Selection of regulatory T cells in the thymus

Chyi-Song Hsieh[1], Hyang-Mi Lee[1] and Chan-Wang J. Lio[1,2]

Abstract | The generation of regulatory T (T_{Reg}) cells in the thymus is crucial for immune homeostasis and self-tolerance. Recent discoveries have revealed the cellular and molecular mechanisms that govern the differentiation of a subset of developing thymocytes into natural T_{Reg} cells. Several models, centred on the self-reactivity of the T cell receptor (TCR), have been proposed to explain the generation of a T_{Reg} cell population that is cognizant of self. Several molecular pathways link TCR and cytokine signalling with the expression of the T_{Reg} cell-associated transcription factor forkhead box P3 (FOXP3). Moreover, interplay between thymocytes and thymic antigen-presenting cells is also involved in T_{Reg} cell generation.

During the last decade, it has become clear that regulatory T (T_{Reg}) cells, which constitute approximately 10% of peripheral $CD4^+$ T cells, are required for the maintenance of immune homeostasis[1,2]. Humans with a mutation in the forkhead box P3 (*FOXP3*) gene — which encodes a transcription factor that is required for T_{Reg} cell development and function — develop IPEX syndrome (immunodysregulation, polyendocrinopathy, enteropathy, X-linked syndrome). This is a severe multiorgan autoimmune disease that requires treatment with bone marrow transplantation in early childhood[3]. Similarly, scurfy mice, which lack FOXP3 expression, develop a lethal autoimmune syndrome[4]. T_{Reg} cells are needed throughout life; indeed, experimental depletion of $FOXP3^+$ T_{Reg} cells in healthy adult mice results in overwhelming autoimmunity and death in a matter of weeks[5,6]. Thus, the generation of T_{Reg} cells is important for immune tolerance and the prevention of spontaneous autoimmunity.

The first clue that T_{Reg} cells are generated in the thymus originated from the classic neonatal thymectomy experiment, in which the removal of the thymus on day 3, but not day 7, after birth results in the spontaneous development of a variety of autoimmune pathologies[7]. This serendipitous observation became one of the principle foundations of the T_{Reg} cell field, as it demonstrated that thymus-derived T_{Reg} cells that migrate to the periphery after day 3 are essential for self-tolerance[8,9]. In addition to the identification of these thymus-derived $FOXP3^+CD4^+$ T_{Reg} cells, which are also known as natural T_{Reg} cells, it has become clear that conventional naive $FOXP3^-CD4^+$ T cells can differentiate in the periphery to become $FOXP3^+$ cells[10] that are known as induced T_{Reg} cells. Induced T_{Reg} cells may have an important role in tolerance to foreign antigens, such as those derived from commensal bacteria in the gut[11]. The mechanisms of development and the antigen specificities of natural T_{Reg} and induced T_{Reg} cells are likely to differ. We focus here on the development of thymic natural T_{Reg} cells.

In this Review, we focus on self-reactivity as the most likely primary determinant of T_{Reg} cell differentiation in the thymus. In detail, we review current models on the role of the affinity of the T cell receptor (TCR) for thymic self antigens in natural T_{Reg} cell selection, and we examine how the signalling events downstream of TCR stimulation lead to the subsequent activation of the *Foxp3* locus in a multistep process. In addition, we discuss the role of thymic antigen-presenting cells (APCs), which generate and present the antigenic representation of self in T_{Reg} cell differentiation.

The role of TCR specificity

Although the existence of cells with suppressor activity was proposed several decades ago[12], the current notion of T_{Reg} cells was greatly facilitated by the identification of CD25 as a reasonably sensitive and specific marker[13]. Using this marker, it was shown that $CD4^+CD25^+$ T_{Reg} cells are present in the thymus of wild-type mice, but are absent from the thymus of transgenic mice that express only the DO11.10 transgenic TCR, which recognizes the foreign antigen chicken ovalbumin[14]. Thus, these data provided the first hint that TCR specificity is important for thymic T_{Reg} cell differentiation.

A seminal study by Caton and colleagues suggested that the expression of a TCR specific for self antigens (self-reactivity) was the crucial requirement for thymic T_{Reg} cell differentiation, as T_{Reg} cells developed in the

[1]*Department of Medicine, Division of Rheumatology, Washington University School of Medicine, St. Louis, Missouri 63110, USA.*
[2]*Present address: Department of Signalling and Gene Expression, La Jolla Institute for Allergy and Immunology, La Jolla, California 92037, USA.*
Correspondence to C.-S.H.
e-mail: chsieh@wustl.edu
doi:10.1038/nri3155
Published online
10 February 2012

thymus of TCR-transgenic mice only when the cognate antigen was also expressed via a second transgene[15]. This has now been observed in several TCR–cognate antigen double-transgenic mouse systems[16,17]. Although in wild-type mice the αβ TCR is first expressed by thymocytes at the $CD4^+CD8^+$ double-positive (DP) stage, TCR expression by most transgenic lines occurs in $CD4^-CD8^-$ double-negative (DN) thymocytes, allowing for abnormally early TCR–cognate antigen interactions, which may affect T cell development[18]. However, data from TCR-transgenic mouse studies formed the basis for the current notion that natural T_{Reg} cell development occurs when the TCR avidity for self antigens lies between the TCR avidities that drive positive selection and negative selection[19].

Further support for this hypothesis came from studies that used mice with a limited TCR diversity to permit analyses of TCR repertoires at the individual TCR level. In these mice, it was observed that the TCR repertoires of T_{Reg} cells and conventional $CD4^+$ T cells are different, with only a small overlap[20–22]. Moreover, T cells that were engineered to express TCRs isolated from the T_{Reg} cell subset were more likely to proliferate in a lymphopenic host[22], consistent with the hypothesis that there is a higher level of self-reactivity in T_{Reg} cell-derived TCRs than there is in TCRs from other T cells. Together with the TCR-transgenic mouse studies, these data suggested that TCR specificity has an important role in the thymic selection of T_{Reg} cells (FIG. 1).

Controversy regarding the role of TCR specificity. Although the studies mentioned above favoured an important role for TCR specificity in the thymic selection of T_{Reg} cells, others argued against this notion. For example, it was observed that thymocytes expressing either 3A9 or KRN transgenic TCRs underwent negative selection rather than differentiating into T_{Reg} cells after encounter with their cognate antigens, which were also expressed as transgenes[23]. Moreover, in AND TCR-transgenic mice that co-expressed the cognate antigen (moth cytochrome *c*) in a doxycycline-inducible manner, increasing the amount of antigen did not result in increased absolute numbers of T_{Reg} cells but in a higher proportion of T_{Reg} cells owing to the deletion of non-T_{Reg} cells[24]. Although the use of an endogenous TCR α-chain might alter TCR specificity and permit the selection of T_{Reg} cells in this model, recognition of cognate antigen by the AND TCR did not directly facilitate thymic T_{Reg} cell generation but increased the relative proportion of T_{Reg} cells, as T_{Reg} cells can resist TCR-induced clonal deletion.

Another study suggested that T_{Reg} cell lineage decisions may be affected by events at the $CD4^-CD8^-$ stage of thymic development[25]. As this stage occurs before the genetic rearrangement of the TCR is completed, these findings implied that T_{Reg} cell selection may be at least partly independent of TCR specificity. Moreover, a TCR repertoire study of T_{Reg} cells suggested that the TCRs used by T_{Reg} cells and conventional T cells are largely overlapping[26]. Finally, hybridoma cells expressing either T_{Reg} cell-derived or conventional $CD4^+$ T cell-derived TCRs responded comparably to stimulation by autologous APCs[26]. Thus, there was a period of time when there was no consensus regarding the importance of TCR specificity in thymic T_{Reg} cell differentiation, and multiple competing models were proposed (FIG. 1).

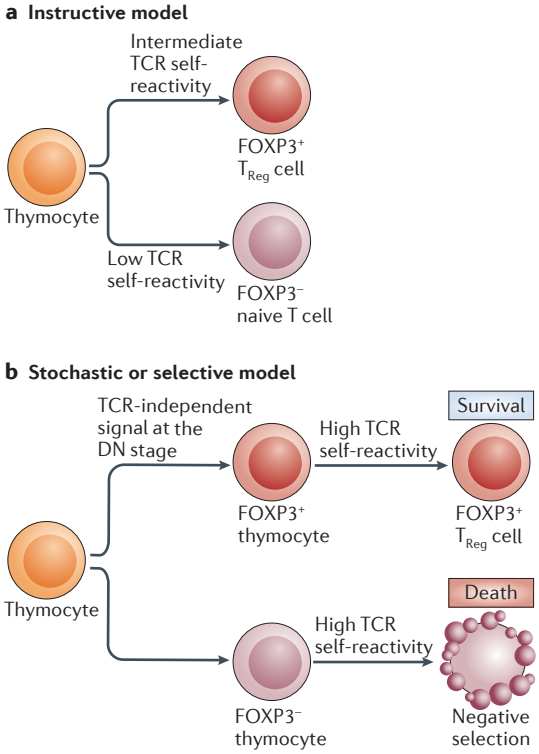


Figure 1 | **Models for thymic T_{Reg} cell development.** **a** | According to the instructive model, cell fate determination is based on the strength of T cell receptor (TCR) stimulation: intermediate levels of TCR stimulation induce forkhead box P3 (FOXP3) expression, whereas higher levels induce negative selection (not shown). Low-level TCR signalling allows the cells to mature and emigrate as conventional naive T cells. **b** | According to the stochastic or selective model, the induction of FOXP3 expression is attributable to a TCR-independent signal, perhaps at the early stage of $CD4^-CD8^-$ double-negative (DN) thymic progenitors. Compared with $FOXP3^-$ thymocytes, $FOXP3^+$ cells are relatively resistant to negative selection, which is induced by a high level of self-reactivity of the TCR. Thus, these self-reactive $FOXP3^+$ thymocytes survive to generate the regulatory T (T_{Reg}) cell population.

The 'niche' hypothesis for the development of natural T_{Reg} cells. In the past few years, several studies have renewed support for the notion that TCR specificity is important for thymic T_{Reg} cell differentiation[27–30], primarily owing to the generation of transgenic mouse lines that express TCRs derived from natural T_{Reg} cells. However, the first reported transgenic mouse line expressing a natural T_{Reg} cell-derived TCR showed negative selection but no T_{Reg} cell generation in the thymus. As this particular TCR was derived from a T_{Reg} cell clone that was isolated from OT-II αβ TCR-transgenic mice[31], it is likely that, when expressed as a transgene in a non-OT-II transgenic mouse, its higher expression levels led to negative

TCR avidity
The combined strength of interaction between the antigen receptors on a single T cell and multiple peptide–MHC complexes on the antigen-presenting cell. The avidity can be broadly described as a function of the TCR affinity and the number of peptide–MHC complexes.

Positive selection
The process by which immature $CD4^+CD8^+$ double-positive thymocytes expressing T cell receptors that are able to recognize self-peptide–MHC complexes can proceed during the T cell maturation process into $CD4^+$ or $CD8^+$ single-positive thymocytes. This selection process is important for the generation of T cells that are restricted to the host's MHC molecules.

Negative selection
The process by which developing T cells expressing T cell receptors that are highly reactive to self antigens presented on thymic antigen-presenting cells are eliminated via apoptosis.

selection. Another two transgenic mouse lines were independently generated by two groups[27,29] using TCRs derived from naturally arising T_{Reg} cells, as determined by TCR repertoire analyses of two different transgenic mouse strains with fixed TCR β-chains (described above). In contrast to what was expected, virtually no T_{Reg} cells were observed in the TCR-transgenic mice when they were bred to a recombination activating gene (RAG)-deficient background[27,29]. This outcome was so unexpected that experimental artefacts — such as an inappropriate transgene integration site or early expression of the transgenic TCR[18] — were initially suspected.

After excluding these possibilities[27], it became clear through the use of intrathymic injection and mixed bone marrow chimaeras that the rarity of thymic T_{Reg} cell development was due to the experimental artefact of a thymus in which all thymocytes expressed TCRs with the same antigen specificity. It was observed that the frequency of natural T_{Reg} cells was inversely related to the clonal frequency of the TCR-transgenic thymocytes (FIG. 2a), implying that intraclonal competition for a limited resource was affecting T_{Reg} cell development[29]. Moreover, the absolute number of T_{Reg} cells reached a plateau that was much lower than the number of $CD4^+$ single-positive (SP) cells that could be generated by positive selection (FIG. 2b), suggesting that the factors required for the development of natural T_{Reg} cells are much more limiting than the factors required for positive selection[27,29]. In addition, the number of T_{Reg} cells developing in the thymus varied considerably depending on the specificity of the TCR, an indication that TCRs facilitate thymic T_{Reg} cell development via a quantitative, rather than qualitative, mechanism. Although the limiting factors for T_{Reg} cell development in the thymus remain to be directly defined, the role of intraclonal competition in this process implies that the limiting factor may be related to the number of APCs with sufficient antigens to trigger T_{Reg} cell development.

The plateau in the number of natural T_{Reg} cells that can be generated at high clonal frequencies suggests that thymic T_{Reg} cell differentiation depends on a single thymocyte–APC encounter, which is subject to intraclonal competition. By contrast, if thymic T_{Reg} cell differentiation required multiple thymocyte–APC encounters that were subject to intraclonal competition, the number of thymic T_{Reg} cells generated would be predicted to continuously decrease with increasing clonal frequency (FIG. 2b). As the niche for natural T_{Reg} cell development appears to be restricted, the ligands for natural T_{Reg} cell selection are likely to be rare and tissue-specific antigens, rather than ubiquitously expressed antigens. Consistent with this notion, a recently characterized TCR that drives thymic T_{Reg} cell development appears to be specific for a skin antigen[32]. Altogether, the results obtained using TCRs isolated from naturally occurring T_{Reg} cells support the notion that thymic T_{Reg} cell development requires distinct TCR specificities. Moreover, these data show that T_{Reg} cell development is typically governed by intraclonal competition for an antigen-specific 'niche', suggesting that the antigens themselves are likely to be rare and tissue specific.

The 'buddy' hypothesis. One consistent observation in the TCR–cognate antigen double-transgenic mouse models was that the proportion of T_{Reg} cells within the total $CD4^+$ T cell population was commonly less than 50%, suggesting that the TCR-mediated selection of T_{Reg} cells is relatively inefficient. This may not be a substantial issue for maintaining tolerance, as the numbers of natural T_{Reg} cells in these mice were sufficient to prevent the induction of autoimmunity by conventional $CD4^+$ T cells bearing the same TCR specificities[15]. This indicates the crucial role of natural T_{Reg} cells in immune tolerance, because if negative selection was the only tolerance mechanism then the escape of any autoreactive T cells could result in autoimmune pathology. Thus, the data from the TCR–cognate antigen double-transgenic mouse models suggested that autoreactive conventional T cells would have a T_{Reg} cell 'buddy' with the same antigen specificity, which would prevent unwanted T cell activation and autoimmunity.

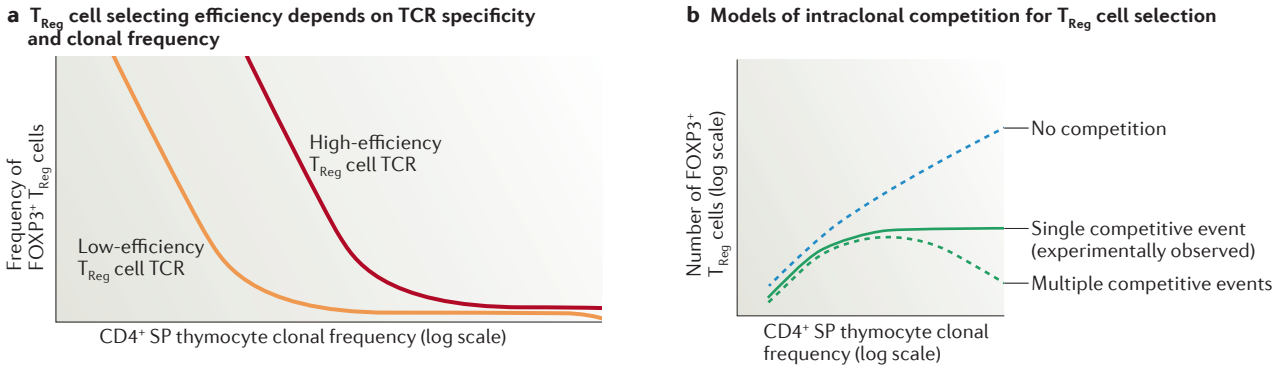
In contrast with the TCR–cognate antigen double-transgenic mouse studies[15,16], TCR repertoire analyses suggested that most of the TCRs found on T_{Reg} cells efficiently facilitate thymic T_{Reg} cell differentiation, as many TCRs that were found in the T_{Reg} cell subset were not present (or were present at frequencies below the limit of detection) in the non-T_{Reg} cell subset[20,21,33]. The niche phenomenon that was suggested by the analysis of natural T_{Reg} cell TCRs may resolve this discrepancy, as the high clonal frequencies in the early TCR–cognate antigen double-transgenic mouse studies would have lowered the frequency of cells undergoing T_{Reg} cell selection[27,29] (FIG. 2a). Although it is not possible to experimentally achieve the low clonal frequencies found during normal T cell development, extrapolation of the available data from the use of transgenic T_{Reg} cell TCRs suggests that T_{Reg} cell differentiation can be very efficient[34]. Thus, the data suggest that most T_{Reg} cells express TCRs with specificities that favour T_{Reg} cell development in the thymus and that do not overlap with the TCR specificities of conventional $CD4^+$ T cells.

However, there are some TCRs that clearly fit the 'buddy' hypothesis, as they are found in both T_{Reg} and non-T_{Reg} cell subsets. Although these TCR specificities that inefficiently drive natural T_{Reg} cell development in the thymus have not been studied, their characterization may provide important information regarding the criteria for TCR-driven selection. It is likely that such TCRs recognize self antigens with low affinity, or are specific for self antigens that are poorly expressed or presented by thymic APCs. Because these TCRs are self-reactive but drive inefficient T_{Reg} cell selection, we speculate that conventional $CD4^+$ T cells expressing these TCRs may be the primary drivers of autoimmune disease when immune regulation is perturbed.

In vivo quantification of TCR signals in thymic T_{Reg} cells. Another line of support for the notion that self-reactivity drives thymic T_{Reg} cell differentiation comes from the newly described *Nur77*–GFP transgenic mouse line. In these mice, expression of green

fluorescent protein (GFP) is driven by the *Nur77* promoter, which is activated in response to TCR stimulation[30]. Interestingly, the expression of GFP was found to be substantially higher in polyclonal thymic CD4$^+$ T cells that expressed FOXP3 than in those that did not, consistent with the hypothesis that increased TCR self-reactivity leads to T_{Reg} cell development. Moreover, intraclonal competition was visualized as an increase in the proportion of G113 T cells that received strong TCR stimulation (as assessed by the levels of GFP expression) as the clonal frequency of T cells expressing the T_{Reg} cell-derived TCR G113 was decreased. This finding is consistent with the notion that competition for rare self antigens is the limiting event in the thymic selection of natural T_{Reg} cells.

In summary, although once controversial, the preponderance of current data supports the hypothesis that self-reactivity is the primary determinant that directs developing thymocytes to undergo thymic T_{Reg} cell differentiation. The self antigens for many T_{Reg} cells



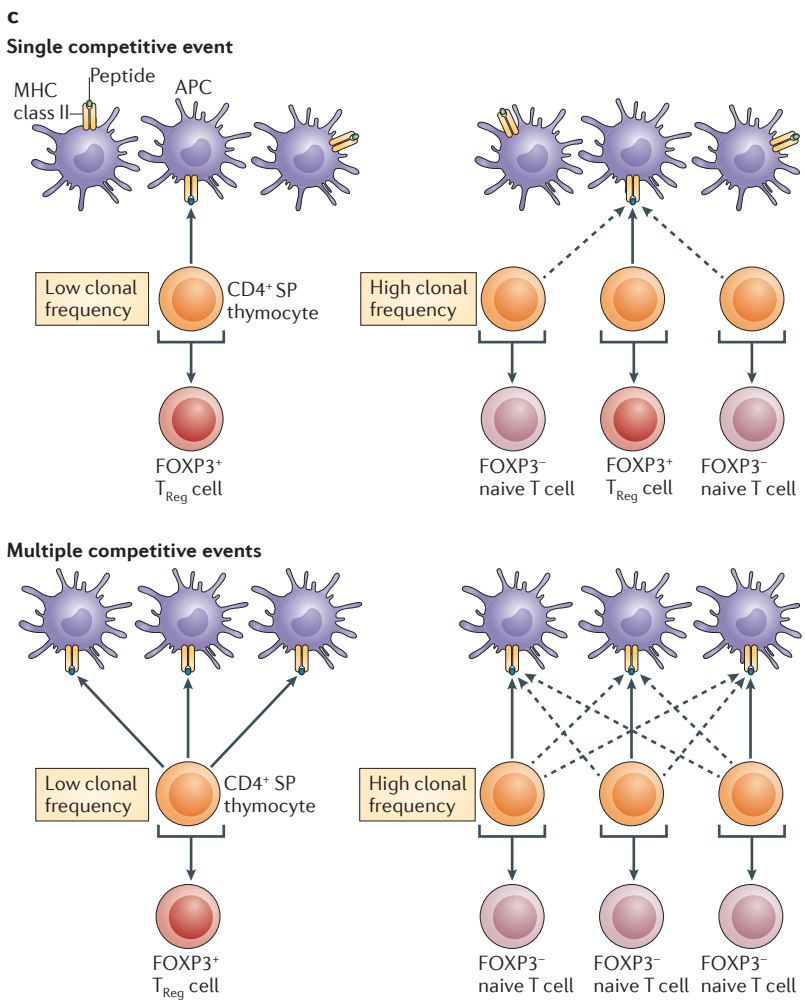


Figure 2 | **Niche hypothesis for T_{Reg} cell development.** **a** | There is an inverse relationship between the clonal frequency of CD4$^+$ single-positive (SP) thymocytes with the same T cell receptor (TCR) specificity and the percentage of thymic regulatory T (T_{Reg}) cells among this CD4$^+$ SP thymocyte population. For example, T_{Reg} cells are rare (<0.1% of the T cell population) in the thymus of transgenic recombination activating gene (RAG)-deficient mice in which all thymocytes express a natural T_{Reg} cell-derived TCR. Moreover, the decrease in the clonal frequency of TCR-transgenic thymocytes in mixed bone marrow chimaeras results in an increased T_{Reg} cell frequency. Note that the clonal frequencies in the normal CD4$^+$ SP thymocyte population are extremely low and therefore T_{Reg} cell development is likely to be more efficient than what can be observed experimentally using TCR-transgenic cells. TCR specificity also influences the selection of T_{Reg} cells in the thymus. The expression of TCRs derived from non-T_{Reg} cells fails to induce forkhead box P3 (FOXP3) expression. T_{Reg} cell-derived TCRs differ quantitatively in their ability to facilitate T_{Reg} cell development (depicted here as low- and high-efficiency T_{Reg} cell-derived TCRs), presumably owing to different sizes of the T_{Reg} cell-selecting 'niche'. **b** | If T_{Reg} cell selection depended only on TCR specificity, the number of T_{Reg} cells would be directly correlated with the number of CD4$^+$ SP thymocytes (dashed blue line), similar to what was observed for the number of positively selected thymocytes with a given TCR specificity (not shown). However, what was experimentally observed for thymocytes expressing T_{Reg} cell-derived TCRs is that the number of T_{Reg} cells reaches a plateau (green line), suggesting a role for intraclonal competition. **c** | One hypothesis is that CD4$^+$ SP thymocytes compete for a single interaction with thymic antigen-presenting cells (APCs) to become T_{Reg} cells. Another hypothesis is that multiple T cell–APC events occurring in series are required for the induction of FOXP3 expression. However, this would be predicted to decrease the number of T_{Reg} cells generated with increasing clonal frequency, and this is not consistent with the experimental data.

Medullary thymic epithelial cells
(mTECs). A specialized type of epithelial cell located in the thymic medulla that is capable of expressing and presenting tissue-specific antigens via an AIRE-dependent mechanism. mTECs have been implicated in the establishment of self-tolerance.

TCR affinity
The strength of interaction between the T cell receptor and a single peptide–MHC complex.

appear to be uncommon, rather than ubiquitous, based on the observation that thymocytes with the same TCR specificity undergo intraclonal competition for a small T_{Reg} cell developmental 'niche'.

Strength of the TCR signal to self

Assuming that TCR self-reactivity drives the thymic selection of T_{Reg} cells, the next question is what level of self-reactivity is required. In this scenario, the answer would have important implications regarding how T_{Reg} cell-mediated recognition of self antigens in the periphery compares with the recognition of such antigens by autoreactive naive T cells that escape thymic negative selection or T_{Reg} cell differentiation. Although positive selection is driven by a low degree of TCR self-reactivity, with evidence for peptide specificity[35,36], this level of self-recognition does not appear to be sufficient to induce thymic T_{Reg} cell generation, based on TCR repertoire studies and analyses of several TCR-transgenic mouse lines[14,15,27,29,37].

By contrast, TCR interactions that lead to negative selection are likely to impose an upper limit on T_{Reg} cell development, as, for example, an excess of antigen appears to overcome the resistance to deletion that is associated with the expression of FOXP3 (REFS 15,23,24,38,39). Although T_{Reg} cell selection has been observed to be coincident with negative selection, it may also occur at a level of TCR self-reactivity below that driving negative selection, as determined in TCR-transgenic mouse models[15,16] or following intrathymic transfer of cells expressing T_{Reg} cell-derived TCRs[29]. Moreover, experimental diminishment of MHC class II expression on medullary thymic epithelial cells (mTECs) revealed a shift from negative selection to T_{Reg} cell differentiation of DO11.10 TCR-transgenic thymocytes in mice expressing ovalbumin under the control of the autoimmune regulator (*Aire*) promoter[40]. Similar conclusions were obtained following *in vivo* and *in vitro* stimulation of thymocytes expressing transgenic TCRs with titrated amounts of their cognate antigens[28,41]. Interestingly, the dose response to a cognate antigen and thymic T_{Reg} cell development appear to also depend on the genetic background of the mice[41,42]. Nonetheless, data from these studies suggest that thymic T_{Reg} cell selection is driven by a level of TCR self-reactivity that is below the level that leads to negative selection and above the level that ensures positive selection.

Affinity, per cell avidity and thymic APC numbers. Whereas the strength of the TCR signal required for negative selection has been quantitatively studied in $CD8^+$ T cells[43,44], TCR signal requirements for thymic T_{Reg} cell development have not been examined as closely. A recent study investigated the relationship between T_{Reg} cell development and TCR affinity and avidity using two transgenic mouse lines that expressed two different haemagglutinin-specific TCRs. These transgenic mouse lines also expressed two different haemagglutinin antigens, which were recognized by the transgenic TCRs with either low or high affinity[38]. Interestingly, the antigens that were recognized with low affinity by the transgenic TCRs could not induce thymic T_{Reg} cell differentiation, even when they were expressed at a level that was sufficient to drive negative selection. Thus, similarly to in a study of T_{Reg} cell induction in the periphery[45], it was suggested that TCR avidity cannot always compensate for TCR affinity in generating appropriate TCR interactions for the induction of thymic T_{Reg} cell selection.

Along with the notion of niche size discussed above, the number of thymic APCs that present self antigens and express appropriate co-stimulators (such as CD80 and CD86) is another factor that is likely to determine T_{Reg} cell generation. T_{Reg} cell development may therefore depend on multiple levels of TCR–antigen interaction (FIG. 3). First, the strength of the interaction between a TCR and an antigenic peptide–MHC class II complex is commonly referred to as the affinity, and this determines the signalling from one TCR complex. Second, the amount of cognate antigen per thymic APC, in conjunction with the affinity of this antigen for the TCR, would affect the overall strength of TCR signalling in one T cell. This parameter is known as the avidity of TCR stimulation (per cell avidity). Third, the number of thymic APCs presenting the cognate antigen and expressing co-stimulatory molecules at sufficient levels would determine, together with the affinity and avidity, the integrated TCR signal over space and time, and this may be abstractly encompassed by the notion of a T_{Reg} cell developmental 'niche'.

In the above-mentioned model, changing the amount of antigen in the thymus can affect per cell avidity as well as the frequency and quality of cognate antigen-presenting thymic APCs, and all of these factors may influence T_{Reg} cell differentiation. For example, antigen

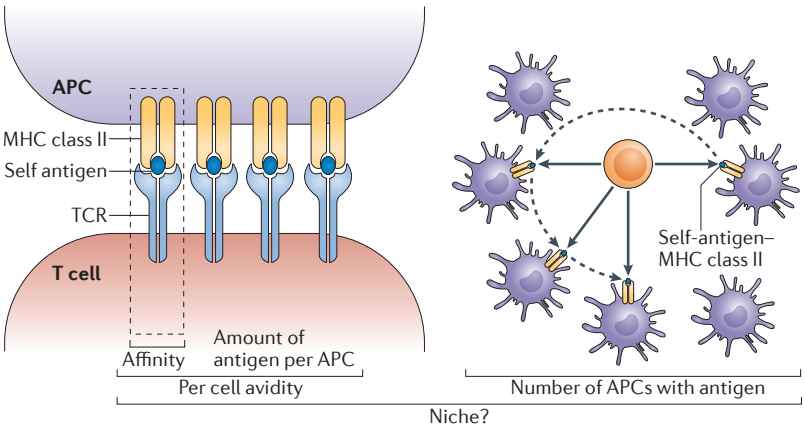


Figure 3 | **Factors that determine T_{Reg} cell development based on self-reactivity.** The T cell receptor (TCR) interactions that determine regulatory T (T_{Reg}) cell development may be affected by several factors. The first factor is the affinity of a single TCR molecule for a single self-peptide–MHC class II complex presented by a thymic antigen-presenting cell (APC). The second is the avidity of a single T cell–APC interaction, which is determined by the number of peptide–MHC ligands on the APC in conjunction with the TCR affinity. Third, the size of the antigen-specific T_{Reg} cell 'niche' — which is likely to be determined by the total number of APCs presenting a given self antigen, together with the affinity and avidity — affects the probability of T cell–APC encounter and thus determines the number of T_{Reg} cells that can differentiate at a given time. Finally, it is possible that T cell interactions with multiple APCs favour different cell fate decisions, such as negative selection rather than T_{Reg} cell differentiation.

presentation by APCs that lack co-stimulatory molecules may favour negative selection rather than T_{Reg} cell generation[46]. Although it remains to be tested, we speculate that, in addition to the signals derived from a single thymocyte–APC interaction, serial encounters of a single thymocyte with multiple thymic APCs may affect T cell fate. It has been suggested using two-photon microscopy that negative selection involves multiple T cell–APC encounters[47]. By contrast, our interpretation of the data supporting the niche hypothesis is that T_{Reg} cell differentiation is based on a single competitive antigen-dependent event (FIG. 2b). This notion is reminiscent of the observation that the induction of FOXP3 expression in peripheral T cells *in vitro* is facilitated by the withdrawal of, rather than continuous, TCR signalling[48]. Future studies will be required to quantify the affinity and the amount of antigen required for thymic T_{Reg} cell selection, and to determine whether the frequency of T cell–APC encounters can alter cell fate decisions between T_{Reg} cell generation and negative selection.

The data from the *Nur77*–GFP reporter mice suggest that the signal strength required for T_{Reg} cell differentiation is substantially higher than that experienced by most of the FOXP3$^-$ T cell population[30]. Moreover, a tenfold reduction in the amount of the agonistic antigen ovalbumin presented by mTECs results in a shift from negative selection to T_{Reg} cell generation in DO11.10 thymocytes[40]. Thus, the range of TCR self-reactivity (which broadly encompasses affinity, avidity and antigen–APC availability) that is permissive for T_{Reg} cell development appears to be greater than that represented by a Gaussian distribution, which has been previously proposed as a mathematical model for T cell development[19]. Moreover, as the frequency of T_{Reg} cells in the thymus is small (approximately 3% of CD4$^+$ SP cells), the range of TCR self-reactivity that drives negative selection rather than T_{Reg} cell generation is extremely small on a Gaussian distribution when drawn to scale. Thus, we propose that a Poisson distribution of the strength of TCR self-reactivity may better approximate the TCR repertoire that is present after positive selection, as it would represent the small proportion of self-reactive thymocytes as an extended tail (FIG. 4).

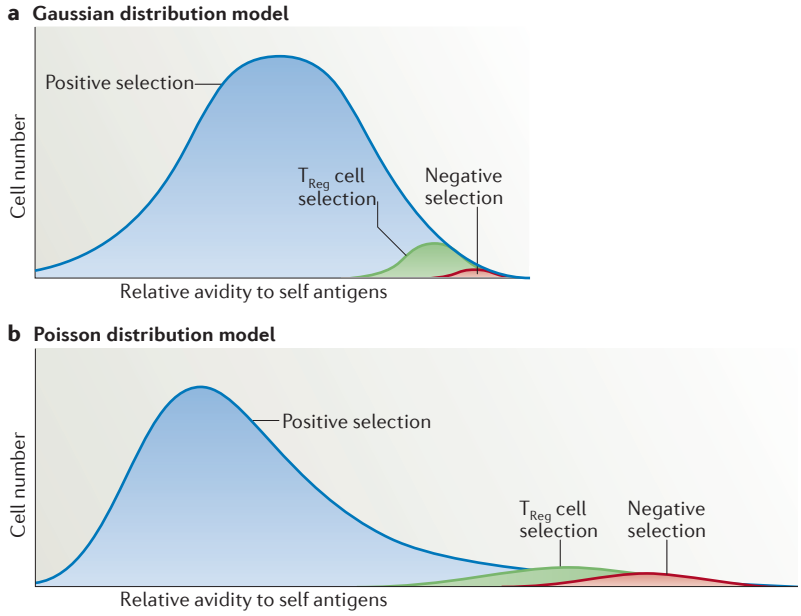


Figure 4 | **Models of self-reactivity and T_{Reg} cell generation. a** | The figure shows the classic Gaussian distribution model as it was first proposed by Maloy and Powrie[19]. The graph depicts the relationship between the relative avidity to self antigens (*x* axis) and the selected cell number (*y* axis). The distribution of positively selected CD4$^+$ single-positive (SP) cells is shown in blue, with the alternative cell fates of regulatory T (T_{Reg}) cell selection and negative selection shown in green and red, respectively. **b** | In a Poisson distribution model, most of the positively selected CD4$^+$ SP cells have the same avidity to self antigens as in the Gaussian model, but the range of self-reactivity that induces T_{Reg} cell selection and negative selection is much greater. Thus, the difference between the levels of self-reactivity that induce positive selection and those that result in T_{Reg} cell selection or negative selection is much larger in the Poisson model than in the Gaussian model.

Shaping of the TCR repertoire of natural T_{Reg} cells in the periphery. The end result of thymic T_{Reg} cell differentiation based on TCR self-reactivity is the release of a population of T cells that might be considered to have a 'memory' of the self-antigen repertoire presented in the thymus. However, unlike conventional memory T cells, which persist in the absence of antigens, the maintenance of FOXP3$^+$ natural T_{Reg} cells that have left the thymus appears to depend on the presence of self antigens in peripheral tissues[49–52]. This notion is supported by a recent study demonstrating that antigen-mediated activation of peripheral T_{Reg} cells leads to their proliferation and prolonged persistence in the tissue from which the antigen originated, even if antigen expression is no longer detectable[53]. Thus, T_{Reg} cells can develop features ascribed to conventional memory T cells. Future studies are required to quantify the similarities and differences between conventional memory T cells and 'memory' T_{Reg} cells, and to determine the proportion of 'memory' versus 'naive' peripheral T_{Reg} cells. However, it appears that the recognition of self antigens in the periphery by natural T_{Reg} cells allows these cells to persist and respond dynamically to self antigens that are released after injury, thereby preventing the induction of autoimmunity.

Molecular signals downstream of the TCR

The role of the NF-κB pathway in natural T_{Reg} cell development. TCR engagement stimulates a variety of downstream signalling molecules and transcription factors, including AKT, mammalian target of rapamycin (mTOR), nuclear factor of activated T cells (NFAT) and nuclear factor-κB (NF-κB). Although Ca^{2+} signalling seems to be involved in thymic T_{Reg} cell development, a role for NFAT — which is activated downstream of Ca^{2+} signalling — has not been clearly demonstrated[54,55]. Moreover, activation of the AKT–mTOR pathway is inhibitory to T_{Reg} cell differentiation[48,56,57]; this may occur via the inhibition of forkhead box O (FOXO) transcription factors, which have recently been shown to be required for T_{Reg} cell development[58–60]. Because TCR activation can induce conflicting signals for FOXP3 induction, an interesting future question will be to determine whether AKT–mTOR signalling is differentially regulated so that T_{Reg} cell development occurs more efficiently in the thymus than in the periphery[61].

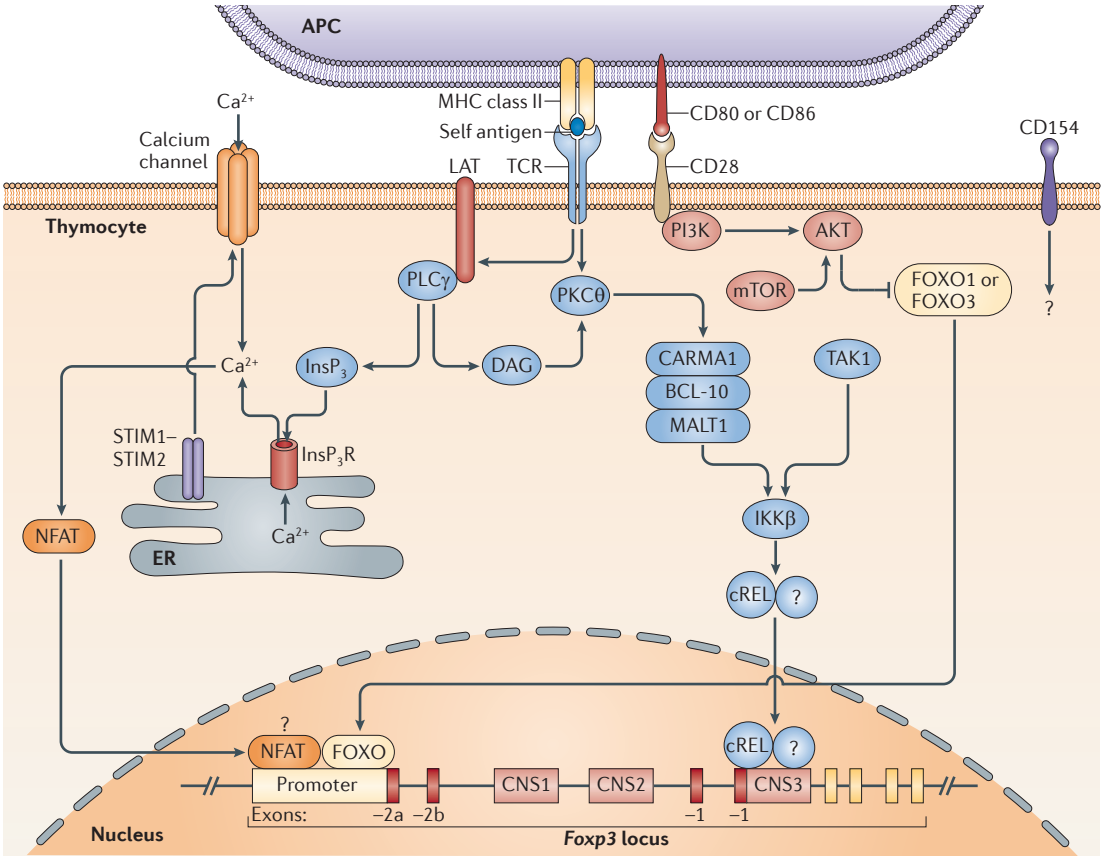


Figure 5 | **Molecular mechanisms linking TCR specificity to FOXP3 expression.** The recognition of a self-antigen–MHC class II complex by the T cell receptor (TCR) triggers several downstream signalling pathways. The primary mechanism for the induction of forkhead box P3 (FOXP3) in developing thymic regulatory T (T_{Reg}) cells appears to be the activation of the nuclear factor-κB (NF-κB) pathway, via the CARMA1–BCL-10–MALT1 complex. IκB kinase-β (IKKβ) is activated by this complex and by TGFβ-activated kinase 1 (TAK1) and then phosphorylates NF-κB inhibitor (IκB), leading to the dissociation of IκB from NF-κB and the subsequent degradation of IκB (not shown). Once released, the NF-κB family transcription factor cREL translocates to the nucleus and binds to the conserved non-coding sequence 3 (CNS3) region of the *Foxp3* locus. Although less well characterized, the nuclear factor of activated T cells (NFAT) pathway, which is activated downstream of Ca^{2+} signalling, may also have a positive role in the induction of FOXP3. Interestingly, the AKT–mTOR (mammalian target of rapamycin) pathway may be a negative regulator of FOXP3 induction through the phosphorylation and inhibition of forkhead box O (FOXO) transcription factors, which facilitate the expression of *Foxp3*. The role of AKT–mTOR signalling in the thymus in inhibiting T_{Reg} cell differentiation remains to be clarified. APC, antigen-presenting cell; BCL-10, B cell lymphoma 10; CARMA1, CARD-containing MAGUK protein 1; DAG, diacylglycerol; ER, endoplasmic reticulum; $InsP_3$, inositol-1,4,5-trisphosphate; $InsP_3R$, $InsP_3$ receptor; LAT, linker for activation of T cells; MALT1, mucosa-associated lymphoid tissue lymphoma translocation protein 1; PI3K, phosphoinositide 3-kinase; PKCθ, protein kinase Cθ; PLCγ, phospholipase Cγ; STIM, stromal interaction molecule.

Moreover, it is still unclear whether there is dynamic variation in the level of AKT–mTOR activation depending on the strength of the TCR–antigen interaction or on the activation status of the APCs.

Among the multiple pathways downstream of the TCR, the NF-κB pathway appears to be the main one involved in thymic T_{Reg} cell differentiation[37,62,63] (FIG. 5). This was first suggested by analyses of mice with mutations in genes encoding components of this signalling cascade, such as protein kinase Cθ (PKCθ), CARD-containing MAGUK protein 1 (CARMA1), B cell lymphoma 10 (BCL-10), TGFβ-activated kinase 1 (TAK1) and IκB kinase-β (IKKβ). These mice displayed dramatic decreases in the frequency of thymic T_{Reg} cells (reviewed in REF. 64). Moreover, it was observed that enforced NF-κB activation via overexpression of a constitutively active form of IKKβ was sufficient to bypass the requirement for TCR-mediated recognition of self, as it resulted in the development of $FOXP3^+$ T_{Reg} cells in the thymus of RAG-deficient TCR-transgenic mice (expressing either the MHC class II-restricted OT-II TCR or the MHC class I-restricted P14 TCR), even though neither of these mouse lines normally generates T_{Reg} cells[37]. Taken together, these data suggest that NF-κB signalling is both necessary and sufficient for thymic T_{Reg} cell differentiation.

Of the NF-κB family of transcription factors, it appears that cREL is the most important for the induction of FOXP3 expression, although there is some debate as to whether it acts as a homodimer or pairs with other

NF-κB family members[37,62,63]. cREL has been reported to bind to the conserved non-coding sequence 3 (CNS3) region of the *Foxp3* locus, which is marked by a permissive histone modification (H3K4me1) even as early as in $CD4^+CD8^+$ DP thymocytes[63]. Thus, it has been proposed that cREL is a pioneer transcription factor that links TCR engagement with the opening of the *Foxp3* locus.

The role of co-stimulation in natural T_{Reg} cell development. CD28 has an important cell-intrinsic role in the generation of thymic T_{Reg} cells, as CD28-deficient mice have an approximately 80% reduction in the frequency of thymic T_{Reg} cells[65,66]. Although it was predicted that CD28 contributes to the overall TCR signalling strength to promote selection into the T_{Reg} cell lineage, recent studies by two independent groups have suggested that this is not the case. Instead, it was observed that the T_{Reg} cell TCR repertoires of CD28-sufficient and -deficient mice were not dramatically different[67]. Studies using TCR–cognate antigen double-transgenic mouse models have supported this notion[46]. Thus, it was proposed that the primary role of CD28 is to enhance either the efficiency of T_{Reg} cell development and/or the survival of thymocytes undergoing T_{Reg} cell differentiation[46,67]. Interestingly, another co-stimulatory molecule, CD40, which binds to CD154 expressed on activated T cells[68], may also have a role in the expansion, rather than selection, of T_{Reg} cell populations[69].

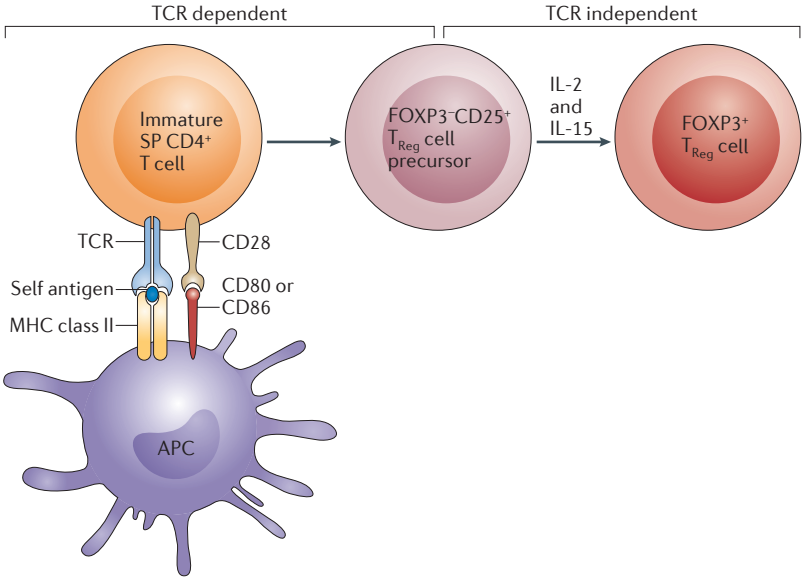


Figure 6 | **A two-step model for T_{Reg} cell development.** When thymocytes recognize self-peptide–MHC class II complexes in the presence of co-stimulatory molecules (such as CD80 or CD86) and with sufficiently high per cell avidity for regulatory T (T_{Reg}) cell selection, some of these cells are selected as $FOXP3^-CD25^+$ T_{Reg} cell precursors. Presumably the T cell receptor (TCR) signal leads to the activation of several downstream pathways, including nuclear factor-κB (NF-κB) activation, and results in the remodelling of the forkhead box P3 (*Foxp3*) locus, rendering it permissive to the induction of FOXP3 expression by interleukin-2 (IL-2) signalling. At this point, the T_{Reg} cell precursor does not require further TCR stimulation for the expression of FOXP3. Instead, cytokine signals mediated by IL-2, or to a lesser extent IL-15, facilitate the induction of FOXP3 expression. APC, antigen-presenting cell; SP, single-positive.

Stages in natural T_{Reg} cell development and the role of cytokines. The neonatal thymectomy experiments suggested that T_{Reg} cell differentiation is delayed compared with conventional T cell maturation[8]. Interestingly, it was noted that a population of $FOXP3^-CD25^+CD4^+$ SP cells temporally preceded the development of $FOXP3^+CD25^+$ T_{Reg} cells[9]. It was subsequently found that the $FOXP3^-CD25^+CD4^+$ SP cell population was enriched in cells that developed into $FOXP3^+CD4^+$ T cells after intrathymic transfer[70]. Notably, these T_{Reg} cell precursors did not require further TCR stimulation to upregulate FOXP3, suggesting that T_{Reg} cell differentiation could be divided into TCR-dependent and -independent steps (FIG. 6).

It was possible that the TCR-independent step reflected merely a lag in the time between the TCR signal and FOXP3 expression. However, short-term culture of the $FOXP3^-CD25^+CD4^+$ SP thymocytes *in vitro* suggested that this was not the case, and that additional signals were required for the upregulation of FOXP3. As cytokines had been shown to be important for the development and maintenance of the thymic T_{Reg} cell population[71–73], and given that the T_{Reg} cell precursors are found in a population of cells that express CD25 (the α-chain of the high-affinity interleukin-2 (IL-2) receptor), it was hypothesized that cytokines such as IL-2 are involved in this second step[70]. Indeed, *in vitro* culture of $FOXP3^-CD25^+$ thymocytes with IL-2, and to a lesser extent with IL-15, was sufficient to rapidly induce FOXP3 expression[70], potentially through the binding of signal transducer and activator of transcription 5 (STAT5) directly to the *Foxp3* locus[73–75].

Consistent with this *in vitro* observation, expression of a hyperactive form of STAT5 increased the *in vivo* frequency of T_{Reg} cells in the thymus and skewed the T_{Reg} cell TCR repertoire to include TCRs that are normally found on conventional $FOXP3^-$ T cells[76]. The level of IL-2 may therefore represent an antigen-independent 'niche' involved in T_{Reg} cell generation and survival[66,72], as opposed to the antigen-specific 'niche' discussed above. As TCR stimulation upregulates CD25 expression through NF-κB activation, the expression of TCRs with high affinity to self antigens may favour the differentiation of T_{Reg} cells by facilitating the generation of $CD25^+$ T_{Reg} cell precursors. Thus, the existence of multiple steps (TCR signalling and cytokine signalling) in T_{Reg} cell development may increase the dependence of T_{Reg} cell selection on high TCR self-reactivity (FIG. 6).

Whereas the current evidence supports the notion that T_{Reg} cell differentiation is a multistep process in both the thymus and the periphery[48,61,77], many questions remain. First, although the $FOXP3^-CD25^+$ thymocyte subset is enriched in cytokine-responsive T_{Reg} cell precursors, CD25 does not represent a specific marker for these cells. FOXP3-independent T_{Reg} cell-associated genes, such as the transcription factor Helios, may be better markers for T_{Reg} cell precursors[78–80]. Second, the relative contribution of cytokines to promoting T_{Reg} cell differentiation versus survival remains to be clarified. For example, transforming growth factor-β (TGFβ) has been suggested to be directly involved in FOXP3 induction during thymic T_{Reg} cell development. However, its

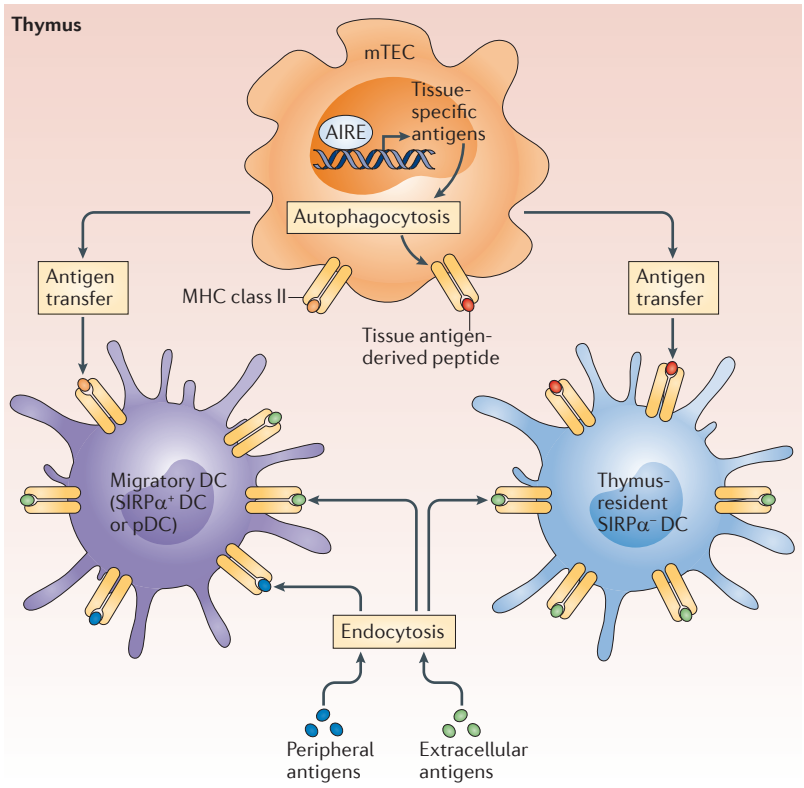


Figure 7 | **The antigenic repertoire presented by thymic medullary APCs.** Multiple thymic antigen-presenting cell (APC) types are capable of facilitating thymic regulatory T (T_{Reg}) cell differentiation in the medulla. Stromal APCs, including medullary thymic epithelial cells (mTECs), can express and present tissue-specific antigens that are induced by autoimmune regulator (AIRE). Tissue-specific antigens are processed by autophagosomes and presented on the cell surface as peptide–MHC class II complexes. Haematopoietic APCs include dendritic cells (DCs), macrophages and B cells. However, the role of macrophages and B cells is unclear (not shown). The DC subsets include plasmacytoid DCs (pDCs) and SIRPα$^+$ conventional DCs, which both migrate from the periphery and thus could potentially present extracellular antigens captured from the peripheral microenvironment. By contrast, resident SIRPα$^-$ conventional DCs originate in the thymus and thus probably present antigens from the thymus. In addition to presenting extracellular antigens, DCs can present mTEC-expressed antigens following antigenic transfer.

role has been controversial, as it may relate more to survival than to selection[81,82]. Finally, formal proof is still required for the notion that cytokine-responsive FOXP3$^-$ T_{Reg} cell precursors are an essential intermediate in the process of T_{Reg} cell differentiation. Future experiments are therefore needed to define T_{Reg} cell precursors with specific markers, as well as to understand the molecular status of the 'TCR-primed' *Foxp3* locus.

Thymic APC subsets in T_{Reg} cell development

As both TCR specificity and co-stimulatory molecules have an important role in T_{Reg} cell selection, the APCs encountered by developing thymocytes may shape the resulting T_{Reg} cell population (FIG. 7). The thymus contains a complex network of APCs that supports the various stages of T cell development; these APCs include cortical thymic epithelial cells (cTECs), mTECs and dendritic cells (DCs). Macrophages and B cells are also present, but their role in thymic T_{Reg} cell development has not been established[83].

Cortical thymic epithelial cells
(cTECs). Epithelial cells located in the thymic cortex that are able to positively select immature double-positive thymocytes.

Initial studies suggested that thymic T_{Reg} cell development takes place in the cortex, as restricted expression of MHC class II molecules on cTECs still resulted in the generation of thymic T_{Reg} cells[84]. This was supported by studies showing that FOXP3$^+$ DP thymocytes could be found in wild-type mice[85–87] and in TCR–cognate antigen double-transgenic mice[15,88]. However, recent reports have argued that the frequency of FOXP3$^+$ DP cells within the thymic T_{Reg} cell population is below 1%, suggesting a minor role for cTECs in T_{Reg} cell generation in a normal thymus[77,89]. Instead, it was proposed that most thymic T_{Reg} cells differentiate during the immature HSA^{hi}CD4$^+$ SP stage.

Among the medullary APCs, mTECs were originally thought to be crucial for T_{Reg} cell selection, as deletion of the MHC class II locus in bone marrow-derived APCs (including DCs) in bone marrow chimaeras had little effect on the number of T_{Reg} cells generated[86,90]. However, recent studies have suggested that bone marrow-derived APCs can also generate normal numbers of thymic T_{Reg} cells. Reciprocal bone marrow chimaeras had equivalent numbers of T_{Reg} cells irrespective of whether CD80 and CD86, or CD40, were expressed only on thymic epithelial cells or only on bone marrow-derived APCs[69,91]. Also, the knockdown of MHC class II expression on mTECs did not affect the frequency of thymic T_{Reg} cells[40]. In addition, transgenic expression of antigens on either cell type was sufficient for selecting TCR-transgenic T_{Reg} cells[15,16,90]. Thus, these results indicate that both bone marrow-derived APCs and mTECs can facilitate thymic T_{Reg} cell differentiation, and either subset alone may be sufficient for the generation of normal T_{Reg} cell numbers *in vivo*.

These data suggest that the various APC subsets are individually dispensable for the generation of normal numbers of thymic T_{Reg} cells. However, it remains unknown whether each type of thymic APC is responsible for the generation of T_{Reg} cells with unique TCR specificities, as mTECs and the various DC subsets differ in their ability to express, capture and present antigens for thymic T_{Reg} cell differentiation[92]. For example, it has been shown that plasmacytoid DCs (pDCs), as well as one-third of thymic conventional DCs (SIRPα$^+$ DCs), are derived from the periphery[93], potentially allowing them to present peripheral antigens in the thymus. By contrast, two-thirds of thymic conventional DCs (SIRPα$^-$ DCs) originate in the thymus[93]. In addition, these DC subsets differ in their capacity to select T_{Reg} cells *in vitro*[28,61], supporting the notion that each DC subset may select for T_{Reg} cells with differing TCR specificities, some of which may be unique to a particular DC subset.

mTECs are also likely to present different antigens from DCs owing to the stochastic expression of tissue-specific antigens regulated by the transcription factor AIRE[90,94,95]. However, transfer of antigens from mTECs to DCs may blur the differences in the antigen repertoire[95–99]. Also, the importance of AIRE-dependent antigens in selecting the T_{Reg} cell population has recently been questioned[100]. Thus, future studies are required to address the hypothesis that the various thymic APCs select for T_{Reg} cell populations with unique TCR specificities to prevent 'holes' in the T_{Reg} cell repertoire that might lead to tissue-specific autoimmunity.

Open questions

Although the past few years have seen substantial progress in understanding the process of thymic T_{Reg} cell selection, there are still many questions. TCR specificity to self antigens appears to be the primary driver for T_{Reg} cell selection, but the endogenous ligands for T_{Reg} cell TCRs are unknown. What is the threshold for self-reactivity that allows escape from T_{Reg} cell selection, and does this still permit peripheral immune responses to self? Are all of the different thymic APC types required to generate an effective T_{Reg} cell population? Does genetic background affect TCR signalling, or does it affect other, as yet unknown, mechanisms involved in thymic T_{Reg} cell selection? Answering these questions would provide a deeper understanding of how thymus-derived T_{Reg} cells prevent spontaneous autoimmunity in the periphery.

cREL appears to be an important molecular link between TCR activation and FOXP3 expression, but NF-κB activation occurs in many cell types, including peripheral T cells. One important question therefore is why TCR activation in the periphery does not typically result in the induction of FOXP3. What are the molecular differences between immature $CD4^+$ SP thymocytes and naive peripheral T cells? Is it the differential regulation of the AKT–mTOR pathway? Why is FOXP3 predominantly expressed by $CD4^+$, and not $CD8^+$, SP thymocytes? What are the molecular characteristics of T_{Reg} cell precursors that render their *Foxp3* locus permissive to binding by transcription factors induced by cytokine signalling? Understanding these molecular mechanisms may allow therapeutic treatment of autoimmune or inflammatory disorders using T_{Reg} cells that are generated from peripheral T cells but that have the stability and functional abilities of thymus-derived natural T_{Reg} cells, which is currently not achievable.

Conclusion

The dependence of thymic T_{Reg} cell selection on self-reactivity is remarkable on many levels. First, it limits the export of self-reactive conventional $CD4^+$ T cells to the periphery. Second, it creates a T cell subset that, at the population level, can distinguish self. Whereas self-reactive T cells would be biased towards the natural T_{Reg} cell subset, T cells recognizing foreign antigens would not have that bias, resulting in a form of self–non-self discrimination. Finally, the principle of clonal expansion is likely to be as crucial for T_{Reg} cell function as it is for conventional $CD4^+$ T cells, allowing T_{Reg} cells to dynamically respond to changes in self-antigen presentation owing to trauma or homeostatic cell death processes.

1. Wing, K. & Sakaguchi, S. Regulatory T cells exert checks and balances on self tolerance and autoimmunity. *Nature Immunol.* **11**, 7–13 (2010).
2. Rudensky, A. Y. Regulatory T cells and Foxp3. *Immunol. Rev.* **241**, 260–268 (2011).
3. d'Hennezel, E. *et al.* FOXP3 forkhead domain mutation and regulatory T cells in the IPEX syndrome. *N. Engl. J. Med.* **361**, 1710–1713 (2009).
4. Ramsdell, F. Foxp3 and natural regulatory T cells: key to a cell lineage? *Immunity* **19**, 165–168 (2003).
5. Lahl, K. *et al.* Selective depletion of Foxp3$^+$ regulatory T cells induces a scurfy-like disease. *J. Exp. Med.* **204**, 57–63 (2007).
6. Kim, J. M., Rasmussen, J. P. & Rudensky, A. Y. Regulatory T cells prevent catastrophic autoimmunity throughout the lifespan of mice. *Nature Immunol.* **8**, 191–197 (2007).
7. Nishizuka, Y. & Sakakura, T. Thymus and reproduction: sex-linked dysgenesia of the gonad after neonatal thymectomy in mice. *Science* **166**, 753–755 (1969).
8. Asano, M., Toda, M., Sakaguchi, N. & Sakaguchi, S. Autoimmune disease as a consequence of developmental abnormality of a T cell subpopulation. *J. Exp. Med.* **184**, 387–396 (1996).
9. Fontenot, J. D., Dooley, J. L., Farr, A. G. & Rudensky, A. Y. Developmental regulation of Foxp3 expression during ontogeny. *J. Exp. Med.* **202**, 901–906 (2005).
10. Apostolou, I. & von Boehmer, H. *In vivo* instruction of suppressor commitment in naive T cells. *J. Exp. Med.* **199**, 1401–1408 (2004).
11. Haribhai, D. *et al.* A requisite role for induced regulatory T cells in tolerance based on expanding antigen receptor diversity. *Immunity* **35**, 109–122 (2011).
12. Gershon, R. K. & Kondo, K. Cell interactions in the induction of tolerance: the role of thymic lymphocytes. *Immunology* **18**, 723–737 (1970).
13. Sakaguchi, S., Sakaguchi, N., Asano, M., Itoh, M. & Toda, M. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor α-chains (CD25). *J. Immunol.* **155**, 1151–1164 (1995).
14. Itoh, M. *et al.* Thymus and autoimmunity: production of CD25$^+$CD4$^+$ naturally anergic and suppressive T cells as a key function of the thymus in maintaining immunologic self-tolerance. *J. Immunol.* **162**, 5317–5326 (1999).
15. Jordan, M. S. *et al.* Thymic selection of CD4$^+$CD25$^+$ regulatory T cells induced by an agonist self-peptide. *Nature Immunol.* **2**, 301–306 (2001).
16. Apostolou, I., Sarukhan, A., Klein, L. & von Boehmer, H. Origin of regulatory T cells with known specificity for antigen. *Nature Immunol.* **3**, 756–763 (2002).
17. Knoechel, B., Lohr, J., Kahn, E., Bluestone, J. A. & Abbas, A. K. Sequential development of interleukin 2-dependent effector and regulatory T cells in response to endogenous systemic antigen. *J. Exp. Med.* **202**, 1375–1386 (2005).
18. Baldwin, T. A., Sandau, M. M., Jameson, S. C. & Hogquist, K. A. The timing of TCRα expression critically influences T cell development and selection. *J. Exp. Med.* **202**, 111–121 (2005).
19. Maloy, K. J. & Powrie, F. Regulatory T cells in the control of immune pathology. *Nature Immunol.* **2**, 816–822 (2001).
20. Pacholczyk, R., Ignatowicz, H., Kraj, P. & Ignatowicz, L. Origin and T cell receptor diversity of Foxp3$^+$CD4$^+$ CD25$^+$ T cells. *Immunity* **25**, 249–259 (2006).
21. Wong, J. *et al.* Adaptation of TCR repertoires to self-peptides in regulatory and nonregulatory CD4$^+$ T cells. *J. Immunol.* **178**, 7032–7041 (2007).
22. Hsieh, C.-S. *et al.* Recognition of the peripheral self by naturally arising CD25$^+$ CD4$^+$ T cell receptors. *Immunity* **21**, 267–277 (2004).
23. Shih, F. F., Mandik-Nayak, L., Wipke, B. T. & Allen, P. M. Massive thymic deletion results in systemic autoimmunity through elimination of CD4$^+$ CD25$^+$ T regulatory cells. *J. Exp. Med.* **199**, 323–335 (2004).
24. Van Santen, H.-M., Benoist, C. & Mathis, D. Number of T reg cells that differentiate does not increase upon encounter of agonist ligand on thymic epithelial cells. *J. Exp. Med.* **200**, 1221–1230 (2004).
25. Pennington, D. J. *et al.* Early events in the thymus affect the balance of effector and regulatory T cells. *Nature* **444**, 1073–1077 (2006).
26. Pacholczyk, R. *et al.* Nonself-antigens are the cognate specificities of Foxp3$^+$ regulatory T cells. *Immunity* **27**, 493–504 (2007).
27. Leung, M. W., Shen, S. & Lafaille, J. J. TCR-dependent differentiation of thymic Foxp3$^+$ cells is limited to small clonal sizes. *J. Exp. Med.* **206**, 2121–2130 (2009).
28. Atibalentja, D. F., Byersdorfer, C. A. & Unanue, E. R. Thymus–blood protein interactions are highly effective in negative selection and regulatory T cell induction. *J. Immunol.* **183**, 7909–7918 (2009).
29. Bautista, J. L. *et al.* Intraclonal competition limits the fate determination of regulatory T cells in the thymus. *Nature Immunol.* **10**, 610–617 (2009).
30. Moran, A. E. *et al.* T cell receptor signal strength in Treg and iNKT cell development demonstrated by a novel fluorescent reporter mouse. *J. Exp. Med.* **208**, 1279–1289 (2011).
By using *Nur77*–GFP as a reporter for TCR stimulation, this study correlated the level of TCR signal received with the potential for T_{Reg} cell differentiation.
31. Dipaolo, R. J. & Shevach, E. M. CD4$^+$ T-cell development in a mouse expressing a transgenic TCR derived from a Treg. *Eur. J. Immunol.* **39**, 234–240 (2008).
32. Killebrew, J. R. *et al.* A self-reactive TCR drives the development of Foxp3$^+$ regulatory T cells that prevent autoimmune disease. *J. Immunol.* **187**, 861–869 (2011).
33. Hsieh, C. S., Zheng, Y., Liang, Y., Fontenot, J. D. & Rudensky, A. Y. An intersection between the self-reactive regulatory and nonregulatory T cell receptor repertoires. *Nature Immunol.* **7**, 401–410 (2006).
34. Lio, C. W. & Hsieh, C. S. Becoming self-aware: the thymic education of regulatory T cells. *Curr. Opin. Immunol.* **23**, 213–219 (2011).
35. Lo, W. L. *et al.* An endogenous peptide positively selects and augments the activation and survival of peripheral CD4$^+$ T cells. *Nature Immunol.* **10**, 1155–1161 (2009).
36. Ebert, P. J., Jiang, S., Xie, J., Li, Q. J. & Davis, M. M. An endogenous positively selecting peptide enhances mature T cell responses and becomes an autoantigen in the absence of microRNA miR-181a. *Nature Immunol.* **10**, 1162–1169 (2009).
37. Long, M., Park, S. G., Strickland, I., Hayden, M. S. & Ghosh, S. Nuclear factor-κB modulates regulatory T cell development by directly regulating expression of Foxp3 transcription factor. *Immunity* **31**, 921–931 (2009).
38. Cozzo Picca, C. *et al.* CD4$^+$CD25$^+$Foxp3$^+$ regulatory T cell formation requires more specific recognition of a self-peptide than thymocyte deletion. *Proc. Natl Acad. Sci. USA* **108**, 14890–14895 (2011).
This study demonstrated that, in addition to avidity, the quality or affinity of the interaction between a TCR and a peptide–MHC complex is important for thymic T_{Reg} cell selection.
39. Riley, M. P. *et al.* Graded deletion and virus-induced activation of autoreactive CD4$^+$ T cells. *J. Immunol.* **165**, 4870–4876 (2000).

40. Hinterberger, M. *et al.* Autonomous role of medullary thymic epithelial cells in central CD4$^+$ T cell tolerance. *Nature Immunol.* **11**, 512–519 (2010).
By diminishing antigen presentation by mTECs using shRNA-mediated knockdown of CIITA expression, this group showed that decreasing the avidity of the mTEC–thymocyte interaction can shift the cell fate from deletion to T_{Reg} cell selection.
41. Feuerer, M. *et al.* Enhanced thymic selection of FoxP3$^+$ regulatory T cells in the NOD mouse model of autoimmune diabetes. *Proc. Natl Acad. Sci. USA* **104**, 18181–18186 (2007).
42. Romagnoli, P., Tellier, J. & van Meerwijk, J. P. Genetic control of thymic development of CD4$^+$CD25$^+$FoxP3$^+$ regulatory T lymphocytes. *Eur. J. Immunol.* **35**, 3525–3532 (2005).
43. Daniels, M. A. *et al.* Thymic selection threshold defined by compartmentalization of Ras/MAPK signalling. *Nature* **444**, 724–729 (2006).
44. Palmer, E. & Naeher, D. Affinity threshold for thymic selection through a T-cell receptor–co-receptor zipper. *Nature Rev. Immunol.* **9**, 207–213 (2009).
45. Gottschalk, R. A., Corse, E. & Allison, J. P. TCR ligand density and affinity determine peripheral induction of Foxp3 *in vivo*. *J. Exp. Med.* **207**, 1701–1711 (2010).
46. Hinterberger, M., Wirnsberger, G. & Klein, L. B7/CD28 in central tolerance: costimulation promotes maturation of regulatory T cell precursors and prevents their clonal deletion. *Front. Immunol.* **2**, 1–12 (2011).
47. Le Borgne, M. *et al.* The impact of negative selection on thymocyte migration in the medulla. *Nature Immunol.* **10**, 823–830 (2009).
48. Sauer, S. *et al.* T cell receptor signaling controls Foxp3 expression via PI3K, Akt, and mTOR. *Proc. Natl Acad. Sci. USA* **105**, 7797–7802 (2008).
49. Lathrop, S. K., Santacruz, N. A., Pham, D., Luo, J. & Hsieh, C. S. Antigen-specific peripheral shaping of the natural regulatory T cell population. *J. Exp. Med.* **205**, 3105–3117 (2008).
50. Taguchi, O. *et al.* Tissue-specific suppressor T cells involved in self-tolerance are activated extrathymically by self-antigens. *Immunology* **82**, 365–369 (1994).
51. Seddon, B. & Mason, D. Peripheral autoantigen induces regulatory T cells that prevent autoimmunity. *J. Exp. Med.* **189**, 877–882 (1999).
52. Wheeler, K. M., Samy, E. T. & Tung, K. S. Cutting edge: normal regional lymph node enrichment of antigen-specific regulatory T cells with autoimmune disease-suppressive capacity. *J. Immunol.* **183**, 7635–7638 (2009).
53. Rosenblum, M. D. *et al.* Response to self antigen imprints regulatory memory in tissues. *Nature* **480**, 538–542 (2011).
This study showed that T_{Reg} cells can differentiate into cells with 'memory' characteristics that reside in the tissue and diminish recurrent autoimmune responses.
54. Bopp, T. *et al.* NFATc2 and NFATc3 transcription factors play a crucial role in suppression of CD4$^+$ T lymphocytes by CD4$^+$ CD25$^+$ regulatory T cells. *J. Exp. Med.* **201**, 181–187 (2005).
55. Oh-Hora, M. *et al.* Dual functions for the endoplasmic reticulum calcium sensors STIM1 and STIM2 in T cell activation and tolerance. *Nature Immunol.* **9**, 432–443 (2008).
56. Haxhinasto, S., Mathis, D. & Benoist, C. The AKT–mTOR axis regulates *de novo* differentiation of CD4$^+$Foxp3$^+$ cells. *J. Exp. Med.* **205**, 565–574 (2008).
57. Delgoffe, G. M. *et al.* The kinase mTOR regulates the differentiation of helper T cells through the selective activation of signaling by mTORC1 and mTORC2. *Nature Immunol.* **12**, 295–303 (2011).
58. Ouyang, W. *et al.* Foxo proteins cooperatively control the differentiation of Foxp3$^+$ regulatory T cells. *Nature Immunol.* **11**, 618–627 (2010).
59. Kerdiles, Y. M. *et al.* Foxo transcription factors control regulatory T cell development and function. *Immunity* **33**, 890–904 (2010).
60. Harada, Y. *et al.* Transcription factors Foxo3a and Foxo1 couple the E3 ligase Cbl-b to the induction of Foxp3 expression in induced regulatory T cells. *J. Exp. Med.* **207**, 1381–1391 (2010).
References 58–60 were three independent studies that demonstrated the importance of FOXO in the thymic development of T_{Reg} cells.
61. Wirnsberger, G., Mair, F. & Klein, L. Regulatory T cell differentiation of thymocytes does not require a dedicated antigen-presenting cell but is under T cell-intrinsic developmental control. *Proc. Natl Acad. Sci. USA* **106**, 10278–10283 (2009).
62. Ruan, Q. *et al.* Development of Foxp3$^+$ regulatory T cells is driven by the c-Rel enhanceosome. *Immunity* **31**, 932–940 (2009).
63. Zheng, Y. *et al.* Role of conserved non-coding DNA elements in the *Foxp3* gene in regulatory T-cell fate. *Nature* **463**, 808–812 (2010).
References 37, 62 and 63 demonstrated that the NF-κB factor cREL is necessary and sufficient for inducing the differentiation of thymic T_{Reg} cells. In addition, the analysis of the *Foxp3* locus in this study revealed a conserved non-coding sequence (CNS3) as an important *cis*-element for the induction of *Foxp3* expression during thymic T_{Reg} cell development.
64. Feuerer, M., Hill, J. A., Mathis, D. & Benoist, C. Foxp3$^+$ regulatory T cells: differentiation, specification, subphenotypes. *Nature Immunol.* **10**, 689–695 (2009).
65. Lohr, J., Knoechel, B., Kahn, E. C. & Abbas, A. K. Role of B7 in T cell tolerance. *J. Immunol.* **173**, 5028–5035 (2004).
66. Tai, X., Cowan, M., Feigenbaum, L. & Singer, A. CD28 costimulation of developing thymocytes induces Foxp3 expression and regulatory T cell differentiation independently of interleukin 2. *Nature Immunol.* **6**, 152–162 (2005).
67. Lio, C. W., Dodson, L. F., Deppong, C. M., Hsieh, C. S. & Green, J. M. CD28 facilitates the generation of Foxp3$^-$ cytokine responsive regulatory T cell precursors. *J. Immunol.* **184**, 6007–6013 (2010).
68. Grewal, I. S. & Flavell, R. A. CD40 and CD154 in cell-mediated immunity. *Annu. Rev. Immunol.* **16**, 111–135 (1998).
69. Spence, P. J. & Green, E. A. Foxp3$^+$ regulatory T cells promiscuously accept thymic signals critical for their development. *Proc. Natl Acad. Sci. USA* **105**, 973–978 (2008).
70. Lio, C. W. & Hsieh, C. S. A two-step process for thymic regulatory T cell development. *Immunity* **28**, 100–111 (2008).
71. Fontenot, J. D., Rasmussen, J. P., Gavin, M. A. & Rudensky, A. Y. A function for interleukin 2 in Foxp3-expressing regulatory T cells. *Nature Immunol.* **6**, 1142–1151 (2005).
72. Malek, T. R., Yu, A., Vincek, V., Scibelli, P. & Kong, L. CD4 regulatory T cells prevent lethal autoimmunity in IL-2Rβ-deficient mice. Implications for the nonredundant function of IL-2. *Immunity* **17**, 167–178 (2002).
73. Burchill, M. A., Yang, J., Vogtenhuber, C., Blazar, B. R. & Farrar, M. A. IL-2 receptor β-dependent STAT5 activation is required for the development of Foxp3$^+$ regulatory T cells. *J. Immunol.* **178**, 280–290 (2007).
Together with reference 70, this study demonstrates that the development of thymic T_{Reg} cells can be divided into at least two steps based on the dependence on TCR stimulation: a proper TCR signal probably instructs thymocytes to develop into FOXP3$^-$ T_{Reg} cell precursors, which then express FOXP3 after acquiring an IL-2 signal without a continuous TCR signal.
74. Zorn, E. *et al.* IL-2 regulates FOXP3 expression in human CD4$^+$CD25$^+$ regulatory T cells through a STAT-dependent mechanism and induces the expansion of these cells *in vivo*. *Blood* **108**, 1571–1579 (2006).
75. Yao, Z. *et al.* Nonredundant roles for Stat5a/b in directly regulating Foxp3. *Blood* **109**, 4368–4375 (2007).
76. Burchill, M. A. *et al.* Linked T cell receptor and cytokine signaling govern the development of the regulatory T cell repertoire. *Immunity* **28**, 112–121 (2008).
77. Schallenberg, S., Tsai, P. Y., Riewaldt, J. & Kretschmer, K. Identification of an immediate Foxp3$^-$ precursor to Foxp3$^+$ regulatory T cells in peripheral lymphoid organs of nonmanipulated mice. *J. Exp. Med.* **207**, 1393–1407 (2010).
78. Gavin, M. A. *et al.* Foxp3-dependent programme of regulatory T-cell differentiation. *Nature* **445**, 771–775 (2007).
79. Hill, J. A. *et al.* Foxp3 transcription-factor-dependent and -independent regulation of the regulatory T cell transcriptional signature. *Immunity* **27**, 786–800 (2007).
80. Thornton, A. M. *et al.* Expression of Helios, an Ikaros transcription factor family member, differentiates thymic-derived from peripherally induced Foxp3$^+$ T regulatory cells. *J. Immunol.* **184**, 3433–3441 (2010).
81. Liu, Y. *et al.* A critical function for TGF-β signaling in the development of natural CD4$^+$CD25$^+$Foxp3$^+$ regulatory T cells. *Nature Immunol.* **9**, 632–640 (2008).
82. Ouyang, W., Beckett, O., Ma, Q. & Li, M. O. Transforming growth factor-β signaling curbs thymic negative selection promoting regulatory T cell development. *Immunity* **32**, 642–653 (2010).
83. Wirnsberger, G., Hinterberger, M. & Klein, L. Regulatory T-cell differentiation versus clonal deletion of autoreactive thymocytes. *Immunol. Cell Biol.* **89**, 45–53 (2011).
84. Bensinger, S. J., Bandeira, A., Jordan, M. S., Caton, A. J. & Laufer, T. M. Major histocompatibility complex class II-positive cortical epithelium mediates the selection of CD4$^+$ CD25$^+$ immunoregulatory T cells. *J. Exp. Med.* **194**, 427–438 (2001).
85. Fontenot, J. D. *et al.* Regulatory T cell lineage specification by the forkhead transcription factor Foxp3. *Immunity* **22**, 329–341 (2005).
86. Liston, A. *et al.* Differentiation of regulatory Foxp3$^+$ T cells in the thymic cortex. *Proc. Natl Acad. Sci. USA* **105**, 11903–11908 (2008).
87. Wan, Y. Y. & Flavell, R. A. Identifying Foxp3-expressing suppressor T cells with a bicistronic reporter. *Proc. Natl Acad. Sci. USA* **102**, 5126–5131 (2005).
88. Cabarrocas, J. *et al.* Foxp3$^+$ CD25$^+$ regulatory T cells specific for a neo-self-antigen develop at the double-positive thymic stage. *Proc. Natl Acad. Sci. USA* **103**, 8453–8458 (2006).
89. Lee, H. M. & Hsieh, C. S. Rare development of Foxp3$^+$ thymocytes in the CD4$^+$CD8$^+$ subset. *J. Immunol.* **183**, 2261–2266 (2009).
90. Aschenbrenner, K. *et al.* Selection of Foxp3$^+$ regulatory T cells specific for self antigen expressed and presented by Aire$^+$ medullary thymic epithelial cells. *Nature Immunol.* **8**, 351–358 (2007).
91. Proietto, A. I. *et al.* Dendritic cells in the thymus contribute to T-regulatory cell induction. *Proc. Natl Acad. Sci. USA* **105**, 19869–19874 (2008).
92. Klein, L., Hinterberger, M., Wirnsberger, G. & Kyewski, B. Antigen presentation in the thymus for positive selection and central tolerance induction. *Nature Rev. Immunol.* **9**, 833–844 (2009).
93. Li, J., Park, J., Foss, D. & Goldschneider, I. Thymus-homing peripheral dendritic cells constitute two of the three major subsets of dendritic cells in the steady-state thymus. *J. Exp. Med.* **206**, 607–622 (2009).
94. Mathis, D. & Benoist, C. Aire. *Annu. Rev. Immunol.* **27**, 287–312 (2009).
95. Nedjic, J., Aichinger, M., Emmerich, J., Mizushima, N. & Klein, L. Autophagy in thymic epithelium shapes the T-cell repertoire and is essential for tolerance. *Nature* **455**, 396–400 (2008).
96. Koble, C. & Kyewski, B. The thymic medulla: a unique microenvironment for intercellular self-antigen transfer. *J. Exp. Med.* **206**, 1505–1513 (2009).
97. Klein, L., Hinterberger, M., von Rohrscheidt, J. & Aichinger, M. Autonomous versus dendritic cell-dependent contributions of medullary thymic epithelial cells to central tolerance. *Trends Immunol.* **32**, 188–193 (2011).
98. Hubert, F. X. *et al.* Aire regulates the transfer of antigen from mTECs to dendritic cells for induction of thymic tolerance. *Blood* **118**, 2462–2472 (2011).
99. Gallegos, A. M. & Bevan, M. J. Central tolerance to tissue-specific antigens mediated by direct and indirect antigen presentation. *J. Exp. Med.* **200**, 1039–1049 (2004).
100. Daniely, D., Kern, J., Cebula, A. & Ignatowicz, L. Diversity of TCRs on natural Foxp3$^+$ T cells in mice lacking Aire expression. *J. Immunol.* **184**, 6865–6873 (2010).

Competing interests statement
The authors declare no competing financial interests.