The Journal of Immunology

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J Immunol 2012; 189:3831-3837; Prepublished online 17 September 2012;
doi: 10.4049/jimmunol.1201564
http://www.jimmunol.org/content/189/8/3831

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

The Thymic Niche Does Not Limit Development of the Naturally Diverse Population of Mouse Regulatory T Lymphocytes

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Thymus-derived CD4$^+$Foxp3$^+$ regulatory T lymphocytes (Tregs) play a central role in the suppression of immune responses to self-antigens and thus avoid autoimmune disorders. It remains unclear if the specialized thymic niche controls the number of differentiating Tregs. We investigated development of murine Tregs from precursors expressing the naturally very large repertoire of TCRs. By analyzing their developmental kinetics, we observed that differentiating Tregs dwell in the thymus ~1 d longer than their conventional T cell counterparts. By generating hematopoietic chimeras with very low proportions of trackable precursors, we could follow individual waves of developing T cells in the thymus. We observed strongly increased proportions of Tregs at the end of the waves, confirming that these cells are the last to leave the thymus. To assess whether the thymic niche limits Treg development, we generated hematopoietic chimeras in which very few T cell precursors could develop. The substantial increase in the proportion of Tregs we found in these mice suggested a limiting role of the thymic niche; however, this increase was accounted for entirely by the prolonged thymic dwell time of Tregs. We conclude that, when precursors express a naturally diverse TCR repertoire, the thymic niche does not limit differentiation of Tregs. ***The Journal of Immunology*****, 2012, 189: 3831–3837.**

During the lifetime of mammals, hematopoietic progenitor cells constantly migrate from the bone marrow (BM) to the thymus, where they undergo a complex process of differentiation to produce mature T lymphocytes. T cells express a receptor (the TCR for Ag) that recognizes peptide Ags presented by highly polymorphic MHC molecules on the surface of APCs. The random nature of *Tcr* gene rearrangements, which generates an enormous diversity of TCRs capable of recognizing a wide variety of pathogens, inevitably produces T cells that react with self-peptide Ags presented by self-MHC molecules. The majority of these "dangerous" autospecific T cells are neutralized during thymocyte development by deletion and/or induction of anergy, a process called negative selection.

Autospecific T cells that escape negative selection and enter the peripheral circulation are actively controlled by regulatory T lymphocytes (Tregs), thus preventing autoimmune disorders. Like other T cells, most of these Tregs differentiate in the thymus. The most extensively studied Treg population is characterized by expression of the coreceptor CD4 and the transcription factor Foxp3 (CD4$^+$Foxp3$^+$) (1). The Treg repertoire is enriched in autospecific cells (2, 3), and it is therefore different from the conventional T cell (Tconv) repertoire. These findings raise several important questions about the mechanisms involved in selection of these two T cell populations in the thymus. Like Tconvs, positive selection of Treg precursors requires their interaction with epithelial cells in the cortical region of the thymus (4–6). Unlike Tconvs, however, Tregs are quite resistant to negative selection in the medulla (7–9); in TCR transgenic mice and other experimental systems, expression of the Ag recognized by the TCR even enhanced development of Tregs (10–14). The absolute numbers of Tregs developing in these experimental systems was, however, very small and hardly exceeded that found in wild-type (wt) animals (15). This suggests that the number of Tregs that develop in an animal is strictly limited.

In mice in which thymocytes expressed only a single transgenic TCR derived from a Treg, very few Tregs developed (16–18). It was shown that a saturable "selecting" niche in the thymus controls the size of developing Treg clones. Whether the thymic microenvironment limits development of Tregs from the naturally very diverse repertoire of T cell precursors (19–21), however, remains unclear. It has previously been shown that costimulatory and other cell-surface molecules, as well as cytokines and lipid mediators, are involved in Treg development (reviewed in Ref. 22). Limited availability of such factors, which are provided by the specialized thymic niche, may therefore control the development of the naturally diverse repertoire of Tregs.

Studies addressing a potentially limiting role of the thymic niche can be performed by generating hematopoietic chimeras in which the number of wt selectable precursors is reduced by dilution with mutant nonselectable precursors (23, 24). Because nonselectable precursors do not compete for the occupancy of the thymic

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Received for publication June 8, 2012. Accepted for publication August 18, 2012.

This work was supported by institutional funds from INSERM, Centre National de la Recherche Scientifique, and Université Paul Sabatier; the Agence Nationale pour la Recherche, Grant ANR-08-BLAN-0187; Région Midi-Pyrénées Grant 08004389; and the Vlaams Instituut voor Biotechnologie.

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The online version of this article contains supplemental material.

Abbreviations used in this article: BM, bone marrow; CD4SP, CD4$^+$CD8$^-$ single positive thymocyte; CD8SP, CD4$^-$CD8$^+$ single positive thymocyte; DN, CD4$^-$CD8$^-$ double-negative thymocyte; DP, CD4$^+$CD8$^+$ double positive thymocyte; EGFP, enhanced GFP; Tconv, conventional T lymphocyte; Treg, regulatory T lymphocyte; wt, wild-type.

selecting niche in the reported experimental models, positive selection of wt precursors is increased. We hypothesized, however, that the developmental kinetics of Tconvs and Tregs might influence the output of such analyses. Fewer than 10 precursor cells home from the BM to the thymus each day (25–27). These immigrants to the thymus will divide and differentiate, giving rise to a wave of T cell development lasting ∼4 wk (28, 29). At very low levels of chimerism (i.e., the proportion of tracked cells among bone marrow precursors in mixed hematopoietic chimeras), these tracked precursors will colonize the thymus only very infrequently, potentially giving rise to distinguishable waves of developing T cells. We developed this approach and then, first, used it to study the kinetics of Treg development. Then, we assessed the potentially limiting role of the thymic niche in the development of Tregs by generating mixed hematopoietic chimeras in which low numbers of selectable T cell precursors developed in the thymus.

Materials and Methods

Mice

Wt C57BL/6 mice were purchased from the Centre de Recherche et d'Elevage Janvier (Le Genest St. Isle, France). C57BL/6 Thy1.1 and C57BL/6 CD45.1 mice were purchased from Charles River Laboratories (L'Arbresle, France). C57BL/6 mice deficient in TCRα (30) were obtained from the Centre de Développement des Techniques Avancées (Orléans, France). B6.Cg-*Foxp3*^{sf} mice were purchased from Jackson Laboratory (Sacramento, CA). C57BL/6-Tg(CAG-EGFP)C15-001-FJ001Osb mice were provided by Dr. Béatrice Cousin (Toulouse, France). *Rag2-Gfp* transgenic mice (31) were supplied by Dr. Pamela Fink (Department of Immunology, University of Washington, Seattle, WA) (32). *Foxp3*$^{Thy1.1}$ knockin mice were provided by Dr. Alexander Rudensky (Howard Hughes Medical Institute and Immunology Program, Sloan-Kettering Institute, New York, NY) (6). All experiments involving animals were performed in compliance with relevant laws and institutional guidelines (regional approval no. 31-13, ethical review no. MP/02/32/10/03; license LA1210570, project P019-2009).

BM chimeras

Chimeras were generated as previously described (13).

Calculation of the "age" postpositive selection of thymocyte populations in Rag2-Gfp *mice*

In *Rag2-gfp* transgenic mice, the *Gfp* transgene is expressed as long as the *Rag2* promoter is active. After shutdown of this promoter at positive selection, GFP degrades with the half-life $t_{1/2}$. Therefore, the GFP fluorescence F remaining at time t, $F(t)$, equals $2^{-(t/t1/2)} \times F(0)$:

$$F\,(t) \;=\; 2^{-\,(t/t1/2)} \times F(0).$$

It follows:

$$\log_2\,[F\,(t)/F(0)] = -\left(t/t_{1/2}\right)$$

$$-\,t_{1/2} \times \log_2\,[F(t)/F(0)] \;=\; t.$$

Because $t_{1/2} = 56\ \text{h} = 2.3\ \text{d}$ (29):

$$t = -\,2.3 \times \log_2\,[F(t)/F(0)]\ \text{d}.$$

Analysis of early, mid, and late waves in hematopoietic chimeras

All data obtained from the 61 chimeric mice reconstituted with 2% test and 98% fill BM were used in this analysis. We first calculated the means and the standard deviations of the percentages of $CD4^+CD8^+$ double positive thymocytes (DP) (85.6 ± 2.4%) and $CD4^+CD8^-$ single positive thymocytes (CD4SP) (8.3 ± 1.4%) among fill thymocytes of the chimeras. High percentages of test cells mean that they exceeded the mean fill percentages by more than 2 SD; low percentages of test cells mean that they were lower than 2 SD below the mean percentage of fill cells. In early wave chimeras, the percentage of test DP cells was high, and that of CD4SP cells was low; in midwave chimeras, the percentages of DP and CD4SP cells were between the high and low values; and in late wave chimeras, the percentage of DP cells was low, and that of CD4SP cells was high.

Results

Individual waves of developing T cells can be visualized in hematopoietic chimeras

We devised a way to observe individual waves of T cell development. We generated chimeric mice by reconstituting lethally irradiated hosts with a mixed population of 98% wt BM cells (we call them fill cells) and 2% BM cells (the test cells) that could be tracked by their expression of transgenic enhanced GFP (EGFP) (21 chimeras) or allelic forms of Thy1 (30 chimeras) or CD45 (10 chimeras) (Supplemental Fig. 1A). We hypothesized that if the proportion of test cells in the reconstituting population were sufficiently low, the very low frequency at which they enter the thymus would allow us to visualize distinct waves of test T cell development. To assess the validity of this hypothesis, 5 wk after injection of the BM cells we analyzed by flow cytometry the fate of the test precursor cells that had migrated to the thymus (Supplemental Fig. 1A).

Highly variable proportions of cells of test bone marrow origin (i.e., test chimerism) were found in the thymi of the chimeras generated (Fig. 1A, 1B). To gain insight into the cause of this variability, we analyzed the level of test chimerism in the thymus and in the spleen of our experimental animals. As shown in Fig. 1A, in the spleens of 2% EGFP-test + 98% fill → wt chimeras, 1.9 ± 0.7% (coefficient of variation = 37.7%) of B cells expressed EGFP. This percentage corresponded closely to the proportion of EGFP-transgenic BM precursors injected. By contrast, test chimerism among total thymocytes was 0.8 ± 0.6% (coefficient of variation = 75.7%), which was substantially lower and much more variable than the splenic B cell test chimerism (Fig. 1A). Similarly low and very variable levels of thymic test chimerism were observed in the other chimeras (Fig. 1B). We therefore conclude that despite the apparently homogeneous reconstitution of stem cells in the BM in the distinct chimeras (as shown by the constant levels of splenic B cell chimerism), the test chimerism in the thymus was highly variable. This variability might be due to the infrequent seeding of the thymus with progenitors of test origin. If, for example, five precursor cells enter the thymus daily and 2% of them are of test origin in our hematopoietic chimeras, then, on average, one test cell will enter the thymus every 10 d. Because the day of entry of the test precursor is random and therefore different in each mouse, in the distinct chimeras the waves of test thymocytes should be at different stages of development. As thymocytes undergo differentiation from $CD4^-CD8^-$ double negative thymocytes (DN) through DP to CD4SP or $CD4^-CD8^+$ single positive thymocytes (CD8SP), the CD4/CD8 profiles of test thymocytes should vary among different chimeric animals. Indeed, we observed very atypical and dissimilar CD4/CD8 profiles among the test thymocytes in individual chimeric animals (examples shown in Fig. 1C). Some mice had high levels of very immature DN cells (7% of the chimeras analyzed), others had higher levels of immature DP cells (15% of the chimeras analyzed) or more mature CD8SP or CD4SP (48% of the chimeras analyzed), and some had normal proportions of these populations (30% of the chimeras analyzed). Thus, in the 61 individual chimeric animals we analyzed (in which test and fill thymocytes were distinguished on the basis of transgenic EGFP expression or expression of distinct CD45 or Thy1 alleles), the proportions of DN, DP, CD4SP, and CD8SP cells among test thymocytes varied dramatically (Fig. 1D).

In contrast to the highly variable CD4/CD8 profiles of test thymocytes, the CD4/CD8 profiles of fill thymocytes were similar

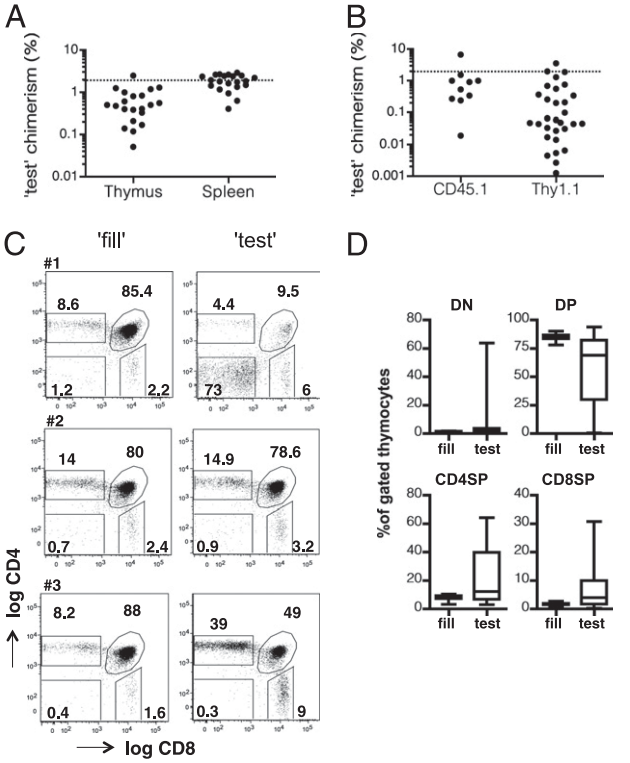

FIGURE 1. Visualization of distinct waves of T cell development in hematopoietic chimeras. (**A**) Lethally irradiated wt mice were reconstituted with 2% EGFP-expressing test and 98% wt fill BM cells and were analyzed by flow cytometry 5–6 wk after hematopoietic reconstitution. The percentage of test (i.e., $EGFP^+$) cells among total thymocytes or splenic B cells (test chimerism) is depicted. The dotted lines indicate the expected 2% test chimerism. (**B**) As in (A), but test cells were distinguished from fill thymocytes by their expression of CD45 or Thy1 allelic markers. Test chimerism in the thymus is depicted. (**C**) Five weeks after reconstitution, thymi from CD45 chimeras [as in (B)] were analyzed by flow cytometry. Plots of CD8 versus CD4 expression by electronically gated $CD45.2^+$ fill and $CD45.1^+$ test thymocytes are shown for three individual chimeric mice. Numbers in or near depicted electronic gates indicate percentages of cells within these gates. The progression of the test T cell development wave from the DN (mouse 1) to DP (mouse 2) and then to single positive stages (mouse 3) of T cell differentiation is clearly visible. (**D**) Variations in the proportions of DN or DP, and CD4SP and CD8SP, cells among the fill and test populations were calculated for all of the chimeras described in this figure (n = 61), using the gates depicted in (C). Box and whisker plots show the median, 25th and 75th centiles (box), and minimal and maximal values (whiskers).

in all the chimeras analyzed (Fig. 1C, 1D). This is to be expected, as large numbers of fill precursors regularly migrate to the thymus and give rise to many asynchronous waves of developing T cells (Supplemental Fig. 1B). Taken together, the results of these three analyses demonstrate that we can observe in our hematopoietic chimeras distinct waves of test T lymphocytes developing in a normal thymus (Supplemental Fig. 1B).

Tregs dwell longer in the thymus than do Tconvs

We next used transgenic mice expressing GFP under the control of the *Rag2* promoter to assess the thymic dwell time of Treg and Tconv cells (31, 32). The RAG-2 protein is involved in *Tcr* gene rearrangements. The gene is highly expressed at the DP stage of T cell development and is shut off upon positive selection (33). In thymocytes, GFP is slowly degraded with a half-life of ~2.3 d (29). In *Rag2-Gfp* mice, the level of GFP, therefore, strictly correlates with how much time has passed since shut-off of the *Rag2* promoter at positive selection.

As expected, DP thymocytes from *Rag2-Gfp* mice all expressed high levels of GFP. Whereas part of the $Foxp3^-$ CD4SP Tconv and $Foxp3^+$ Treg populations displayed lower levels, part of these populations were negative for GFP (Fig. 2A). Activated peripheral Tconvs and Tregs can recirculate back to the thymus (34, 35). Previous studies have shown that the GFP^{neg} population represents such recirculating T lymphocytes and that the GFP^{low} population consists of newly developing cells (29). We took into account only newly developing GFP^{low} thymocytes. Considering the 2.3-d half-life of GFP, the difference in median GFP fluorescence intensities between DP and CD4SP cells indicated that the median age (postpositive selection) of $Foxp3^-$ CD4SP Tconvs was 2.4 d and that of $Foxp3^+$ CD4SP Tregs was 4.3 d, a difference of 1.9 d (Fig. 2B; for calculation, see *Materials and Methods*). Because only the most mature T cells leave the thymus and the CD4SP population is heterogeneous (29), it was important to assess the age, after termination of *Rag2* gene expression, of the most mature cells. CD69 is an activation marker that is rapidly expressed upon positive selection and still present on the surface of immature, but not mature, single positive thymocytes (36). Whereas practically all thymic $Foxp3^+$ CD4SP Tregs display a very mature $CD69^-$ phenotype, in the $Foxp3^-$ CD4SP Tconv population immature $CD69^+$ and mature $CD69^-$ cells can be observed (37). We therefore analyzed GFP levels in the latter two populations (Fig. 2A). On the basis of the median GFP levels of GFP^{low} cells and the 2.3-d half-life of this fluorescent molecule, we calculated that the average age of immature $CD69^+$ $Foxp3^-$ CD4SP Tconvs was 1.6 d after shutdown of the *Rag2-Gfp* gene (Fig. 2B). The age of the most mature $CD69^-$ $Foxp3^-$ CD4SP Tconv population, ready to leave the thymus, was 3.3 d. Given that the $Foxp3^+$ CD4SP Treg population had an average age of 4.3 d, these data suggest that the thymic dwell time of $Foxp3^+$ CD4SP Tregs is ~1 d longer than that of $Foxp3^-$ Tconvs.

An alternative interpretation is that $Foxp3^+$ CD4SP cells may divide more than their $Foxp3^-$ counterparts and thus lose their GFP fluorescence. To corroborate our findings, therefore, we analyzed Ki67 expression in $Foxp3^+$ and $Foxp3^-$ CD4SP cells. Ki67 is a nuclear Ag expressed by cells in the mitotic division cycle. Fewer $Foxp3^+$ than $Foxp3^-$ CD4SP thymocytes expressed this Ag (Fig. 2C). The lower GFP levels detected on $Foxp3^+$ (as compared with $Foxp3^-$) CD4SP cells therefore cannot be explained by increased proliferation.

Large proportions of Foxp3$^+$ CD4SP Tregs are found at the end of a developmental wave

If $Foxp3^+$ CD4SP Tregs dwell in the thymus 1 d longer than their conventional $Foxp3^-$ counterparts, one would predict that the trailing end of waves of T cell development would be composed of mature CD4SP thymocytes strongly enriched in Tregs. To test this prediction, we analyzed the proportion of $Foxp3^+$ thymocytes in the 2% test + 98% fill → wt hematopoietic chimeras described above. We divided our 61 chimeric animals into three groups (early wave, mid wave, and late wave) based on their relative proportions of DP and CD4SP cells (as indicated in *Materials and Methods*). We then analyzed the proportion of $Foxp3^+$ Tregs among CD4SP thymocytes in all the samples. In the early wave group, when DP cells were abundant and few CD4SP thymocytes were present, the proportion of $Foxp3^+$ cells among CD4SPs was generally low (0.2 ± 0.2%; Fig. 3A, 3B), indicating that Tregs develop later or more slowly than Tconvs. In the mid wave group, when the proportion of DP thymocytes had diminished and that of mature CD4SP cells had increased (both within the range of values found for fill thymocytes), the percentage of $Foxp3^+$ cells among CD4SPs had increased to normal levels when compared

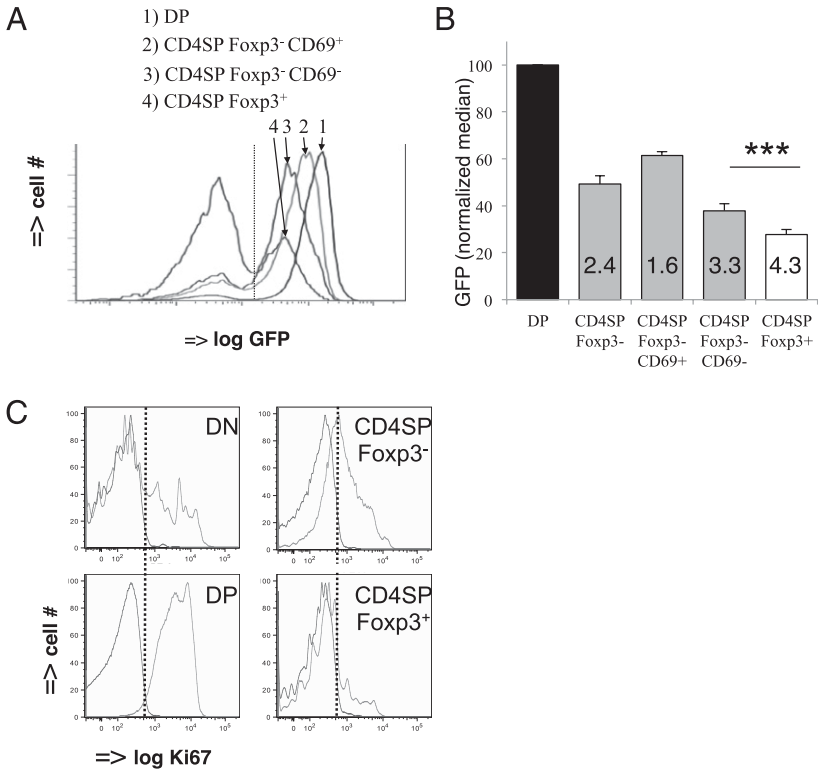

FIGURE 2. Longer thymic dwell time of Foxp3$^+$ than of Foxp3$^-$ CD4SP thymocytes. (**A**) Thymocytes from *Rag2-Gfp/Foxp3-Thy1.1* mutant mice were analyzed by flow cytometry. GFP expression by electronically gated populations is depicted. (**B**) For the indicated populations, median values of GFP fluorescence among GFP$^+$ cells [i.e., expressing GFP at levels above the dotted line in (A)] were calculated. For the four distinct CD4SP populations, these values were normalized to that found on the DP population in the same mouse. Depicted are the mean thus normalized values ± SD (n = 6). The values depicted in the bars indicate the time, in days, that has passed since shutdown of *Rag2* gene expression, calculated as described in *Materials and Methods*. ***$p <$ 0.0001, Student t test. (**C**) The expression level of Ki67 (gray histogram) was analyzed in electronically gated CD4 and CD8 DN or DP, as well as Foxp3$^-$ and Foxp3$^+$ CD4SP thymocytes, of wt B6 mice. The black histograms represent the isotype-matched control.

with the fill Tregs (6.9 ± 5.0%, Fig. 3A, 3B). Interestingly, in the late stages of the development wave, when the proportion of CD4SP cells was much increased and that of DP cells dramatically diminished, the percentage of Foxp3$^+$ cells among CD4SP cells was substantially larger (19.1 ± 20.7%, Fig. 3A, 3B). As expected, the proportion of Foxp3$^+$ Tregs among fill thymocytes was constant in all the chimeras, confirming that T cell development was altogether normal in these chimeras (Supplemental Fig. 2A, 2B). Foxp3$^+$ Tregs are, therefore, abundant in the late stages of the development wave, when a proportion of the Foxp3$^-$ CD4SP Tconvs has already emigrated from the thymus. These data confirm our conclusion that Tregs have a longer dwell time in the thymus than do Tconvs.

Development of the naturally diverse Treg population is not limited by the specialized thymic niche

To assess whether the thymic niche limits development of Tregs from a precursor population that expresses the naturally very diverse TCR repertoire, we analyzed the generation of Tregs from a few wt progenitors developing in a thymus filled with thymocytes that could not compete for the niche. Thymocytes lacking the TCRα-chain (TCRα^0) cannot be positively selected because they fail to express the TCRαβ heterodimer, and their differentiation is arrested at the DP stage of T cell development (30). These cells are unlikely to occupy the thymic niche that is specialized in Treg development for two reasons: First, in the absence of the TCRαβ heterodimer, they clearly cannot occupy selecting niches, and, second, these cells do not migrate to the medulla, the thymic compartment thought to be specialized in Treg development (14). By taking a similar approach in which a few wt precursors developed in a thymus filled with TCRα^0 cells, one of us previously showed that the thymic niche limits development of CD8SP T cells (24).

We lethally irradiated wt host mice and reconstituted them with a mix of wt test and TCRα^0 fill BM cells at various ratios. We used the Thy1 allelic marker to distinguish between selectable wt (Thy1.1) and nonselectable TCRα^0 (Thy1.2) thymocyte populations. At 5–6 wk after hematopoietic reconstitution, we analyzed thymocytes from these wt test + TCRα^0 fill → wt chimeras. Flow cytometry demonstrated that differentiation of TCRα^0 thymocytes was blocked at the DP stage, as expected, and the wt thymocytes developed into mature CD4SP and CD8SP cells (Supplemental Fig. 3A). At low levels of test chimerism, the ratio of mature to immature thymocytes increased, as previously observed in similar chimeras (38). The percentages of Foxp3$^+$ Tregs among CD4SP cells were highly variable from animal to animal (Fig. 4A, Supplemental Fig. 3B), with an inverse correlation between the level of test chimerism of the thymus and the proportion of Foxp3$^+$ cells: In the chimeras with fewest wt precursors, we found strikingly high percentages of Foxp3$^+$ Tregs among the CD4SP thymocytes—up to 70% in some mice (Fig. 4A, Supplemental Fig. 3B).

At first sight, the observation that decreasing the number of selectable precursors in these mixed chimeras apparently improves the relative efficiency of Treg generation suggests that the size of the thymic niche dedicated to differentiation of these cells is limiting and that developing thymocytes compete for its occupancy. Yet, in control chimeras, reconstituted with a wt fill population, we observed similar increases in the proportion of test (but not fill) Tregs in the mice with fewest wt precursors (Fig. 4B, Supplemental Fig. 4B).

During the very strict selection processes operating when thymocytes mature from the DP to the single positive stages, a substantial number of cells are lost (39–41). When the test wave is at the early (i.e., DP) stage of development, the test chimerism among total thymocytes must therefore be higher than when it is at the late (i.e., single positive) stage. Indeed, we found that during progression through development of test waves (as indicated by increasing ratios of CD4SP to DP thymocytes), test chimerism became progressively lower (Supplemental Fig. 4B). Low levels of test chimerism therefore corresponded to very late time points in the developmental waves at the time of analysis. The strongly

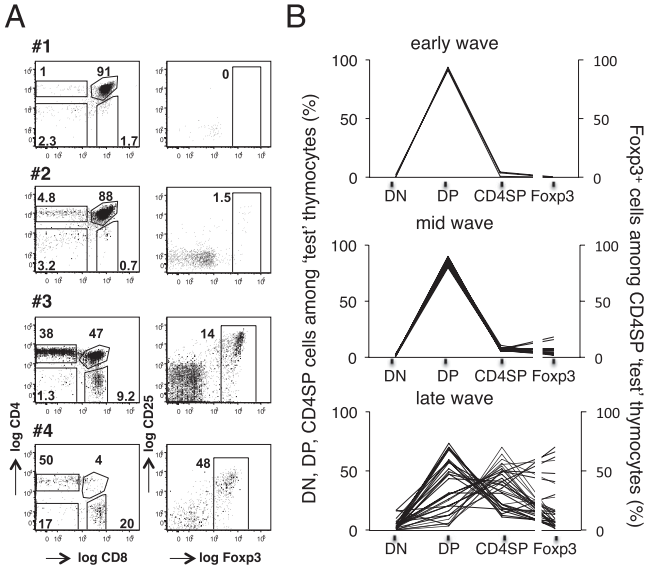

FIGURE 3. Strongly increased proportions of Foxp3$^+$ Tregs at the trailing ends of distinguishable waves of T cell development. (**A**) At 5 wk after hematopoietic reconstitution, thymocytes from lethally irradiated wt (Thy1.2) mice reconstituted with 2% Thy1.1 test and 98% Thy1.2 fill BM cells were analyzed by flow cytometry. Thy1.1$^+$ test thymocytes were electronically gated, and CD8/CD4 plots of total test cells and Foxp3/CD25 plots of electronically gated CD4$^+$CD8$^-$ test cells are depicted. The gradual progression of the T cell development wave from the DP to single positive stages of T cell differentiation, accompanied by substantial increases in the proportion of Foxp3$^+$ cells among CD4SP thymocytes, is clearly visible in the thymi of the four depicted mice. Numbers near or in depicted electronic gates represent percentages of cells within these gates. Analysis of the corresponding Thy1.2 fill population is shown in Supplemental Fig. 2A. (**B**) The 61 chimeras analyzed were divided into three groups according to the stage of development (early, mid, or late wave) of test thymocytes, as described in *Materials and Methods*. Within each group, for every individual mouse the percentages of DN or DP and CD4SP test thymocytes, and of Foxp3$^+$ cells among CD4SP (Foxp3) test cells, are depicted; one line corresponds to one mouse. DN, DP, and CD4SP: *left-hand* y-*axis*; Foxp3: *right-hand* y-*axis*.

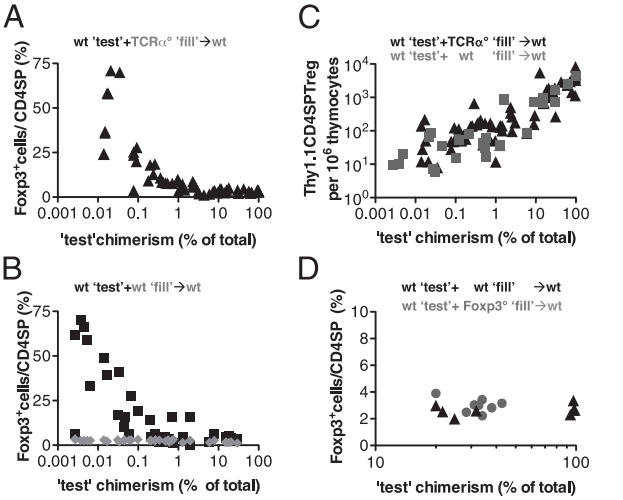

FIGURE 4. The thymic niche does not limit Foxp3$^+$ Treg development. (**A**) Lethally irradiated wt (Thy1.2) mice were reconstituted with a mix of Thy1.1 wt test and Thy1.2 TCRα^0 fill BM cells at various ratios. At 5 wk after reconstitution, thymocytes were analyzed by flow cytometry. The plot shows, for each individual chimera, the percentage of Foxp3$^+$ cells among Thy1.1$^+$ test CD4SP thymocytes (for the gates applied, see Supplemental Fig. 3A) as a function of test chimerism (i.e., percentage of test cells among total thymocytes). (**B**) As in (A), but for chimeras in which fill cells were of wt (Thy1.2) origin. The same data depicted as a function of test chimerism at the CD4SP stage are shown in Supplemental Fig. 4A. Black square, test cells; diamond, fill cells. (**C**) Numbers of test Thy1.1 CD4SP Foxp3$^+$ cells per 10^6 total thymocytes from the chimeric mice described in (A) (TCRα^0 fill, triangle) and (B) (wt fill, gray square) are shown as a function of test chimerism in total thymocytes. Each symbol corresponds to one mouse. Using the one-way ANOVA test, we found no statistically significant difference between the two groups of mice. (**D**) Lethally irradiated wt Thy1.2 mice were reconstituted with a mixture of 30% wt Thy1.1 test and 70% Foxp3-deficient (Foxp3^0, circle) or wt (diamond) Thy1.2 fill BM cells. At 5 wk after reconstitution, thymocytes were analyzed by flow cytometry, as in (A). The percentage of Foxp3$^+$ cells among Thy1.1$^+$ test CD4SP thymocytes is shown as a function of test (Thy1.1) chimerism in total thymocytes. The same data depicted as a function of test chimerism at the CD4SP stage are shown in Supplemental Fig. 4C.

increased proportions of Tregs we found at low levels of test chimerism thus confirm our conclusion that Tregs dwell in the thymus longer than do Tconvs.

Foxp3-expressing cells are found not only among CD4SP thymocytes but also among DP cells (42). Of interest, an increased proportion of Foxp3$^+$ among DP test thymocytes was also observed at low levels of test chimerism (Supplemental Fig. 4C). The increase in the proportion of Foxp3$^+$ CD4SP and DP cells was observed at similar levels of test chimerism, consistent with the idea that Foxp3-expressing DP cells are not the precursors of CD4SP Tregs (42).

If the thymic niche limits Treg development, the numbers of Tregs that develop from wt precursors in the wt test + TCRα^0 fill → wt chimeras should be superior to that in the wt test + wt fill → wt control chimeras. We calculated the number of wt test Tregs developing in these two types of chimeras (Fig. 4C). With decreasing levels of test chimerism, the number of developing Foxp3$^+$ Tregs also decreased, which is inconsistent with the idea that the thymic niche limits Treg development. In chimeras with very low levels of test thymocytes, the numbers of Foxp3$^+$ Tregs that developed were highly variable from animal to animal. Crucially, the numbers of test cells that differentiated into Foxp3$^+$ Tregs in the control chimeras were similar to the numbers of test cells that differentiated into Foxp3$^+$ Tregs in the chimeras that received TCRα^0 fill cells. Reducing the number of selectable T cell precursors, therefore, clearly does not enhance development of Tregs. We found no evidence from these experiments for a limiting role of the thymic niche in Treg development.

CD4SP thymocytes undergo negative selection through their interaction with epithelial cells in the medulla, and, in turn, this interaction provides signals required for full maturation of the medulla (43). One might argue, therefore, that in our experiments with the TCRα^0 fill chimeras, fewer of these signals are generated and that, as a consequence, the medulla cannot mature properly and cannot fully support Treg development. However, using TCR-transgenic but RAG-sufficient animals, researchers showed that very low levels of endogenous TCR-expressing CD4SP thymocytes were sufficient for maturation of medullary epithelial cells (43). It appears, therefore, rather unlikely that this hypothesis explains why we did not see an increase in Foxp3$^+$ Tregs proportional to the reduction of test chimerism in our chimeras. Nevertheless, to formally test this possibility, we analyzed the differentiation of wt test Tregs in thymi filled with thymocytes that can undergo negative selection upon interaction with medulla epithelial cells but that cannot differentiate into Foxp3$^+$ Tregs. To perform this experiment, we generated chimeras with 70% Foxp3-deficient and 30% wt BM cells, which provided sufficiently large numbers of wt cells to prevent autoimmune disorders due to Treg

deficiency, but sufficiently few wt cells to see any potential effect due to a limiting role of the thymic niche. Among wt thymocytes in these chimeras, the proportions of Foxp3$^+$ cells among CD4SP thymocytes were no different from those in the control chimeras (Fig. 4D, and Supplemental Fig. 4D). On the basis of data we have obtained using several experimental systems, we conclude that no evidence exists to support the idea that thymic niche size limits development of the polyclonal Treg repertoire.

Discussion

In this study, we have addressed the question of whether the thymic niche limits development of Tregs from precursors with a naturally diverse TCR repertoire. In BM chimeras in which very few selectable T cell precursors developed, we observed a much larger proportion of Foxp3$^+$ Tregs among mature CD4SP thymocytes than was seen in normal mice. This increase was not due to a limitation imposed by the thymic niche, however, but could be accounted for entirely by a delay in the egress of Foxp3$^+$ Tregs (as compared with Foxp3$^-$ Tconvs) from the thymus. It therefore appears that natural levels of factors involved in Treg development in the specialized thymic microenvironment (i.e., the thymic niche) do not limit differentiation of this important immunoregulatory population.

It was previously shown that very few T cell precursors home to the thymus every day (25–27). Given that these cells will proliferate but do not have self-renewing capacity, each precursor will give rise to a single wave of developing T cells. By generating hematopoietic chimeras in which trackable precursors enter the thymus very infrequently, we could thus visualize distinct waves of developing T cells. This allowed us to discover that thymic dwell times of conventional and regulatory CD4 single positive mature T cells differ substantially: Foxp3$^+$ Tregs dwell ~1 d longer in the thymus after positive selection (as assessed by shutdown of the *Rag2* gene) than Tconvs do. This experimental design will be a powerful tool to further investigate the kinetics of thymic entry of precursor cells and of their progression through the distinct stages of development.

Thymic differentiation of Tregs is a highly flexible and accommodating process. It must, however, presumably be controlled with care to avoid an overproduction of these cells, which could otherwise potentially dampen peripheral immune responses (44). Selection of a Treg precursor is favored by expression of the agonist ligand for its TCR (8, 10–13). Expression of autospecific TCRs or availability of ligand, or both, therefore potentially limit Treg development (16–18). It has been shown, however, that the population of immature T cell precursors comprises up to two-thirds autospecific cells (41); thus, it appears unlikely that expression of TCRs with high affinity for self-MHC–peptide ligands is a rate-limiting factor in Treg development. Even in mice in which a very large proportion of precursors expressed a transgenic TCR and also expressed high levels of the agonist ligand for this TCR, still only very few Tregs developed (reviewed in Ref. 15). Our data reported in this article demonstrate that the selecting niche does not limit development of Tregs from the naturally very diverse repertoire of T cell precursors. It therefore appears that neither the number of precursors expressing high-avidity TCR nor the availability of selecting ligand limits development of the Treg repertoire in wt mice.

Factors other than those linked to TCR specificity (e.g., cytokines and costimulatory molecules) are involved in Treg development (22). If limiting amounts of these factors restrict the numbers of Tregs differentiating in the thymus, we would have seen an increase in Treg development proportional to the reduction in the number of selectable precursors in our hematopoietic chimeras. However, we saw no increased proportions of Tregs at intermediate levels of test chimerism. This observation is therefore incompatible with a limiting role of the thymic niche.

We thus conclude that the thymic niche is not a limiting factor in Treg development. Importantly, we have reached this conclusion using an experimental system in which Treg precursors had a naturally diverse TCR repertoire and in which the thymic stroma expressed a normal repertoire and density of selecting MHC/peptide ligands. It has been shown, however, that various proportions (and absolute numbers) of Tregs develop in the thymus, depending on the strain of mouse investigated and that factors acting in a thymocyte-intrinsic manner are responsible for this phenotype (45–47). Identification of the polymorphic genetic factors limiting Treg differentiation should shed light on the mechanisms that strictly control the number of Tregs developing in the thymus. Finally, our data also reveal that newly developing Tregs linger in the thymus longer than do Tconvs. The mechanisms involved and the physiological consequences of this curious phenomenon remain a matter of investigation.

Acknowledgments

We thank Drs. Sylvie Guerder, Eric Espinosa, Lucette Pelletier, Christophe Viret, Jean-Charles Guery, and Etienne Joly for critical reading of this manuscript; Drs. Fatima-Ezzahra L'Faqihi-Olive and Valérie Duplan-Eche (INSERM U1043 flow cytometry facility) and the personnel of the INSERM US006 Analyse et Exploration Fonctionnelle Centre Régional d'Exploration Fonctionnelle et Ressources Expérimentales animal facility—in particular, Guillaume Morera and Maryline Calise—for expert technical assistance; Drs. Masaru Okabe, Béatrice Cousin, Louis Casteilla, and Pamela Fink for providing transgenic mice; and Dr. Alexander Rudensky for supplying *Foxp3$^{Thy1.1}$* mice and advice on experimental design.

Disclosures

The authors have no financial conflicts of interest.

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