Published in final edited form as:

Eur J Immunol. 2014 September ; 44(9): 2712–2720. doi:10.1002/eji.201444529.

Both retention and recirculation contribute to long-lived regulatory T cell accumulation in the thymus

EnJun Yang^{1,*}, Tao Zou^{1,*}, Theresa M. Lechner¹, Shirley L. Zhang¹, and Taku Kambayashi¹

¹Department of Pathology and Laboratory Medicine, Perelman School of Medicine at the University of Pennsylvania, Philadelphia, PA 10194

Abstract

Natural regulatory T cells (Tregs) acquire their lineage-determining transcription factor Foxp3 during development in the thymus and play an important role in maintaining immunologic tolerance. Here we analyzed the composition of the thymic Treg pool using RAG2-GFP/FoxP3-RFP dual reporter mice and found that a population of long-lived GFP⁺ Tregs exists in the thymus. These long-lived Tregs substantially increased with age, to a point where these Tregs represent >90% of the total thymic Treg pool at 6 months of age. In contrast, long-lived conventional T cells remained at ~15% of the total thymic pool at 6 months of age. Consistent with these studies, we noticed that host-derived Tregs represented a large fraction (~10%) of the total thymic Treg pool in bone marrow chimeras, suggesting that long-lived Tregs also reside in the thymus of these mice. The pool of long-lived Tregs in the thymus was sustained by retention of Tregs in the thymus and by recirculation of peripheral Tregs back into the thymus. These long-lived Tregs were functionally similar to wildtype splenic Tregs, and were able to suppress T cell proliferation to an equivalent extent. Together, these data demonstrate that long-lived Tregs accumulate in the thymus by both retention and recirculation.

Keywords

Treg Cells; Thymus; Treg Homeostasis

Introduction

Regulatory T cells (Tregs) play a key role in immunological tolerance to self. They are a subset of CD4⁺ T cells that express the lineage-determining transcription factor Foxp3. The importance of Tregs is illustrated in scurfy mice and IPEX patients that lack a functional Foxp3 gene, which results in the absence of Tregs leading to a systemic autoimmune disorder that is often fatal [1].

To whom correspondence should be addressed: Taku Kambayashi, Department of Pathology and Laboratory Medicine, 288 John Morgan Building, 3620 Hamilton Walk, Philadelphia, PA, 19104; taku.kambayashi@uphs.upenn.edu; phone: 215-746-7610; fax: 215-573-9261.

*These authors contributed equally to the manuscript.

Conflicts of Interest Disclosures

The authors declare no conflicts of interest.

Tregs are largely divided into two subsets based on where they are formed. Tregs that develop in the periphery from conventional T cells (Tconvs) are known as inducible Tregs, while those that acquire Foxp3 during development in the thymus are called natural Tregs. During development in the thymus, natural Tregs are thought to be formed when an immature CD4⁺ T cell receives a strong TCR signal [2,3]. To purge overly self-reactive T cells from entering the periphery, most CD4⁺ T cells expressing TCRs with strong affinity for self MHC-peptide are negatively selected. However, as an alternative fate, a fraction of these CD4⁺ T cells are driven toward the Treg lineage and enter the periphery to maintain immunologic tolerance to self [4]. Early in life, thymic output of Tregs is important for populating the periphery with the appropriate number of Tregs. However, recent studies have shown that the thymic output of Tregs decreases rapidly with age, and that the peripheral maintenance of Tregs in young adult mice is largely independent of newly generated Tregs from the thymus [5].

As the role of the thymus is to create new T cells to populate the periphery, it would be expected for T cells to leave the thymus upon maturation. Indeed, it has been estimated that the emigration of Tconvs from the thymus occurs in 4–5 days after becoming a CD4 or CD8 single positive T cell [6]. However, some populations of long-lived T cells appear to linger for prolonged periods of time in the thymus. For example, a subset of NKT cells are retained in the thymus even after full maturation [7], where their presence may affect the development of other T cells such as CD8⁺ innate like lymphocytes [8]. In addition to NKT cells, long-lived Tregs that have developed more than 2 weeks ago are also present in the thymus of adult mice [6,9,10]. However, whether the composition of the long-lived population of Tregs alters with age and how this long-lived Treg population is formed is not known.

In this study, we examined the formation of the Treg pool in the thymus. Studies with RAG2-GFP reporter mice demonstrated that the long-lived Treg population in the thymus increases with age and represents >90% of the thymus by 6 months of age. Using bone marrow (BM)-transplanted mice, we found that a large percentage of host Tregs survived irradiation and represented a large fraction of Tregs in the recipient thymus. Furthermore, using a number of transplant and adoptive transfer approaches, we found that both thymic retention and recirculation of Tregs play an important role in establishing the long-lived Treg population in the thymus, with recirculation playing a lesser role. The long-lived thymic Tregs were equally suppressive as WT splenic Tregs, suggesting that these long-lived Tregs were functional, and may therefore affect thymic selection events.

Results

A long-lived population of Tregs accumulates in the thymus

In order to study the contribution of long-lived Tregs and newly developed Tregs to the Treg pool in the thymus, we utilized RAG2-GFP reporter mice. In these mice, GFP is detected in T cells for up to 2 weeks after RAG2 expression and thus marks recently developed T cells [10]. To better visualize GFP⁺ Tregs, we crossed RAG2-GFP mice to Foxp3-RFP reporter mice. As expected, a high proportion of GFP⁺ cells was observed in thymocytes from 5-week old mice (Fig. 1A and B), with ~60% of thymic RFP⁺ Tregs and almost all CD4⁺

Tconvs expressing GFP. As the mice aged, the total number of CD4⁺ Tconvs but not Tregs in the thymus decreased (Fig. 1C). As expected, the fraction of Tconvs expressing GFP stayed relatively high despite their decreasing cell numbers, with about ~85% still being GFP⁺ at 6 months of age. In contrast, the percentage of Tregs expressing GFP decreased rapidly to ~20% at 13 weeks and down to <10% at 6 months of age (Fig. 1A and B). Given that the total number of Tregs remained relatively constant with age, the increasing fraction of GFP⁺ Tregs suggested that long-lived Tregs were accumulating in the thymus as the mice aged.

Next, test whether these long-lived thymic Tregs were bona fide regulatory T cells with suppressive function, we sorted RFP⁺GFP⁺ Tregs from the thymi of the RAG2-GFP/FoxP3-RFP reporter mice and tested their ability to suppress T cell division in an *in vitro* suppression assay. Compared to splenic RFP⁺GFP⁺ Tregs, we found that the GFP⁺ Tregs from the thymus were as suppressive as WT splenic Tregs (Fig. 1D). Together, these data suggest that long-lived functional Tregs accumulate and contribute greatly to the total thymic Treg pool with increasing age.

Host-derived Tregs can be found in the thymus of bone marrow chimeric mice

To further study the formation of the pool of long-lived Tregs in the thymus, we made BM chimeric mice and examined the residual radioresistant host-derived Treg population in the thymus 9–12 weeks after transplantation. A proportion of host-derived Tregs survived irradiation and represented ~40% of the total splenic Treg pool (Fig. 2A and B). Similarly, as a fraction of memory T cells are also radioresistant, ~20% of the splenic CD4⁺ and CD8⁺ Tconvs remained of host origin. In contrast, CD4⁺ and CD8⁺ Tconvs of host origin were barely detectable in the thymi of the BM chimeric mice (Fig. 2A and B). However, host-derived Tregs still represented a substantial fraction (~10%) of the thymic Treg pool (Fig. 2A and B). The presence of the host-derived Tregs in the thymus was not due to ongoing new Treg development, as host-derived double positive (DP) thymocytes were not observed in these mice (Fig. 2A and B). As expected, there were significantly more total CD4⁺ Tconvs cells than Tregs. However, the total number of host-derived Tregs was similar to the total number of host-derived CD4⁺ Tconvs cells, suggesting that a selective enrichment of host-derived Tregs in the thymus was occurring (Fig. 2C). Together, these findings suggest that a pool of long-lived Tregs was maintained in the thymus of BM chimeric mice.

Peripheral Tregs recirculate back into the thymus

We next investigated the mechanisms by which long-lived Tregs accumulate in the thymus. The presence of long-lived Tregs in the thymus could be potentially explained by the recirculation of Tregs from the periphery back into the thymus. To directly evaluate this possibility in the BM transplant setting, we adoptively transferred congenically disparate CD4⁺ T cells at the time of BM transplant. 9–12 weeks later, the spleen and thymi were analyzed for the presence of Tregs. Indeed, Tregs of adoptive transfer origin were detected in the spleen and in the thymi of the BM chimeras (Fig. 3A). Moreover, we found a significant positive correlation between the percentages of adoptively transferred Tregs in the thymus and spleen (Fig. 3B). A large number of DPs almost all of donor BM origin were found in the thymi of BM-transplanted mice, suggesting that BM reconstitution was

successful (Supporting Fig. 2). Next, to test whether Tregs could recirculate back into the thymus of unirradiated hosts, congenically disparate CD4⁺ T cells were adoptively transferred into RAG1 KO mice. As expected, CD4⁺ T cells of adoptive transfer origin could be found in the spleens of these mice after 6 weeks (Fig. 3C). The total number of adoptively transferred Tregs in the thymi of RAG1 KO mice and BM-transplanted mice were similar (Fig. 3C and D), suggesting that irradiation was not necessary for these Tregs to reenter the thymus. Together, these data suggest that peripheral Tregs have the ability to recirculate back into the thymus.

A fraction of Tregs are retained in the thymus

Given that only a small number of adoptively transferred Tregs was detected in the thymus, we explored other possibilities that could also give rise to long-lived Treg accumulation in the thymus. One possibility was that a fraction of newly developed Tregs was being retained in the thymus. To test this hypothesis, congenically disparate neonatal thymi were transplanted into the axilla of mice and allowed to engraft. 6 weeks later, the engrafted thymus was examined for the presence of donor-derived cells. All DP thymocytes were of host origin in the donor thymus, confirming that successful engraftment of the donor thymus had occurred (Fig. 4A). In addition, the absence of donor-derived DP thymocytes suggested that ongoing T cell development of donor origin was no longer occurring. Despite the absence of donor-derived DP thymocytes, Tregs of donor origin still represented ~1% of all Tregs in the thymus and was significantly higher than the fraction of donor-derived CD4⁺ Tconvs. In addition to the thymus, ~1% of splenic Tregs (Fig. 4A) were also of donor origin, again raising the possibility that the donor-derived Tregs in the donor thymi could represent recirculating Tregs from the spleen. However, a significantly smaller fraction and absolute number of donor-derived Tregs were detected in the endogenous host thymus compared to the engrafted donor thymus (Fig. 4B and C). Since the donor-derived Tregs could only seed the host thymus by recirculation from the periphery, and a higher fraction and total number of donor-derived Tregs were found in the donor thymus, these data suggest that a proportion of Tregs are retained by the thymus after development.

Retention plays a larger role than recirculation in establishing the pool of long-lived Tregs in the thymus of BM chimeric mice

We next sought to estimate the relative contribution of recirculation and retention to the establishment of the long-lived Treg pool in the thymus. To this end, we injected congenically disparate WT CD4⁺ T cells and BM from RAG2-GFP/Foxp3-RFP reporter mice into lethally irradiated recipients (Fig. 5A). For these calculations, the BM-derived GFP⁺ Tregs (long-lived Tregs) in the thymus were assumed to be formed by both retention and recirculation, while the adoptively transferred Tregs were assumed to seed the thymus only by recirculation. Additionally, it was assumed that equilibrium between the periphery and the thymus was established at the time of analysis and that the BM-derived Tregs and adoptively transferred Tregs possessed equal opportunity at recirculating into the thymus. Finally, based on our data from Fig. 3B, we assumed a linear correlation between the fraction of Tregs in the periphery and recirculation into the thymus (See Supporting Figure 3 for details on calculations). Based on these assumptions, we calculated that retention plays a

larger role than recirculation in forming the long-lived thymic Treg pool in the BM chimeric mice, with an average of ~3:1 retention:recirculation ratio (Fig. 5B).

Discussion

In this study, we examined the composition of the Treg pool in the thymus. The RAG2-GFP reporter mouse has been useful to distinguish newly developing T cells from older T cells by marking cells that have recently undergone TCR rearrangement. It has been estimated that GFP is no longer detected in T cells ~2 weeks after RAG expression has ceased [10]. Similar to previously reported studies [6,9], we initially examined newly developed vs. older Tregs in the thymi of RAG2-GFP reporter mice by staining intracellularly for Foxp3 and detecting GFP⁺Foxp3⁺ and GFP⁻Foxp3⁺ cells by flow cytometry. However, it was difficult to interpret our results using this approach, since the fixation process used for staining intracellular Foxp3 led to a large loss of the GFP signal (data not shown). Thus, we were concerned that the fraction of GFP⁺Foxp3⁺ Tregs in the thymi of these mice was being overestimated. To address this issue, we bred the RAG2-GFP mouse to the Foxp3-RFP reporter mouse, which allowed us to detect the GFP signal in Foxp3⁺ Tregs without fixation. As an added benefit, the use of Foxp3-RFP reporter mice also enabled us to perform functional assays on the long-lived GFP⁺Foxp3⁺ Tregs that accumulated in the thymus. Our data from the RAG2-GFP/Foxp3-RFP mice suggest that as opposed to the majority of Tconvs that leave the thymus to populate the periphery after development, an increasingly large proportion of long-lived Tregs accumulated in the thymus with age, reaching >90% of all thymic Tregs at 6 months of age. The pool of long-lived Tregs displayed suppressive function, suggesting that these cells are bona fide functional Tregs.

Although the presence of long-lived Tregs has been demonstrated previously [6,9], how the long-lived Treg pool is established has not been reported. We reasoned that the pool of long-lived Tregs could be formed by retention of Tregs in the thymus, recirculation of peripheral Tregs back into the thymus, or both. Using a combination of BM transplantation and adoptive transfer approaches, we provide data to suggest that the long-lived Treg pool in the thymus is established by both retention and recirculation. In our BM chimera systems, we estimate that thymic retention plays a greater role than recirculation in this process. However, we do not know whether thymic retention plays a more dominant role also in unmanipulated replete mice, since the irradiation of mice is known to affect the thymic environment and progenitor settling processes [11]. Furthermore, while our data from adoptive transfers into RAG1 KO mice suggests that the recirculation of Tregs does not require irradiation, there are several caveats that are also associated with this experiment. First, it does not exclude the possibility that an empty thymus could artificially enhance the recirculation of Tregs into the thymus. Thus, the relative roles of retention and recirculation may change with the emptiness of the thymus, which could be affected by the age of the mice. Second, Tregs are known to localize mainly to the medulla of the thymus [12], but RAG KO mice have a poorly formed medullary region [13]. Thus, the Tregs that recirculated into the RAG KO thymus might be mislocalized in RAG KO mice. However, our data still suggest that peripheral Tregs possess the ability to enter the thymus.

In addition to Tregs, long-lived NKT cells have also been reported to be retained by the thymus [7]. However, the mechanism by which thymic retention of developed T cells occurs is unknown. Thymic retention may involve the expression of a receptor that actively retains the cell in the thymus. Alternatively, it could result from the lack of a receptor that is necessary for these cells to exit the thymus. Thymic egress is largely driven by the sphingosine 1-phosphate (S1P) receptor, S1P1. As developing thymocytes mature into T cells, the expression of the S1P1 receptor increases, allowing them to follow an S1P gradient out of the thymic cortex [14]. Interestingly, the expression of S1P1 antagonizes Treg development by activating the AKT-mTOR pathway [15]. Thus, Tregs likely have lower expression of S1P1 on their cell surface during development. It is possible that Tregs are slow to upregulate S1P1 during after they develop, which retards their egress to the periphery. However, not all Treg exit from the thymus is delayed, since the spleen of the RAG2-GFP/Foxp3-RFP reporter mouse contains Tregs that are GFP⁺. However, it is an intriguing possibility that a fraction of Tregs that fail to upregulate S1P1 are those that are retained.

Our findings are consistent with a previous study showing that activated T cells are capable of recirculating back into the adult thymus [16], as Tregs bear an activated phenotype (CD25^{hi}, CD44^{hi}). One major unanswered question is the physiological role of peripheral recirculation of Tregs in the thymus. Identification of the mechanisms that govern thymic recirculation would be necessary to answer this question. The chemokine receptors CCR7 and CCR9 have been shown to be critical in homing of T cell progenitors to the thymus [17]. Thus, CCR7, which is also expressed on a fraction of Tregs might provide a potential mechanism for their ability to reenter the thymus from the periphery [18]. However, the overlap of chemokine receptor usage in homing to various organs would make it difficult to selectively disrupt Treg recirculation and to test the impact of this process on Treg development and immune homeostasis.

Nevertheless, a steadily accumulating pool of long-lived thymic Tregs may serve to explain why thymic Treg output is severely diminished in adult mice [5]. It is conceivable that the presence of pre-existing thymic Tregs would hamper the development of newly developing Tregs. For example, Treg development is dependent on the cytokines IL-2 or IL-15, as demonstrated by the severely defective Treg development seen in mice lacking IL-2R β [19]. In addition, long-lived Tregs constitutively express the high affinity IL-2 receptor, which is required for their survival and homeostasis [20,21]. Thus, pre-existing Tregs would utilize IL-2 and IL-15 for their survival, potentially making these cytokines less available for new Treg development.

In addition to cytokines, strong TCR stimulation of thymocytes is required for Treg development [3,22,23]. Previous studies have shown that developing thymocytes compete for limiting amounts of antigens presented in the thymus [22,24]. Interestingly, an increase in the precursor frequency of antigen-specific T cells limits Treg development specific to that antigen [24]. This is thought to occur because the competition among antigen-specific T cells blocks any one T cell from interacting with the antigen long enough to trigger sufficient TCR signaling to induce Treg development. Thus, pre-existing Tregs could potentially inhibit the development of new Tregs with overlapping specificity by competing

for antigens. This may serve a purpose to diversify the Treg TCR repertoire so that Tregs of similar antigen specificities are not created in great abundance.

Regardless of their mechanism of action, an increasing subset of long-lived thymic Tregs also suggests that the output of newly developed Tregs from an older thymus is severely limited. Our studies have highlighted two potential routes by which these cells are maintained in the thymus, and may provide a starting point for devising strategies to revitalize thymic Treg production.

Materials and methods

Mice

RAG2-GFP reporter mice (RAG2-NGBAC-GFP) were described previously [25]. C57BL/6 (B6), B6.SJL, and RAG1 KO mice were purchased from The Jackson Laboratory or Taconic Farms. RAG2-GFP reporter mice were bred to the Foxp3-RFP mice. CD45.1/45.2 heterozygous mice were created by breeding B6 mice to B6.SJL mice. Mice were housed in pathogen-free conditions and treated in strict compliance with Institutional Animal Care and Use Committee regulations of the University of Pennsylvania.

Flow cytometry, cell sorting, data analysis, and statistical analysis

Abs for flow cytometry were purchased from BD Pharmingen (San Diego, CA), eBioscience (San Diego, CA), or Molecular Probes, Invitrogen (Carlsbad, CA). Flow cytometry and FACS were performed with an LSR II, FACS Canto, or a FACS Aria cell sorter (BD Biosciences). Data were analyzed with Flowjo software (Tree Star) and all statistical analyses were performed using Prism (Graphpad). All plots shown are pregated on live singlets. Unless otherwise specified, the following conventions are used for gating lymphocyte populations: CD4⁺ Tconvs are CD4⁺ Foxp3⁻CD8⁻, Tregs are CD4⁺Foxp3⁺CD8⁻, CD8 single positive cells are CD8⁺CD4⁻, and DPs are CD4⁺CD8⁺ thymocytes (See Supporting Fig. 1 for representative plots).

BM chimera generation and adoptive transfers

T cell-depleted BM ($2-5 \times 10^6$ cells) from B6 mice were injected i.v. into lethally irradiated congenic host mice (9.5 Gy) with a different CD45 allelic combination. In experiments involving adoptive transfer of T cells, 1.5×10^6 MACS-purified CD4⁺ T cells from congenic mice with a CD45 allelic combination different from the BM and the host were co-injected with the donor BM. Thymi and spleen were taken from the BM chimeras between 9–12 weeks later for analysis. For adoptive transfers into RAG1 KO mice, 1.5×10^6 MACS-purified CD4⁺ T cells from B6.SJL mice were injected i.v. into RAG1 KO (CD45.2⁺) mice. Splenic and thymic T cells were then analyzed 5 weeks after injection.

Thymic transplants

For thymic transplants, thymi from neonatal mice (Day 2–3) were transplanted under the axilla of a congenic mouse with a different CD45 allelic combination. The donor thymi were then allowed to engraft and revascularize for 6 weeks. The donor thymus, host thymus, and spleen were then analyzed for the presence of T cells by flow cytometry.

Treg Suppression Assays

MACS-enriched CD90.2⁺ T cells were sorted by FACS for CD4⁺RFP⁺GFP⁻ thymocytes or splenocytes from FoxP3-RFP Rag2-GFP dual reporter mice. FACS sorting was also used to obtain CD4⁺GFP⁻ Tconvs from WT B6.SJL Foxp3.GFP reporter mice (CD45.1⁺). The Tconvs were labeled with CFSE and cultured at various ratios with Tregs in the presence of irradiated, T cell-depleted CD45.2⁺ feeder cells and soluble anti-CD3 antibody (1 µg/ml). CFSE labeling was performed by resuspending the cells with PBS containing CFSE (5 mM) at 37°C followed by continuous shaking for 9 min. The reaction was then immediately quenched with 100% FBS, and the cells were washed before being cultured. The extent of CFSE dilution by Tconvs (CD4⁺CD45.1⁺) was assessed by flow cytometry after 4 days in culture.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by grants from the National Blood Foundation, the University of Pennsylvania internal funds, and the National Institutes of Health (R01HL111501, R01HL107589).

We thank the Kambayashi, Wu, Behrens, Nichols, and Koretzky lab members for helpful discussions.

Abbreviations used in this article

BM	bone marrow
DP	double positive
Treg	regulatory T cell
Tconv	conventional T cell

References

1. Ohkura N, Kitagawa Y, Sakaguchi S. Development and Maintenance of Regulatory T cells. *Immunity*. 2013; 38:414–423.10.1016/j.immuni.2013.03.002 [PubMed: 23521883]
2. de Lafaille MAC, Lafaille JJ. Natural and Adaptive Foxp3. *Immunity*. 2009; 30:626–635.10.1016/j.immuni.2009.05.002 [PubMed: 19464985]
3. Schmidt AM, Zou T, Joshi RP, Lechner TM, Pimentel MA, Sommers CL, Kambayashi T. Diacylglycerol Kinase Limits the Generation of Natural Regulatory T Cells. *Science Signaling*. 2013; 6:ra101–ra101.10.1126/scisignal.2004411 [PubMed: 24280042]
4. Sakaguchi S. Naturally Arising CD4⁺ Regulatory T Cells for Immunologic Self-Tolerance and Negative Control of Immune Responses. *Annu Rev Immunol*. 2004; 22:531–562.10.1146/annurev.immunol.21.120601.141122 [PubMed: 15032588]
5. Rubtsov YP, Nieuwe RE, Josefowicz S, Li L, Darce J, Mathis D, Benoist C, et al. Stability of the Regulatory T Cell Lineage in Vivo. *Science*. 2010; 329:1667–1671.10.1126/science.1191996 [PubMed: 20929851]
6. McCaughy TM, Wilken MS, Hogquist KA. Thymic emigration revisited. *Journal of Experimental Medicine*. 2007; 204:2513–2520.10.1084/jem.20070601 [PubMed: 17908937]
7. Berzins SP, McNab FW, Jones CM, Smyth MJ, Godfrey DI. Long-term retention of mature NK1.1⁺ NKT cells in the thymus. *J Immunol*. 2006; 176:4059–4065. [PubMed: 16547241]

8. Verykokakis M, Boos MD, Bendelac A, Kee BL. SAP Protein-Dependent Natural Killer T-like Cells Regulate the Development of CD8. *Immunity*. 2010; 33:203–215.10.1016/j.immuni.2010.07.013 [PubMed: 20674402]
9. Cuss SM, Green EA. Abrogation of CD40-CD154 Signaling Impedes the Homeostasis of Thymic Resident Regulatory T Cells by Altering the Levels of IL-2, but Does Not Affect Regulatory T Cell Development. *The Journal of Immunology*. 2012; 189:1717–1725.10.4049/jimmunol.1200588 [PubMed: 22802415]
10. Boursalian TE, Golob J, Soper DM, Cooper CJ, Fink PJ. Continued maturation of thymic emigrants in the periphery. *Nat Immunol*. 2004; 5:418–425.10.1038/ni1049 [PubMed: 14991052]
11. Zlotoff DA, Zhang SL, De Obaldia ME, Hess PR, Todd SP, Logan TD, Bhandoola A. Delivery of progenitors to the thymus limits T-lineage reconstitution after bone marrow transplantation. *Blood*. 2011; 118:1962–1970.10.1182/blood-2010-12-324954 [PubMed: 21659540]
12. Fontenot JD, Rasmussen JP, Williams LM, Dooley JL, Farr AG, Rudensky AY. Regulatory T Cell Lineage Specification by the Forkhead Transcription Factor Foxp3. *Immunity*. 2005; 22:329–341.10.1016/j.immuni.2005.01.016 [PubMed: 15780990]
13. van Ewijk W, Holländer G, Terhorst C, Wang B. Stepwise development of thymic microenvironments in vivo is regulated by thymocyte subsets. *Development*. 2000; 127:1583–1591. [PubMed: 10725235]
14. Matloubian M, Lo CG, Cinamon G, Lesneski MJ, Xu Y, Brinkmann V, Allende ML, et al. Lymphocyte egress from thymus and peripheral lymphoid organs is dependent on S1P receptor 1. *Nature*. 2004; 427:355–360.10.1038/nature02284 [PubMed: 14737169]
15. Liu G, Burns S, Huang G, Boyd K, Proia RL, Flavell RA, Chi H. The receptor S1P. *Nature Publishing Group*. 2009; 10:770–778.10.1038/ni.1743
16. Agus DB, Surh CD, Sprent J. Reentry of T cells to the adult thymus is restricted to activated T cells. *J Exp Med*. 1991; 173:1039–1046. [PubMed: 2022918]
17. Zlotoff DA, Sambandam A, Logan TD, Bell JJ, Schwarz BA, Bhandoola A. CCR7 and CCR9 together recruit hematopoietic progenitors to the adult thymus. *Blood*. 2010; 115:1897–1905.10.1182/blood-2009-08-237784 [PubMed: 19965655]
18. Kocks JR, Davalos-Misslitz ACM, Hintzen G, Ohl L, Forster R. Regulatory T cells interfere with the development of bronchus-associated lymphoid tissue. *Journal of Experimental Medicine*. 2007; 204:723–734.10.1084/jem.20061424 [PubMed: 17371929]
19. Malek TR, Porter BO, Codias EK, Scibelli P, Yu A. Normal lymphoid homeostasis and lack of lethal autoimmunity in mice containing mature T cells with severely impaired IL-2 receptors. *J Immunol*. 2000; 164:2905–2914. [PubMed: 10706676]
20. D'Cruz LM, Klein L. Development and function of agonist-induced CD25+Foxp3+ regulatory T cells in the absence of interleukin 2 signaling. *Nat Immunol*. 2005; 6:1152–1159.10.1038/ni1264 [PubMed: 16227983]
21. Josefowicz SZ, Lu L-F, Rudensky AY. Regulatory T Cells: Mechanisms of Differentiation and Function. *Annu Rev Immunol*. 2012; 30:531–564.10.1146/annurev.immunol.25.022106.141623 [PubMed: 22224781]
22. Jordan MS, Boesteanu A, Reed AJ, Petrone AL, Hohenbeck AE, Lerman MA, Naji A, et al. Thymic selection of CD4+CD25+ regulatory T cells induced by an agonist self-peptide. *Nat Immunol*. 2001; 2:301–306.10.1038/86302 [PubMed: 11276200]
23. Kawahata K, Misaki Y, Yamauchi M, Tsunekawa S, Setoguchi K, Miyazaki J-I, Yamamoto K. Generation of CD4(+)CD25(+) regulatory T cells from autoreactive T cells simultaneously with their negative selection in the thymus and from nonautoreactive T cells by endogenous TCR expression. *J Immunol*. 2002; 168:4399–4405. [PubMed: 11970982]
24. Bautista JL, Lio C-WJ, Lathrop SK, Forbush K, Liang Y, Luo J, Rudensky AY, et al. Intracloal competition limits the fate determination of regulatory T cells in the thymus. *Nat Immunol*. 2009; 10:610–617.10.1038/ni.1739 [PubMed: 19430476]
25. Yu W, Nagaoka H, Jankovic M, Misulovin Z, Suh H, Rolink A, Melchers F, et al. Continued RAG expression in late stages of B cell development and no apparent re-induction after immunization. *Nature*. 1999; 400:682–687.10.1038/23287 [PubMed: 10458165]

FIGURE 1.

Long-lived Tregs increasingly accumulate in the thymus with age. (A) RAG2-GFP/Foxp3-RFP mice were analyzed for the presence of Tregs and CD4⁺ Tconvs at 5 weeks, 8 weeks, 13 weeks, and 6 months (26 weeks) of age. Representative flow cytometric data at 5 weeks and 6 months of age is shown. (B) The % GFP expression of spleen and thymic CD4 Tconvs (CD4⁺RFP⁻), and Tregs (CD4⁺RFP⁺) and (C) the total number of spleen and thymic CD4 Tconvs and Tregs in RAG2-GFP/Foxp3-RFP mice ($n = 3/\text{time point}$) is represented as a scatter plot. (D) Long-lived thymic and splenic Tregs (RFP⁺GFP⁻) from RAG2-GFP/Foxp3-RFP mice were sorted and co-cultured with CFSE-labeled Tconvs at various Treg:Tconv ratios. After anti-CD3 stimulation for 4 days, the dilution of CFSE by Tconvs was analyzed by flow cytometry. The division index of the Tconvs at different Treg:Tconv ratios is plotted. The data are representative of two independent experiments.

FIGURE 2.

Host-derived Tregs are detected in the thymus of mice after BM transplantation. (A) BM chimeric mice were generated and their thymic and splenic T cell populations were analyzed by flow cytometry 9–12 weeks later. Representative flow cytometric plots of DP thymocytes, CD8 single positive (SP) thymocytes, CD8⁺ T cells (CD8s), CD4⁺ Tconvs, and Tregs are shown. Donor cells are of CD45.2⁺ origin, while host cells are CD45.1⁺. (B) Data were compiled from BM chimeric mice from two separate experiments ($n = 8$) and is represented as a scatter plot. (C) Total numbers of all and (D) the number of host-derived Tregs, CD4 Tconvs, and CD8s in the thymus of BM chimeric mice is represented as a scatter plot. *** represents statistical significance of $p < 0.001$ and NS = not significant by Student's paired t-test.

FIGURE 3.

Tregs recirculate into the thymi of BM chimeric and RAG1 KO mice. **(A)** Congenically disparate CD4⁺ T cells were adoptively transferred into BM chimeric mice. Representative flow cytometric data at 9–12 weeks post BM transplant is shown. Donor BM-derived T cells are CD45.1⁺/CD45.2⁺, adoptively transferred T cells are CD45.2⁺, and host T cells are CD45.1⁺. **(B)** The ratio of Tregs of adoptive transfer origin in the spleen was plotted against that of the thymus and linear regression analysis was performed. **(C)** Congenically disparate CD4⁺ T cells were adoptively transferred into RAG1 KO mice. 5 weeks later, the presence of donor-derived CD4⁺ T cells in the recipient thymus and spleen were detected by flow cytometry. Representative flow cytometric data of $n = 7$ mice from two independent experiments are shown. **(D)** Total numbers of adoptive transfer origin in BM chimeric mice and RAG1 KO hosts are represented as a scatter plot. NS = not significant by Student's t-test.

FIGURE 4.

Long-lived Tregs are retained in the thymus after development. WT mice were transplanted with congenically disparate (CD45.1⁺) neonatal thymi. 6 weeks later, the donor thymus, host (endogenous) thymus, and the spleen were analyzed for the presence of CD4⁺ Tconvs, Tregs, and DPs. (A) Representative flow cytometric plots, (B) the % contribution by, and (C) numbers of donor-derived cells to the Treg pool of the donor thymus and host thymus of $n = 5$ mice from two independent experiments are shown. * represents statistical significance of $p < 0.05$ by Student's paired t-test.

FIGURE 5.

Retention contributes more than recirculation to establishment of the long-lived Treg pool in the thymus of BM chimeric mice. (A) Congenically disparate CD4⁺ T cells from WT (CD90.1⁺) mice and BM from RAG2-GFP/Foxp3-RFP mice (CD45.2⁺) were injected into congenically disparate (CD45.2⁻, CD90.1⁺) irradiated recipient WT mice. 9–12 weeks later, the thymus and spleen were analyzed for the presence of host-derived, donor BM-derived, and adoptive transfer-derived CD4⁺ Tconvs, Tregs, and DPs. Representative flow cytometric plots of CD4 single positives (SPs), CD4⁺ Tconvs, Tregs, and DPs and of GFP expression in donor BM-derived Tregs and Tconvs in the thymus and spleen are shown. (B) The relative contribution of recirculation and retention were calculated from data of n = 9 mice from 2 separate experiments. *** represents statistical significance of p<0.001 by Student's paired t-test.