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Dendritic Cells Support Homeostatic Expansion of Foxp3+ Regulatory T Cells in Foxp3.LuciDTR Mice

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Foxp3+CD4+ regulatory T cells (Tregs) are crucial in maintaining self-tolerance and limiting immune responses to pathogens. Shifting the sensitive balance between Tregs and effector T cells requires extensive knowledge of the homeostatic properties of the different T cell populations. For the investigation of Treg homeostatic expansion, we introduce in this study novel BAC transgenic mice, designated Foxp3.LuciDTR, coexpressing enhanced GFP, luciferase for bioluminescence imaging of Tregs, and the diphtheria toxin receptor (DTR) for specific ablation of Tregs. Of several founder lines, Foxp3.LuciDTR-4 mice displayed ~95% Treg depletion following injection of DT, resulting in activation of conventional CD4+ T cells, probably due to lack of control by Tregs. In contrast, Foxp3.LuciDTR-3 mice displayed only ~70% Treg depletion without concomitant activation of CD4+ T cells and represented, therefore, a suitable model to study Treg homeostasis in an environment where other T cell populations were not altered. After depletion, the Treg compartment recovered to its original size within ~2 wk. This recovery was mediated in a thymus-independent fashion by homeostatic proliferation of the surviving, nondepleted Tregs. The proliferating Tregs acquired an activated phenotype and maintained their suppressive capacity. Studies involving DT-mediated depletion of dendritic cells in CD11c.DOG mice showed that dendritic cells were required for optimal Treg homeostasis. In addition, IL-2 was identified as an essential factor for homeostatic recovery of the Treg compartment. These results show that Treg homeostasis is specifically regulated by the size of the Treg compartment and is independent of proliferation of conventional T cells. The Journal of Immunology, 2010, 184: 1810–1820.

Regulatory T cells (Tregs), a subset of CD4+ T cells characterized by the expression of CD25 and the nuclear transcription factor Foxp3, have been shown to play a crucial role in maintaining self-tolerance and limiting immune responses to pathogens (1). Neonatally thymectomized mice that are devoid of Tregs develop multiorgan inflammatory disease, which is mediated by CD4+ T cells and is characterized by inflammatory infiltrates in the affected organs and autoantibody production (2–4). A defective foxp3 gene in so-called scurfy mice results in severe autoagression and fatal lymphoproliferative disease, identifying foxp3 as a key regulator in Treg development and function (5–8). Similarly, the human X-linked immunodeficiency syndrome IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome) is caused by mutations in the FOXP3 gene and is characterized by autoimmune disease in endocrine organs, inflammatory bowel disease, severe allergy, and fatal infection (9).

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Abbreviations used in this paper: BLI, bioluminescence imaging; DC, dendritic cell; DT, diphtheria toxin; DTR, diphtheria toxin receptor; eGFP, enhanced GFP; LN, lymph node; MHC-II, MHC class II; RLU, relative light unit; Treg, regulatory T cell.

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Tregs comprise a functionally distinct cellular lineage and are continuously produced in the thymus by positive selection (10). In the periphery, the homeostatic processes maintaining the pool size of CD4+ and CD8+ T cells have been extensively studied, but the homeostasis of Tregs is not fully understood. Clarifying the mechanisms that control maintenance of the Treg pool size is of importance for clinical applications, where Treg number is reduced after chemotherapy or lymphodepletion during organ transplantation or is dysregulated as in tumor-bearing patients or in patients suffering from autoimmune diseases (11). Manipulation of the Treg compartment is a promising therapeutic strategy. For example, reduction of Treg number or function can promote tumor immunity, whereas Ag-specific expansion of Tregs may support transplantation tolerance and therapy of autoimmune diseases (1). Thus, it is of importance to elucidate how Tregs differ from other T cell subsets in their homeostatic requirements and properties.

Homeostatic proliferation and survival of naive CD4+ and CD8+ T cells is largely dependent on the cytokine IL-7 (12, 13), whereas IL-15 plays an important role in the expansion and maintenance of memory CD8+ T cells (14). In addition, it has been shown that naive T cells require contacts with self-MHC–peptide complexes as homeostatic signals, whereas most memory T cells are MHC-independent (15, 16). Thus, upon transfer into lymphopenic hosts, T cells undergo homeostatic proliferation, which has been attributed to increased availability of MHC ligands and/or IL-7 (15, 16). Tregs have long been thought to have very limited proliferative capacity, as they are anergic in vitro when stimulated through the TCR (17–19) and are hyporesponsive to antigenic stimuli in vivo (19). However, in a lymphopenic environment, Tregs are capable of mounting a strong proliferative response, which is promoted by MHC class II (MHC-II) interactions (19). Whereas IL-7 is an important growth factor for CD4+ and CD8+ T cells, IL-2 appears to play a significant role in both thymic development and
peripheral maintenance of Tregs (20–22). In a bone marrow chimera model, it has been demonstrated that Treg expansion depends on IL-2 mainly produced by conventional CD4+ T cells (23). However, in a different study with cotransfer of Tregs together with conventional T cells into RAG-deficient hosts, IL-2 deficiency was unable to inhibit lymphopenia-induced homeostatic expansion of Tregs (24). These data suggest that homeostatic requirements of Tregs may depend on the host’s state of immunodeficiency and on the activation status of the surrounding cell populations.

Therefore, we investigated in the current study whether and how Tregs are capable to expand by homeostatic proliferation in an environment in which only the size of the Treg compartment is reduced, whereas the pool size and activation status of all other T cell populations remains unaltered. For this purpose, we generated novel BAC transgenic mice, designated Foxp3.LuciDTR, which permit noninvasive bioluminescence imaging of Foxp3+ Tregs via luciferase and in addition allow specific ablation of Tregs utilizing the human diphtheria toxin receptor (DTR) system. We show that following selective ablation of Tregs, the fraction of remaining Tregs underwent strong homeostatic proliferation and filled up again the Treg compartment to its original size in a thymus-independent fashion in ~2 wk. Mechanistically, the homeostatic expansion of Tregs depended strongly on IL-2 and was supported by dendritic cells (DCs).

**Materials and Methods**

**Mice and Treg depletion**

C57BL/6N mice (B6/WT; CD45.2+) were purchased from Charles River Laboratories (Wilmington, MA). B6.SJL-Ptprca Pep3b/BoyJ (CD45.1), CD11c.DOG (25), and RAG2-deficient (Rag2-/-) mice were bred at the central animal facility of the German Cancer Research Center (Heidelberg, Germany). Foxp3EGFP mice (26) were generously provided by B. Mann (Centre Immunologie de Marseille-Luminy, Marseille, France) via I. Prinz (Medizinische Hochschule Hannover, Hannover, Germany). All animals were held under specific pathogen-free conditions, and experiments were conducted according to governmental and institutional guidelines and regulations.

For generation of Foxp3.LuciDTR BAC transgenic mice, a construct composed of the cDNAs for enhanced GFP (eGFP), the human DTR, and CBGr99 luciferase (27) was generated. Coding sequences were separated by a self-cleaving 2A peptide sequence from porcine teschovirus-1 for stoichiometric production of the three individual transgenic proteins (28). The construct was inserted at the start codon (ATG) in the third exon of the foxp3 gene in the BAC RP23-147HS (BACPAC Resource Center, Children’s Hospital Oakland Research Institute, Oakland, CA) using Escherichia coli EL250 (29), excited using NotI, and injected into the pronuclei of fertilized B6 mouse oocytes. The resulting transgenic mouse lines were designated Foxp3.LuciDTR-3, -4, and -5. All experiments were performed using mice heterozygous for the eGFP-DTR-luciferase construct. Typing was carried out either phenotypically by flow cytometric analysis of PBLs for eGFP expression or by PCR from genomic DNA of tail biopsies using the following primers for the DTR: 5’-GCCACCTCAGAAGCTGTCGGCG-3’ and 5’-TCAGTGGAAGTAGCTGTCGCC-3’. For Treg depletion, mice were injected i.p. with 15 mg/kg body weight diphtheria tox in (DT) (Sigma-Aldrich, St. Louis, MO), unless otherwise noted.

**Bioluminescence imaging**

Mice were imaged 5 min after i.p. injection of 4.5 mg d-luciferin (SynChem, Elk Grove Village, IL) using the IVIS 100 imaging system (Xenogen, Hopkinton, MA) as described (27). Analysis was performed with LivingImage software (v2.50, Xenogen). Light output was quantified as relative light units (RLU). Where indicated, organs were removed immediately after whole body imaging and incubated in 1.5 mg/ml d-luciferin in PBS for 2 min at room temperature for imaging of isolated organs.

**Flow cytometry**

The following biotinylated or fluorochrome-conjugated Abs were purchased from BD Biosciences (San Jose, CA) or eBioscience (San Diego, CA): CD4 (RM4-5 or GK1.5), CD8 (53-6.7), Foxp3 (FJK-16s), CD25 (PC61 or 7D4), K67 (B56), CD44 (IM7), CD62L (MEL-14), CD45.1 (A20), CD45.2 (104), CD103 (M290), CD69 (H1.2F3), ICOS (7E.17G9), OX40 (OX-86), PD-1 (J43), GITR (DTRA-1), CTLA-4 (UC10-4B9), CD122 (TM-β1), and CD127 (A7R34). Fluorochrome-labeled streptavidin was obtained from BD Biosciences. Propidium iodide (Sigma-Aldrich) or ethidium monoxide ( Molecular Probes, Eugene, OR) were used as viability dyes. Labeled cells were analyzed on a BD FACSCalibur or BD FACS Canto II flow cytometer (BD Biosciences) and evaluated using FlowJo v.e8.2.2 (Tree Star, Ashland, OR). When the Foxp3 staining set (eBioscience) was used, eGFP staining was no longer visible, thereby preventing simultaneous analysis of Foxp3 and eGFP.

**Adaptive transfer of T cells and in vivo IL-2 neutralization**

CD4+ T cells were purified from spleens and lymph nodes (LN s) of CD45.1 mice using anti-CD4 microbeads (L3T4, Miltenyi Biotec, Auburn, CA) according to the manufacturer’s instructions. Purity of CD4+ T cells was typically >80%. The cells were labeled with 1 μM CFSE (Molecular Probes/Invitrogen), and a total of 5 × 106 cells was transferred i.v. into the indicated hosts. For in vivo IL-2 neutralization, mice were injected i.p. with a daily dose of 1 mg anti–IL-2 Ab S4B6 (BioXCell, Beverly, MA) for the indicated number of days.

**In vitro suppression assay**

CD4+ T cells from spleens and LNs were enriched using anti-CD4 microbeads (L3T4, Miltenyi Biotec) and sorted into CD4+CD25- responder cells and CD4+CD25high Tregs using a BD FACSAria cell sorter to a purity >99%. A total of 103 responder T cells from B6 mice were activated with 5 × 106 irradiated splenocytes and 2 μg/ml anti-CD3ε (145-2C11, eBioscience) in the presence of titrated numbers of Tregs in RPMI 1640 medium containing 10% FCS and 1% glutamine in round-bottom 96-well plates for 90 min. Proliferation was measured by [3H]thymidine incorporation during the last 16–18 h of culture and expressed as cpm.

**Statistical analysis**

Results are shown as mean ± SEM. For statistical analysis, the Student t test was applied to obtain p values as provided in the figure legends.

**Results**

**Generation of BAC transgenic Foxp3.LuciDTR mice for in vivo Treg visualization and depletion**

To study homeostasis of Foxp3+ regulatory T cells in mice, we generated a novel set of BAC transgenic mice, so-called Foxp3.LuciDTR mice, which would allow selective ablation of Tregs without causing major alterations in the conventional CD4+ and CD8+ T cell compartments. In these mice, Tregs coexpress eGFP, the DTR, for depletion of Tregs, and the click-beetle luciferase CBGr99 for in vivo BLI of Tregs (Fig. 1A). In a previous study, the CBGr99 luciferase was determined to be optimal for BLI owing to a high photon yield in the red part of the light emission spectrum (27). To ensure expression of the three proteins as individual moieties at equimolar amounts, a technology was employed in which the coding cDNAs are separated by a self-cleaving 2A viral sequence (27, 28). Several Foxp3-eGFP-2A-DTR-2A-luciferase founder mice were selected for further breeding on the basis of different degrees of Treg depletion. Foxp3.LuciDTR-5 mice displayed >95% Foxp3+ CD4+ T cell depletion, but depletion resulted in wasting disease, possibly due to fatal autoimmunity (data not shown). As will be discussed below, mice from lines Foxp3.LuciDTR-3 and Foxp3.LuciDTR-4 exhibited ∼75% and 90% depletion of Foxp3+CD4+ T cells, respectively, and showed no signs of weight loss.

Upon injection of the luciferase substrate d-luciferin into Foxp3.LuciDTR mice, the measured photon emission peaked at the location of lymphoid organs such as cervical, axillary, and inguinal LNs, thymus, and peritoneal area, whereas wild-type control mice gave no signal (Fig. 1B). The RLUs obtained were 5.21 × 106 RLU for Foxp3.LuciDTR-3 and 1.56 × 107 RLU for Foxp3.LuciDTR-4 mice, indicating a stronger expression of the BAC construct in the latter strain. After DT injection, the bioluminescence signal was strongly
decreased, demonstrating that most luciferase-expressing Foxp3+ Tregs were depleted (Fig. 1B). As tissues can absorb much of the emitted photons, in particular blood-containing organs such as spleen (27), mice were sacrificed and isolated organs imaged by BLI (Fig. 1C). The results confirm the tissue distribution presented in Fig. 1B and show that Tregs are visible mainly in thymus, spleen, peripheral LNs, Peyer’s patches, lung, and skin. Supplemental Fig. 1 shows the BLI signal of skin obtained after longer exposure. At present, it is not clear whether the Tregs reside in the skin parenchyma or in blood vessels. Comparable results were obtained with Foxp3.LuciDTR-4 mice, which yielded stronger BLI signals (Fig. 1C).

Cytofluorometric analysis of LNs (Fig. 1D, 1E, upper panels) and spleen cells (Supplemental Fig. 1B) from Foxp3.LuciDTR-3 and Foxp3.LuciDTR-4 mice demonstrated that application of DT results in almost complete depletion of eGFP+/Foxp3+ cells among all CD4+ cells. Foxp3.LuciDTR-3 mice were treated with DT on 2 consecutive days. Treg visualization by BLI was performed before and at indicated time points after DT treatment. G. Quantification of BLI signals of the mice shown in F. Data are expressed as RLUs. Data indicate the mean ± SEM of three mice. Shown is a representative of three to five experiments.
FIGURE 2. Recovery of the Treg compartment after Treg depletion in Foxp3.LuciDTR-3 mice. 

A, Foxp3.LuciDTR-3 mice were treated with DT on 2 consecutive days and the recovery of eGFP^+DTR^+ Tregs (♦) and total Foxp3^+ Tregs (♦) was determined as frequency of CD4^+ cells in spleen, LNs, and blood (upper graphs). The lower graph displays the absolute number of Foxp3^+CD4^+ Tregs in the spleen. Data represent the mean ± SEM of three mice. 

B, Representative dot plots (left panels) and summary (right panel) of Ki67 staining in LN of mice shown in A. It can be seen from the right-hand graph that most Foxp3^+CD4^+ T cells were proliferating (♦), but not the conventional Foxp3^- CD4^+ T cells (♦). C, Foxp3.LuciDTR-3 mice were thymectomized and received 2 to 3 wk thereafter daily applications of 30 ng/g body weight DT. The frequency of eGFP^+DTR^- Tregs (♦) and total Foxp3^- cells (♦) was determined in spleen, LNs, and blood. Data indicate the mean ± SEM of three mice. D, Representative dot plots (left panels) and summary (right panel) of Ki67 staining in LN of mice shown in C. Experiments were repeated several times.
and ∼90% in Foxp3.LuciDTR-4 mice (Supplemental Fig. 1b, 1c). These findings indicated that the BACs were not expressed in all Foxp3⁺CD4⁺ Tregs, allowing us to investigate the fate of the non-depleted Tregs, which consisted largely of BAC-negative T cells and a residual fraction of BAC-positive (eGFP⁺DTR⁺Luc⁺) Tregs.

**Homeostatic expansion of Tregs in mice with a reduced Treg compartment**

Following depletion by two daily injections of DT, recovery of Tregs was assessed by noninvasive BLI. Fig. 1F and 1G show that the Treg compartment recovered with time in Foxp3.LuciDTR-3 mice. Foxp3.LuciDTR-4 mice gave similar results (data not shown). The kinetics of Treg recovery were confirmed by cytofluorometric analysis of Tregs in spleen, LNs, and blood from Foxp3.LuciDTR-3 mice. DT injection resulted in almost complete depletion of eGFP⁺DTR⁺ Tregs, which comprised ∼65–75% depletion of the total Treg pool (Fig. 2A). The surviving eGFP⁺DTR⁻ Foxp3⁺ Tregs and eGFP⁺DTR⁻Foxp3⁺ Tregs were found to recover with kinetics similar to the data obtained by BLI (Fig. 2A).

Fig. 2A shows the frequency of Tregs and absolute Treg numbers in the spleen. The size of spleen and LNs in DT-treated Foxp3.LuciDTR-3 mice did not increase, which is in contrast to >90% Treg-depleted Foxp3.LuciDTR-4 mice, as will be shown below.

Recovery of the Treg pool may be due to homeostatic expansion of the ∼25% surviving Tregs and/or to de novo thymic output of Tregs.

Upregulation of Ki67, a marker for proliferating cells, suggested that repopulation of the Treg compartment was caused by homeostatic proliferation (Fig. 2B). To more precisely analyze a potential contribution of the thymus, thymectomized Foxp3.LuciDTR-3 mice were used. Daily treatment of thymectomized Foxp3.LuciDTR-3 mice with DT resulted in continuous depletion of the eGFP⁺DTR⁺ Tregs, so that expansion of the surviving eGFP⁺DTR⁻ Tregs could be selectively studied. Fig. 2C demonstrates that Treg number recovered in the spleen and blood with similar kinetics to that found in euthymic mice. Recovery in LNs was somewhat slower. As the recovery occurs in thymectomized animals, we conclude that the Treg compartment expanded mostly by homeostatic proliferation. As observed in euthymic mice (Fig. 2B), Ki67 was found in a large percentage of the expanding Foxp3⁺CD4⁺ T cells in Treg-depleted, thymectomized mice, confirming that the Tregs undergo proliferation and that homeostatic expansion of Tregs is not dependent on thymic output (Fig. 2D). In contrast, in the same animals, only few conventional Foxp3⁻CD4⁺ T cells underwent proliferation, as indicated by Ki67 (Fig. 2B, 2D).

Recovery of Treg numbers was also studied in Foxp3.LuciDTR-4 mice treated with DT. The small surviving fraction of 5–10% Foxp3⁺ Tregs expanded rapidly and filled up the Treg pool both in spleen and LNs within ∼2 wk (Fig. 3A). Thus, a small residual pool of Tregs can also rapidly reconstitute the Treg compartment. However, following >90% depletion of Tregs in Foxp3.LuciDTR-4 mice, the...
The cellularity of spleen (Fig. 3B) and LNs (data not shown) was increased by ∼2-fold after 21 d. The total number of Tregs, CD4+ T cells (Fig. 3B) and CD8+ T cells, B cells, macrophages, and NK cells was also increased, but the ratio of these populations was not much changed, with the exception of DCs, which were slightly decreased (Supplemental Fig. 2). The increase in cell numbers was accompanied by activation of the conventional Foxp3+CD4+ T cells, as indicated by upregulation of the activation marker CD44 and downregulation of

**FIGURE 4.** Selective expansion of Tregs following transfer of total CD4+ T cells into Treg-depleted Foxp3.LuciDTR-3 mice. A, CD45.1+ congenic CD4+ T cells were purified from spleens and LNs of WT donors via MACS, labeled with CFSE, and adoptively transferred into CD45.2+ Foxp3.LuciDTR-3, WT B6, and Rag2−/− recipients. In the Foxp3.LuciDTR-3 recipients, Tregs were depleted with DT on 5 consecutive days. CD4+ T cells were transferred 8 h after the first DT injection. Mice were sacrificed 5 d after cell transfer, donor-derived CD45.1+ lymphocytes were stained for Foxp3, and CFSE profiles were measured. FACS plots show a representative LN staining from each group of recipients. B, Summary of data obtained with two to four mice per group, indicating the percentage of cells that have undergone at least one division as measured by CFSE dilution of Foxp3+ (○) and Foxp3− (♦) CD4+ donor-derived T cells. Lines indicate mean values. One of three comparable experiments is shown.
CD62L (Fig. 3C, 3D and Supplemental Fig. 3). In contrast, as will be shown later in the 75% depleted Foxp3.LuciDTR-3 mice, no activation of conventional CD4+ T cells was observed. To avoid a potential influence of activated conventional CD4+ T cells on the homeostatic expansion of Tregs, we focused on Foxp3.LuciDTR-3 mice for further studies.

**Homeostatic proliferation of Tregs following cell transfer**

The upregulation of Ki67 during Treg recovery suggests selective homeostatic proliferation of Tregs in mice with reduced Treg numbers. To study this more precisely, we investigated proliferation in a cell transfer system (Fig. 4). For this purpose, CFSE-labeled total CD4+ T cells bearing the allelic CD45.1 marker were transferred into Treg-depleted Foxp3.LuciDTR-3 mice, wild-type mice, and Rag2−/− mice. DT treatment was continued for 5 d. The results show that after transfer into Treg-depleted Foxp3.LuciDTR-3 mice, only the Foxp3+CD4+ T cells proliferated vigorously. Of the conventional Foxp3−CD4+ T cells, only a small fraction was found to proliferate, but this fraction was comparable to proliferating CD4+ T cells observed after transfer into wild-type mice (Fig. 4). Following transfer into wild-type mice, no significant proliferation of Tregs was observed, whereas transfer into T cell-deficient Rag2−/− mice resulted in strong proliferation of both Tregs and conventional CD4+ T cells (Fig. 4). Together, these results demonstrate that an incomplete Treg compartment induces selective proliferation of Tregs.

It has been reported that in the periphery, homeostatic expansion of conventional CD4+ T cells under lymphopenic conditions can lead to conversion into Foxp3+CD4+ T cells (30, 31). To see if some of the above-described proliferating Foxp3+ cells are derived from conventional CD4+ cells by conversion, we sorted Foxp3−CD4+ T cells from Foxp3EGFP Thy1.1+ reporter mice (26) and transferred them into Foxp3.LuciDTR-3 reporter mice (Thy1.2+) and wild-type mice as controls. Five days after transfer in both recipient strains, only a small and identical number of Thy1.1+ Foxp3−CD4+ T cells was found (Supplemental Fig. 4). This indicates that the repopulation of the Treg compartment is mainly due to homeostatic proliferation of Tregs and not conversion of CD4+ conventional T cells. As a control, sorted GFP+ Tregs were transferred and found to proliferate in depleted Foxp3.LuciDTR-3 mice (Supplemental Fig. 5), as expected from results obtained following transfer of unfractionated CD4+ T cells (Fig. 5).

**Homeostatically proliferating Tregs suppress conventional T cell responses**

Next, the homeostatically expanding Tregs were investigated for expression of activation markers. A number of activation markers were upregulated on homeostatically proliferating Tregs (Fig. 5 and Supplemental Fig. 6), but no significant production of the cytokines IL-2, IL-4, and IFN-γ by the proliferating Tregs was found (data not shown). The percentage of expanding Tregs expressing the costimulatory molecule ICOS was strongly increased. Because ICOS was suggested to control the pool size of Tregs (32), we studied a potential contribution of ICOS to homeostatic Treg proliferation. However, following transfer into depleted Foxp3.LuciDTR-3 mice, ICOS−/− and wild-type Tregs were found to proliferate equally well (data not shown). Thus, ICOS expression on Tregs does not seem to contribute much to Treg homeostatic proliferation. In contrast to the Tregs, the conventional CD4+ T cells did not undergo activation in Foxp3.LuciDTR-3 mice following DT application (Fig. 5). Likewise, we did not observe alterations in frequency and activation status of B cells, NK cells, granulocytes, macrophages, and DCs (Supplemental Fig. 7).

**FIGURE 5.** Homeostatically proliferating Tregs acquire an activated phenotype and maintain suppressive capacity. A, Foxp3.LuciDTR-3 mice were treated daily with DT, and expression of activation markers was determined in peripheral LNs. Data show the frequency mean fluorescence intensity of cells expressing the indicated molecule of Foxp3−CD4+ Tregs (○) and Foxp3+ CD4+ conventional T cells (●). CD103: αEβ7 integrin; CD122: IL-2R/IL-15R common β-chain; CD127: IL-7R. B, Foxp3.LuciDTR-3 and WT controls were injected daily with DT for 7 d, spleen and LNs cells enriched for CD4+ T cells via MACS, followed by FACS sorting of CD4+CD25high Tregs and CD4+CD25− T responder cells, and suppression assay performed as described in Materials and Methods. T responder cells were derived from WT controls. The same control bar (no Tregs) is shown once in each group for better comparison. Experiments were repeated several times with comparable results.
Tregs require IL-2 for homeostatic proliferation

IL-2 is known to be critical for maintenance and survival of Tregs (24, 33), but homeostatic proliferation of Tregs in lymphopenic RAG-deficient mice was found to be IL-2 independent (24). Therefore, we investigated the role of IL-2 for homeostatic expansion of Tregs in Foxp3.LuciDTR-3 mice. In orientating experiments, wild-type B6 mice were treated for 5 d with anti–IL-2 Ab S4B6 (Fig. 7A). In agreement with previous results (24, 33), this treatment reduced the number of Foxp3+ Tregs in LNs slightly by ∼20% (Fig. 7A, right panel). This decrease was mainly due to a reduction of the naturally proliferating Ki67+ Tregs (Fig. 7A, left panel). Next, depleted Foxp3-LuciDTR-3 mice were treated with anti–IL-2 Ab for 5 d. The frequency of endogenous Tregs was reduced by ∼60% by IL-2 neutralization as compared with Foxp3.LuciDTR-3 mice treated with DT only (Fig. 7B, right panel). This difference was again mainly due to a reduction of proliferating Ki67+ Tregs (Fig. 7B, left panel). Similar observations were made following transfer of CFSE-labeled total CD4+ T cells into Treg-depleted Foxp3.LuciDTR-3 mice. The CFSE profiles presented in Fig. 7C, 7D show that homeostatic proliferation of Tregs was strongly reduced by IL-2 starvation.

DCs promote homeostatic expansion of Tregs

Homeostatic proliferation of T cells (34, 35) and NK cells (25) was reported to depend on DCs, at least to a certain degree. Therefore, we asked whether DCs would also promote homeostatic expansion of Tregs. For this purpose, CD11c.DTR mice (designated CD11c.DOG) (25) that allow continuous depletion of DCs were mated to Foxp3.LuciDTR-3 mice. CFSE-labeled total wild-type CD4+ T cells were transferred into the resulting (CD11c.DOG × Foxp3.LuciDTR-3)F1 mice from which both DCs and Tregs were depleted by DT. For comparison, CD4+ T cells were also transferred into Foxp3.LuciDTR-3 mice from which only the Foxp3+CD4+ T cells were depleted. The results presented in Fig. 8A, 8B show that Foxp3+ cells proliferate more strongly in the Foxp3.LuciDTR-3 mice, which still harbor DCs. These data indicate that DCs promote homeostatic expansion of Tregs, but are not absolutely required.

Discussion

For the investigation of Treg homeostasis, a set of BAC transgenic Foxp3.LuciDTR mice was generated. The use of BACs ensures tissue-specific expression, but often BACs are not expressed in all cells of a particular tissue. Therefore, we expected to obtain Foxp3.LuciDTR mice in which not all Tregs would be deleted by application of DT so that the fate and homeostasis of the remaining Tregs could be studied. Indeed, several founder lines with different degrees of DTR expression were obtained. Foxp3.LuciDTR-5 mice displayed >95% Treg depletion, but died of wasting disease (data not shown), possibly due to autoimmunity. This is reminiscent of Foxp3.DTR knockin mice, which were described to succumb to fatal autoimmunity after Treg depletion (36). Mouse lines Foxp3.LuciDTR-3 and Foxp3.LuciDTR-4 displayed less complete Treg depletion, namely ∼65–75% and 90–95%, respectively (Fig. 1) and showed no signs of pathology, similar to an independent strain of BAC transgenic Foxp3.DTR mice (37). However, the 90–95% depletion in Foxp3.LuciDTR-4 mice resulted in activation of conventional CD4+ T cells, probably due to lack of control by Tregs, whereas after 65–75% depletion in Foxp3.LuciDTR-3 mice, the conventional CD4+ T cells remained quiescent. Therefore, Foxp3.LuciDTR-3 mice were selected for further studies, as they allowed us to investigate Treg homeostasis in a situation where only the Treg compartment was reduced without concomitant major alterations in numbers and activation status of the other T cell populations.

Owing to the expression of luciferase, Tregs could be visualized in live mice by BLI, demonstrating that Tregs were mainly located in lymphoid organs (Fig. 1B, 1C). Successful Treg depletion could be also monitored by BLI, but longitudinal studies showed that the Treg compartment was filled up again to its original size in ∼2 wk. The recovery was due to homeostatic proliferation of the remaining Tregs, as indicated by acquisition of a Ki67+ phenotype. Moreover, we observed similar return kinetics in thymectomized mice, suggesting that the de novo production of Tregs by the thymus did not contribute much to the reconstitution of the Treg compartment (Fig. 2C). Recovery of the Treg pool appeared to be slower in LNs than in spleen or blood (Fig. 2A, 2C). A possible explanation is that homing receptors required for recirculation into LNs may be downregulated as a consequence of proliferation-induced activation, whereas entry into the spleen seems to be less restricted (38). CD62L is probably not responsible for this effect, as it was generally low on Tregs (e.g., see Fig. 4A, day 0 values for CD62L).

Induction of Treg proliferation was also demonstrated in a transfer model. Following adoptive transfer of total CFSE-labeled CD4+ T cells into Treg-depleted Foxp3.LuciDTR-3 mice, the Tregs were found to expand by homeostatic proliferation, indicating that induction of Treg number is sufficient to promote strong Treg proliferation, whereas no significant proliferation was observed after transfer into nondepleted mice (Fig. 5). Comparable proliferation of Tregs was observed after transfer into Treg-depleted and Rag2−/− mice, but the underlying mechanisms appear to be different. Whereas...
Treg expansion in Rag2–/– mice has been reported to be IL-2 independent, as shown by the use of IL-2–deficient Rag2–/– recipients (24). Treg expansion in Treg-depleted mice is clearly dependent on IL-2, as demonstrated in this study by Ab-mediated IL-2 neutralization (Fig. 7). Together, the results indicate that Treg reduction is sufficient to induce proliferation of the vast majority of the remaining Tregs. It is likely that besides IL-2, additional factors are involved in Treg homeostasis. IL-2–deficient as well as IL-2Rα– and IL-2Rβ– deficient mice can produce Tregs, although in decreased numbers and with functional defects, whereas common γ-chain–deficient mice are completely devoid of Tregs, indicating that other γ-chain cytokines are partially able to compensate for the lack of IL-2 (39, 40).

It has been reported that under lymphopenic conditions, homeostatic proliferation of conventional CD4+ T cells can cause conversion into Foxp3+ Tregs (30, 31). This is in contrast to the results presented in this study, which failed to provide evidence for conversion (Supplemental Fig. 4). Possibly this apparent discrepancy can be explained by the different experimental systems used.

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Whereas in the previous studies CD25− or Foxp3− conventional CD4+ T cells were transferred into lymphopenic RAG-deficient mice, where the CD4+ T cells would proliferate vigorously, we performed transfers into Treg-depleted Foxp3LuciDTR-3 mice, in which transferred CD4+ T cells do not proliferate (Fig. 5).

The homeostatic proliferation of Tregs appears to be accompanied by cell death, as suggested by comparison of the proliferation rates and recovery of the Treg compartment. In the adoptive transfer experiments, the division rate of Tregs was at least five times per 5 d (Fig. 5). At this rate, 10–20% of Tregs should have filled up the compartment in ~4 to 5 d, but the actual recovery time was ~2 wk (Figs. 2, 3), suggesting that many proliferating Tregs die, probably due to activation-induced cell death. This is comparable to IL-2–triggered activation-induced cell death in conventional CD4+ T cells (41). The proliferating Tregs acquired an activated phenotype and retained their suppressive activity. In fact, in several experiments, the proliferating Tregs exhibited a higher suppressive activity than Tregs isolated from a nonmanipulated mouse (Fig. 4B).

Previous studies have shown that Ag-bearing DCs induce expansion of Tregs in vivo (42), but a role for DCs in homeostatic proliferation in the absence of Ag has not yet been addressed. Therefore, we asked whether DCs promote homeostatic proliferation of Tregs in the absence of Ag. Transfer of Tregs into (Foxp3LuciDTR-3 × CD11c.DOG)F1 mice, in which not only Tregs but also DCs are depleted by DT, demonstrated that DCs support homeostatic expansion of Tregs (Fig. 8). The molecular mechanisms are not yet clear, but possibly MHC-II recognition is involved, as suggested by the observation that homeostatic proliferation of CD25+CD4+ T cells in RAG-deficient mice is MHC-II dependent (19). It has been reported that DCs can transiently produce IL-2 following activation (43), but it remains to be investigated whether DC-derived IL-2 contributes to Treg expansion. Thus, DCs support lymphopenia-induced homeostatic proliferation not only for T cells and NK cells (25, 34, 35), but also for Tregs. It should be noted that after depletion of Tregs in Foxp3LuciDTR-3 mice, no increase in frequency and activation status of DCs was observed (Supplemental Fig. 7), which is in contrast to Foxp3.DTR knockin mice, where the almost complete depletion of Tregs led to activation and increased numbers of DCs (36).

The rapid homeostatic recovery of the Treg pool described in this paper is likely to be of consequence for the development of therapeutic modulation of Tregs; for example, in tumor therapy, where several strategies aim at reducing Treg numbers, or in autoimmune and transplantation situations, where an increased frequency of Tregs may be desired. In approaches where the Treg-modulating reagent is applied only for a brief time, or in situations where Tregs become resistant to modulation, homeostatic mechanisms may adjust the Treg compartment to its original size, thereby limiting the therapeutic window.

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Disclosures
The authors have no financial conflicts of interest.


Supplemental Figure 1. A, Bioluminescence imaging of Foxp3.LuciDTR-4 skin as compared to WT control skin. B, Flow cytometric analysis of Foxp3.LuciDTR-3 and Foxp3.LuciDTR-4 mice before and 24 hours after two daily i.p. injections. Spleen cells were either stained for CD4 and measured for eGFP (upper panels), or stained for CD4 and Foxp3 (lower panels). The depicted percentages indicate the fraction of eGFP⁺/Foxp3⁺ cells among all CD4⁺ cells. Compare also Fig. 1 for LN staining. C, Frequency of eGFP⁺ (left) or Foxp3⁺ (right) Tregs among CD4⁺ T cells in untreated or DT-treated Foxp3.LuciDTR-3 (top) and Foxp3.LuciDTR-4 (bottom) mice. Numbers indicate mean ± SD of 3 (Foxp3.LuciDTR-3) or 6 (Foxp3.LuciDTR-4) individual mice.

Supplemental Figure 2. Foxp3.LuciDTR-4 mice were treated daily with DT. At the indicated time points, absolute number (left column) and frequency (right column) of different splenic cell subsets were determined (compare Fig 3A,B). Cell populations were measured by flow cytometry as follows: CD4⁺ T cells, CD4⁺CD8⁻; CD8⁺ T cells, CD8⁺CD4⁻; DCs, CD11c⁺CD11c⁺; MHC-II⁺; B cells, CD19⁺; macrophages, F4/80⁺CD11c⁺; granulocytes, Gr-1⁺CD11b⁺; NK cells, NK1.1⁺. Antibodies were purchased from BD Biosciences or Caltag: CD11c (HL3), I-A/I-E (M5/114.15.2), Gr-1 (RB6-8C5), CD11b (M1/70), F4/80 (A3-1), NK1.1 (PK136), CD19 (1D3).

Supplemental Figure 3. Representative flow cytometry diagrams for CD44 and CD62L expression summarized in Figure 3C. Foxp3.LuciDTR-4 mice were treated with DT on two consecutive days and analyzed on day 7 for activation of splenic CD4⁺ T cells. Plots are gated on CD4⁺Foxp3⁻ T cells.
**Supplemental Figure 4.** Conversion of conventional CD4+ T cells does not significantly contribute to the homeostatic recovery of the Treg compartment. CD4+ T cells from spleens and LNs of Foxp3EGFP Thy1.1+ donors were enriched using anti-CD4 microbeads (L3T4, Miltenyi Biotec). From these, CD4+eGFP− conventional T cells were sorted using a BD FACSAria cell sorter to a purity >99%, labeled with CFSE and adoptively transferred into WT B6 or Foxp3.LuciDTR-3 recipients, which received daily DT treatment for 5 days. Recipients were then stained for donor-derived CD4+ and Foxp3+ T cells. Representative dot plots of LN (top) and spleen (bottom), gated on Thy1.1+ donor-derived CD4+ T cells, are shown.

**Supplemental Figure 5.** Purified Tregs proliferate upon transfer into Treg-depleted Foxp3.LuciDTR-3 recipients. CD4+ T cells from spleens and LNs of Foxp3EGFP Thy1.1+ donors were enriched using anti-CD4 microbeads (L3T4, Miltenyi Biotec). From these, CD4+eGFP+ Tregs were sorted using a BD FACSAria cell sorter to a purity >99%, labeled with CFSE and adoptively transferred into WT B6 or Foxp3.LuciDTR-3 recipients, which received daily DT treatment for 5 days. Cells were transferred 8h after the first DT injection. Recipients were then stained for donor-derived CD4+Foxp3+ T cells. Representative histograms of LN gated on Thy1.1+ donor-derived CD4+Foxp3+ Tregs are shown.

**Supplemental Figure 6.** Representative flow cytometry diagrams for the stainings summarized in Figure 5A. Foxp3.LuciDTR-3 mice were treated daily with DT, and expression of activation markers was determined in peripheral lymph nodes. Representative stainings for untreated (day 0) and DT-treated (day 10)
Supplemental Figure 7. Treg depletion in Foxp3.LuciDTR-3 mice does not change numbers of DCs, granulocytes, macrophages, NK cells, B cells, CD4 T cells, CD8 T cells. Foxp3.LuciDTR-3 mice were treated daily with DT and flow cytometry for the indicated leukocyte populations was performed before and on the indicated time points after DT treatment. Populations were as follows: DCs, CD11c^{high}MHC-II^{+}; granulocytes, Gr-1^{high}CD11b^{+}; macrophages, F4/80^{+}CD11c^{−}; NK cells, NK1.1^{+}; B cells, CD19^{+}B220^{+}; CD4 T cells, CD4^{+}CD8^{−}; CD8 T cells, CD8^{+}CD4^{−}. Antibodies were purchased from BD Biosciences or Caltag: CD11c (HL3), I-A/I-E (M5/114.15.2), Gr-1 (RB6-8C5), CD11b (M1/70), F4/80 (A3-1), NK1.1 (PK136), CD19 (1D3), B220 (RA3-6B2).
A

B

C

Frequency eGFP$^+$ of CD4$^+$ cells & Frequency Foxp3$^+$ of CD4$^+$ cells

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<td>DT-</td>
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<td>DT+</td>
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<tr>
<td>lymph nodes</td>
<td>11.43 ± 1.03</td>
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Suffner_Supplemental_Figure_3

Gated on CD4^+Foxp3^+

WT

Foxp3 LuciDTR-4 +DT

CD4

CD4

CD4

CD4
Suffner_Supplemental_Figure_4

LN

spleen

wild type

0.45%

0.56%

Foxp3.LuciDTR-3 +DT

0.37%

0.82%

CD4

Foxp3
Transfer of FACS-sorted GFP+ Tregs

Gated on donor-derived Thy1.1+CD4+Foxp3+

WT + DT

Foxp3.LuciDTR-3 + DT

CFSE

Cell number
Suffner_Supplemental_Figure_7

![Graph depicting the percentage of live splenocytes over time for different cell types: DCs, granulocytes, macrophages, and NK cells.]

![Another graph showing the percentage of live splenocytes for B cells, CD4 T cells, and CD8 T cells.]

![A third graph illustrating the MFI of CD86 on DCs over time.]