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► **To cite this version:**

Magali Irla. Instructive Cues of Thymic T Cell Selection. Annual Review of Immunology, 2022, 40 (1), pp.95-119. 10.1146/annurev-immunol-101320-022432 . hal-03670661

**HAL Id: hal-03670661**

**<https://amu.hal.science/hal-03670661>**

Submitted on 10 Feb 2023

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# Instructive Cues of Thymic T Cell Selection

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## Keywords

thymus, clonal deletion, Foxp3<sup>+</sup> regulatory T cells, T cell receptor, cytokines, thymic antigen-presenting cells

## Abstract

A high diversity of  $\alpha\beta$  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<sup>+</sup> 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  $\alpha\beta$  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 foreign 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  $\beta$ -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

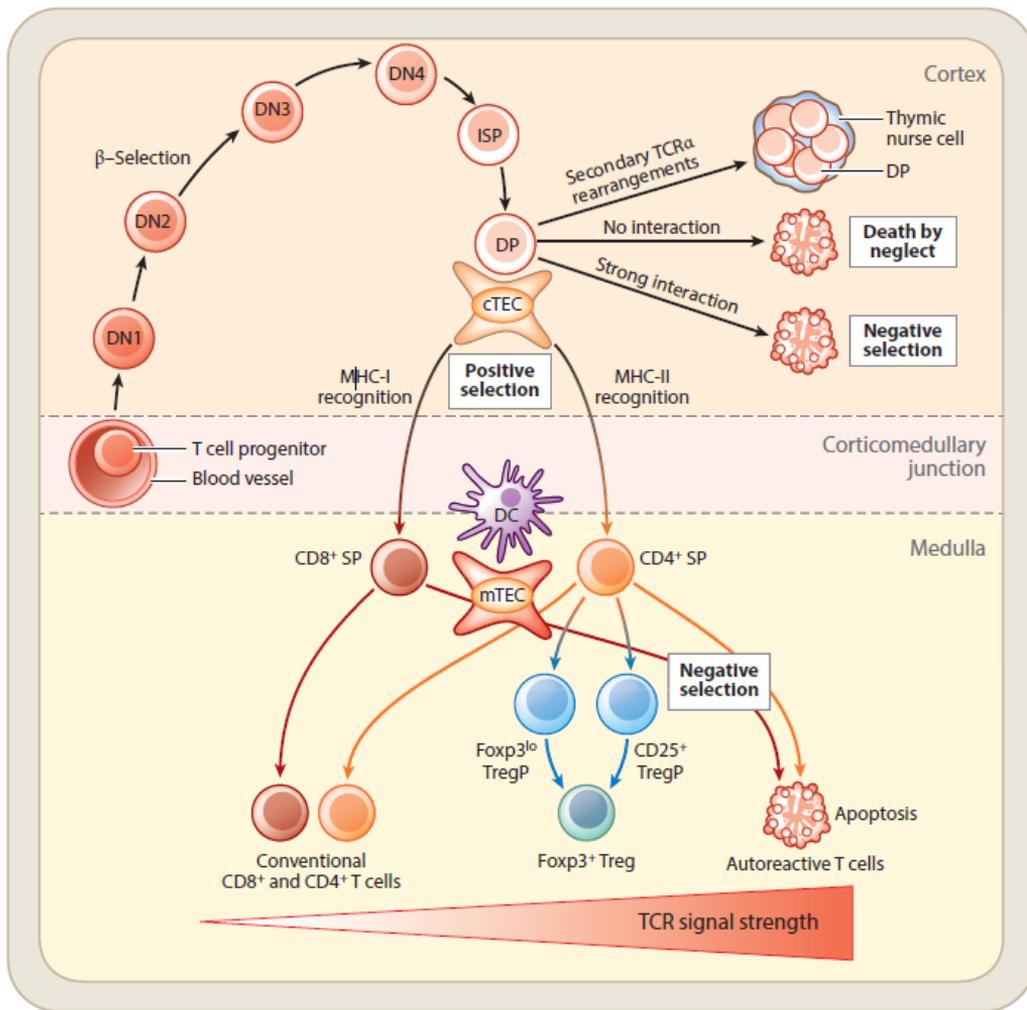
TCR signal, triggered by the recognition of peptide-MHC complexes and by the integration of costimulatory and cytokine signals provided by numerous thymic APCs. Only ~5% of developing thymocytes are selected, and  $\sim 1-4 \times 10^6$  mouse naive T cells are exported daily to the periphery. In this review, I summarize our latest knowledge on intrathymic  $\alpha\beta$  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<sup>-</sup>CD8<sup>-</sup> 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 IL2R $\alpha$  chain) (4–6). DN1 (CD44<sup>+</sup>CD25<sup>-</sup>) 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 ligand and expressed by endothelial cells (7, 8). Early T cell progenitors develop into DN2a (CD44<sup>+</sup>CD25<sup>+</sup>CD117<sup>hi</sup>) and then into DN2b (CD44<sup>+</sup>CD25<sup>+</sup>CD117<sup>lo</sup>) cells. DN2b cells are more committed to the T cell lineage than DN2a cells, although they retain other lineage potentials (9). During DN2-to-DN3 (CD44<sup>-</sup>CD25<sup>+</sup>) 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  $\alpha\beta$  T cell lineage and express a pre-TCR complex at the cell surface by association of the TCR  $\beta$  chain with an invariant pre-TCR  $\alpha$  chain and CD3 molecules. Low-affinity interactions between the TCR  $\beta$  subunit of the pre-TCR and peptide-MHC complexes induce pre-TCR signaling that rescues cells from apoptosis, allowing their development into DN4 (CD44<sup>-</sup>CD25<sup>-</sup>) cells (13, 14). This first major checkpoint, called  $\beta$ -selection, enables amplification of cells with productive TCR $\beta$  rearrangements. These cells upregulate CD4 and CD8 coreceptors, leading to double-positive (DP) cells, which progress through an immature CD8<sup>+</sup>CD4<sup>-</sup>CD3<sup>-</sup> intermediate single-positive (ISP) stage (15). DP thymocytes rearrange their TCR  $\alpha$  chain to produce a cell surface  $\alpha\beta$  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<sup>+</sup>CD4<sup>hi</sup>CD8<sup>lo</sup> intermediate thymocytes. Cells bearing  $\alpha\beta$  TCR that recognize with low to moderate affinity peptides presented by MHC-I or MHC-II are positively selected as CD8<sup>+</sup> or CD4<sup>+</sup> 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 TCR $\alpha$  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<sup>+</sup> and CD4<sup>+</sup> SP thymocytes, respectively. After positive selection, CD8<sup>+</sup> and CD4<sup>+</sup> 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<sup>+</sup> SP thymocytes to the Treg lineage. Strong TCR signals generate CD25<sup>+</sup> Treg precursors, whereas weaker TCR signals generate Foxp3<sup>lo</sup> 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  $\alpha\beta$  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<sup>+</sup> and CD4<sup>+</sup> 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<sup>+</sup> thymocytes with low self-reactivity. Nevertheless, whether the positive selection of CD4<sup>+</sup> thymocytes with low self-reactivity is similar to that of CD8<sup>+</sup> 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<sup>+</sup> thymocytes undergoing positive and negative selection (33). Using Helios, researchers identified two major waves of Bim-mediated negative selection in 55% of cortical CCR7<sup>-</sup>CD24<sup>+</sup>CD4<sup>lo</sup>CD8<sup>lo</sup> DP thymocytes and in 20% of medullary CCR7<sup>+</sup>CD4<sup>+</sup> 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<sup>-</sup>CD24<sup>-</sup> SP thymocytes that proliferate upon TCR stimulation, semimature CD69<sup>+</sup>CD24<sup>+</sup> 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  $\alpha$  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<sup>+</sup> thymocytes depends on the expression pattern of the self-antigen (45). CD4<sup>+</sup> thymocytes specific for easily accessed or highly expressed antigens are massively deleted. In contrast, CD4<sup>+</sup> 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<sup>+</sup> thymocytes are more susceptible to apoptosis than CD4<sup>+</sup> 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<sup>+</sup> T lymphocytes specific for  $\beta$  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<sup>+</sup> T cells specific for the SMCY self-peptide derived 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 escape clonal deletion in males (49). Similarly, a pioneering study using tetramers found that one-third of male-specific Smcy3 CD8<sup>+</sup> 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 polyclonal 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 incomplete, tolerance mechanisms likely rely on thymus-derived, antigen-specific Foxp3<sup>+</sup> Tregs, at least for some TRAs (53).

## Foxp3<sup>+</sup> 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<sup>+</sup> 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 CD8 $\alpha\alpha$ <sup>+</sup> intraepithelial lymphocytes (IELs), a concept reviewed elsewhere (56). Strong TCR signals driving Foxp3<sup>+</sup> 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<sup>+</sup> 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 between 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<sup>+</sup> 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*<sup>-/-</sup>, 3BBM74-*Rag2*<sup>-/-</sup>, B3K508-*Rag1*<sup>-/-</sup>, and OTII-*Rag2*<sup>-/-</sup>, 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<sup>+</sup> thymocytes (68). Furthermore, mixed bone marrow chimeras and the intrathymic transfer of TCR transgenic Tregs into wild-type recipients lead to Foxp3<sup>+</sup> 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<sup>+</sup>Foxp3<sup>-</sup> cells, called CD25<sup>+</sup> Treg precursors. The second step depends on  $\gamma$ -chain cytokines, mainly IL-2 and IL-15, promoting Foxp3 upregulation and CD25<sup>+</sup>Foxp3<sup>+</sup> Treg development. A second Treg precursor lacking CD25 expression and characterized by a low level of Foxp3, called Foxp3<sup>lo</sup> precursor, was subsequently identified (71, 72). Intrathymic transfer of either CD25<sup>+</sup> or Foxp3<sup>lo</sup> precursors leads to CD25<sup>+</sup>Foxp3<sup>+</sup> Treg development (72, 73), indicating that Tregs arise from two developmental pathways. Whereas CD25<sup>+</sup> precursors appear at day 2 after birth, Foxp3<sup>lo</sup> precursors are detectable only at day 4. Furthermore, the TCR repertoire of Foxp3<sup>lo</sup> precursors shows little overlap with that of CD25<sup>+</sup> precursors and resembles that of mature CD25<sup>+</sup>Foxp3<sup>+</sup> Tregs (73). Accordingly, Foxp3<sup>lo</sup> precursors also show a more mature CD69<sup>-</sup>MHC-I<sup>+</sup> 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<sup>lo</sup> (GITR<sup>lo</sup>PD-1<sup>lo</sup>CD25<sup>lo</sup>) and Triple<sup>hi</sup> (GITR<sup>hi</sup>PD-1<sup>hi</sup>CD25<sup>hi</sup>) show a distinct TCR repertoire (74). Triple<sup>hi</sup> Tregs express TCRs with stronger self-reactivity than Triple<sup>lo</sup> Tregs. Although it is tempting to speculate that Triple<sup>hi</sup> and Triple<sup>lo</sup> Tregs would be derived from CD25<sup>+</sup> and Foxp3<sup>lo</sup> precursors, respectively, their developmental relationship remains to be established. Interestingly, CD25<sup>+</sup>Foxp3<sup>+</sup> Tregs have distinct functional activities, with Tregs derived from Foxp3<sup>lo</sup> precursors protecting from T cell-induced colitis and those derived from CD25<sup>+</sup> precursors protecting from experimental autoimmune encephalomyelitis (73, 75). Similarly to Tregs derived from Foxp3<sup>lo</sup> precursors, Triple<sup>lo</sup> Tregs limit T cell-induced colitis, whereas Triple<sup>hi</sup> 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).

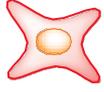
## ANTIGEN-PRESENTING CELLS IN T CELL SELECTION

### 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 reactivity to the lectin *Ulex europaeus* agglutinin 1 (UEA-1). cTECs are thus commonly defined as EpCAM1<sup>+</sup>Ly51<sup>+</sup>CD205<sup>+</sup>UEA-1<sup>-</sup>. 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<sup>lo</sup> and cTEC<sup>hi</sup>, 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*<sup>-/-</sup> 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<sup>+</sup>MHC-II<sup>hi</sup> 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<sup>+</sup> 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 <sup>lo</sup>	EpCAM <sup>+</sup> UEA-1 <sup>-</sup> Ly51 <sup>+</sup> CD205 <sup>+</sup> MHC-II <sup>lo</sup> CD40 <sup>lo</sup> DLL4 <sup>lo</sup>	 Post-Aire mTEC	EpCAM <sup>+</sup> UEA-1 <sup>+</sup> Ly51 <sup>-</sup> MHC-II <sup>lo</sup> CD80 <sup>lo</sup> Aire <sup>-</sup> KRT10 <sup>+</sup> Involucrin <sup>+</sup>
 cTEC <sup>hi</sup>	EpCAM <sup>+</sup> UEA-1 <sup>-</sup> Ly51 <sup>+</sup> CD205 <sup>+</sup> MHC-II <sup>hi</sup> CD40 <sup>hi</sup> DLL4 <sup>hi</sup>	 Tuft-like mTEC	EpCAM <sup>+</sup> UEA-1 <sup>+</sup> Ly51 <sup>-</sup> CCL21 <sup>-</sup> MHC-II <sup>lo</sup> DCLK1 <sup>+</sup> Pou2f3 <sup>+</sup>
 CCL21 <sup>+</sup> mTEC <sup>lo</sup>	EpCAM <sup>+</sup> UEA-1 <sup>+</sup> Ly51 <sup>-</sup> MHC-II <sup>lo</sup> CD80 <sup>lo</sup> CCL21 <sup>+</sup>	 cDC1	CD11c <sup>int</sup> MHC-II <sup>+</sup> CD8α <sup>hi</sup> Sirpα <sup>-</sup> CD11b <sup>-</sup> XCR1 <sup>+</sup>
 Aire <sup>-</sup> mTEC <sup>hi</sup>	EpCAM <sup>+</sup> UEA-1 <sup>+</sup> Ly51 <sup>-</sup> MHC-II <sup>hi</sup> CD80 <sup>hi</sup> Aire <sup>-</sup>	 cDC2	CD11c <sup>int</sup> MHC-II <sup>+</sup> CD8α <sup>lo</sup> Sirpα <sup>+</sup> CD11b <sup>+</sup> XCR1 <sup>-</sup>
 Aire <sup>+</sup> mTEC <sup>hi</sup>	EpCAM <sup>+</sup> UEA-1 <sup>+</sup> Ly51 <sup>-</sup> MHC-II <sup>hi</sup> CD80 <sup>hi</sup> Aire <sup>+</sup>	 pDC	CD11c <sup>int</sup> MHC-II <sup>lo</sup> PDCA1 <sup>+</sup> B220 <sup>+</sup>

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 2a**). They express the thymoproteasome subunit  $\beta 5t$  (encoded by *Psmb11*), which is required for MHC-I presentation of low-affinity self-peptides specialized for CD8<sup>+</sup> thymocyte positive selection (86, 87). *Psmb11*<sup>-/-</sup> mice show defective positive selection of CD8<sup>+</sup> 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<sup>+</sup> T cell positive selection (88, 89). Mice deficient for *Ctsl* have a drastic reduction in CD4<sup>+</sup> 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 TCR $\alpha$  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.

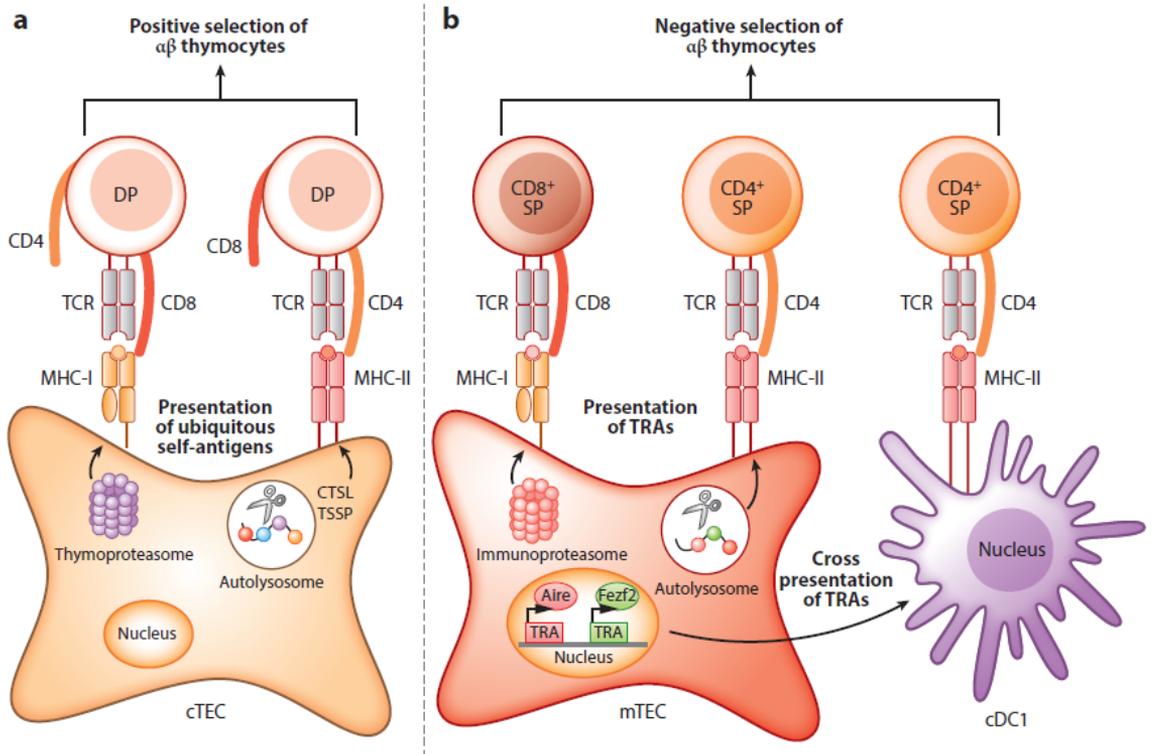
Nevertheless, it remains unknown whether a single cTEC can simultaneously promote all these functions or whether discrete cTEC subsets are specialized for specific functions. Further investigations are required to elucidate cTEC functional heterogeneity.

### 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-III<sup>lo</sup>CD80<sup>lo</sup> (mTEC<sup>lo</sup>) and MHC-III<sup>hi</sup>CD80<sup>hi</sup> (mTEC<sup>hi</sup>), with mTEC<sup>hi</sup> derived from progenitors included in the mTEC<sup>lo</sup> 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 mTEC<sup>hi</sup> 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<sup>+</sup> 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<sup>+</sup> thymocytes from clonal deletion with enhanced Treg selection, demonstrating the autonomous contribution of mTECs in CD4<sup>+</sup> 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  $\beta 5t$  subunit that produces a unique set of MHC-I-associated peptides for the selection of CD8<sup>+</sup> SP thymocytes. By expressing CTSL and TSSP, they generate MHC-II-associated self-peptides required for the positive selection of CD4<sup>+</sup> 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<sup>+</sup> SP and CD4<sup>+</sup> 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<sup>+</sup> 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<sup>lo</sup> include mTEC<sup>hi</sup> progenitors and a CCL21<sup>+</sup> 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<sup>hi</sup> include two subsets, Aire<sup>-</sup>Fezf2<sup>+</sup> and Aire<sup>+</sup>Fezf2<sup>+</sup>. 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 induction 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<sup>+</sup> thymocytes play a dominant role in this process, as illustrated by impaired differentiation of Aire<sup>+</sup> mTECs and an underdeveloped medulla in mice deficient in CD4<sup>+</sup> thymocytes (66, 67). By providing RANKL (encoded by *Tnfsf11*) and CD40L (encoded by *Cd40lg*), CD4<sup>+</sup> 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 coupled 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<sup>+</sup> thymocytes and leads to spontaneous fatal autoimmunity (120). There are three main subsets of thymic DCs: two subsets of CD11c<sup>hi</sup> conventional DCs (cDCs) and CD11c<sup>int</sup> plasmacytoid DCs (pDCs) (40, 121) (**Table 1**). Approximately 70% of cDCs are resident cells of intrathymic origin (also called cDC1s; CD11c<sup>hi</sup>CD8 $\alpha$ <sup>hi</sup>Sirp $\alpha$ <sup>-</sup>), and ~30% are migratory cells of extrathymic origin (also called cDC2s; CD11c<sup>hi</sup>CD8 $\alpha$ <sup>lo</sup>Sirp $\alpha$ <sup>+</sup>). Three thymic DC counterparts have been identified in humans: DC1s (XCR1<sup>+</sup>CLEC9A<sup>+</sup>CD11b<sup>-</sup>), DC2s (SIRPA<sup>+</sup>CLEC10A<sup>+</sup>CD11b<sup>+</sup>), and pDCs (IL3RA<sup>+</sup>CLEC4C<sup>+</sup>) (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<sup>+</sup> 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<sup>+</sup> and CD4<sup>+</sup> 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*<sup>-/-</sup> mice) and mTECs (*Traf6*<sup>1</sup>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<sup>+</sup> thymocytes and Treg selection (133, 134, 138). However, their contribution in CD8<sup>+</sup> 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*<sup>-/-</sup> mice (140, 141). The recruitment of pDCs into the thymus likely relies on both CCR9 and CCR2, although the generation of *Ccr9*<sup>-/-</sup> × *Ccr2*<sup>-/-</sup> 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<sup>+</sup> thymocytes (132), their role in CD8<sup>+</sup> T cell and Treg selection remains unclear. pDCs were recently found to be capable of migrating 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<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup> thymocytes regulate the recruitment of peripheral DCs into the thymus. Upon these interactions, lymphotoxin  $\alpha$  (LT $\alpha$ ), upregulated in CD4<sup>+</sup> thymocytes, negatively regulates the expression of CCR2 ligands, fine-tuning cDC2 and pDC entry and clonal deletion (66, 136). Consequently, *Lta*<sup>-/-</sup> 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<sup>+</sup> 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*<sup>-/-</sup> and *Cd80*<sup>-/-</sup> × *Cd86*<sup>-/-</sup> 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<sup>+</sup> thymocytes bearing TCRs of low to intermediate affinity. Furthermore, 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<sup>+</sup>CD8<sup>lo</sup> intermediate stage of thymocyte development. Moreover, in the absence of CD28, surviving thymocytes are diverted to anergic TCRαβ<sup>+</sup> DN cells that then migrate to the intestine, where they become CD8αα<sup>+</sup> IELs. Thus, CD28 costimulation is required for clonal deletion, and CD8αα<sup>+</sup> IELs probably derive from autoreactive thymocytes that survive negative selection. A recent study revisited the role of CD28-CD80/CD86 costimulation in clonal deletion (153). *Cd80*<sup>-/-</sup> × *Cd86*<sup>-/-</sup> mice have increased numbers of CD4<sup>+</sup> and CD8<sup>+</sup> thymocytes, a phenomenon observed from the CD4<sup>+</sup>CD8<sup>lo</sup> 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*<sup>-/-</sup> × *Cd86*<sup>-/-</sup> mice. Peptide-MHC-II tetramers used to identify endogenous antigen-specific polyclonal CD4<sup>+</sup> thymocytes showed that CD28-CD80/CD86 costimulation is important for clonal deletion and Treg selection of TRA-specific CD4<sup>+</sup> 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<sup>+</sup> 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*<sup>-/-</sup> 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 superantigens (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<sup>+</sup> 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<sup>+</sup>CD25<sup>+</sup> 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<sup>+</sup>CD25<sup>+</sup> 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- $\alpha$  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.

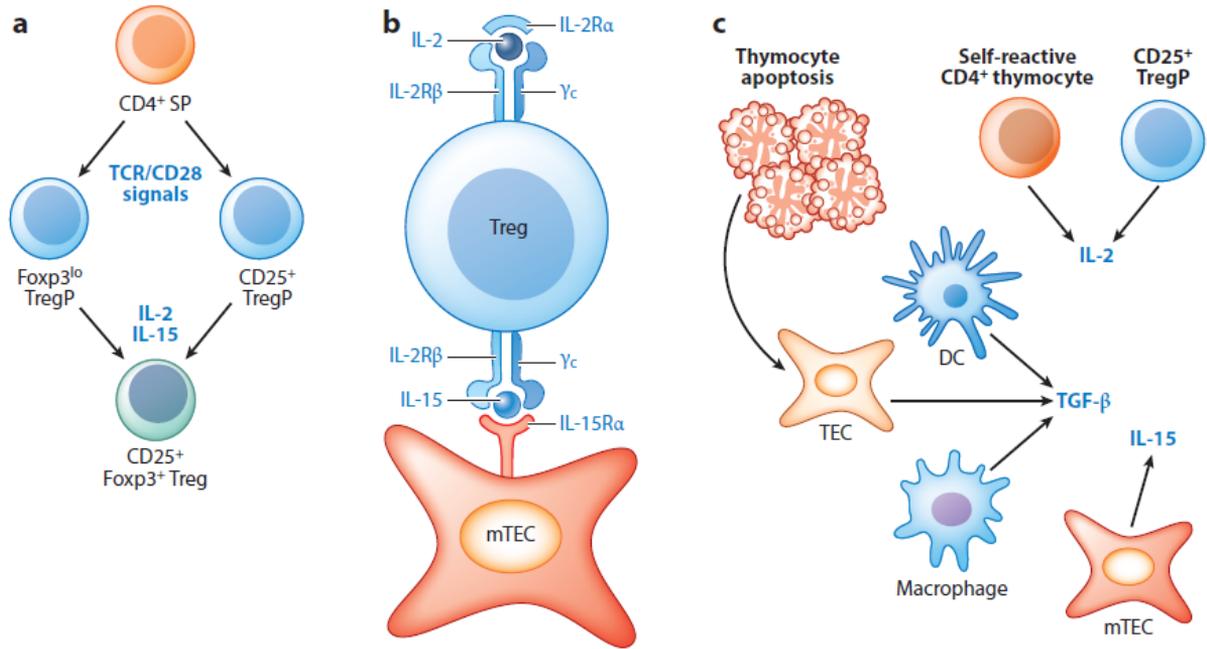
## CYTOKINES IN T CELL SELECTION IL-2 and IL-15

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

Thymic Foxp3<sup>+</sup> Tregs express the IL-2R $\beta$ , IL-4R $\alpha$ , IL-7R $\alpha$ , IL-15R $\alpha$ , and IL-21R $\alpha$  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*<sup>-/-</sup>, *Il15*<sup>-/-</sup>, or *Il2ra*<sup>-/-</sup> display a modest reduction in Foxp3<sup>+</sup> Tregs in their thymuses (169, 171, 172). By contrast, 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<sup>+</sup> Tregs (169, 171–173) (Figure 3b). In vitro stimulation of CD25<sup>+</sup> Treg precursors with IL-2 or IL-15 induces their differentiation into Foxp3<sup>+</sup>CD25<sup>+</sup> 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-15R $\alpha$  expressed by mTECs enhances the efficiency of IL-15 to induce Foxp3 (Figure 3b). IL-2 stimulation of Foxp3<sup>lo</sup> Treg precursors induces their differentiation into Foxp3<sup>+</sup>CD25<sup>+</sup> cells more efficiently than IL-2 stimulation of CD25<sup>+</sup> precursors (71, 72). In humans, IL-2 and IL-15 also drive CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>+</sup> Treg precursor differentiation into FOXP3<sup>+</sup> cells and promote their proliferation and survival (174). Furthermore, in the absence of IL-15R $\alpha$  but not IL-2, the generation of Foxp3<sup>lo</sup> 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<sup>+</sup>Foxp3<sup>+</sup> Treg cell development. (a) TCR/CD28 signals drive generation of Foxp3<sup>lo</sup> and CD25<sup>+</sup> TregPs, while IL-2 and IL-15 cytokine signal integration promotes their development into mature CD25<sup>+</sup>Foxp3<sup>+</sup> Tregs. (b) The heterotrimeric IL-2 and IL-15 receptors share two subunits: IL-2R $\beta$  and the common cytokine receptor gamma chain ( $\gamma_c$ ). They have distinct  $\alpha$  subunits: IL-2R $\alpha$  and IL-15R $\alpha$ , 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-15R $\alpha$  of mTECs. (c) Several cells secrete cytokines implicated in CD25<sup>+</sup>Foxp3<sup>+</sup> Treg development. IL-2 is mainly provided by self-reactive CD4<sup>+</sup> thymocytes and also likely by CD25<sup>+</sup> TregPs, whereas IL-15 is mainly produced by mTECs. The production of TGF- $\beta$  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<sup>+</sup> thymocytes, including 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 question of whether IL-2 production by self-reactive CD4<sup>+</sup> 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 provided by stromal cells supports the development of Foxp3<sup>lo</sup> precursors and CD25<sup>+</sup>Foxp3<sup>+</sup> 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 $\beta$**

Similar to findings for *Tgfb1*<sup>-/-</sup> mice, T cell conditional deletion of *Tgfb1* or *Tgfb2* results in an early fatal inflammatory syndrome (182–184), demonstrating that TGF- $\beta$  signaling crucially controls T cell tolerance. The level of active TGF- $\beta$ 1 increases during the neonatal period, concomitantly with medulla development, and TGF- $\beta$  receptor II (TGF- $\beta$ RII) is upregulated during the transition from DP to SP thymocytes. These observations suggest that TGF- $\beta$  signaling might regulate T cell selection (179, 185). Although it is widely accepted that TGF- $\beta$  controls Foxp3 induction in naive CD4<sup>+</sup>CD25<sup>-</sup>

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 *Tgfb2* (*Cd4Cre*  $\times$  *Tgfb2*<sup>fl/fl</sup> mice) suggested that TGF- $\beta$  signaling is dispensable for Treg development in 12- to 16-day-old mice (182, 183). However, a subsequent study showed that mice deficient for *Tgfb1* specifically in T cells (*LckproximalCre*  $\times$  *Tgfb1*<sup>fl/fl</sup> mice) lack CD25<sup>+</sup>Foxp3<sup>+</sup> Tregs at postnatal days 3–5, showing that TGF- $\beta$  signaling in thymocytes controls the emergence of thymic Tregs (187). Notably, although at this early stage CD25<sup>+</sup> Treg precursors are unaffected, both Foxp3<sup>+</sup> Treg precursors and mature CD25<sup>+</sup>Foxp3<sup>+</sup> Tregs are almost absent, which could be in line with a role in Foxp3 induction. The proportion of thymic CD25<sup>+</sup>Foxp3<sup>+</sup> 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<sup>+</sup> Tregs. Thymic Tregs from *Cd4Cre*  $\times$  *Tgfb2*<sup>fl/fl</sup> 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<sup>+</sup> Treg numbers in four-day-old thymuses, indicating that TGF- $\beta$  protects Tregs from *Bim*-induced cell death.

Interestingly, the production of TGF- $\beta$  by DCs, macrophages, and TECs is stimulated by thymocyte apoptosis (179) (Figure 3c). Thymocyte apoptosis enhanced by treatment with dexamethasone, gamma irradiation, or anti-CD3 antibody increases TGF- $\beta$  production. Furthermore, gamma irradiation after birth increases thymocyte apoptosis, TGF- $\beta$  levels, and Treg frequencies. Conversely, decreased thymocyte apoptosis in gamma-irradiated *Bim*<sup>-/-</sup> mice leads to reduced

TGF- $\beta$  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 thymocytes. Thus, the negative selection of autoreactive thymocytes seems to control Treg production

efficiency. Although further studies are required to clarify the role of TGF- $\beta$  in Treg selection, it is likely that TGF- $\beta$  signaling acts as both a differentiation and a survival factor for thymic Tregs. Few studies have attempted to investigate the role of TGF- $\beta$  signaling in the negative selection 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 TCR $\beta^{\text{hi}}$  OTII thymocytes was enhanced on a *Tgfb2*<sup>-/-</sup> background in eight-day-old mice (185). This phenotype was accompanied by reduced survival of these cells, suggesting that TGF- $\beta$  signaling protects thymocytes from negative selection. The identification of Helios as a marker of autoreactive conventional CD4<sup>+</sup> thymocytes has helped in evaluating the proportion of thymocytes undergoing negative selection in an unskewed polyclonal repertoire (33). *Cd4*<sup>Cre</sup>  $\times$  *Tgfb2*<sup>fl/fl</sup> mice show an increased proportion of Helios<sup>+</sup> cortical and medullary CD4<sup>+</sup> 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<sup>+</sup> thymocytes express the proapoptotic factor Bim and the active caspase 3, indicating that TGF- $\beta$  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- $\beta$  signaling upregulates RANKL expression in autoreactive CD4<sup>+</sup> thymocytes, known to trigger Aire<sup>+</sup> mTEC differentiation (114). TGF- $\beta$  by itself is inefficient at inducing RANKL expression and requires synergy with TCR stimulation (189). Accordingly, RANKL levels in Helios<sup>+</sup> autoreactive CD4<sup>+</sup> thymocytes are reduced in *Cd4*<sup>Cre</sup>  $\times$  *Tgfb2*<sup>fl/fl</sup> mice (188). Thus, highly autoreactive thymocytes also escape from negative selection by failing to provide RANKL signals to mTECs, resulting in altered Aire<sup>+</sup> mTEC differentiation and self-antigen expression. Although TGF- $\beta$  signaling is likely implicated in both Treg selection and the negative selection of CD4<sup>+</sup> thymocytes, its involvement in the selection of CD8<sup>+</sup> thymocytes remains to be established.

## **FUTURE ISSUES**

Advances of the last decade have revealed a high heterogeneity in the stromal compartment, opening 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 understanding of T cell selection, it would be interesting to determine whether these ligands are delivered 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 allow 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<sup>+</sup> and Foxp3<sup>lo</sup> 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 tissue regenerative potential remains to be established.

## **DISCLOSURE STATEMENT**

The author is not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

## ACKNOWLEDGMENTS

The Immune Tolerance and T-Cell Differentiation laboratory received funding from the Agence Nationale de la Recherche (grant ANR-19-CE18-0021-01, RANKLthym to M.I.); the Initiative d'Excellence d'Aix-Marseille Université A\*MIDEX, a French Programme d'Investissements d'Avenir (LT $\alpha$ -Treg to M.I.); and Canceropôle, Institut National du Cancer, and Région Sud Provence-Alpes-Côte d'Azur (ThymMap to M.I.). My laboratory is also supported by institutional grants from Institut National de la Santé et de la Recherche Médicale (INSERM), Centre National de la Recherche Scientifique (CNRS), and Aix-Marseille Université. I gratefully thank Prof. Arnaud Sergé (LAI, Marseille) for critical reading of the manuscript.

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