Differentiation of regulatory Foxp3⁺ T cells in the thymic cortex

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Regulatory Foxp3⁺ T cells (T_R) are indispensable for preventing autoimmune pathology in multiple organs and tissues. During thymic differentiation T cell receptor (TCR)–ligand interactions within a certain increased affinity range, in conjunction with γ C-containing cytokine receptor signals, induce Foxp3 expression and thereby commit developing thymocytes to the T_R lineage. The contribution of distinct MHC class II–expressing accessory cell types to the differentiation process of Foxp3⁺ thymocytes remains controversial, because a unique role in this process has been ascribed to either thymic dendritic cells (tDC) or to medullary thymic epithelial cells (mTEC). Furthermore, it was suggested that the thymic medulla, where the bulk of the negative selection of self-reactive thymocytes takes place, provides a specialized microenvironment supporting T_R differentiation. Here, we report that the cortex, as defined by cortical thymic epithelial cells (cTEC), is sufficient for supporting T_R differentiation. MHC class II expression restricted to both cTEC and mTEC or to cTEC alone did not significantly affect the numbers of Foxp3⁺ thymocytes. Furthermore, genetic or pharmacologic blockade of thymocyte migration resulted in a prominent accumulation of Foxp3⁺ thymocytes in the cortex, demonstrating that secondary signals required for Foxp3 up-regulation exist in the cortex. Our results suggest that mTEC or tDC do not serve as a cell type singularly responsible for T_R differentiation and that neither the cortex nor the medulla exclusively provides an environment suitable for Foxp3 induction. Instead, multiple accessory cell types probably contribute to the thymic generation of regulatory Foxp3⁺ T cells.

immune tolerance | selection | thymus

Regulatory T cells (T_R) are indispensable for suppression of autoimmunity mediated by self-reactive T cells (1). Most peripheral T_R cells arise in the thymus, where up-regulation of the transcription factor Foxp3 is necessary for a subset of thymocytes to commit to the regulatory T cell lineage (2, 3). Foxp3 functions by regulating a broad set of genes required for T_R suppressor activity and for proliferative and metabolic fitness (3, 4) and by repressing alternative T cell differentiation fates (5). T_R cells originate from thymocytes expressing T cell antigen receptors (TCRs) with an increased affinity for self-peptide–MHC complexes (6). Although activated Foxp3⁺ T_R cells suppress immune responses in an antigen-nonspecific fashion, induction of the suppressor function by T_R cells seems to require antigen-specific stimulation through their TCR (7). These observations suggest that TCR specificity for tissue-restricted “self” antigens confers on the T_R cell the ability to prevent immune-mediated inflammation in the corresponding tissue (8).

Different types of antigen-presenting cells (APC) in the thymus display distinct repertoires of endogenous peptide–MHC complexes, in part because of differences in proteolytic processing machinery. For example, cortical thymic epithelial cells (cTEC), a cell type responsible for the bulk of positive selection but thought to be rather ineffectual at negative selection, use lysosomal cysteine proteinase cathepsin L (CatL) for MHC class II maturation and antigen processing and a unique proteasome subunit for MHC class I antigen processing (9). In contrast, tDC and medullary thymic

epithelial cells (mTEC) key APC-mediating negative selection of self-reactive thymocytes, and peripheral APC rely primarily on cathepsin S but not on CatL for MHC class II maturation (9). Importantly, mTEC, but not cTEC or tDC, are capable of expressing a broad range of tissue-restricted antigens via a poorly defined transcriptional mechanism dependent on nuclear factor Aire (10). This feature of mTEC led to the idea that selection of Foxp3⁺ T_R precursors on tissue-specific self-antigens displayed by mTEC is requisite for preventing tissue-specific autoimmunity (11). In support of this idea, thymocytes co-expressing a transgenic TCR differentiate into Foxp3⁺ T_R cells on encounter with its cognate ligand encoded by a transgene expressed in Aire⁺ mTEC (12).

T_R cells do not differentiate solely in response to a certain TCR cue but also require additional signals through IL-2R and CD28 (13, 14). The insufficiency of the TCR signal alone is demonstrated by studies in transgenic mice featuring a single TCR specificity and in experimental models relying on analyses of diverse TCR repertoires that revealed identical self-reactive TCR expressed by both Foxp3⁺ and Foxp3[−] cells in the thymus and in the periphery (15). The requirement for individual secondary signals seems to be contextual rather than absolute. For example, CD28 signals can be replaced by constitutive activation of the γ C-cytokine signaling target Stat5b (16). A requirement for TCR and an accessory signal suggests that particular thymic microenvironments or accessory cell types might be needed to support T_R lineage commitment. This issue has become further complicated by recent studies showing a 2-step process for T_R commitment, whereby γ C-cytokine signals can be received after TCR stimulation and still lead to induction of Foxp3 expression (16, 17).

Most Foxp3⁺ thymocytes are localized in the medulla in unmanipulated mice (18). Based on these observations and the aforementioned mTEC-driven up-regulation of Foxp3 in TCR-transgenic T_R cells, mTEC were proposed to serve as the key accessory cell for T_R differentiation. Alternatively, another recent study proposed that the purported differentiation of Foxp3⁺ T cells in the medulla did not result from reliance on mTEC but rather from the dense network of tDC. In this model, tDC gain the capacity to induce expression of Foxp3 in differentiating thymocytes on exposure to thymic stromal lymphopoietin (TSLP) (19). In contradiction to the previously mentioned studies, however, CD25⁺ and Foxp3⁺ CD4⁺ T cells with a suppressive capacity were found in transgenic mice with MHC class II expression restricted to cTEC (20, 21). A caveat for these studies, however, was that they did not ascertain whether the commitment of differentiating thymocytes to

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Fig. 1. Hemopoietic MHC class II expression is dispensable for commitment to the Foxp3⁺ lineage or gain of suppressor function. (a) B6 mice were irradiated and reconstituted with either Foxp3^{GFP} or MHC class II-deficient Foxp3^{GFP} BM. Eight weeks after reconstitution, commitment to the Foxp3⁺ lineage was analyzed by flow cytometry; representative flow profiles are shown ($n = 10, 6$). (b) Percentages of SP thymocytes that express Foxp3 and (c) absolute number of Foxp3⁺ SP thymocytes from Foxp3^{GFP} → B6 and *Ab1^{null}* Foxp3^{GFP} → B6 chimeras (mean $\pm$ standard deviation). (d) CD11c-Cre transgenic mice crossed with *IA^b flx/flx* mice showed highly efficient ablation of MHC class II expression on thymic CD11c⁺ cells (black line = Cre⁻; red line = Cre⁺). Representative profiles ($n = 3$). (e) flow cytometric analysis of the absolute number of CD4 SP thymocytes, (f) the percentage of CD4 SP thymocytes that express Foxp3, and (g) the absolute number of Foxp3⁺ SP cells (mean $\pm$ standard deviation).

T_R lineage occurred in the cortex; in line with the aforementioned 2-step process of T_R differentiation, one can envision a scenario in which TCR stimulation occurs in the cortex, but Foxp3 expression requires medullary production of cytokines. Additional studies have demonstrated that, on early expression of a TCR transgene with a high affinity to self, some double positive (DP) cells can be induced to express Foxp3 on antigen stimulation (22, 23). However, these studies did not address the anatomical location of these cells or whether these observations can be extended to physiological settings. Thus, a role for the thymic cortex versus medulla as the site of T_R lineage commitment and the roles of mTEC, cTEC, or tDC as essential accessory cells in this process remained obscure.

Here, we revisit the issue of the location of T_R lineage commitment (i.e., Foxp3 up-regulation) within the thymus to test a model of a dedicated role for thymic medulla in this process. Our studies demonstrated the competence of the thymic cortex to provide both TCR-dependent and TCR-independent signals to facilitate T_R lineage commitment and support the idea that the cortex is a site of generation of a substantial proportion of Foxp3⁺ thymocytes in normal mice.

Results

MHC Class II Expression by Thymic Dendritic Cells Is Dispensable for Foxp3⁺ T_R Lineage Commitment. Recent *in vitro* studies suggested that tDC exposed to TSLP in the thymic medulla gain the ability to facilitate generation of Foxp3⁺ T_R cells from naïve thymocytes (19). However, genetic evidence in support of a unique role for TSLP in T_R differentiation is lacking, because murine TLSP deficiency does not result in a detectable reduction in the numbers of Foxp3⁺ thymocytes and fails to induce a disease phenotype characteristic of T_R-cell deficiency (24). To test the numerical contribution of bone marrow (BM)-derived APC cells to the generation of the Foxp3⁺ thymocytes, we transferred MHC class II-sufficient *Ab1^{WT}* Foxp3^{GFP} or MHC class II-deficient *Ab1^{null}* Foxp3^{GFP} BM into irradiated RAG-1-deficient recipient mice. Flow cytometric anal-

ysis of MHC class II expression 8–10 weeks after BM transfer confirmed essentially complete reconstitution of BM-derived APC by cells of donor origin. Analysis of *Ab1^{null}* Foxp3^{GFP} BM-reconstituted mice showed a characteristic increase in the proportion of single-positive (SP) thymocytes resulting from deficient negative selection (Fig. 1a). Within the CD4⁺ SP thymocyte subset, a minor reduction in the proportion of Foxp3⁺ cells was observed (Fig. 1a and b). However, because of the numerical increase in size of the SP thymocyte subset, there was no reduction in the absolute numbers of Foxp3⁺ thymocytes in the absence of MHC class II expression by BM-derived APC (Fig. 1c). Based on the expression of cell-surface marker, Foxp3⁺ thymocytes selected with or without MHC class II on BM-derived APC were indistinguishable (data not shown). These results suggested that thymic BM-derived APC, primarily tDC, are dispensable for thymic differentiation of T_R cells. This conclusion is conditional upon the assumption that the requirements for thymic T_R differentiation are similar in irradiated BM chimeras and intact animals. To address this potential issue, we crossed mice harboring a conditional *IA^b flx/flx* allele with recently described mice expressing Cre recombinase transgene under the control of the CD11c promoter to induce an MHC class II deletion in DC (25, 26). We found essentially complete deletion of MHC class II in thymic dendritic cells (tDC), but its expression on thymic epithelial cells and thymic B cells was spared (Fig. 1d; data not shown). In agreement with the analysis of BM chimeras, we found a numerical increase in SP numbers in *IA^b flx/flx* CD11c-Cre mice (Fig. 1e) with a concomitant decrease in the percentage, but not in the absolute number, of Foxp3⁺ SP (Fig. 1f and g). Together, these results indicate that presentation of MHC class II by tDC was dispensable for Foxp3⁺ T_R lineage commitment.

Subset of Foxp3⁺ Thymocytes Localized to the Thymic Cortex. Previously, cTEC-restricted expression of MHC class II molecules was accomplished by introducing an A β transgene under the keratin 14 (K14) promoter into MHC class II-deficient *Ab1^{null}* mice (K14-A β)

Fig. 3. DP Foxp3⁺ cells are postselection. (a) Foxp3⁺ DP cells (line) compared with Foxp3⁻ DP cells (shaded) for CD69 expression. (b) Proportion of Foxp3⁺ cells among DP CD69⁻ cells (Left) and DP CD69⁺ cells (Right). Results are representative of three experiments. (c) Proportion of Foxp3⁺ cells among CCR7⁻ DP cells (Left) and CCR7⁺ DP cells (Right). (d) Percentage of CCR7⁻ DP and CCR7⁺ DP cells that are Foxp3⁺ (mean $\pm$ standard deviation, $n = 5$). (e) Representative histograms and (f) average (mean $\pm$ standard deviation, $n = 5$) of CCR7 expression on Foxp3⁻ DP cells (solid gray area), Foxp3⁺ DP cells (black line), Foxp3⁻ SP cells (blue line), and Foxp3⁺ SP cells (red line). (g) CD25 expression on Foxp3⁻ DP (red line), Foxp3⁻ SP (blue line), Foxp3⁺ SP (black line), and Foxp3⁺ DP (solid gray area) cells (representative of $n = 10$).

thymocytes from the thymic cortex to the medulla (28, 29). Although only 0.03% of CCR7^{lo} cells expressed Foxp3, the frequency of Foxp3⁺ cells within the CCR7^{hi} DP subset was increased by ≈ 70 -fold, reaching a proportion similar to that of Foxp3⁺ cells within the CD4 SP subset (Fig. 2 *c* and *d*). Furthermore, the level of CCR7 expression by Foxp3⁺ DP thymocytes was as high as that of SP thymocytes (Fig. 3 *e* and *f*). By contrast, the level of CD25 on DP Foxp3⁺ cells was intermediate between naïve thymocytes and SP Foxp3⁺ thymocytes (Fig. 3 *g*). These results suggest that DP Foxp3⁺ cells in the cortex are enriched within the small fraction of DP thymocytes that have up-regulated CCR7 enabling their migration from the cortex to the medulla.

Cortical Microenvironment Is Sufficient for Induction of Foxp3 Expression in Thymocytes. The notion of efficient differentiation of Foxp3-expressing thymocytes in the cortex was seemingly at odds with the observation that in both K14-A β^b *AbI*^{null} mice with the cTEC-restricted MHC class II expression and in WT mice most Foxp3⁺ thymocytes were found in the medulla (Fig. 2). A possible explanation for this discrepancy was that the up-regulation of Foxp3 in thymocytes in K14-A β^b *AbI*^{null} mice was induced in the cortex, but thereafter these Foxp3⁺ thymocytes migrated rapidly to the medulla. This migration would produce a scenario similar to the step

of DP-SP differentiation in positive selection, which is known to occur in the cortex, but SP thymocytes localize exclusively in the medulla because of the tight coupling of differentiation and CCR7 up-regulation (28, 29). Alternatively, in a model analogous to negative selection, the up-regulation of Foxp3 in thymocytes in K14-A β^b *AbI*^{null} mice might occur in 2 steps, in which TCR-MHC interactions in the cortex are required but are not sufficient to induce Foxp3 expression, and in which the medullary microenvironment is required to “complete” the process initiated in the cortex by providing a required second signal (17).

To distinguish formally between these 2 models, we inhibited G protein-coupled receptor signaling including chemokine receptors by short-term treatment of Foxp3^{GFP} mice with pertussis toxin (PT) at a concentration capable of blocking the migration of newly generated SP thymocytes from the cortex to the medulla (30). Mice treated with PT showed no increase in Foxp3⁺ DP cells (Fig. 4*a*) but had a dramatic increase in the number of Foxp3⁺ thymocytes in the cortex (Fig. 4*b*), indicating that the transition of late DP to the Foxp3⁺ SP thymocytes was not impaired in the presence of PT. Thus, these results demonstrate that the second step of the suggested 2-step T_R cell differentiation process (16, 17) is not limited to the medulla.

It was thought likely that PT treatment inhibits the migration of newly developing SP cells to the medulla through the inhibition of CCR7 signaling. To test this notion, we examined the localization of Foxp3⁺ thymocytes to the thymic cortex and medulla in CCR7-deficient mice. We observed an increased frequency of Foxp3⁺ cells in the cortex (Fig. 4*c*). To exclude potential effects of CCR7 deficiency on thymic epithelial cells and to examine the cortico-medullary distribution of CCR7-deficient and -sufficient Foxp3⁺ thymocytes in the same environment, we generated a series of BM chimeras. Specifically, irradiated *Rag1*^{null} recipients were reconstituted with Ly5.1 *Ccr7*^{wt} Foxp3^{GFP} or Ly5.2 *Ccr7*^{-/-} Foxp3^{wt} BM cells or with their mixture at a 1:1 ratio. This combination of BM donors allowed discrimination between *Ccr7*^{wt} and *Ccr7*^{-/-} thymocytes by flow cytometric analysis of Ly5.1 and Ly5.2 expression. Immunofluorescence analysis of Foxp3 and GFP expression distinguished between WT Foxp3⁺ cells (Foxp3⁺GFP⁺) and *Ccr7*^{-/-} Foxp3⁺ cells (Foxp3⁺GFP⁻). Although *Ccr7*^{-/-} thymocytes exhibit diminished migration to the medulla (28, 29), flow cytometric analysis revealed overall normal development of Ly5.1 and Ly5.2 thymocytes and a comparable size of Foxp3⁺ thymocyte subsets originating from WT and *Ccr7*^{-/-} BM (Fig. 4*c*). To validate our approach, we next examined the presence of Foxp3- and GFP-expressing cells in control chimeric mice reconstituted with only *Ccr7*^{wt} Foxp3^{GFP} or *Ccr7*^{-/-} Foxp3^{wt} BM. In the former mice, Foxp3 and GFP antibody staining was essentially overlapping, whereas in the latter Foxp3⁺ cells were present, but GFP⁺ cells were not (Fig. 4*e*). Similar analysis of GFP and Foxp3 immunofluorescence of thymuses in the mixed BM chimeras revealed that the rates of *Ccr7*^{-/-} Foxp3⁺ cells in the cortex were 10-fold higher than the rates of *Ccr7*^{wt} Foxp3⁺ cells (Fig. 4*e* and *f*). Together, the analyses of K14-A β^b *AbI*^{null} mice, PT-treated mice, and *Ccr7*^{-/-} mice demonstrated that the cortex is sufficient, whereas the medulla is dispensable for differentiation of Foxp3⁺ thymocytes and that predominant medullary localization of Foxp3⁺ thymocytes probably results from rapid CCR7-dependent migration following Foxp3 induction.

Our observation that cortical Foxp3⁺ DP cells represent one fourth of all Foxp3⁺ thymocytes (Table S2) and express high levels of CD69 and CCR7 is consistent with the idea that in WT animals Foxp3⁺ DP thymocytes also can differentiate into Foxp3⁺ SP thymocytes and contribute substantially to the Foxp3⁺T_R population. Previous studies of the differentiation of Foxp3⁺ thymocytes in neonates demonstrated that Foxp3 induction is delayed significantly (31). We found an analogous phenomenon occurs during the reconstitution of an irradiated thymus (Fig. S2). Because this time frame is much longer than the DP or SP thymic dwell time,

Fig. 4. Retention of SP cells in the thymic cortex does not impede Fxp3 commitment. The thymuses of PT-treated mice and CCR7-deficient mice were analyzed for Fxp3⁺ T cell location. (a) CD4-CD8 profiles and Fxp3 expression within DP and SP populations for untreated and PT-treated mice (representative sections, $n = 6$). (b) Thymic sections from untreated and PT-treated mice stained for Fxp3 (green) and CDR1/6C3 (cortex, blue) (representative sections, $n = 6$). (c) Thymic sections from wild type and *Ccr7*^{-/-} mice stained for Fxp3 (green) and CDR1/6C3 (cortex, blue). (d) Number of Fxp3⁺ thymocytes in Ly5.1 *Fxp3*^{GFP} BM, Ly5.1 *Fxp3*^{GFP} + Ly5.2 *Ccr7*^{-/-} mixed BM, and Ly5.2 *Ccr7*^{-/-} BM chimeras, for Ly5.1 (wild type, black bar) and Ly5.2 (*Ccr7*^{-/-}, white bar) cells, corrected for degree of BM chimerism. (e) Number of Fxp3⁺ cells in the cortex of Ly5.1 *Fxp3*^{GFP} BM, Ly5.1 *Fxp3*^{GFP} + Ly5.2 *Ccr7*^{-/-} mixed BM, and Ly5.2 *Ccr7*^{-/-} BM chimeras, using Fxp3 (Left) or GFP (Right). $n = 5$. (f) Thymic sections from Ly5.1 *Fxp3*^{GFP} + Ly5.2 *Ccr7*^{-/-} mixed BM chimeras, stained for CDR1/6C3 (cortex, blue) and either GFP (green; left) or Fxp3 (green; right) (representative sections, $n = 5$).

reconstitution of an irradiated thymus cannot be used to determine the temporal relationship between the Fxp3-expressing DP and SP populations. To establish the temporal relationship between the appearance of Fxp3-expressing DP and SP thymocyte subsets, we monitored the kinetics of their homeostatic regeneration following ablation in *Fxp3*^{DTR} mice. These knockin mice harbor “ablatable” thymic and peripheral Fxp3⁺ T cell populations because of the expression of a human diphtheria toxin receptor (DTR)-GFP fusion protein (1). In heterozygous female *Fxp3*^{wt/DTR-GFP} mice, random X chromosome inactivation leads to generation of 2 Fxp3⁺ T_R subsets of approximately equal size: a GFP⁺Fxp3⁺ T_R subset expressing DTR and, therefore, sensitive to diphtheria toxin (DT)-induced ablation, and a GFP⁻Fxp3⁺ T_R subset lacking DTR which is resistant. In agreement with our previous report, *Fxp3*^{wt/DTR-GFP} mice lost thymic and peripheral GFP⁺Fxp3⁺ T_R cells within 48 h of DT treatment, but these mice were fully protected from immune-mediated inflammation by DT-resistant GFP⁻Fxp3⁺ T_R cells because the expression of T cell activation markers or cytokine production remained unchanged (data not shown). Interestingly, we observed restoration of normal numbers of DP GFP⁺ Fxp3⁺ cells 1 day after cessation of DT treatment, and the SP Fxp3⁺ subset was fully restored to its original size 2 to 3 days later (Fig. 5). The rebound was not caused by a niche-filling mechanism, because compensation does not occur in *Fxp3*^{+/-} heterozygous females (Fig. S3). The delay in the rebound of Fxp3⁺ SP thymocytes compared with Fxp3⁺ DP thymocytes supports the notion that Fxp3⁺ DP thymocytes make a substantial numerical contribution to the Fxp3⁺ SP subset.

To test the precursor-product relationship between Fxp3⁺ DP and SP directly, we exploited a Fxp3 reporter enabling magnetic

Fig. 5. Product-precursor relationship between DP and SP Fxp3⁺ thymocytes. (a) Representative flow profiles of regenerated GFP⁺ cells in *Fxp3*^{wt/DTR-GFP} mice treated with two doses of DT at day -1 and day 0 and traced for reconstitution of GFP⁺ cells. (b) Mean $\pm$ standard deviation ($n = 3, 4, 4, 4, 3, 2$) for the absolute number of Fxp3⁺ DP and Fxp3⁺ SP cells. Day 0.1 represents mice injected with DT at day -1 and at -2 h. Short and long dashes indicate numbers of SP and DP GFP⁺ cells, respectively, in uninjected mice. (c) Expression of CD8 (Left) and Fxp3^{Thy1.1} (Right) on Ly5.1⁺Ly5.2 thymocytes (shaded) and purified Ly5.2 DP Fxp3^{Thy1.1} thymocytes 18 h after intrathymic injection into *Ly5.1* mice.

bead enrichment of Foxp3⁺CD25^{low} DP thymocytes. Thy1.1 was expressed on the surface of Foxp3⁺ T_R cells on insertion of the corresponding DNA sequence equipped with an internal ribosome entry site into the 3' UTR of the Foxp3 sequence (Fig. S4a).

We found tight coexpression of Foxp3 and the Thy1.1 reporter in both the thymus and peripheral tissue (Fig. S4 b–d). The combination of anti-Thy1.1 bead enrichment followed by FACS sorting allowed efficient purification of DP Foxp3^{Thy1.1+} cells (>90%) (Fig. S4e). Sorted DP Foxp3^{Thy1.1+} cells were injected into congenic Ly5.1 mice and 18 h later were found to have progressed to CD4⁺CD8[−]Foxp3^{Thy1.1+} SP thymocytes, in agreement with a precursor–product relationship between DP Foxp3⁺ cells and SP Foxp3⁺ cells. Taken together, our results strongly suggest that DP Foxp3⁺ cells differentiate into SP Foxp3⁺ thymocytes.

Discussion

Contrary to models ascribing a dedicated role for the thymic medulla, and for mTEC or tDC in particular, in inducing Foxp3 expression and, therefore, T_R lineage commitment (12, 19), we found the thymic cortex to be fully capable of supporting T_R differentiation. Indeed, a very modest decrease in the overall number of Foxp3⁺ thymocytes was observed when MHC class II was not expressed in mTEC and tDC. Furthermore, kinetic analysis of Foxp3⁺ thymocyte generation was consistent with a scenario that a sizeable proportion of SP Foxp3⁺ cells acquired Foxp3 expression as cortical DP thymocytes. Because the expression of CD69 and CCR7/Foxp3⁺ was increased in DP thymocytes, it seems likely that the TCR signaling leading to Foxp3 up-regulation in DP thymocytes was either coincident with or subsequent to positive selection.

Although these results show that the thymic cortex is sufficient for Foxp3 induction, they by no means argue against the ability of medulla and MHC class II⁺ tDC and mTEC to support differentiation of T_R cells. Indeed, it has been observed that flu HA-specific TCR transgenic thymocytes are able to commit to the Foxp3⁺ lineage on direct presentation of the transgene-encoded HA antigen expressed by Aire⁺ mTEC (12). Recent data demonstrating the ability of temporally discrete signals to induce Foxp3 (16, 17) also raise the possibility that some DP cells are primed through TCR signaling in the cortex and become Foxp3⁺ only upon later TCR-independent stimulation as SP cells in the medulla. Therefore, it is likely that Foxp3 induction is not limited to a single anatomical location and that multiple APC types including cTEC, mTEC, and

tDC are able to support the generation of Foxp3⁺ thymocytes and contribute to the peripheral T_R cell pool in normal animals. These findings raise a question about the TCR specificity of T_R cells selected by different APC types and their potency in preventing autoimmunity in different tissues. However, a definitive answer will require the development of new genetic models.

Materials and Methods

Mice. Foxp3^{GFP} (18), Foxp3^{KO} (2), Foxp3^{GFP-DTR} (1), Foxp3^{Thy1.1}, CD11c-Cre (25), Ab1^{tm1Gru} (Taconic), IAb^{fixdflx} (26), K14-IAb, and Ccr7^{tm1Dgen} (Jackson) mice were on the B6 background. Foxp3^{GFP} mice also were used on the B6.Ly5.1 background. BM chimeras were constructed using 7 × 10⁶ BM cells per recipient, injected i.v. into irradiated (900 rads) 6- to 10-week-old hosts. PT treatment consisted of 15 μg of PT administered i.p. 2.5 days before analysis. Intrathymic injection was performed on mice under tribromoethanol anesthesia. Thymocytes for intrathymic injection were enriched with MACS using anti-Thy1.1 with the magnetic activated cell sorting LS column system (Miltenyi Biotec) followed by FACS of CD4⁺CD8⁺Foxp3^{Thy1.1+} cells. 1.5 × 10⁶ cells were intrathymically injected in 40 μl (20 μl/lobe) and extracted for analysis at 18 h. Experimental mice were age- and sex-matched and were housed under specific pathogen-free conditions in accordance with guidelines from the Institutional Animal Care Committee of the University of Washington.

Flow Cytometry and Immunofluorescence. Five to 10-week-old mice were analyzed using the following antibodies: CD4-PerCP (PharMingen), CD8-PE-Cy7, CD25-PE, CD69-PE, Ly5.1-PE, Ly5.2-APC, MHC class II-APC, Foxp3-APC, and CCR7-APC (eBioscience). For CCR7 staining, cells were incubated for 60 min at 37°C before staining. For tDC staining, the thymus was minced and treated with 2 mg/ml collagenase D and 15 μg/ml DNase I (Roche) for 30 min at 37°C and treated with 5 mM EDTA/5% FBS/HBSS for 5 min at 37°C.

Thymic sections were prepared and stained as described in ref. 18, using polyclonal IgG anti-Foxp3 antibodies (2), polyclonal anti-GFP antibodies (Rockland), and anti-CDR1/6C3 (cortex). Estimations of cortex: medulla ratios were performed by analysis of serial sections (every 10th section through the thymus) using immunohistochemical staining with ER-TR5 supernatant. Estimations of frequency of cortical and medullary Foxp3⁺ cells were performed by immunofluorescence analysis of random 100 μm² sections of cortex and medulla in serial sections (every 25th section).

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