

TCR-dependent differentiation of thymic Foxp3⁺ cells is limited to small clonal sizes

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Numerous studies have highlighted the importance of high-affinity interactions between T cell receptors (TCRs) and their ligands in the selection of Foxp3⁺ regulatory T cells (T reg cells). To determine the role of the TCR in directing T cells into the Foxp3⁺ lineage, we generated transgenic (Tg) mice expressing TCRs from Foxp3⁺ cells. Initial analyses of the TCR Tg mice crossed with RAG-deficient mice showed that the percentage of Foxp3⁺ cells was very low. However, intrathymic injection and bone marrow chimera experiments showed a saturable increase of the Foxp3⁺ population when T reg TCR Tg cells were present in low numbers. Furthermore, when analyzing whole thymi of T reg TCR Tg RAG-deficient mice, we found significantly more Foxp3⁺ cells than in conventional T cell TCR Tg mice. Our results indicate that although the TCR has an instructive role in determining Foxp3 expression, selection of Foxp3⁺ individual clones in the thymus is limited by a very small niche.

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Abbreviations used: cDNA, complementary DNA; DN, double negative; DP, double positive; GTR, glucocorticoid-induced tumor necrosis factor receptor; MBP, myelin basic protein; SP, single positive; T conv, conventional T cell; T reg cell, regulatory T cell; Tg, transgenic.

Foxp3⁺ regulatory T cells (T reg cells) are one of the major cell types involved in the control of excessive T cell-mediated inflammatory responses (Zheng and Rudensky, 2007; Sakaguchi et al., 2008), as indicated by the lymphoproliferation and severe autoimmunity found in mice and humans deficient in FOXP3 (Chatila et al., 2000; Bennett et al., 2001a,b).

Interest in the TCR utilization of T reg cells surfaced when it was noted that myelin basic protein (MBP)-specific TCR transgenic (Tg) mice crossed to RAG-deficient mice developed spontaneous autoimmune encephalomyelitis, which did not occur in RAG⁺ MBP-specific TCR Tg mice (Lafaille et al., 1994). We postulated that lymphocytes that were selected through incomplete allelic exclusion in RAG⁺ MBP-specific TCR Tg mice but could not be selected in RAG-deficient MBP-specific TCR Tg mice had a protective effect on the development of spontaneous autoimmune disease (Lafaille et al., 1994). Our experiments showed that regulatory CD4⁺ T cells expressing endogenous TCR chains protected MBP-specific Tg RAG⁺ mice from disease (Olivares-Villagómez et al., 1998). Much later, when flow cytometry assays for Foxp3 expression became available, we showed that, indeed,

Foxp3⁺ T cells were missing from TCR Tg RAG-deficient mice (Shen et al., 2005). The surface expression of two TCR α chains was found in \sim 10% of MBP TCR Tg RAG⁺ T cells and was, by definition, absent in TCR Tg RAG-deficient T cells. However, we showed that the expression of two TCRs, with the subsequent changes in signaling which it entails, was not a prerequisite for T reg cell development (Olivares-Villagómez et al., 2000). Our results supported the view that T reg cells cannot be selected in the thymus unless they had the appropriate TCR (Olivares-Villagómez et al., 1998, 2000; Furtado et al., 2001).

Studies from several laboratories showed that TCR Tg RAG-deficient mice could develop T reg cells if the high-affinity ligands recognized by their TCR were present (Bensinger et al., 2001; Jordan et al., 2001; Apostolou et al., 2002). Previously, Modigliani et al. (1996) had presented a model whereby high-affinity selection on thymic epithelial cells was a requisite for selection of T reg cells that protected from rejection of allogeneic skin grafts.

Finally, TCR- α repertoire analysis on TCR- β chain Tg mice (Hsieh et al., 2004, 2006) or TCR- β Tg mice crossed with minilocus

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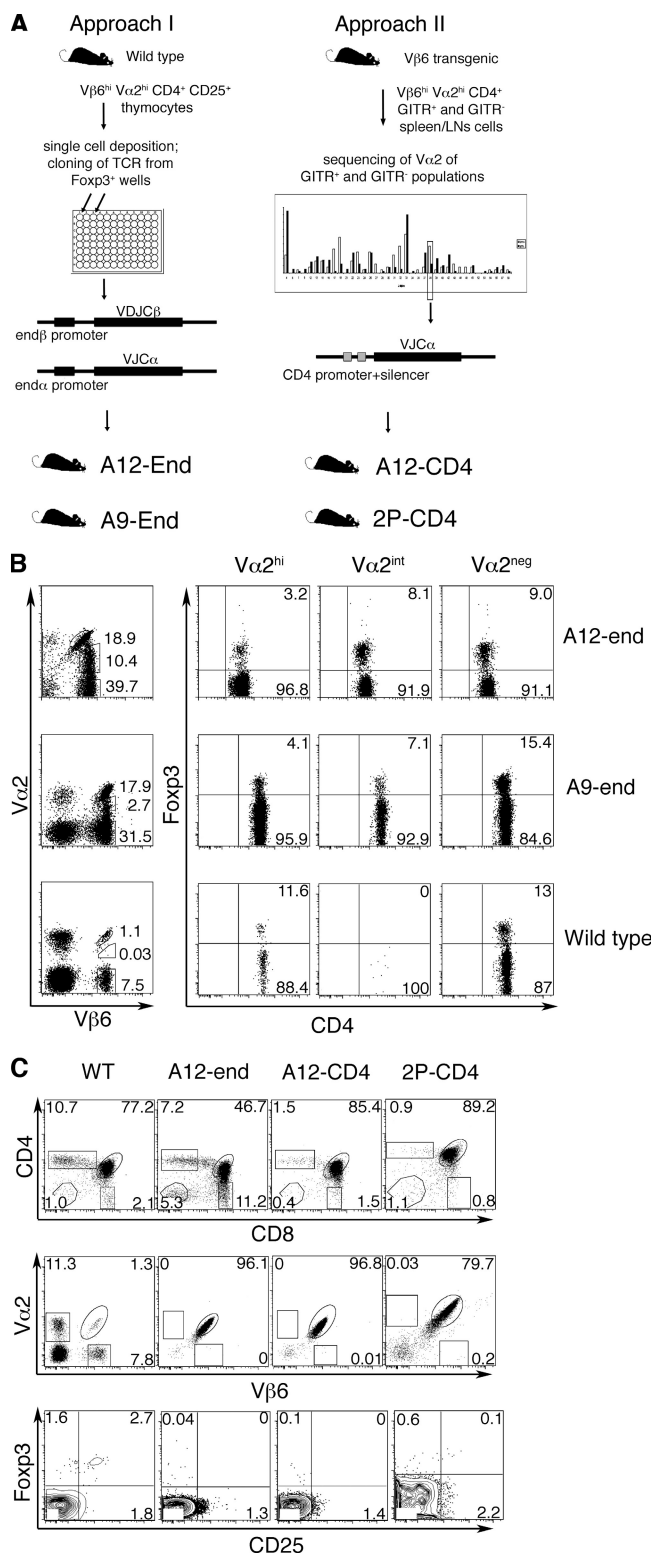


Figure 1. T reg TCR Tg mice have a low percentage of Foxp3⁺ cells in the thymus. (A) Schematic of two approaches used to choose T reg TCR for generating Tg mice (see Material and methods for more details). (B) Foxp3 flow cytometry analysis of A12-end and A9-end RAG⁺ splenocytes expressing different levels of Vα2. (C) Analysis of thymic Foxp3⁺ cells in T reg TCR RAG-deficient Tg lines. Foxp3 analysis was gated on

TCR-α Tg mice (Pacholczyk et al., 2006; Wong et al., 2007) showed that the repertoires of T reg cells and CD4⁺ conventional T cells (T conv cells) was largely different, although some degree of overlap was found in all studies. The possible nature of the repertoire overlap will be discussed later in this manuscript.

Based upon these data and those derived from the analysis of a large number of TCR Tg mice made from a variety of T cell types, we generated TCR Tg mice from Foxp3⁺ T cells. We speculated that TCR Tg made from a T reg cell-derived TCR would have two possible outcomes: the T reg cells would amount to a high percentage of all the T cells, as happens in most monospecific TCR Tg mice, or they would be limited to 3–5%, which would fill the putative Foxp3⁺ T cell niche to the size of a normal mouse T reg cell compartment.

In this manuscript, we describe the generation of TCR Tg mice with T reg cell-derived TCR and show that thymic selection of Foxp3⁺ T reg cells supported neither of the two predicted outcomes. Although the TCR instructed T cells into the T reg cell lineage as it does in non-T reg cells (e.g., CD8⁺ cells, CD4⁺ cells, γδT cells, and NKT cells), the size of the clonal T reg cell compartment was much smaller than clonal compartments of T conv cells. When introduced as transgenes, TCR from T conv cells lead to large clone size, sometimes reaching 40 or 50 million monospecific CD4 single-positive (SP) cells. In contrast, the size of the T reg cell clones reached a plateau at a very small number, with only a few thousand clonal Foxp3⁺ cells per thymus. We propose that the availability of high-affinity ligands for T reg cell selection is strongly limiting and thus restricts the number of clonal Foxp3⁺ cells.

RESULTS AND DISCUSSION

T reg TCR Tg mice have few clonal thymic Foxp3⁺ cells

To study the selection of Foxp3⁺ T cells, we generated TCR Tg mice, a method that has produced fundamental information on T cell selection, activation, and homeostasis, as well as the role of T reg cells in the control of autoimmune diseases. Three T reg TCR clones, A9, A12, and 2P, were independently selected by two different approaches summarized in Fig. 1 A. The T reg TCR Tg mice that we studied in detail were designated A9-end (A9 T cell with TCR-α expression under endogenous TCR-α promoter/enhancer; Kouskoff et al., 1995), A12-end (A12 T cell with TCR-α expression under endogenous TCR-α promoter/enhancer), A12-CD4 (A12 T cell with TCR-α expression under CD4 promoter/enhancer), and 2P-CD4 (2P T cell with TCR-α expression under CD4 promoter/enhancer). Table I summarizes all the T reg TCR Tg mice as well as the T conv TCR Tg mice used in this study.

CD4⁺ CD8[−] Vβ6⁺ Vα2⁺ cells. Numbers in the quadrant represent the percentage of cells of the indicated quadrant. Results are representative of at least three independent experiments with three or more mice per group.

Table 1. List of T reg and T conv TCR Tg mice

Tg lines	Type	TCR- β (promoter)	TCR- α (promoter)	TCR- β CDR3	TCR- α CDR3
A9-end	T reg	A9 V β 6 (endogenous)	V α 2.3 J α 12 (endogenous)	SISPRHTGQLY	SGTGGYKVV
A12-end	T reg	A12 V β 6 (endogenous)	V α 2.3 J α 28 (endogenous)	SRTGYHNSPLY	SVPGTGSNRLT
A12-CD4	T reg	A12 V β 6 (endogenous)	V α 2.3 J α 28 (CD4)	SRTGYHNSPLY	SVPGTGSNRLT
2P-CD4	T reg	A12 V β 6 (endogenous)	V α 2.7 J α 38(CD4)	SRTGYHNSPLY	LVGDNSKLI
MBP	T conv	MBP V β 8.2 (endogenous)	V α 4.3 J α 48 (endogenous)	SGDASGGNTLY	SENYGNEKIT
TB	T conv	OVA V β 8.2 (endogenous)	V α 13 J α 21 (endogenous)	GTTNTEVF	SPNYNVLY

When we analyzed A9-end and A12-end TCR Tg RAG⁺ H-2^u mice for V β 6, V α 2, CD4, and Foxp3 expression, we observed the expected dominant V β 6 expression together with the three populations of V α 2 (Fig. 1 B): V α 2-negative cells that express TCR- α chains encoded by the endogenous TCR loci; V α 2 intermediate cells that express two TCR- α chains, one encoded by the V α 2 transgene and the other by a non-V α 2 endogenous TCR- α chain; and V α 2-high cells that contain the majority of single TCR transgene-expressing V β 6 and V α 2 cells, together with some V α 2⁺ cells expressing endogenously arranged V α 2 loci. Foxp3 staining of the three V α 2 populations gave approximately WT frequencies in V α 2 negative and V α 2 intermediate populations; however, the frequency of Foxp3-expressing T cells in the V α 2 high population was remarkably low considering that this population contained the highest percentage of cells expressing the TCR- α and - β chains derived from Foxp3⁺ T cells (Fig. 1 B). Although the V α 2^{hi} V β 6⁺ population in TCR Tg RAG⁺ mice is by no means monoclonal, the paucity of Foxp3⁺ T cells in a population enriched for the T reg $\alpha\beta$ TCR suggested that the upper limit for selection of clonal T reg cells may be quite low. The subsequent analysis of RAG-deficient A9 and A12 mice confirmed our suspicion. Indeed, when we crossed A9-end and A12-end TCR Tg mice with RAG-deficient mice and analyzed their thymi, we found a very low frequency of Foxp3⁺ cells in A9-end (not depicted) and A12-end mice (Fig. 1 C).

In TCR Tg mice in which the TCR- α chain is expressed under the endogenous TCR transcription regulatory elements, the TCR- α chain is expressed very early during T cell development. As a consequence of this accelerated development, pre-TCR selection (β selection) is bypassed (Trop et al., 2000; Borowski et al., 2004), lineage selection is altered (Fritsch et al., 1998; Terrence et al., 2000; Lacorazza et al., 2001; Egawa et al., 2008), and thymus structure is abnormal (Goverman et al., 1997). These problems can be corrected by delaying expression of the TCR (Baldwin et al., 2005). As it was possible that the accelerated TCR expression would impinge on the capacity of the thymic cells to acquire Foxp3 expression, we generated new lines of T reg TCR Tg mice with TCR- α chain expressed under the CD4 promoter, enhancer, and silencer. This promoter/enhancer/silencer was expected to provide a more physiological timing of TCR- α expression than the endogenous TCR- α cassette.

We studied two CD4-driven TCR Tg lines designated A12-CD4 and 2P-CD4 (Fig. 1 A). As intended, the timing of TCR expression in the A12-CD4 and 2P-CD4 mouse lines was normal, with no expression during the double-negative (DN) stage (Fig. S1, A and B). TCR^{hi}-expressing cells were only found in the CD4⁺ SP population, and a TCR intermediate population was present and composed of CD4⁺CD8⁺ double-positive (DP) and CD8⁺ immature SP. This normal timing of development contrasts sharply with the accelerated expression of the TCR in the A9-end and A12-end TCR Tg mouse lines where a great proportion of TCR^{hi} cells are in the DN stage (Fig. S2 A). Finally, we characterized the distribution of DN1 to DN4 populations of A12-CD4 and 2P-CD4 Tg mice and found that, although the proportion of the DN1-DN4 cells did not exactly match that of WT mice, like WT mice, the predominant population was DN3, a stage which is poorly represented in A12-end and A9-end Tg mice (Fig. S2 B).

Having recapitulated a more physiological T cell development, we analyzed A12-CD4 and 2P-CD4 RAG-deficient H-2^{u/u} mice for the presence of Foxp3⁺ T cells in the thymus. In spite of the late TCR expression, A12-CD4 and 2P-CD4 RAG^{-/-} mice also harbored a very small population of Foxp3⁺ cells (Fig. 1 C).

The absolute number of TCR Tg Foxp3⁺ cells saturates at a low plateau

The low frequency of thymic Foxp3⁺ T cells found in all T reg TCR Tg mice that we generated suggested that Foxp3⁺ T cell thymic selection is unique, opening the possibility that the TCR could only play a limited role in the selection of this lineage. However, TCR Tg mice often have abnormal thymic structures (Goverman et al., 1997). To study the development of Foxp3⁺ T cells in a normal thymic environment, we used two approaches, intrathymic injections and BM chimeras.

We intrathymically injected T reg Tg RAG^{-/-} BM cells into WT thymi to obtain low repopulation of T reg TCR Tg cells developing in the midst of a large number of WT cells. Indeed, we obtained an exceedingly low frequency of T reg Tg-derived T cells. Importantly, we observed the first instance of a high frequency of Foxp3⁺ T cells among the T reg TCR Tg RAG^{-/-} CD4⁺ SP cells (range from 14 to 65%; Fig. 2 A). Furthermore, the total number of CD4⁺ Foxp3⁺

cells appeared to reach a plateau, suggesting the saturation of the Foxp3⁺ T cell generation (Fig. 2 A).

We also performed intrathymic injections of total thymocytes from Thy1.2 T reg and T conv TCR Tg RAG^{-/-} mice into Thy1.1 WT thymi. We consistently obtained a higher number and frequency of Foxp3⁺ cells derived from the T reg TCR Tg than from T conv TCR Tg cells. The frequency of 2P-CD4-derived Foxp3⁺ cells was the highest, followed by A12-end (Fig. 2 B). In contrast, under identical circumstances, thymocytes from the two T conv TCR Tg lines produced virtually no Foxp3⁺ cells (Fig. 2 B). Our results confirmed that T cells from T conv TCR Tg RAG-deficient mice do not differentiate along the Foxp3 pathway,

indicating that the TCR plays an important role in Foxp3⁺ lineage selection but that other factors are crucial because the majority of T reg TCR Tg cells still remained Foxp3⁻.

Next, we generated BM chimeras with TCR Tg RAG KO and WT cells. BM chimeras were established by transfer of A12-CD4 Thy1.2 cells into sublethally irradiated WT Thy1.1 hosts or by transfer of a 3:1 ratio of WT Thy1.1/A12-CD4 Thy1.2 BM mixture into RAG-deficient recipients (mixed BM chimeras). A wide range of T reg TCR Tg cells reconstitution was established in the hosts' thymi. Although there was not a high frequency of Foxp3⁺ T cells derived from the T reg Tg precursors recovered from a recipient's thymus compared with WT polyclonal cells, we

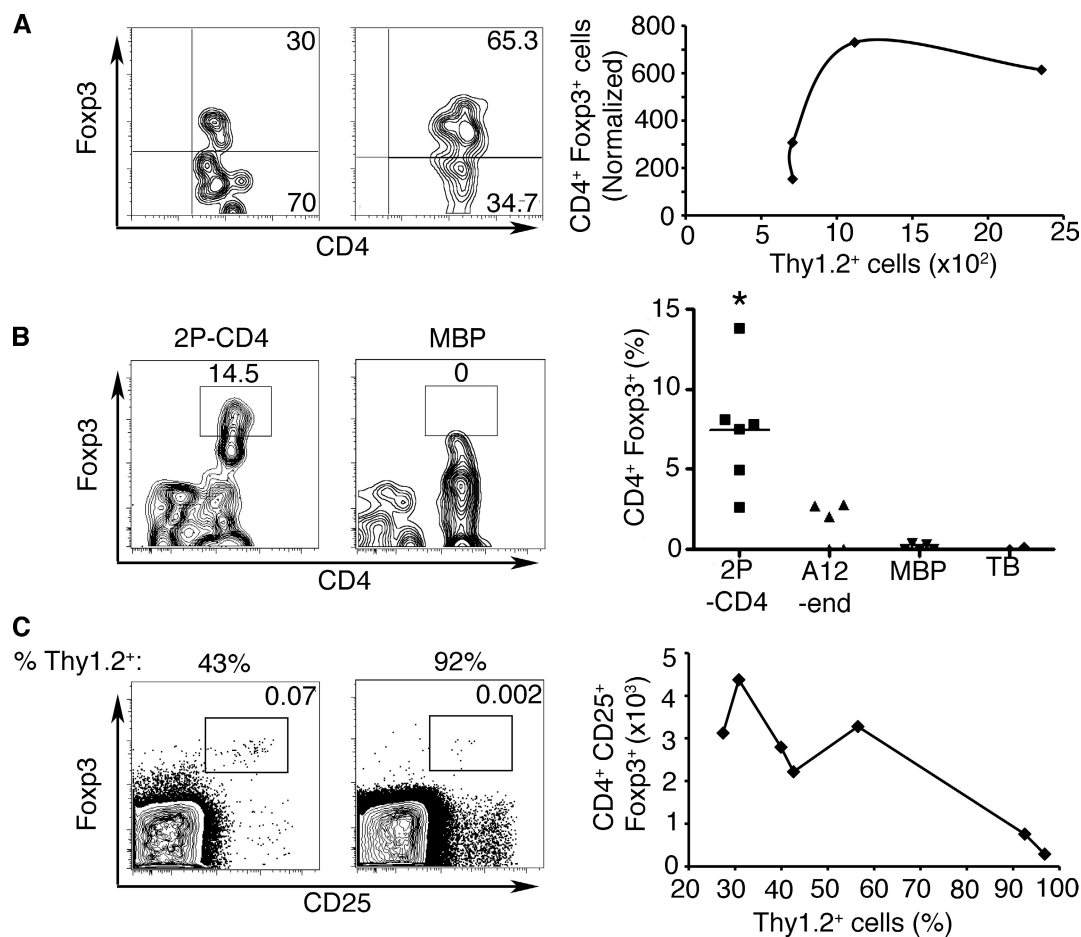


Figure 2. T reg monoclonal cells are present in high percentages but low absolute numbers upon intrathymic injection or generation of BM chimeras. (A) $\sim 5 \times 10^6$ Thy1.2 A12-end BM cells were injected intrathymically into 4–6-wk-old Thy1.1 WT hosts. 14 d after transfer, the mice were sacrificed and 10% of the thymus was analyzed. Representative Foxp3 stainings of gated on Thy1.2⁺ cells are shown on the left, and the quantification of recovered Foxp3⁺ cell numbers is shown on the right. (B) $\sim 50 \times 10^6$ cells total thymocytes were injected intrathymically into 4–6-wk-old Thy1.1 WT hosts. 14–20 d after transfer, individual thymus single cell suspensions were depleted with anti-CD8 beads before analyzed with flow cytometry. Representative Foxp3 staining of Thy1.2⁺ gated cells are shown on the left, and the quantification of Thy1.2⁺ CD4⁺ Foxp3⁺ cells from respective donor Tg lines is shown on the right. MBP and TB are T conv TCR Tg, whereas 2P-CD4 and A12-end are T reg TCR Tg. Asterisk indicates P < 0.05 compared with MBP. Each symbol represents an individual mouse from two independent experiments. The bar indicates the mean percentage of CD4⁺ Foxp3⁺ cells. (C) Mixed BM chimeras at a 3:1 ratio of Thy1.1 WT/Thy1.2 A12-CD4 RAG-deficient BM cells into RAG-deficient hosts were analyzed 10 wk after injection; 10% of the thymus was analyzed in the FACS. Representative Foxp3 staining gated on Thy1.2⁺ cells from different levels of reconstitution hosts are shown on the left, and the quantification of CD4⁺ CD25⁺ Foxp3⁺ cells relative to total Thy1.2⁺ cells recovered is shown on the right. Results are representatives of two independent experiments.

noticed that there was an inverse relationship between the frequency of reconstituted T reg Tg-derived T cells and the number of Foxp3⁺ T cells. When reconstitution was lower, there was a higher number of Foxp3⁺ cells (Fig. 2 C). It appeared that a plateau was reached at ~4,000 A12-CD4 Foxp3⁺ cells. Results essentially similar to the mixed chimeras were obtained with the simpler sublethal irradiation chimeras (unpublished data).

T reg TCR Tg RAG-deficient mice harbor limited but consistent number of Foxp3⁺ cells

Given the results in the preceding section showing that a small but consistent number of cells expressing the T reg TCR, but not T conv TCR, gave rise to Foxp3⁺ T cells, we reanalyzed the T reg TCR Tg RAG-deficient mice to assess whether or not T reg cells were reliably present in these mice, even if their frequency was low. To accurately count the number of thymic Foxp3⁺ cells derived from each T reg TCR clone, we analyzed 5% of the total thymic cells of T reg TCR Tg RAG^{-/-} mice, with the remaining 95% being used in its entirety to analyze the CD4⁺ SP population after CD8 depletion with beads (Moon et al., 2007). The analysis of the unfractionated cells (5%) allowed us to assess any losses that might have occurred during depletion of CD8⁺ cells. Because we passed the whole thymic CD4⁺ SP compartment through the flow cytometer, we could determine the total number of Foxp3⁺ cells in T reg TCR Tg A12-CD4, 2P-CD4 and A12-end, and T conv TCR Tg MBP and T-Bmc mice (Curotto de Lafaille et al., 2001). In addition, we analyzed T-Bmc mice crossed with Foxp3-deficient *scurfy* mice to ensure a more stringent negative control in case there were some exceedingly rare “real” Foxp3⁺ in thymi of T-B mc mice (Fig. 3 C). All the T reg TCR Tg RAG-deficient mice had significantly more Foxp3⁺ cells than the T conv TCR Tg RAG-deficient controls (Fig. 3, A and C). Foxp3⁺ cells generated in the thymus of T reg TCR Tg mice were CD69⁺ and CD24^{lo}, which is the same phenotype of WT Foxp3⁺ cells (Fig. 3 B).

Interestingly, when the number of Vβ6⁺Vα2⁺Foxp3⁺ cells in the WT thymus was determined, they were present at the same order of magnitude as the Vβ6⁺Vα2⁺Foxp3⁺ monoclonal populations of A12-CD4 and 2P-CD4 mice (Fig. 3 C). Considering that the Vβ6⁺Vα2⁺Foxp3⁺ population in the WT thymus is polyclonal, our results indicate that the transgenesis procedure does allow for an increase in the clonal size of Foxp3⁺ cells, although to a much lower degree than TCR transgenesis increases the clonal size of T conv cells. Finally, thymi from A12-CD4 mice had significantly more Foxp3⁺ cells than thymi from A12-end mice, suggesting that the late expression of the identical TCR-α chain improves the generation of Foxp3⁺ cells. This is interesting because it has been shown that pre-Tα-deficient mice have a higher frequency of T reg cells, indicating that lack of pre-Tα signaling has a higher impact on T conv cells than on T reg cells (Pennington et al., 2006). It is clear that late expression of the TCR-α chain allows a more normal pre-Tα signaling than the early expression of the same TCR.

The small number of Foxp3⁺ A12 and 2P Tg cells suggests that, unlike other selecting T cells, there is a strong intraclonal competition for limiting factors in Foxp3⁺ T cell development. Our data, collectively with the striking increase in T reg frequency observed when high-affinity ligands were expressed as transgenes in the thymus in previous studies (Bensinger et al., 2001; Jordan et al., 2001; Apostolou et al., 2002), suggest that the high-affinity natural ligands for A9, A12, and 2P are scarce and limiting, whereas low-affinity interactions contribute to the development of A9, A12, and 2P T cells into the conventional CD4 T cell compartment are abundant.

A12-CD4 and 2P-CD4 T reg TCR Tg RAG-deficient mice accumulate a large proportion of Foxp3⁺ T cells in the periphery

We also studied the distribution of Foxp3⁺ cells in the peripheral lymphoid organs of T reg TCR Tg RAG-deficient mice. Remarkably, the frequency of Foxp3⁺ cells was greatly increased in 2P-CD4 Tg compared with WT mice. A12-CD4 RAG-deficient mice had lower frequency of Foxp3⁺ cells compared with WT but a higher frequency than in A12-end and T conv TCR Tg mice (Fig. 4, A–C).

T reg cells have been considered to display enhanced autoreactivity compared with T conv cells, and the T reg cell autoreactivity does not result in pathogenicity, largely because of Foxp3 expression in these autoreactive T cells (Hsieh et al., 2004, 2006). However, a later study has questioned these conclusions (Pacholczyk et al., 2007). We did not find any evidence of enhanced autoreactivity in the T reg TCR Tg mice, which should have been more conspicuous given the fact that the majority of the T cells in the T reg TCR Tg RAG-deficient mice do not express Foxp3. Of all our founders studied, one A12-end founder had weight loss and diarrhea, but this was not restricted to the selecting H-2^u haplotype, and all other T reg TCR Tg founders made from the A12 TCR remained healthy. We do not know the specificity of the three T cells whose TCRs were used in this study; hence, it is possible that their pathogenic autoreactivity would not be apparent in the tissues that we examined.

To assess the activation state of the T cells in peripheral lymphoid organs, we stained peripheral T cells for activation markers and found that peripheral Foxp3⁺ cells in T reg TCR Tg RAG-deficient mice maintained a naive phenotype (Fig. 4 C). Furthermore, we labeled CD25⁺ cells from T reg TCR Tg RAG-deficient mice with CFSE and noticed that the peripheral expansion in lymphopenic hosts was normal. Moreover, acquisition of Foxp3 expression under lymphopenic conditions was not enhanced compared with WT mice (Fig. 4 D). It is also possible that the T reg TCR Tg cells are indeed more autoreactive, but the small number of Foxp3⁺ cells present in the T reg TCR Tg mice prevented the activation of the remaining of the cells.

From the thymic analysis of Vα2 transgenes expressed under the CD4 promoter, a reduction in number and frequency of mature CD4 SP cells was apparent (Fig. 1 C), and these mice displayed an augmented monoclonal Foxp3⁺ T cell population in the peripheral lymphoid organs (Fig. 4 A).

It is possible that these peripheral Foxp3^+ cells were either expanded from the few thymic Foxp3^+ cells whose presence we showed in this study or that they resulted from peripheral conversion of Foxp3^- cells, which we also showed. Indeed, most peripheral Foxp3^+ cells are CD44^{hi} and CD62L^{lo} (Fig. 4 C). DiPaolo and Shevach (2009) recently generated T reg TCR Tg mice with one TCR- α chain obtained from OTII TCR Tg mice. This is different from our case, as the TCR selected by DiPaolo and Shevach was the endogenous TCR- α chain from a T reg cell that coexpressed the OTII TCR- α chain. Therefore, the total TCR signaling of the original Foxp3^+ cell during thymic selection had contributions from the two

TCRs. Those authors observed a reduced number of CD4^+ SP thymocytes, which could be interpreted as a late deletion of the TCR Tg cells or perhaps a weak positive selection of the T cells expressing this TCR. Interestingly, the CD4 number could not be rescued by expressing the OTII TCR, a situation that recreated the dual TCR situation of the original T cell. As is the case in the TCR Tg mice described in the present study, there was a poor selection of Foxp3^+ cells when the TCR Tg mice were crossed to RAG-deficient animals (DiPaolo and Shevach, 2009).

Our data revealed dramatic differences in the thymic selection of T reg and T conv cells. The first conclusion is that

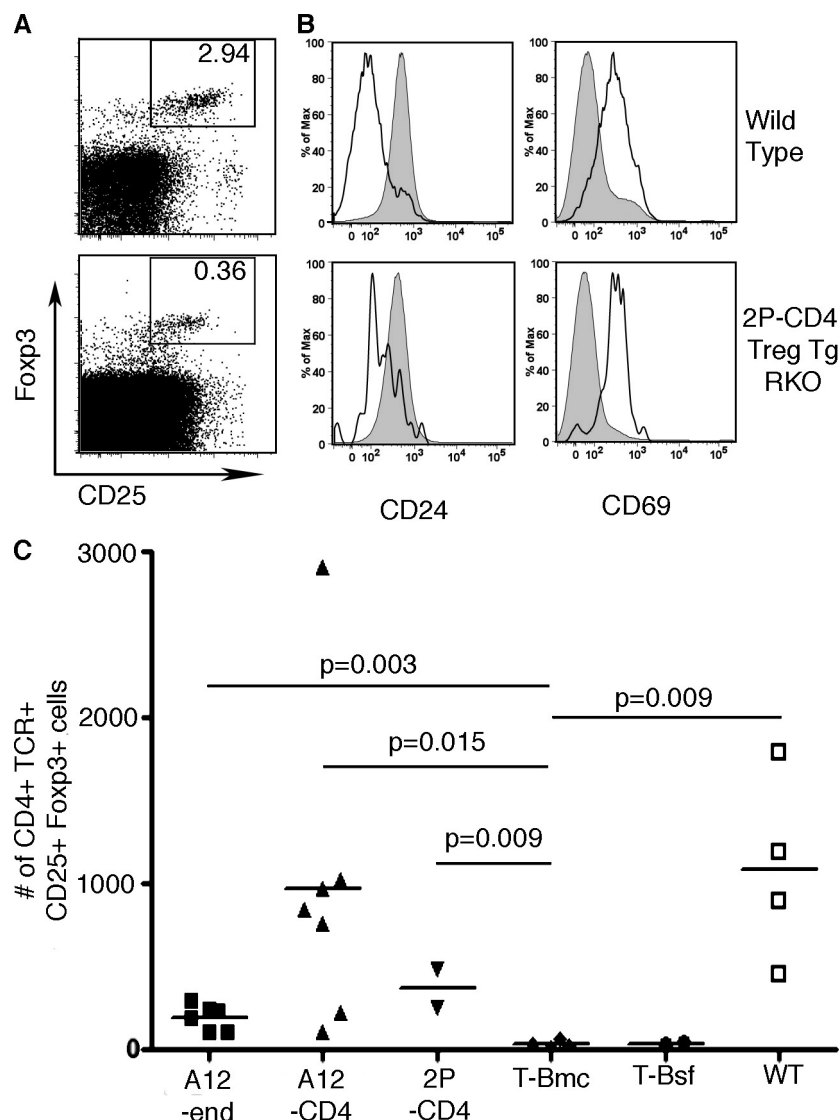


Figure 3. Foxp3^+ cells are present in the thymus of T reg TCR Tg mice at higher numbers than in T conv TCR Tg mice. 95% of whole thymi were depleted with CD8 beads before being analyzed using flow cytometry, whereas the remaining 5% was stained and analyzed undepleted. (A) Representative Foxp3 staining of WT and T reg TCR Tg RAG-deficient (2P-CD4) mice gated on $\text{CD4}^+ \text{V}\beta 6^+ \text{V}\alpha 2^+$ thymocytes after CD8 depletion. (B) CD24 and CD69 staining of $\text{CD4}^+ \text{Foxp3}^+$ (line) and $\text{CD4}^+ \text{Foxp3}^-$ (shaded) populations of WT and T reg TCR Tg RAG-deficient (2P-CD4) mice. (C) Quantification of absolute numbers of thymic $\text{TCR}^+ \text{CD4}^+ \text{CD25}^+ \text{Foxp3}^+$ cells in respective T reg TCR Tg RAG-deficient. A two-tailed Student's *t* test was used to calculate the indicated *p*-values. Each symbol represents an individual mouse from four independent experiments (A12-end, $n = 6$; A12-CD4, $n = 7$; 2P-CD4, $n = 2$; T-Bmc, $n = 4$; T-Bsf, $n = 3$; WT, $n = 4$). Bars indicate the mean number of $\text{CD4}^+ \text{TCR}^+ \text{CD25}^+ \text{Foxp3}^+$ cells in each group.

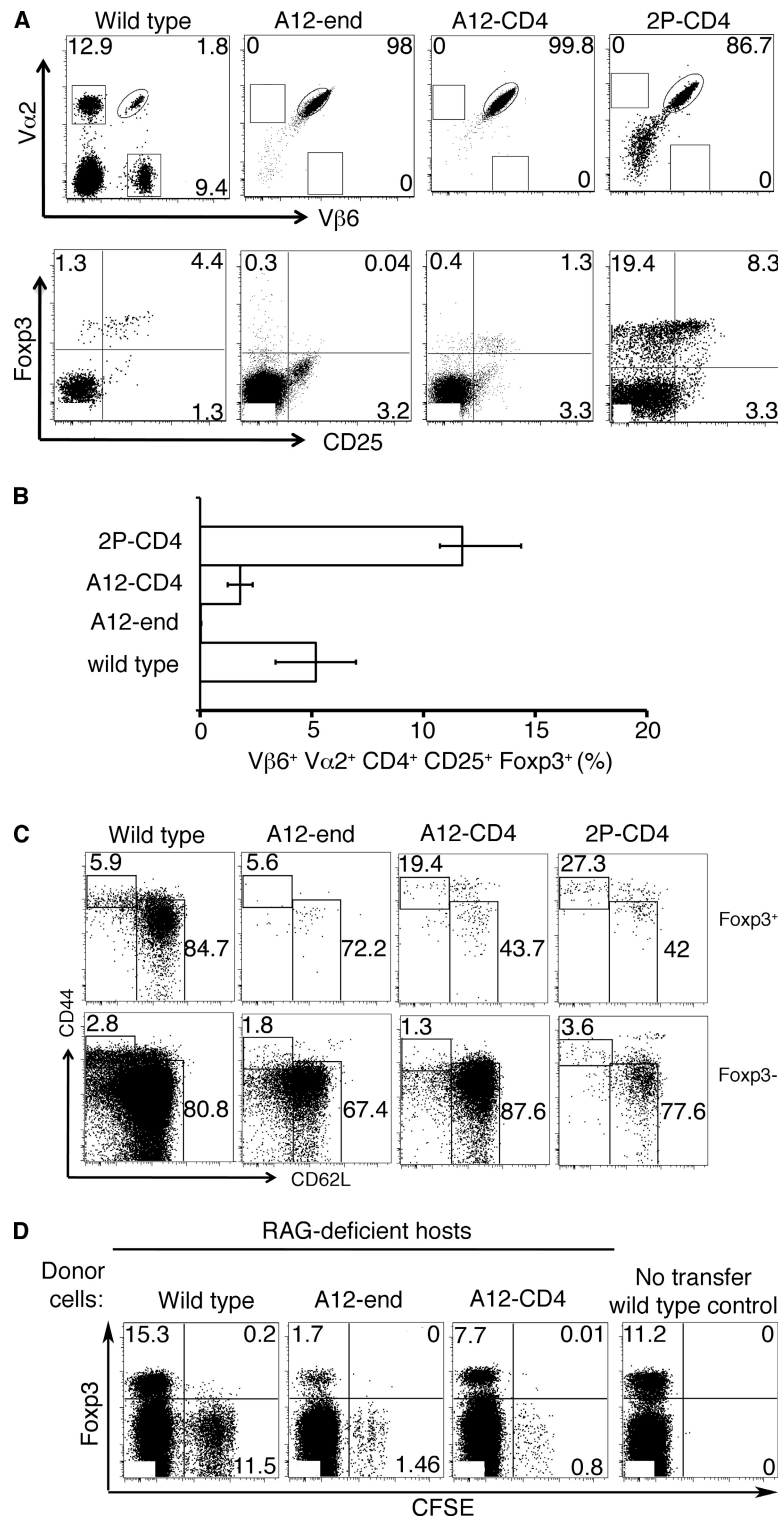


Figure 4. T reg TCR Tg mice generated using the CD4 constructs have increased numbers of Foxp3⁺ T cells in the peripheral lymphoid organs. (A) Analysis of Foxp3⁺ cells in T reg TCR Tg lines in pooled inguinal/axillary/brachial peripheral lymph nodes. Foxp3 analysis was gated on Vβ6⁺ Vα2⁺ CD4⁺ B220[−] cells. (B) Percentage of Vβ6⁺ Vα2⁺ CD4⁺ CD25⁺ Foxp3⁺ cells in T reg TCR Tg lymph nodes. At least three independent experiments of $n = 3$ per group are represented. (C) CD62L and CD44 staining of Vβ6⁺ Vα2⁺ CD4⁺ Foxp3[−] and Foxp3⁺ cells in peripheral blood. Results are representative of three independent experiments. (D) CD25[−] T cells from WT and T reg TCR Tg mice were labeled with CFSE and transferred into RAG1-deficient recipients. After 2 wk, pooled lymph nodes were collected and Foxp3 expression was analyzed by flow cytometry. Data is representative of three independent experiments with $n = 3$ per group.

there is an intense intraclonal competition for the ligands that induce Foxp3 expression and this competition results in a very small clone size for Foxp3⁺ T cells. Overflow created by a large excess of T cells with identical T reg TCR results in the development of Foxp3⁺CD4⁺ SP cells.

Our results indicate that the selection of T conv cells and T reg T cells has important differences. T conv cells are selected with the contribution of several low-affinity interactions, and several combinations of MHC-peptide complexes can achieve the required low avidity to select a given T conv clone. The space originated in the absence of one T conv TCR clone is readily occupied by other T conv clones. In the extreme, when only one T conv TCR clone is present as a result of TCR Tg expression, the size of this T conv TCR clone in the thymus is of the same order of magnitude as the size of the WT polyclonal T conv cells combined. The thymus of T conv TCR Tg mice is capable of selecting a large number of monoclonal T helper or CTL cell precursors. In contrast, for a developing T cell to express Foxp3, there is a much more defined ligand requirement. The ligands that induce Foxp3 expression in one TCR clone cannot readily induce Foxp3 expression in another TCR clone. It thus appears that repertoire of T reg cells in WT mice comprises by a sum of small clonal populations generated more independently than the clones of T conv cells. In Foxp3⁺ T reg selection there is strong intraclonal competition but less interclonal competition than in T conv cells. A question that remains to be answered is whether the different T reg cell clones behave in an additive (or quasi additive) manner, as our model will predict. In conclusion, our studies suggest thymic Foxp3⁺ cells selection is instructed by the TCR and is limited by a small niche.

MATERIALS AND METHODS

Mice. All T reg TCR Tg mice (A9-end, A12-end, A12-CD4, and 2P-CD4) were generated at the New York University Tg facility. A9 and A12 TCR- β and TCR- α genes were cloned into pT β and pT α cassettes provided by D. Mathis (Harvard Medical School, Boston, MA; Kouskoff et al., 1995). TCR- β and TCR- α constructs were injected into B6 and FVB/N fertilized eggs to generate A9-end and A12-end lines. We obtained eight different TCR- $\alpha\beta$ Tg founders in pure C57BL/6 (B6), two in FVB/N for A12-end, and four TCR- $\alpha\beta$ B6 founders for the A9-end. These mice were then backcrossed into a B10.PL H-2^b RAG^{-/-} background. 2P and A12 TCR- α genes were cloned into the CD4 promoter/silencer cassette provided by D.R. Littman (New York University School of Medicine, New York, NY). The TCR- α constructs were injected into A12-V β 6 Tg H-2^b RAG^{+/-} fertilized eggs to generate four A12-CD4 and two 2P-CD4 Tg founders.

MBP Ac1-11-specific TCR Tg $\alpha\beta$ KO mice have been described previously (Furtado et al., 2001). T-Bmc mice have D011.10 OVA-specific TCR transgenes and 17/9 influenza hemagglutinin-specific immunoglobulin knockin genes on a RAG-deficient background (Curotto de Lafaille et al., 2001). T-Bmc was crossed with Foxp3-deficient *scuffy* mice to generate T-Bsf (Curotto de Lafaille et al., 2008).

All mice were kept under specific pathogen-free conditions. All animal protocols were approved by New York University's Institutional and Animal Care and Use Committee.

T reg TCR clones selection criteria for generation of T reg TCR Tg mice. We used two different approaches and T reg surrogate markers in selecting TCR clones for generating T reg TCR Tg mice. Our first

approach was to sort single V β 6^{hi} V α 2^{hi} CD4⁺CD25⁺CD8⁻ SP thymic cells from WT C57BL/10.PL mice into each well of a 96-well plate. Single-cell PCR techniques were used to determine Foxp3 expression and the junctional V β 6 and V α 2 sequences. Two cells (designated A12 and A9), among the several Foxp3⁺ cells that yielded in-frame sequences for both V β 6 and V α 2 TCR, were selected for the first set of Tg constructs. In addition, we obtained an A12 V β 6-only TCR Tg line that was used to study the V α 2 repertoire of T reg and T conv cells, instrumental in picking our third T reg TCR (2P, see subsequent paragraphs).

Our second approach for the isolation of T reg TCR was the characterization of the V α 2 TCR repertoire of T reg and T conv T cells in V β 6-only TCR Tg RAG⁺ mice using glucocorticoid-induced tumor necrosis factor receptor (GITR) as a T reg surface marker. GITR expression correlates well with Foxp3 expression in V β 6-only Tg mice (Fig. S3 A). V β 6-only TCR Tg RAG⁺ mice express the TCR- β chain of A12 cells mentioned in the previous paragraph and have normal percentage of Foxp3⁺ cells (Fig. S3 B). We sorted V β 6^{hi} V α 2^{hi} CD4⁺ GITR^{hi} and GITR^{neg} and analyzed 310 TCR- α sequences, 117 from GITR^{hi} V α 2^{hi} cells and 193 from GITR⁻ V α 2^{hi} cells. To make Tg mice, we selected a prototypical V α 2 sequence that repeated in the GITR⁺ population and was not found in the GITR⁻ population of four mice. In these V β 6-only TCR Tg mice, the usage of the different J α segments by V α 2^{hi} cells was different between GITR⁺ and GITR⁻ cells, as determined by real time PCR (Fig. S4); furthermore, the real-time PCR data correlated well with J α usage observed by junctional sequencing. The TCR- α gene from typical GITR⁺ cells was designated 2P.

We generated two lines of T reg TCR Tg mice with TCR- α chain expressed under the CD4 promoter, enhancer, and silencer. This promoter is expected to allow TCR expression in the CD4⁺CD8⁺ DP cells and CD4 SP cells but not at early stages of development or on CD8 SP cells. Two T reg TCR Tgs generated with the CD4 promoter were TCR- α of A12, selected from single cell cloning, and 2P, a prototypical T reg TCR- α chain as determined by sequence analysis of GITR⁺ peripheral T reg cells and GITR⁻ T conv cells in A12 V β 6-only TCR Tg mice. It is noteworthy that both TCR- α chains (A12 and 2P) naturally pair with the same A12 V β 6 chain. We designated the CD4-driven TCR $\alpha\beta$ Tg lines as A12-CD4 and 2P-CD4.

Single cell deposition for T reg TCR cloning. V β 6⁺ V α 2⁺ CD4⁺CD25^{hi} thymocytes were sorted from WT B10.PL mice by a MoFlo single cell depositor (Dako) into 96-well plates. Complementary DNA (cDNA) was synthesized and real-time PCR was used to confirm Foxp3 expression in each well. To clone V β 6 and V α 2 TCR genes, high-fidelity PCR reactions were performed with Ex-Taq (Takara) and the following primers: V α 2, 5'-CAGGTCGAACATGGACAAGATCCTGACAGCA-3'; C α 2, 5'-CAGGTCAGTACGACTCAACTGGACCACCACAGCC-TCA-3'; V β 6, 5'-CTGTACAGTAGGCCGGGTG-3'; and C β , 5'-CTCCAACTATGAAGAAGTG-3'.

V α 2 repertoire studies by DNA sequencing. To study V α repertoire, we sorted V β 6^{hi} V α 2^{hi} CD4⁺ GITR^{hi} (T reg) cells and V β 6^{hi} V α 2^{hi} CD4⁺ GITR^{neg} (T conv) cells from A12-V β 6 Tg-pooled splenocytes/lymph nodes. cDNA was synthesized and V α 2 chains were amplified by high-fidelity Ex-Taq with V α 2-specific primers. PCR products were then inserted into pCR 2.1 vectors for sequencing (Macrogen). CDR3 amino acid sequences were compared between GITR⁺ and GITR⁻ populations. 2P was chosen for its absence in GITR⁻ population and repeated appearance in GITR⁺ population in four individual experiments.

Real-time PCR. V β 6 splenocytes were labeled with CD4, V α 6, V α 2 and GITR. GITR^{hi} and GITR^{neg} populations were Mo-Flo sorted, RNA was extracted, and cDNA was synthesized. Primers were V α 2 sense (5'-TCTCCTTGACATCAGAGACTC-3') paired with J α 4R (5'-AGTCTGGTCCCT-GCTCCAA-3'), J α 28R (5'-GTGCCTTTCCCAAAAGTGAG-3'), J α 32R (5'-TCCCAATTCCCAATGAGC-3'), J α 33R (5'-GGTTCCTCCAGAGCCCCAGAT-3'), and J α 38R (5'-TACCAGAGACTTGTCCCAAGC-3') to analyze respective J α quantitatively.

Intrathymic injection. After animals were anesthetized with acepromazine, ketamine, and xylazine, the sternum was cut open to access the thymus. $\sim 5 \times 10^6$ BM cells or $\sim 5 \times 10^7$ total thymocytes were injected in 10 μ l suspension into the thymus of 4–6-wk-old WT B10.PL Thy1.1 mice. Animals were placed on a heat pad until they regained consciousness. After the surgery, all animals were given antibiotic in drinking water until the time of sacrifice.

BM chimera. BM chimeras were generated using two methods. WT B10.PL Thy1.1 mice were sublethally irradiated and reconstituted with BM from A12-end Thy1.2 RAG^{-/-} mice. Alternatively, WT B10.PL Thy1.1 BM cells were mixed with A12-CD4 Thy1.2 RAG-deficient BM cells at a 3:1 ratio and coinjected into RAG^{-/-} mice to generate mixed BM chimera. After 2 mo, mice were sacrificed and thymic and peripheral lymphoid organs were removed for analysis of Foxp3 expression.

Whole thymus FACS analysis. Single cell suspensions were prepared from whole thymic and 95% of thymic were depleted of CD8 cells by labeling cells with anti-CD8 microbeads and passing through magnetic LD columns (Miltenyi Biotec). Total sample of the CD8-depleted fractions and the 5% unmanipulated fractions were acquired on LSRII machine.

Adoptive cell transfer. Cells from peripheral lymph nodes and spleens were labeled with anti-B220, CD8, CD25, CD49b, and CD11b PE antibodies and depleted of these labeled cells with anti-PE microbeads. The flow through fraction from magnetic LD column were labeled with CFSE at 37°C for 10 min and washed with cold PBS before 10^6 CD4 cells were transferred into RAG-1 deficient mice. After 2 wk, pooled secondary lymph organs were analyzed by flow cytometry.

Online supplemental material. Fig. S1 depicts V β 6 and V α 2 expression in DN stages of T reg TCR Tg. Fig. S2 shows that T reg TCR Tg mice with TCR- α under CD4 promoter have dominant DN3 population and TCR^{hi}-expressing cells in CD4⁺ SP but not in CD4⁺ CD8⁺ DN cells. Fig. S3 shows that Foxp3 expression in V β 6 Tg R⁺ mice is comparable to WT and correlates well with GTR. Fig. S4 shows J α usage of the V β 6⁺ V α 2⁺ CD4⁺ GTR⁺ and GTR⁻ population. Online supplemental material is available at <http://www.jem.org/cgi/content/full/jem.20091033/DC1>.

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