaling is likely implicated in both Treg selection and the negative selection of CD4^{+} thymocytes, its involvement in the selection of CD8^{+} thymocytes remains to be established.

FUTURE ISSUES

Advances of the last decade have revealed a high heterogeneity in the stromal compartment, open- ing new exciting questions on T cell selection. The respective roles of the newly identified discrete subsets of APCs in clonal deletion and Treg selection remain to be defined. The identification of self-antigens presented and costimulatory molecules expressed by each subset and their cytokine environment may help in addressing this important question. In particular, to improve our under- standing of T cell selection, it would be interesting to determine whether these ligands are de- livered simultaneously or sequentially during their trafficking in the thymus. Although single-cell RNA sequencing identified new stromal cell subsets, their lineage relationships, tissue localization, and neighboring cells remain unknown. The recently developed revolutionary techniques that al- low visualization of the transcriptome within tissue are expected to shed new light on specialized stromal niches that dictate T cell fate. Furthermore, deciphering the developmental signals that drive the generation of CD25^{+} and Foxp3^{lo} Treg precursors is of importance for Treg therapy given that they harbor a distinct TCR repertoire. The ability of Tregs derived from these two precursors to treat and/or prevent distinct autoimmune and inflammatory disorders and their tis- sue regenerative potential remains to be established.

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LITERATURE CITED

1. Zarnitsyna VI, Evavold BD, Schoettle LN, Blattman JN, Antia R. 2013. Estimating the diversity, com- pleteness, and cross-reactivity of the T cell repertoire. *Front. Immunol.* 4:485
2. Nikolich-Žugich J, Slifka MK, Messaoudi I. 2004. The many important facets of T-cell repertoire diversity. *Nat. Rev. Immunol.* 4(2):123–32
3. Petrie HT, Zúñiga-Pflücker JC. 2007. Zoned out: functional mapping of stromal signaling microenvi- ronments in the thymus. *Annu. Rev. Immunol.* 25:649–79
4. Lind EF, Prockop SE, Porritt HE, Petrie HT. 2001. Mapping precursor movement through the postnatal thymus reveals specific microenvironments supporting defined stages of early lymphoid development.
   J. Exp. Med. 194(2):127–34
5. Porritt HE, Gordon K, Petrie HT. 2003. Kinetics of steady-state differentiation and mapping of intrathymic-signaling environments by stem cell transplantation in nonirradiated mice. *J. Exp. Med.* 198(6):957–62
6. Godfrey DI, Kennedy J, Suda T, Zlotnik A. 1993. A developmental pathway involving four phenotyp- ically and functionally distinct subsets of $CD3^{-}CD4^{-}CD8^{-}$ triple-negative adult mouse thymocytes defined by CD44 and CD25 expression. *J. Immunol.* 150(10):4244–52
7. Cumano A, Berthault C, Ramond C, Petit M, Golub R, et al. 2019. New molecular insights into immune cell development. *Annu. Rev. Immunol.* 37:497–519
8. Buono M, Facchini R, Matsuoka S, Thongjuea S, Waithe D, et al. 2016. A dynamic niche provides Kit ligand in a stage-specific manner to the earliest thymocyte progenitors. *Nat. Cell Biol.* 18(2):157–67
9. Yui MA, Feng N, Rothenberg EV. 2010. Fine-scale staging of T cell lineage commitment in adult mouse thymus. *J. Immunol.* 185(1):284–93
10. Hozumi K, Mailhos C, Negishi N, Hirano KI, Yahata T, et al. 2008. Delta-like 4 is indispensable in thymic environment specific for T cell development. *J. Exp. Med.* 205(11):2507–13
11. Koch U, Fiorini E, Benedito R, Besseyrias V, Schuster-Gossler K, et al. 2008. Delta-like 4 is the essential, nonredundant ligand for Notch1 during thymic T cell lineage commitment. *J. Exp. Med.* 205(11):2515– 23
12. Hosokawa H, Rothenberg EV. 2021. How transcription factors drive choice of the T cell fate. *Nat. Rev. Immunol.* 21:162–76

13. Kreslavsky T, Gleimer M, Miyazaki M, Choi Y, Gagnon E, et al. 2012. β-Selection-induced proliferation is required for αβ T cell differentiation. *Immunity* 37(5):840–53
14. Mallis RJ, Bai K, Arthanari H, Hussey RE, Handley M, et al. 2015. Pre-TCR ligand binding impacts thymocyte development before αβTCR expression. *PNAS* 112(27):8373–78
15. MacDonald HR, Budd RC, Howe RC. 1988. A $CD3^-$ subset of $CD4^-8^+$ thymocytes: a rapidly cycling
intermediate in the generation of $CD4^+8^+$ cells. *Eur. J. Immunol.* 18(4):519–24
16. Singer A, Adoro S, Park JH. 2008. Lineage fate and intense debate: myths, models and mechanisms of CD4- versus CD8-lineage choice. *Nat. Rev. Immunol.* 8:788–801
17. Surh CD, Sprent J. 1994. T-cell apoptosis detected in situ during positive and negative selection in the thymus. *Nature* 372(6501):100–3
18. McDonald BD, Bunker JJ, Erickson SA, Oh-Hora M, Bendelac A. 2015. Crossreactive αβ T cell receptors are the predominant targets of thymocyte negative selection. *Immunity* 43(5):859–69
19. thymocytes can occur in the cortex with no involvement of the medulla. J. Exp. Med. 205(11):2575–84
20. Daley SR, Hu DY, Goodnow CC. 2013. Helios marks strongly autoreactive CD4+ T cells in two major
21. waves of thymic deletion distinguished by induction of PD-1 or NF-κB. J. Exp. Med. 210(2):269–85
22. Daley SR, Teh C, Hu DY, Strasser A, Gray DHD. 2017. Cell death and thymic tolerance. Immunol. Rev.
23. 277:9–20
24. Bouillet P, Purton JF, Godfrey DI, Zhang LC, Coultas L, et al. 2002. BH3-only Bcl-2 family member Bim is required for apoptosis of autoreactive thymocytes. Nature 415(6874):922–26
25. Stritesky GL, Xing Y, Erickson JR, Kalekar LA, Wang X, et al. 2013. Murine thymic selection quantified using a unique method to capture deleted T cells. PNAS 110(12):4679–84
26. Gray DHD, Kupresanin F, Berzins SP, Herold MJ, O'Reilly LA, et al. 2012. The BH3-only proteins Bim and Puma cooperate to impose deletional tolerance of organ-specific antigens. Immunity 37(3):451–62
27. Kwan J, Killeen N. 2004. CCR7 directs the migration of thymocytes into the thymic medulla. J. Immunol. 172(7):3999–4007
28. Ueno T, Saito F, Gray DHD, Kuse S, Hieshima K, et al. 2004. CCR7 signals are essential for cortex-medulla migration of developing thymocytes. J. Exp. Med. 200(4):493–505
29. Lopes N, Sergé A, Ferrier P, Irla M. 2015. Thymic crosstalk coordinates medulla organization and T-cell tolerance induction. Front. Immunol. 6:365
30. Hogquist KA, Xing Y, Hsu F-C, Shapiro VS. 2015. T cell adolescence: maturation events beyond positive selection. J. Immunol. 195(4):1351–57
31. Kisielow P, Blüthmann H, Staerz UD, Steinmetz M, Von Boehmer H. 1988. Tolerance in T-cell-receptor transgenic mice involves deletion of nonmature CD4+8+ thymocytes. Nature 333(6175):742–46

32. Baldwin TA, Sandau MM, Jameson SC, Hogquist KA. 2005. The timing of TCRα expression critically influences T cell development and selection. *J. Exp. Med.* 202(1):111–21
33. Moon JJ, Dash P, Oguin TH, McClaren JL, Chu HH, et al. 2011. Quantitative impact of thymic selection on Foxp3$^+$ and Foxp3$^-$ subsets of self-peptide/MHC class II-specific CD4$^+$ T cells. *PNAS* 108(35):14602–7
34. Malhotra D, Linehan JL, Dileepan T, Lee YJ, Purtha WE, et al. 2016. Tolerance is established in polyclonal CD4$^+$ T cells by distinct mechanisms, according to self-peptide expression patterns. *Nat. Immunol.* 17(2):187–95
35. Sinclair C, Bains I, Yates AJ, Seddon B. 2013. Asymmetric thymocyte death underlies the CD4:CD8 T-cell ratio in the adaptive immune system. *PNAS* 110(31):E2905–14
36. Gonzalez-Duque S, Azoury ME, Colli ML, Afonso G, Turatsinze JV, et al. 2018. Conventional and neo- antigenic peptides presented by β cells are targeted by circulating naïve CD8$^+$ T cells in type 1 diabetic and healthy donors. *Cell Metab*. 28(6):946–60.e6
37. Culina S, Lalanne AI, Afonso G, Cerosaletti K, Pinto S, et al. 2018. Islet-reactive CD8$^+$ T cell
frequencies in the pancreas, but not in blood, distinguish type 1 diabetic patients from healthy donors.
Sci. Immunol. 3(20):4013
38. Yu W, Jiang N, Ebert PJR, Kidd BA, Müller S, et al. 2015. Clonal deletion prunes but does not eliminate self-specific αβ CD8$^+$ T lymphocytes. *Immunity* 42(5):929–41
39. Bouneaud C, Kourilsky P, Bousso P. 2000. Impact of negative selection on the T cell repertoire reactive to a self-peptide: A large fraction of T cell clones escapes clonal deletion. *Immunity* 13(6):829–40
40. Kuchroo VK, Anderson AC, Waldner H, Munder M, Bettelli E, Nicholson LB. 2002. T cell response in experimental autoimmune encephalomyelitis (EAE): role of self and cross-reactive antigens in shaping, tuning, and regulating the autopathogenic T cell repertoire. *Annu. Rev. Immunol.* 20:101–23
41. Klein L, Klugmann M, Nave KA, Tuohy VK, Kyewski B. 2000. Shaping of the autoreactive T-cell reper- toire by a splice variant of self protein expressed in thymic epithelial cells. *Nat. Med.* 6(1):56–61
42. Legoux FP, Lim JB, Cauley AW, Dikiy S, Ertelt J, et al. 2015. CD4$^+$ T cell tolerance to tissue-restricted
self antigens is mediated by antigen-specific regulatory T cells rather than deletion. *Immunity* 43(5):896– 908
43. Hsieh CS, Lee HM, Lio CWJ. 2012. Selection of regulatory T cells in the thymus. *Nat. Rev. Immunol.*
12:157–67
44. Klein L, Robey EA, Hsieh CS. 2019. Central CD4$^+$ T cell tolerance: deletion versus regulatory T cell differentiation. *Nat. Rev. Immunol.* 19:7–18
45. Hogquist KA, Jameson SC. 2014. The self-obsession of T cells: how TCR signaling thresholds affect fate "decisions" and effector function. *Nat. Immunol.* 15(9):815–23
46. Jordan MS, Boesteanu A, Reed AJ, Petrone AL, Holenbeck AE, et al. 2001. Thymic selection of CD4$^+$CD25$^+$ regulatory T cells induced by an agonist self-peptide. *Nat.*

Immunol. 2(4):301–6
47. Larkin J, Rankin AL, Picca CC, Riley MP, Jenks SA, et al. 2008. $CD4^+$ $CD25^+$ regulatory T cell
repertoire formation shaped by differential presentation of peptides from a self-antigen. *J. Immunol.*
180(4):2149–57
48. Walker LSK, Chodos A, Eggena M, Dooms H, Abbas AK. 2003. Antigen-dependent proliferation of $CD4^+$ $CD25^+$ regulatory T cells in vivo. *J. Exp. Med.* 198(2):249–58
49. Aschenbrenner K, D'Cruz LM, Vollmann EH, Hinterberger M, Emmerich J, et al. 2007. Selection of $Foxp3^+$ regulatory T cells specific for self antigen expressed and presented by $Aire^+$ medullary thymic epithelial cells. *Nat. Immunol.* 8(4):351–58
50. Kawahata K, Misaki Y, Yamauchi M, Tsunekawa S, Setoguchi K, et al. 2002. Generation of $CD4^+$ $CD25^+$
regulatory T cells from autoreactive T cells simultaneously with their negative selection in the thymus and from nonautoreactive T cells by endogenous TCR expression. *J. Immunol.* 168(9):4399–405
51. Hsieh CS, Zheng Y, Liang Y, Fontenot JD, Rudensky AY. 2006. An intersection between the self-reactive regulatory and nonregulatory T cell receptor repertoires. Nat. Immunol. 7(4):401–10
52. Klawon DEJ, Gilmore DC, Leonard JD, Miller CH, Chao JL, et al. 2021. Altered selection on a single self-ligand promotes susceptibility to organ-specific T cell infiltration. J. Exp. Med. 218(6):e20200701
53. Leung MWL, Shen S, Lafaille JJ. 2009. TCR-dependent differentiation of thymic Foxp3+ cells is limited
54. to small clonal sizes. J. Exp. Med. 206(10):2121–30
55. Bautista JL, Lio CWJ, Lathrop SK, Forbush K, Liang Y, et al. 2009. Intraclonal competition limits the fate determination of regulatory T cells in the thymus. Nat. Immunol. 10(6):610–17
56. Irla M, Guerri L, Guenot J, Sergé A, Lantz O, et al. 2012. Antigen recognition by autoreactive CD4+
57. thymocytes drives homeostasis of the thymic medulla. PLOS ONE 7(12):e52591
58. Irla M, Hugues S, Gill J, Nitta T, Hikosaka Y, et al. 2008. Autoantigen-specific interactions with CD4+ thymocytes control mature medullary thymic epithelial cell cellularity. Immunity 29(3):451–63
59. Hemmers S, Schizas M, Azizi E, Dikiy S, Zhong Y, et al. 2019. IL-2 production by self-reactive CD4 thymocytes scales regulatory T cell generation in the thymus. J. Exp. Med. 216(11):2466–78
60. Lio CWJ, Hsieh CS. 2008. A two-step process for thymic regulatory T cell development. Immunity
61. 28(1):100–11

62. Burchill MA, Yang J, Vang KB, Moon JJ, Chu HH, et al. 2008. Linked T cell receptor and cytokine signaling govern the development of the regulatory T cell repertoire. Immunity 28(1):112–21

63. Tai X, Erman B, Alag A, Mu J, Kimura M, et al. 2013. Foxp3 transcription factor is proapoptotic and lethal to developing regulatory T cells unless counterbalanced by cytokine survival signals. Immunity 38(6):1116–28

64. Marshall D, Sinclair C, Tung S, Seddon B. 2014. Differential requirement for IL-2 and IL-15 during bifurcated development of thymic regulatory T cells. J. Immunol. 193(11):5525–33

65. Owen DL, Mahmud SA, Sjaastad LE, Williams JB, Spanier JA, et al. 2019. Thymic regulatory T cells arise via two distinct developmental programs. Nat. Immunol. 20(2):195–205

66. Wyss L, Stadinski BD, King CG, Schallenberg S, McCarthy NI, et al. 2016. Affinity for self antigen selects Treg cells with distinct functional properties. Nat. Immunol. 17(9):1093–101

67. Santamaria JC, Borelli A, Irla M. 2021. Regulatory T cell heterogeneity in the thymus: impact on their functional activities. Front. Immunol. 12:643153

68. Raffin C, Vo LT, Bluestone JA. 2020. Treg cell-based therapies: challenges and perspectives. Nat. Rev. Immunol. 20:158–72

69. Ferreira LMR, Muller YD, Bluestone JA, Tang Q. 2019. Next-generation regulatory T cell therapy. Nat. Rev. Drug Discov. 18(10):749–69

70. Dijke IE, Hoeppli RE, Ellis T, Pearcey J, Huang Q, et al. 2016. Discarded human thymus is a novel source of stable and long-lived therapeutic regulatory T cells. Am. J. Transplant. 16(1):58–71

71. Bautista JL, Cramer NT, Miller CN, Chavez J, Berrios DI, et al. 2021. Single-cell transcriptional profil- ing of human thymic stroma uncovers novel cellular heterogeneity in the thymic medulla. Nat. Commun. 12(1):1096

72. Holländer GA, Wang B, Nichogiannopoulou A, Platenburg PP, Van Ewijk W, et al. 1995. Developmen- tal control point in induction of thymic cortex regulated by a subpopulation of prothymocytes. Nature 373(6512):350–53

73. Shakib S, Desanti GE, Jenkinson WE, Parnell SM, Jenkinson EJ, Anderson G. 2009. Checkpoints in the development of thymic cortical epithelial cells. J. Immunol. 182(1):130–37

74. Gossens K, Naus S, Corbel SY, Lin S, Rossi FMV, et al. 2009. Thymic progenitor homing and lymphocyte homeostasis are linked via S1P-controlled expression of thymic P-selectin/CCL25. J. Exp. Med. 206(4):761–78

75. Plotkin J, Prockop SE, Lepique A, Petrie HT. 2003. Critical role for CXCR4 signaling in progenitor localization and T cell differentiation in the postnatal thymus. J. Immunol. 171(9):4521–27

76. Alves NL, Richard-Le Goff O, Huntington ND, Sousa AP, Ribeiro VSG, et al. 2009. Characterization of the thymic IL-7 niche in vivo. PNAS 106(5):1512–17

77. Laufer TM, DeKoning J, Markowitz JS, Lo D, Glimcher LH. 1996. Unopposed positive selection and autoreactivity in mice expressing class II MHC only on thymic cortex. Nature

383(6595):81–85

78. Xing Y, Jameson SC, Hogquist KA. 2013. Thymoproteasome subunit-β5T generates peptide-MHC complexes specialized for positive selection. PNAS 110(17):6979–84
79. Sasaki K, Takada K, Ohte Y, Kondo H, Sorimachi H, et al. 2015. Thymoproteasomes produce unique peptide motifs for positive selection of CD8+ T cells. Nat. Commun. 6:7484
80. Honey K, Nakagawa T, Peters C, Rudensky A. 2002. Cathepsin L regulates CD4+ T cell selection
81. independently of its effect on invariant chain: a role in the generation of positively selecting peptide ligands. J. Exp. Med. 195(10):1349–58
82. Gommeaux J, Grégoire C, Nguessan P, Richelme M, Malissen M, et al. 2009. Thymus-specific serine protease regulates positive selection of a subset of $CD4^+$ thymocytes. *Eur. J. Immunol.* 39(4):956–64
83. Nedjic J, Aichinger M, Emmerich J, Mizushima N, Klein L. 2008. Autophagy in thymic epithelium shapes the T-cell repertoire and is essential for tolerance. *Nature* 455(7211):396–400
84. Wekerle H, Ketelsen UP, Ernst M. 1980. Thymic nurse cells: lymphoepithelial cell complexes in murine thymuses; morphological and serological characterization. *J. Exp. Med.* 151(4):925–44
85. Nakagawa Y, Ohigashi I, Nitta T, Sakata M, Tanaka K, et al. 2012. Thymic nurse cells provide microenvi- ronment for secondary T cell receptor α rearrangement in cortical thymocytes. *PNAS* 109(50):20572–77
86. Gray DHD, Seach N, Ueno T, Milton MK, Liston A, et al. 2006. Developmental kinetics, turnover, and stimulatory capacity of thymic epithelial cells. *Blood* 108(12):3777–85
87. Gäbler J, Arnold J, Kyewski B. 2007. Promiscuous gene expression and the developmental dynamics of medullary thymic epithelial cells. *Eur. J. Immunol.* 37(12):3363–72
88. Smith KM, Olson DC, Hirose R, Hanahan D. 1997. Pancreatic gene expression in rare cells of thymic medulla: evidence for functional contribution to T cell tolerance. *Int. Immunol.* 9(9):1355–65
89. Jolicoeur C, Hanahan D, Smith KM. 1994. T-cell tolerance toward a transgenic beta-cell antigen and transcription of endogenous pancreatic genes in thymus. *PNAS* 91(14):6707–11
90. Egwuagu CE, Charukamnoetkanok P, Gery I. 1997. Thymic expression of autoantigens correlates with resistance to autoimmune disease. *J. Immunol*. 159(7):3109–12
91. Derbinski J, Schulte A, Kyewski B, Klein L. 2001. Promiscuous gene expression in medullary thymic epithelial cells mirrors the peripheral self. *Nat. Immunol.* 2(11):1032–39
92. Sansom SN, Shikama-Dorn N, Zhanybekova S, Nusspaumer G, Macaulay IC, et al. 2014. Population and single-cell genomics reveal the Aire dependency, relief from Polycomb silencing, and distribution of self-antigen expression in thymic epithelia. *Genome Res*. 24(12):1918–31
93. Anderson MS, Venanzi ES, Klein L, Chen Z, Berzins SP, et al. 2002. Projection of an immunological self shadow within the thymus by the aire protein. *Science* 298(5597):1395–401

94. Takaba H, Morishita Y, Tomofuji Y, Danks L, Nitta T, et al. 2015. Fezf2 orchestrates a thymic program of self-antigen expression for immune tolerance. *Cell* 163(4):975–87
95. Tomofuji Y, Takaba H, Suzuki HI, Benlaribi R, Martinez CDP, et al. 2020. Chd4 choreographs self- antigen expression for central immune tolerance. *Nat. Immunol.* 21(8):892–901
96. Klein L, Kyewski B, Allen PM, Hogquist KA. 2014. Positive and negative selection of the T cell repertoire: what thymocytes see (and don't see). *Nat. Rev. Immunol.* 14:377–91
97. Gallegos AM, Bevan MJ. 2004. Central tolerance to tissue-specific antigens mediated by direct and indirect antigen presentation. *J. Exp. Med.* 200(8):1039–49
98. Aichinger M, Wu C, Nedjic J, Klein L. 2013. Macroautophagy substrates are loaded onto MHC class II of medullary thymic epithelial cells for central tolerance. *J. Exp. Med.* 210(2):287–300
99. Hinterberger M, Aichinger M, Da Costa OP, Voehringer D, Hoffmann R, Klein L. 2010. Autonomous role of medullary thymic epithelial cells in central $CD4^+$ T cell tolerance. *Nat. Immunol.* 11(6):512–19
100. Akiyama N, Shinzawa M, Miyauchi M, Yanai H, Tateishi R, et al. 2014. Limitation of immune tolerance- inducing thymic epithelial cell development by Spi-B-mediated negative feedback regulation. *J. Exp. Med.* 211(12):2425–38
101. Hauri-Hohl M, Zuklys S, Holländer GA, Ziegler SF. 2014. A regulatory role for TGF-β signaling in the establishment and function of the thymic medulla. Nat. Immunol. 15(6):554–61
102. Mouri Y, Nishijima H, Kawano H, Hirota F, Sakaguchi N, et al. 2014. NF-κB-inducing kinase in thymic stroma establishes central tolerance by orchestrating cross-talk with not only thymocytes but also dendritic cells. J. Immunol. 193(9):4356–67
103. Lin J, Yang L, Silva HM, Trzeciak A, Choi Y, et al. 2016. Increased generation of Foxp3+ regulatory
104. T cells by manipulating antigen presentation in the thymus. Nat. Commun. 7:10562
105. Cowan JE, Parnell SM, Nakamura K, Caamano JH, Lane PJL, et al. 2013. The thymic medulla is required for Foxp3+ regulatory but not conventional CD4+ thymocyte development. J. Exp. Med. 210(4):675–81
106. Coquet JM, Ribot JC, Bąbala N, Middendorp S, van der Horst G, et al. 2013. Epithelial and dendritic cells in the thymic medulla promote CD4+Foxp3+ regulatory T cell development via the CD27-CD70 pathway. J. Exp. Med. 210(4):715–28
107. Kadouri N, Nevo S, Goldfarb Y, Abramson J. 2020. Thymic epithelial cell heterogeneity: TEC by TEC.
108. Nat. Rev. Immunol. 20:239–53
109. Irla M. 2021. RANK signaling in the differentiation and regeneration of thymic epithelial cells. Front. Immunol. 11:623265
110. Park J-E, Botting RA, Domínguez Conde C, Popescu D-M, Lavaert M, et al. 2020. A cell atlas of human thymic development defines T cell repertoire formation. Science 367(6480):eaay3224
111. van Ewijk W, Shores EW, Singer A. 1994. Crosstalk in the mouse thymus. Immunol. Today 15(5):214–17

112. Irla M, Hollander G, Reith W. 2010. Control of central self-tolerance induction by autoreactive CD4+ thymocytes. Trends Immunol. 31(2):71–79
113. Asano T, Okamoto K, Nakai Y, Tsutsumi M, Muro R, et al. 2019. Soluble RANKL is physiologically dispensable but accelerates tumour metastasis to bone. Nat. Metab. 1:868–75
114. Le Borgne M, Ladi E, Dzhagalov I, Herzmark P, Liao YF, et al. 2009. The impact of negative selection on thymocyte migration in the medulla. Nat. Immunol. 10(8):823–30
115. Ohnmacht C, Pullner A, King SBS, Drexler I, Meier S, et al. 2009. Constitutive ablation of dendritic cells breaks self-tolerance of CD4 T cells and results in spontaneous fatal autoimmunity. J. Exp. Med. 206(3):549–59
116. Wu L, Shortman K. 2005. Heterogeneity of thymic dendritic cells. Semin. Immunol. 17(4):304–12
117. Lei Y, Ripen AM, Ishimaru N, Ohigashi I, Nagasawa T, et al. 2011. Aire-dependent production of XCL1 mediates medullary accumulation of thymic dendritic cells and contributes to regulatory T cell devel- opment. J. Exp. Med. 208(2):383–94
118. Koble C, Kyewski B. 2009. The thymic medulla: a unique microenvironment for intercellular self-antigen transfer. J. Exp. Med. 206(7):1505–13
119. Kroger CJ, Spidale NA, Wang B, Tisch R. 2017. Thymic dendritic cell subsets display distinct efficiencies and mechanisms of intercellular MHC transfer. J. Immunol. 198(1):249–56
120. Gray D, Abramson J, Benoist C, Mathis D. 2007. Proliferative arrest and rapid turnover of thymic epithelial cells expressing Aire. J. Exp. Med. 204(11):2521–28
121. Hubert FX, Kinkel SA, Davey GM, Phipson B, Mueller SN, et al. 2011. Aire regulates the transfer of antigen from mTECs to dendritic cells for induction of thymic tolerance. Blood 118(9):2462–72
122. Skogberg G, Lundberg V, Berglund M, Gudmundsdottir J, Telemo E, et al. 2015. Human thymic epithe- lial primary cells produce exosomes carrying tissue-restricted antigens. Immunol. Cell Biol. 93(8):727–34
123. Perry JSA, Russler-Germain EV, Zhou YW, Purtha W, Cooper ML, et al. 2018. CD36 mediates cell-surface antigens to promote thymic development of the regulatory T cell receptor repertoire and allo-tolerance. Immunity 48(5):923–36.e4
124. Herbin O, Bonito AJ, Jeong S, Weinstein EG, Rahman AH, et al. 2016. Medullary thymic epithelial cells and CD8α+ dendritic cells coordinately regulate central tolerance but CD8α+ cells are dispensable for thymic regulatory T cell production. J. Autoimmun. 75:141–49
125. Perry JSA, Lio CWJ, Kau AL, Nutsch K, Yang Z, et al. 2014. Distinct contributions of Aire and antigen- presenting-cell subsets to the generation of self-tolerance in the thymus. Immunity 41(3):414–26
126. Bonasio R, Scimone ML, Schaerli P, Grabie N, Lichtman AH, von Andrian UH. 2006. Clonal deletion of thymocytes by circulating dendritic cells homing to the thymus. Nat. Immunol. 7(10):1092–100
127. Hadeiba H, Lahl K, Edalati A, Oderup C, Habtezion A, et al. 2012. Plasmacytoid dendritic cells

transport peripheral antigens to the thymus to promote central tolerance. Immunity 36(3):438–50
128. Proietto AI, Van Dommelen S, Zhou P, Rizzitelli A, D'Amico A, et al. 2008. Dendritic cells in the thymus contribute to T-regulatory cell induction. PNAS 105(50):19869–74
129. Baba T, Nakamoto Y, Mukaida N. 2009. Crucial contribution of thymic Sirpα+ conventional
130. dendritic cells to central tolerance against blood-borne antigens in a CCR2-dependent manner.
131. J. Immunol. 183(5):3053–63
132. Atibalentja DF, Murphy KM, Unanue ER. 2011. Functional redundancy between thymic CD8α+ and Sirpα+ conventional dendritic cells in presentation of blood-derived lysozyme by MHC class II proteins.
133. J. Immunol. 186(3):1421–31
134. Lopes N, Charaix J, Cédile O, Sergé A, Irla M. 2018. Lymphotoxin α fine-tunes T cell clonal deletion by regulating thymic entry of antigen-presenting cells. Nat. Commun. 9(1):1262
135. Lancaster JN, Thyagarajan HM, Srinivasan J, Li Y, Hu Z, Ehrlich LIR. 2019. Live-cell imaging reveals the relative contributions of antigen-presenting cell subsets to thymic central tolerance. Nat. Commun. 10(1):2220
136. Atibalentja DF, Byersdorfer CA, Unanue ER. 2009. Thymus-blood protein interactions are highly effective in negative selection and regulatory T cell induction. J. Immunol. 183(12):7909–18
137. Li J, Park J, Foss D, Goldschneider I. 2009. Thymus-homing peripheral dendritic cells constitute two of the three major subsets of dendritic cells in the steady-state thymus. J. Exp. Med. 206(3):607–22
138. Cédile O, Løbner M, Toft-Hansen H, Frank I, Wlodarczyk A, et al. 2014. Thymic CCL2 influences induction of T-cell tolerance. J. Autoimmun. 55(1):73–85
139. Cédile O, Jørgensen LØ, Frank I, Wlodarczyk A, Owens T. 2017. The chemokine receptor CCR2 maintains plasmacytoid dendritic cell homeostasis. Immunol. Lett. 192:72–78
140. Ennamorati M, Vasudevan C, Clerkin K, Halvorsen S, Verma S, et al. 2020. Intestinal microbes influence development of thymic lymphocytes in early life. PNAS 117(5):2570–78
141. Cebula A, Seweryn M, Rempala GA, Pabla SS, McIndoe RA, et al. 2013. Thymus-derived regulatory T cells contribute to tolerance to commensal microbiota. Nature 497(7448):258–62
142. Oh J, Wu N, Barczak AJ, Barbeau R, Erle DJ, Shin J-S. 2018. CD40 mediates maturation of thymic dendritic cells driven by self-reactive CD4+ thymocytes and supports development of natural regulatory T cells. J. Immunol. 200(4):1399–412
143. Salomon B, Lenschow DJ, Rhee L, Ashourian N, Singh B, et al. 2000. B7/CD28 costimulation is essential for the homeostasis of the CD4+CD25+ immunoregulatory T cells that control autoimmune diabetes. Immunity 12(4):431–40
144. Vang KB, Yang J, Pagán AJ, Li L-X, Wang J, et al. 2010. Cutting edge: CD28 and c-Rel-dependent pathways initiate regulatory T cell development. J. Immunol. 184(8):4074–77

145. Tai X, Cowan M, Feigenbaum L, Singer A. 2005. CD28 costimulation of developing thymocytes induces Foxp3 expression and regulatory T cell differentiation independently of interleukin 2. Nat. Immunol. 6(2):152–62
146. Oh H, Grinberg-Bleyer Y, Liao W, Maloney D, Wang P, et al. 2017. An NF-κB transcription-factor- dependent lineage-specific transcriptional program promotes regulatory T cell identity and function. Immunity 47(3):450–65.e5
147. Schuster M, Plaza-Sirvent C, Visekruna A, Huehn J, Schmitz I. 2019. Generation of Foxp3+CD25−
148. regulatory T-cell precursors requires c-Rel and IκBNS. Front. Immunol. 10:1583
149. Fulford TS, Grumont R, Wirasinha RC, Ellis D, Barugahare A, et al. 2021. c-Rel employs multiple mechanisms to promote the thymic development and peripheral function of regulatory T cells in mice. Eur. J. Immunol. 51(8):2006–26
150. Isomura I, Palmer S, Grumont RJ, Bunting K, Hoyne G, et al. 2009. c-Rel is required for the development of thymic Foxp3+ CD4 regulatory T cells. J. Exp. Med. 206(13):3001–14
151. Pobezinsky LA, Angelov GS, Tai X, Jeurling S, Van Laethem F, et al. 2012. Clonal deletion and the fate of autoreactive thymocytes that survive negative selection. Nat. Immunol. 13(6):569–78
152. Watanabe M, Lu Y, Breen M, Hodes RJ. 2020. B7-CD28 co-stimulation modulates central tolerance via thymic clonal deletion and Treg generation through distinct mechanisms. Nat. Commun. 11(1):6264
153. Breed ER, Watanabe M, Hogquist KA. 2019. Measuring thymic clonal deletion at the population level.
154. J. Immunol. 202(11):3226–33
155. Hikosaka Y, Nitta T, Ohigashi I, Yano K, Ishimaru N, et al. 2008. The cytokine RANKL produced by positively selected thymocytes fosters medullary thymic epithelial cells that express autoimmune regulator. Immunity 29(3):438–50
156. Spence PJ, Green EA. 2008. Foxp3+ regulatory T cells promiscuously accept thymic signals critical for
157. their development. PNAS 105(3):973–78
158. Cuss SM, Green EA. 2012. Abrogation of CD40-CD154 signaling impedes the homeostasis of thymic resident regulatory T cells by altering the levels of IL-2, but does not affect regulatory T cell development. J. Immunol. 189(4):1717–25
159. Walters SN, Webster KE, Daley S, Grey ST. 2014. A role for intrathymic B cells in the generation of natural regulatory T cells. J. Immunol. 193(1):170–76
160. Foy TM, Page DM, Waldschmidt TJ, Schoneveld A, Laman JD, et al. 1995. An essential role for gp39, the ligand for CD40, in thymic selection. J. Exp. Med. 182(5):1377–88
161. Williams JA, Sharrow SO, Adams AJ, Hodes RJ. 2002. CD40 ligand functions non-cell autonomously to promote deletion of self-reactive thymocytes. J. Immunol. 168(6):2759–65
162. Irla M, Guenot J, Sealy G, Reith W, Imhof BA, Sergé A. 2013. Three-dimensional visualization of the mouse thymus organization in health and immunodeficiency. J. Immunol. 190(2):586–

163. Williams JA, Zhang J, Jeon H, Nitta T, Ohigashi I, et al. 2014. Thymic medullary epithelium and thy- mocyte self-tolerance require cooperation between CD28-CD80/86 and CD40-CD40L costimulatory pathways. J. Immunol. 192(2):630–40
164. Spidale NA, Wang B, Tisch R. 2014. Cutting edge: Antigen-specific thymocyte feedback regulates homeostatic thymic conventional dendritic cell maturation. J. Immunol. 193(1):21–25
165. Fujihara C, Williams JA, Watanabe M, Jeon H, Sharrow SO, Hodes RJ. 2014. T cell-B cell thymic cross-talk: Maintenance and function of thymic B cells requires cognate CD40-CD40 ligand interaction.
166. J. Immunol. 193(11):5534–44
167. Yamano T, Nedjic J, Hinterberger M, Steinert M, Koser S, et al. 2015. Thymic B cells are licensed to present self antigens for central T cell tolerance induction. Immunity 42(6):1048–61
168. Mahmud SA, Manlove LS, Schmitz HM, Xing Y, Wang Y, et al. 2014. Costimulation via the tumor-necrosis factor receptor superfamily couples TCR signal strength to the thymic differentiation of regulatory T cells. Nat. Immunol. 15(5):473–81
169. Klinger M, Kim JK, Chmura SA, Barczak A, Erle DJ, Killeen N. 2009. Thymic OX40 expression discriminates cells undergoing strong responses to selection ligands. J. Immunol. 182(8):4581–89
170. Hu DY, Wirasinha RC, Goodnow CC, Daley SR. 2017. IL-2 prevents deletion of developing T-regulatory cells in the thymus. Cell Death Differ. 24(6):1007–16
171. Fontenot JD, Rasmussen JP, Gavin MA, Rudensky AY. 2005. A function for interleukin 2 in Foxp3-expressing regulatory T cells. Nat. Immunol. 6(11):1142–51
172. Vang KB, Yang J, Mahmud SA, Burchill MA, Vegoe AL, Farrar MA. 2008. IL-2, -7, and -15, but not thymic stromal lymphopoeitin, redundantly govern CD4+ Foxp3+ regulatory T cell development.
173. J. Immunol. 181(5):3285–90
174. Burchill MA, Yang J, Vogtenhuber C, Blazar BR, Farrar MA. 2007. IL-2 receptor β-dependent STAT5 activation is required for the development of Foxp3+ regulatory T cells. J. Immunol. 178(1):280–90
175. Soper DM, Kasprowicz DJ, Ziegler SF. 2007. IL-2Rβ links IL-2R signaling with Foxp3 expression. Eur.
176. J. Immunol. 37(7):1817–26
177. Malek TR, Yu A, Vincek V, Scibelli P, Kong L. 2002. CD4 regulatory T cells prevent lethal autoimmu- nity in IL-2Rβ-deficient mice: implications for the nonredundant function of IL-2. Immunity 17(2):167– 78
178. Caramalho I, Nunes-Silva V, Pires AR, Mota C, Pinto AI, et al. 2015. Human regulatory T-cell de- velopment is dictated by interleukin-2 and -15 expressed in a non-overlapping pattern in the thymus.

179. J. Autoimmun. 56:98–110
180. Yao Z, Kanno Y, Kerenyi M, Stephens G, Durant L, et al. 2007. Nonredundant roles for Stat5a/b in directly regulating Foxp. Blood 109(10):4368–75
181. Dikiy S, Li J, Bai L, Jiang M, Janke L, et al. 2021. A distal Foxp3 enhancer enables interleukin-2 depen- dent thymic Treg cell lineage commitment for robust immune tolerance. Immunity 54(5):931–46.e11
182. Weist BM, Kurd N, Boussier J, Chan SW, Robey EA. 2015. Thymic regulatory T cell niche size is dictated by limiting IL-2 from antigen-bearing dendritic cells and feedback competition. Nat. Immunol. 16(6):635–41
183. Owen DL, Mahmud SA, Vang KB, Kelly RM, Blazar BR, et al. 2018. Identification of cellular sources of IL-2 needed for regulatory T cell development and homeostasis. J. Immunol. 200(12):3926–33
184. Konkel JE, Jin W, Abbatiello B, Grainger JR, Chen W. 2014. Thymocyte apoptosis drives the intrathymic generation of regulatory T cells. PNAS 111(4):E465–73
185. Thiault N, Darrigues J, Adoue V, Gros M, Binet B, et al. 2015. Peripheral regulatory T lymphocytes recirculating to the thymus suppress the development of their precursors. Nat. Immunol. 16(6):628–34
186. Cui G, Hara T, Simmons S, Wagatsuma K, Abe A, et al. 2014. Characterization of the IL-15 niche in primary and secondary lymphoid organs in vivo. PNAS 111(5):1915–20
187. Li MO, Sanjabi S, Flavell RAA. 2006. Transforming growth factor-β controls development, homeosta- sis, and tolerance of T cells by regulatory T cell-dependent and -independent mechanisms. Immunity 25(3):455–71
188. Marie JC, Liggitt D, Rudensky AY. 2006. Cellular mechanisms of fatal early-onset autoimmunity in mice with the T cell-specific targeting of transforming growth factor-β receptor. Immunity 25(3):441–54
189. Diebold RJ, Eis MJ, Yin M, Ormsby I, Boivini GP, et al. 1995. Early-onset multifocal inflammation in the transforming growth factor β1-null mouse is lymphocyte mediated. PNAS 92(26):12215–19
190. Ouyang W, Beckett O, Ma Q, Li MO. 2010. Transforming growth factor-β signaling curbs thymic negative selection promoting regulatory T cell development. Immunity 32(5):642–53
191. Chen WJ, Jin W, Hardegen N, Lei KJ, Li L, et al. 2003. Conversion of peripheral CD4+CD25− naive
192. T cells to CD4+CD25+ regulatory T cells by TGF-β induction of transcription factor Foxp3. J. Exp. Med. 198(12):1875–86
193. Liu Y, Zhang P, Li J, Kulkarni AB, Perruche S, Chen WJ. 2008. A critical function for TGF-β signaling in the development of natural CD4+CD25+Foxp3+ regulatory T cells. Nat. Immunol. 9(6):632–40
194. McCarron MJ, Irla M, Sergé A, Soudja SMH, Marie JC. 2019. Transforming growth factor-beta signaling in αβ thymocytes promotes negative selection. Nat. Commun. 10(1):5690
195. Wang R, Zhang L, Zhang X, Moreno J, Celluzzi C, et al. 2002. Regulation of activation-induced

receptor activator of NF-κB ligand (RANKL) expression in T cells. Eur. J. Immunol. 32(4):1090–98