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Differential Postselection Proliferation Dynamics of \( \alpha\beta \) T Cells, Foxp3\(^+\) Regulatory T Cells, and Invariant NKT Cells Monitored by Genetic Pulse Labeling

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The thymus generates two divergent types of lymphocytes, innate and adaptive T cells. Innate T cells such as invariant NKT cells provide immediate immune defense, whereas adaptive T cells require a phase of expansion and functional differentiation outside the thymus. Naive adaptive T lymphocytes should not proliferate much after positive selection in the thymus to ensure a highly diverse TCR repertoire. In contrast, oligoclonal innate lymphocyte populations are efficiently expanded through intrathymic proliferation. For CD4\(^+\)Foxp3\(^+\) regulatory T cells (Tregs), which are thought to be generated by agonist recognition, it is not clear whether they proliferate upon thymic selection. In this study, we investigated thymic and peripheral T cell proliferation by genetic pulse labeling. To this end, we used a mouse model in which all developing \( \alpha\beta \) thymocytes were marked by expression of a histone 2B–enhanced GFP (H2BeGFP) fusion-protein located within the Tcrd locus (Tcrdh2BeGFP). This reporter gene was excised during TCR \( \alpha \)-chain VJ-recombination, and the retained H2BeGFP signal was thus diluted upon cell proliferation. We found that innate T cells such as CD1d-restricted invariant NKT cells all underwent a phase of intense intrathymic proliferation, whereas adaptive CD4\(^+\) and CD8\(^+\) single-positive thymocytes including thymic Tregs cycled, on average, only once after final selection. After thymic exit, retention or loss of very stable H2BeGFP signal indicated the proliferative history of peripheral \( \alpha\beta \) T cells. There, peripheral Tregs showed lower levels of H2BeGFP compared with CD4\(^+\)Foxp3\(^+\) T cells. This further supports the hypothesis that the Treg repertoire is shaped by self-Ag recognition in the steady-state. The Journal of Immunology, 2013, 191: 000–000.

Proliferation of \( \alpha\beta \) T cells occurs at different stages during their development and differentiation. The earliest CD4\(^-\)CD8\(^-\) double-negative (DN) T cell precursors are proliferative, presumably to compensate for the few thymus seeding progenitors that can enter the thymus at a time (1, 2). Next, at the CD4\(^-\)CD8\(^-\) DN CD25\(^+\) stage, selection of productively rearranged Tcrb genes induces a phase of strong proliferation that is called \( \beta \)-selection and serves to expand clones expressing a TCR \( \beta \)-chain (3), and that is also required for \( \beta \)-lineage commitment and progression to the CD4\(^+\)CD8\(^-\) double-positive (DP) stage (4). Then, proliferation is terminated before Tcra rearrangement and positive selection. It is not clear to what extent DP thymocytes divide after positive selection of their \( \alpha\beta \) TCR. In theory, the lack of further proliferation after final \( \alpha\beta \) thymocyte selection would maximize the diversity of \( \alpha\beta \) T cells. However, it is conceivable that a few intrathymic cell divisions support efficient generation of functional T cells whereas maintaining a still highly diverse TCR repertoire (5, 6). For late DP and single-positive (SP) thymocytes, eventual encounter of agonistic TCR ligands can either lead to negative selection by apoptosis, thereby ensuring central tolerance, or induce survival by a process called agonist selection. The best studied agonist selection is the differentiation of invariant NKT (iNKT) cells (7). After recognition of instructive signals during TCR engagement by CD1d-expressing cortical thymocytes, intrathymic proliferation generates large numbers of oligoclonal innate T cell populations with limited TCR diversity (8). In contrast, the role of agonist selection for thymic differentiation of Foxp3\(^+\) regulatory T cells (Tregs) is currently controversial (9–12), and it is not known whether self-Ag recognition induces intrathymic proliferation of Tregs.

There are numerous methods for measurement of cell proliferation, including quantification of cells that incorporated \(^{3}H\) thymidine or BrdU (13, 14), CFSE labeling (15), or direct detection of recently divided cells by Ki-67 expression (16). Earlier studies using these methods suggested intrathymic expansion of mature thymocytes before export to the periphery (17). More recently, BAC-transgenic mice with multiple copies of GFP under the control of the Rag2 promoter (18) were used as a genetic tool to monitor the dynamics of thymic maturation, emigration (19), and to directly identify recent thymic emigrants (RTEs) (20, 21). In this Rag2p-GFP system, GFP-reporter transcription terminates after Tcra rearrangement in DP cells, but GFP fluorescence is detectable in peripheral T cells because of the considerable half-life of GFP in mouse T cells estimated to range between 16 and 18 h (20) and 54 and 56 h (19). Thus, the observation that the GFP signal in Rag2p-GFP mice lingers in SP thymocytes and RTEs...
further supports the idea that conventional αβ T cells do not cycle much after positive selection.

In this study, we used a genetic system in which a histone 2B–enhanced GFP (H2BeGFP) was introduced into the 3′ untranslated region of the Tcrd constant gene (22). In these TcrdH2BeGFP mice, all γδ T cells are marked by high expression of a very stable H2BeGFP–fusion protein. In addition, the reporter gene is transcribed in all DN thymocytes but excised during Vα-Jα recombination in DP thymocytes. Because the estimated half-life of H2BeGFP is in the range of several months (23), it is retained in nonproliferating postselection αβ T cells and allows to track their proliferative history in vivo. Using this genetic system for pulsing DP thymocytes, we monitored the differential dynamics of conventional T cells, Foxp3+ Tregs, and iNKT cells. We observed that all developing iNKT cells completely lost H2BeGFP fluorescence at an early developmental stage (8), thereby validating the proliferation-reporter system. In contrast, CD4+ and CD8+ SP cells still displayed intermediate H2BeGFP levels after thymic maturation and, therefore, had proliferated only to a minor degree. After thymic exit, the sole RTE showing proliferation were CD8+, which was in line with results from Fink and colleagues (20). In the periphery, resting, naive CD4+ or CD8+ T cells continued to retain an intermediate H2BeGFP signal. Even massive Ag-specific T cell immune responses to viral infection did not induce bystander proliferation among the peripheral naïve T cell pool. Rather, homeostatic proliferation of naïve T cells was observed only under lymphopenic conditions, for example, after adoptive transfer into Rag2−/−γc−/− recipients. Furthermore, nuclear localization of the H2BeGFP reporter within the chromatin facilitated simultaneous detection of intracellular Foxp3 protein and monitoring of proliferative history. Although Foxp3+CD4+ thymocytes and mature Foxp3+CD4+ and CD8+ SP cells showed similar intrathymic proliferation, Foxp3+Tregs in the periphery displayed lower H2BeGFP levels than Foxp3+CD4+T cells. This was consistent with the hypothesis that Tregs proliferate after recognizing self-Ag in the periphery and undergo organ-specific shaping of their TCR repertoire (24, 25). In summary, we present new data on the different postselection proliferation dynamics of innate αβ T cells such as iNKT cells as compared with adaptive αβ T cell populations, which include Foxp3+Tregs.

Materials and Methods

Mice

TcrdH2BeGFP mice have been described previously (22). C57BL/6J mice were obtained from Charles River (Germany). Mice were bred and housed under specific pathogen-free conditions in the central animal facilities of Hannover Medical School or Centre d’Immunologie de Marseille-Luminy and used at 6–12 wk of age. All experiments were approved by local institutional animal care and research committees, and authorized by local governments. This study was conducted in accordance with the European Communities Directive86/609/EEC for the protection of animals used for experimental purposes.

Flow cytometry and intracellular staining

Before staining, cells were incubated at 4°C for 5 min with Fc-blocking reagent (mAb 2.4G2). For multicolor staining, cells were incubated with conjugate Abs for 15 min at 4°C, washed with PBS/3% FCS, and, if required, incubated with secondary Ab streptavidin–PE-Cy7 for 10 min at 4°C and washed with PBS/3% FCS. For CCR7 mAb staining, cells were incubated with anti–CCR7–PE Ab in T cell medium for 30 min at 37°C, washed with PBS/3% FCS, and incubated with surface Abs for 15 min at 4°C. Intracellular Foxp3 staining was performed using Foxp3 staining buffer (eBioscience) according to the manufacturer’s protocol. The same buffer system was used for staining of intracellular helios with anti-helios mAb clone 2F6 conjugated with Alexa Fluor 647 (Biolegend). For Neuropilin-1 (NRP1) staining, we used biotinylated polyclonal goat anti-Nrp1 IgG (R&D Systems), followed by PE-Cy7–conjugated streptavidin. In some experiments, thymic iNKT cells were enriched by labeling the cells with anti-CD1d–GalCer tetramer–allophycocyanin and subsequent magnetic enrichment with anti–allophycocyanin–Microbeads (Miltenyi). Flow cytometric analysis was performed on a LSR cytometer (BD Bioscience) equipped with the BD FACSDiva software. Because ~1% of all αβ thymocytes show mononuclear Tcra rearrangement, a fraction of those retained H2BeGFP levels comparable with γδ T cells (unpublished data). Therefore, we excluded H2BeGFP+CD4+ and CD8+ T cells from our analysis. The validity of this approach was confirmed by analysis of F1 offspring from Tcrd-H2BeGFP reporter mice crossed to Tcra-deficient mice as described previously (26). In experiments where data are shown as fold change of H2BeGFP fluorescence intensity, we always included separate analyses of the respective cell populations from nonfluorescent C57BL/6 control mice to calculate relative values compared with WT control. Further gating strategies are described in the figure legends. Postacquisition analyses were conducted using the FlowJo software (TreeStar).

Intrathymic biotin–OSu injection

For intrathymic injection of d-biotin–N-hydroxysuccinimide ester (biotin-OSu; Anaspec) to mark RTEs, mice were anesthetized and 10 μl biotin-OSu solution (2.5 μg/μl in DMSO/PBS) was injected into one thymic lobe. All mice were analyzed after 20 h. Biotin+ cells were identified by streptavidin–Cy5 conjugation.

Adaptive cell transfer and thymectomy

To investigate homeostatic proliferation under lymphopenic conditions, we adoptively transferred 5 × 105 TcrdH2BeGFP lymphocytes derived from a filtered peripheral lymph node suspension into congenic Rag2−/−γc−/− (C57BL/6) recipients via the lateral tail vein. For thymectomy, 4- to 6-wk-old TcrdH2BeGFP mice were anesthetized and thymi removed by suction as described elsewhere (27).

Murine CMV infection

Infections were initiated by i.p. delivery of 5 × 104 PFU thawed murine CMV (MCMV) stocks (28). Smith strain salivary gland extracts were prepared as described previously (29). Infections were initiated on different days so that each animal in a given experiment was sacrificed on the same day.

Statistical analysis

Group data were compared with one-way ANOVA or repeated-measures ANOVA with Tukey posttest (GraphPad Prism) to generate p values.

Results

αβ T cells in TcrdH2BeGFP mice receive a thymic pulse H2BeGFP labeling

Developing thymocytes open the Tcra locus at the transition from DN1b to DN2 stage, regardless of their final commitment to αβ or γδ lineage (22). Later at the CD4+CD8+ DP stage, the Tcra locus is excised during Vα to Jα rearrangement (30). Thus, in TcrdH2BeGFP mice, the H2BeGFP reporter gene within the Tcra locus is transiently transcribed during CD4+CD8−DN to CD4+CD8+DP stages, but removed during Tcra rearrangement (Supplemental Fig. 1A). After positive selection and excision of the reporter gene, the very stable H2BeGFP is retained and legacy fluorescence is thus distributed among daughter cells upon proliferation (Supplemental Fig. 1B). To control that loss of H2BeGFP correlated with cell proliferation, we stimulated T cells derived from spleen and peripheral lymph nodes in an in vitro proliferation assay and double-checked with BrdU staining. As shown in Supplemental Fig. 2A, in vivo proliferated BrdU+ CD4+ and CD8+ T cells possessed significantly lower H2BeGFP levels compared with BrdU− cells. These data demonstrate a direct link between proliferation and H2BeGFP loss. To exclude that TCR rearrangement excision circles might still drive H2BeGFP expression in αβ T cells, we analyzed Tcra transcription of sorted H2BeGFP+ and H2BeGFP− αβ T cells. No transcription of rearranged Tcra genes was detected, suggesting that H2BeGFP+ αβ T cells do not transcribe H2BeGFP mRNA (Supplemental Fig. 2B). Next, we monitored how dilution of eFluor...
Within the thymus, we observed even more intense labeling of all DP developing αβ T cells as compared with peripheral T cells. DP green fluorescence could be quantified as 30-fold of the background in C57BL/6 mice (fold median H2BeGFP fluorescence intensity [MFI] of C57BL/6 control throughout the article) in DP thymocytes (Fig. 1). This qualified DP thymocytes as H2BeGFP intermediate with regard to the ~10-fold higher fluorescence observed in γδ T cells. Thymic CD4+ and CD8+ SP T cells showed H2BeGFP signal of 15- and 10-fold MFI, respectively (Fig. 1). Because signal intensity principally decreases through cell proliferation, 50% loss of fluorescence between DP and SP stage is consistent with the occurrence, on average, of one cell division.

**Intrathymic proliferation of naive T cell subsets**

DP thymocytes require (at least weak) stimulation of their TCR to proliferation. To further distinguish such DP thymocytes from mature SP T cells and TCRdH2BeGFP+Tcells in unchallenged C57BL/6 mice (fold median H2BeGFP fluorescence intensity). Representative and combined data from three different experiments (n = 15) are shown.

![Figure 1](https://example.com/figure1.png)

**Figure 1.** Thymic αβ T cells retain intermediate H2BeGFP fluorescence in TcrdH2BeGFP mice. Histograms show the H2BeGFP level, and graph shows quantification H2BeGFP MFI fold change of γδ (CD4+ CD8- GL3+H2BeGFP+), DP (CD4+CD8+), CD4SP (CD4+CD8- CD25+ NK1.1+), and CD8SP (CD8+CD4- CD25+ NK1.1-) T cells in unchallenged TcrdH2BeGFP mice compared with C57BL/6 controls (gray filled histograms). Representative and combined data from three different experiments (n = 15) are shown.

different maturation steps of CD4+ and CD8+ T thymocytes, we gated them into CD24-CD69- (semimature) and CD24+CD69- (mature) thymocytes. We observed that CD4+ and CD8+ SP cells lost further H2BeGFP signal during thymic maturation (Fig. 2). Together, these observations provide fresh evidence that thymocytes undergo one to two cell divisions after positive selection. Such duplication of the naive T cell pool may warrant a trade-off between maximal TCR repertoire diversity and the presence of more than one representative of a crucial T cell clone. Our results are in line with previous studies that showed late intrathymic proliferation of SP T cells before thymic exit (5, 6, 17).

**Postthymic maturation and peripheral proliferation of adaptive T cells**

Next, we asked whether T cells continued to proliferate in the periphery after thymic exit. To this end, TcrdH2BeGFP mice were intrathymically injected with a reactive bovine derivative, and peripheral T cells were analyzed 20 h later. At this time point, 0.30 ± 0.19% of peripheral CD4+ T cells and 0.23 ± 0.15% of peripheral CD8+ T cells were identified as RTEs by surface bovine labeling (Fig. 3). Compared with non-RTEs, RTEs displayed slightly higher H2BeGFP levels. Furthermore, CD8+ RTEs, but not CD4+ RTEs, showed lower H2BeGFP intensity in comparison with mature SP thymocytes, indicative of fewer turnovers after or during thymic exit (Fig. 3). To address how H2BeGFP levels were distributed among peripheral T cell subsets in steady-state, we correlated evidence that nonactivated, naive CD4+ and CD8+ T cells show surprisingly little turnover under steady-state and inflammatory conditions, whereas homeostatic proliferation can maintain the peripheral T cell pool if necessary because of lymphopenia.

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Innate lymphocytes proliferate during thymic development

In addition to producing a diverse repertoire of adaptive T cells, the thymus generates innate T cell populations such as iNKT with limited TCR diversity. After final TCR rearrangement, iNKT undergo intrathymic proliferation to efficiently produce an oligoclonal T cell population (33). In this study, we investigated intrathymic proliferation during the developmental stages of CD1d-restricted iNKT cells as an example for agonist-selected αβ T cells. Thereby, iNKT cell precursors (stage 0, defined as CD1d/aGC+CD44loCD24hiNK1.12) (8) are thought to be agonist activated by endogenous CD1d-bound lipid ligands, and thus show a strong TCR activation (7, 34). Stage 0 iNKT cells from TcrdH2BeGFP mice were showing fluorescence levels similar to CD4+ SP T cells (Fig. 6). But already at stage 1 (CD1d/aGC+CD44loCD24loNK1.12), all developing iNKT cells had completely lost their H2BeGFP signal, indicating that progression from stage 0 to stage 1 is stringently linked to intrathymic cell division (Fig. 6). Consistently, subsequent iNKT stages 2 and 3

FIGURE 2. Proliferation of postselection thymocytes quantified by loss of H2BeGFP. CD4+CD8+ DP thymocytes before and after positive selection were gated as CD69+TCRβ+ and CD69+TCRβ+, respectively (upper left panel). During selection, CD4+ and CD8+ SP T cells lose CD24 and CD69 expression; therefore, CD24+CD69+ SP T cells were defined as semimature and CD24+CD69+ SP T cells as mature. Graph shows fold change of H2BeGFP MFI of the indicated thymocyte populations compared with C57BL/6 mice. Combined data from three different experiments (n = 12) are shown. Statistical analysis was performed with one-way repeated-measures ANOVA with Tukey posttest. ***p < 0.001.

FIGURE 3. CD8+ RTEs, but not CD4+ RTEs, proliferate upon thymic exit. Twenty hours after intrathymic injection of reactive biotin, CD62L+biotin+ T cells in peripheral lymph nodes were identified and gated as RTEs. Right panels show fold change of H2BeGFP MFI of the indicated CD4+ (upper panels) or CD8+ (lower panels) T cells compared with C57BL/6 control mice. Combined data from two different experiments (n = 6–8) are shown. Statistical analysis was performed with one-way repeated-measures ANOVA with Tukey posttest. ***p < 0.001.

FIGURE 4. Activation of peripheral T cells correlates with loss of H2BeGFP. (A) Histograms show CD62L+CD44+ (blue), CD62L-CD44+ (green), CD62L+CD44- (red), and CD62L-CD44- (orange) subsets of CD4+ (upper panel) and CD8+ (lower panel) T cells of peripheral lymph nodes from TcrdH2BeGFP mice compared with C57BL/6 (filled gray). Graphs show fold change of H2BeGFP MFI for all different CD4+ (left) and CD8+ (right) T cell subsets compared with C57BL/6 mice. Lower panels show fold change of H2BeGFP MFI of (B) CD69+ and CD69- and (C) CCR7- and CCR7+ subsets of CD4+ and CD8+ T cells derived from TcrdH2BeGFP compared with C57BL/6 mice. Representative and combined data from one (B) CD69; n = 4 or three [A, B] CCR7; n = 12] experiments are shown. Statistical analysis was performed with one-way repeated-measures ANOVA with Tukey posttest (A) and Student t test (B). ***p < 0.001.
also showed no reappearance of the lost H2BeGFP label. Therefore, iNKT development validated the TcrdH2BeGFP reporter system as a reliable genetic tool for in vivo monitoring of post-selection T cell proliferation within the thymus.

Proliferation of Tregs during thymic development

Tregs are another T cell subset that is discussed to be agonist selected (11, 34, 35). Therefore, we asked whether recognition of self-peptides induced proliferation within developing Tregs when compared with naive CD4+Foxp3+ T cells or iNKT cells. In this study, intracellular Foxp3 staining was possible because chromatin-linked H2BeGFP fluorescence was not lost during cell permeabilization (21). Still, H2BeGFP intensities in fixed/permeabilized cells were found to be lower than in untreated cells. Thymic Treg precursors, defined as CD4+ SP Foxp3- CD25+CD122+ (36), showed a 2-fold decline in H2BeGFP levels compared with semimature CD4+Foxp3+ SP cells, but similar levels compared with mature CD4+Foxp3+ SP (Fig. 7). This loss of H2BeGFP signal translates into one thymic cell division per cell after passing the CD4+CD8+ DP stage. Further maturation of thymic Tregs, characterized by Foxp3 induction, was not accompanied by a significant loss of H2BeGFP levels (Fig. 7). These observations are consistent with
the idea that agonistic activation results in proliferation at Foxp3− Treg precursor stage. However, taking into account that H2BeGFP levels of CD4+ SP T cells were also divided in half during thymic maturation, this means that mature CD4+Foxp3− T cells and mature CD4+Foxp3+ Tregs leave the thymus with comparable H2BeGFP intensities, and thus with a similar proliferation history.

**Proliferation history of peripheral Treg subsets**

Next, we analyzed proliferation of Tregs in the periphery in correlation to their anatomical location. In lymph nodes and spleen, Tregs showed lower H2BeGFP levels compared with CD4+Foxp3− T cells (Fig. 8). This suggests that, on average, peripheral Tregs in secondary lymphoid organs had proliferated more than CD4+Foxp3− T cells. This matches our previous findings that the peripheral Treg repertoire is very diverse, albeit less diverse than naive αβ T cells (25). In contrast, in small intestine and colon, Tregs and CD4+Foxp3− T cells completely lost their H2BeGFP, indicating that they proliferated more than two times after thymic exit (Fig. 8). It was proposed that different Treg subsets display distinct phenotypes and homeostasis in normal unmanipulated mice (37). In the steady-state, some Tregs remain quiescent and have a long life span, in the order of months, whereas the other Tregs are dividing extensively and express multiple activation markers. Therefore, we mapped Treg proliferation according to expression of activation markers. First, analogous to CD4+Foxp3− and CD8+ T cells, Tregs with a CD62L−CD44+ phenotype had proliferated most (Fig. 9A), which is in line with previous findings (38). Next, we sought to investigate the proliferation history of Treg populations defined by Treg-specific markers. The transcription factor Helios (39) and the surface molecule Nrp1 (40, 41) were recently proposed to discriminate thymic-derived and peripherally induced Tregs. As revealed by H2BeGFP levels of the respective Treg populations in secondary lymphoid organs, Helios+ and Nrp1high Tregs had proliferated more than Helios− or Nrp1low Tregs (Fig. 9B, 9C). An interesting exception was the mesenteric LN, where the proliferation history of Nrp1high and Nrp1low Tregs did not differ. In addition, activated FR4high Tregs (42) showed significantly lower H2BeGFP levels compared with FR4low and FR4intermediate Tregs (Fig. 9D). Taken together, expression of Helios, Nrp1, and FR4 correlated with postselection proliferation of Tregs, and thus likely indicated Treg activation.
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FIGURE 8. Peripheral Tregs proliferate more than CD4⁺Foxp³⁻ T cells. Histograms show H2BeGFP level, and graph shows H2BeGFP MFI fold change of CD4⁺Foxp³⁻ (gray) and CD4⁺Foxp³⁺ (black) T cells in peripheral lymph nodes and small intestine of TcrdH2BeGFP mice compared with C57BL6 control (gray filled). Representative dot plot of CD4⁺ T cells of peripheral LNs from TcrdH2BeGFP mice. Graph shows quantification of fold change H2BeGFP MFI in CD4⁺Foxp³⁻ (open) and CD4⁺Foxp³⁺ (filled) T cells derived from indicated organs compared with C57BL6 control. Representative data from five different experiments (n = 19) are shown. Statistical analysis was performed with Student t test. ***p < 0.001.

Discussion

In this study, TcrdH2BeGFP mice were used to directly track the proliferative history of βα T cells by transient genetic pulse labeling during thymic development. This novel tool allowed us to revisit the contrasting postselection dynamics of mainstream βα T cells, Foxp³⁻ Tregs, and iNKT cells. The latter, identified by α-galactosylceramide bound to CD1d (43), are representative innate T cells. Agonistic TCR selection of an invariant Vα14-Jα18 TCRo-chain (44), predominantly paired with Vβ8.2, Vβ7, and Vβ2, propagates intrathymic proliferation via a strong TCR signal (34). Recent data suggest that miR-181a/b-1 expression in DP thymocytes supports iNKT agonist selection and subsequent efficient expansion through intrathymic proliferation (45). In contrast, adaptive βα T cells should maintain a highly diverse TCR repertoire to be prepared for recognition of any foreign Ag. Thus, it is conceivable that adaptive T cells should not proliferate unless they encounter their cognate ligands. Indeed, retention of H2BeGFP signal in naive peripheral CD4⁺ and CD8⁺ T cells indicated that these cells had, on average, undergone less than three cell divisions after positive selection and less than two divisions after thymic exit. Even in the highly inflammatory milieu of a fulminating primary immune response to MCMV infection, nonparticipating naive bystander T cells did not proliferate. This situation may be different in neonates, when the secondary lymphoid organs of mice are filling rapidly. The argument that little proliferation is a means of guaranteeing diversity of the TCR repertoire is valid for adult mice but may not be so for neonates. In adult mice, only strong lymphopenia after adoptive transfer into Rag⁻/⁻ recipients induced homeostatic proliferation; thereby CD8⁺ T cells appeared to be more responsive to homeostatic proliferation as described previously (46). Also, studies in Rag²p-GFP transgenic mice showed that, in particular, CD8⁺ RTEs undergo a process of postthymic maturation before they are incorporated into the pool of mature peripheral T cells (20). In RTEs, the CD4/CD8 ratio is higher compared with mature peripheral T cells, and adjustment of the CD4/CD8 ratio includes increased proliferation of CD8⁺ RTEs. In this study, we confirmed these results by demonstrating H2BeGFP loss during thymic exit solely for CD8⁺ and not for CD4⁺ RTEs. It was speculated that CD8⁺ thymocytes in Rag2p-GFP transgenic mice showed lower GFP levels because their DP to SP transition took longer time than in CD4⁺ thymocytes (19). In this study, we observed that decline of H2BeGFP reporter intensity was greater in CD8⁺ compared with CD4⁺ T cells. Because H2BeGFP is very stable, we therefore suggest slightly more H2BeGFP retention by CD8⁺ thymocytes in TcrdH2BeGFP mice compared with C57BL6 control mice. This result should be confirmed by repeated experiments.

H2BeGFP loss during thymic exit solely for CD8⁺ and not for CD4⁺ RTEs. It was speculated that CD8⁺ thymocytes in Rag2p-GFP transgenic mice showed lower GFP levels because their DP to SP transition took longer time than in CD4⁺ thymocytes (19). In this study, we observed that decline of H2BeGFP reporter intensity was greater in CD8⁺ compared with CD4⁺ T cells. Because H2BeGFP is very stable, we therefore suggest slightly more proliferation during CD8⁺ DP-to-SP transition. Nevertheless, we observed proliferation in CD4⁺ as well as CD8⁺ SP thymocytes. Although H2BeGFP may be partially lost because of rebuilding of thymocyte differentiation, one might speculate that one to two thymic cell divisions produce a “backup” of the naive TCR repertoire.

It is currently controversial whether self-Ag recognition by thymic Treg precursors equals agonist selection and to what extent this Ag encounter induces proliferation (9, 10, 12, 34, 35).
Hogquist’s group (34) showed with Nur77EGFP reporter mice that TregS, upon thymic selection, perceived a stronger TCR stimulation compared with CD4+Foxp3+ T cells. Unexpectedly, thymic Tregs in TcrdH2BE GFP mice displayed reporter fluorescence levels similar to mature CD4+Foxp3+ SP cells. This supports the idea that thymus re-entry or long-term thymic retention, as described for agonist-selected iNKT (47) or γδ NK cells (48), does not chiefly contribute to the thymic Treg pool.

Thymus-derived Tregs make up the largest part of the peripheral Treg compartment. However, Tregs can also be induced from CD4+Foxp3- peripheral T cells. Up to now, no solid marker to discriminate thymus-derived and peripherally induced Tregs was found. Recently, Nrp-1 was proposed to be expressed at high levels on thymus-derived opposed to peripherally induced Tregs (40, 41). In this study, we found lower H2BEGFP levels in Nrp1high compared with Nrp1low Tregs. Furthermore, Helios, an Ikaros transcription factor family member, was proposed to be expressed by thymus-derived but not peripherally induced Tregs (39, 49, 50). However, Helios might be rather a marker for T cell activation and proliferation (51). In line with the latter notion, we could show that Helios+ Tregs are significantly more proliferated than their Helios- counterparts. Together, assuming that lack of Nrp-1 and Helios expression identifies peripherally induced Tregs, their Foxp3 induction would not be accompanied by proliferation.

In conclusion, these studies further illustrate that the thymus maintains two fundamentally different production lines: innate and adaptive T cells. On one hand, agonist-selected iNKT cells display a limited TCR repertoire, and they are efficiently produced via intrathymic proliferation. On the other hand, adaptive CD4+ T cells hardly proliferate during thymic development and maintain a highly diverse TCR repertoire. Thymic Tregs, although possibly activated by TCR agonist selection, do not proliferate more than CD4+Foxp3 thymocytes and also sustain a highly diverse TCR repertoire. In future work, being able to track the proliferative history of αβ T cells at the individual cell level in TcrdH2BE GFP mice should be useful to study homeostatic proliferation and loss of clonal diversity in aged mice.

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Disclosures

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