Homeostasis and Effector Function of Lymphopenia-Induced "Memory-Like" T Cells in Constitutively T Cell-Depleted Mice

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Homeostasis and Effector Function of Lymphopenia-Induced “Memory-Like” T Cells in Constitutively T Cell-Depleted Mice

David Voehringer,2*,† Hong-Erh Liang,*, and Richard M. Locksley2*,

Naive T lymphocytes acquire a phenotype similar to Ag-experienced memory T cells as a result of proliferation under lymphopenic conditions. Such “memory-like” T (TML) cells constitute a large fraction of the peripheral T cell pool in patients recovering from T cell ablative therapies, HIV patients under highly active antiretroviral therapy, and in the elderly population. To generate a model that allows characterization of TML cells without adoptive transfer, irradiation, or thymectomy, we developed genetically modified mice that express diphtheria toxin A under control of a loxP-flanked stop cassette (R-DTA mice). Crossing these mice to CD4Cre mice resulted in efficient ablation of CD4 single-positive thymocytes, whereas double-positive and CD8 single-positive thymocytes were only partially ablated. In the periphery the pool of naive (CD44hiCD62Llow) T cells was depleted. However, some T cells were resistant to Cre activity, escaped deletion in the thymus, and underwent lymphopenia-induced proliferation resulting in a pool of TML cells that was similar in size and turnover to the pool of CD44hiCD62Llow “memory phenotype” T cells in control mice. CD4Cre/R-DTA mice remained lymphopenic despite the large available immunological “space” and normal Ag-induced T cell proliferation. CD4Cre/R-DTA mice showed a biased TCR repertoire indicating oligoclonal T cell expansion. Infection with the helminth *Nippostrongylus brasiliensis* resulted in diminished effector cell recruitment and impaired worm expulsion, demonstrating that TML cells are not sufficient to mediate an effective immune response. *The Journal of Immunology*, 2008, 180: 4742–4753.

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2 Address correspondence and reprint requests to Dr. R. M. Locksley, University of California, Box 0654, S 1032B, 513 Parnassus Avenue, San Francisco, CA 94143. E-mail address: locksley@medicine.ucsf.edu or Dr. David Voehringer, Institute for Immunology, University of Munich, Goethestrasse 31, Munich, D-80336 Germany. E-mail address: david.voehringer@med.uni-muenchen.de

3 Abbreviations used in this paper: TML, “memory phenotype” T; TML, “memory-like” T; DTA, diphtheria toxin A; Hprt, hypoxanthine phosphoribosyltransferase; DN, double negative; DP, double positive; SP, single positive; Treg, regulatory T; KLRG1, killer cell lectin-like receptor G1; Siglec, sialic acid-binding Ig-like lectin.

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adoptive transfers of mature T cells into lymphopenic hosts that either genetically lacked endogenous T cells or were irradiated before transfer (9, 14–16). So far there are no mouse models for constant and spontaneous lymphopenia without experimental manipulations that could be used to study certain aspects of immune responses under lymphopenic conditions. Spontaneous mouse models for lymphopenia would be helpful to develop more efficient vaccination strategies for the elderly population and to study peripheral tolerance mechanisms that control the onset of autoimmunity, which is often associated with lymphopenic conditions (12).

Herein, we describe a newly generated mouse strain that encodes diphtheria toxin A (DTA) under control of a loxp-flanked stop cassette in the ubiquitously expressed ROSA26 locus. Thereby, it can be used in combination with tissue-specific and/or inducible Cre-expressing mouse strains to achieve toxin-mediated cell ablation in vivo. ROSA-DTA mice were crossed to CD4Cre mice to generate a spontaneous lymphopenic mouse model with an oligoclonal repertoire of T<sub>Mi</sub> cells. We determined their phenotype and turnover under steady-state conditions and analyzed the immune response upon infection with the helminth Nippostrongylus brasiliensis.

Materials and Methods

Mice

To generate R-DTA mice, the diphtheria toxin α-chain (DTA) was amplified by PCR from pKO SelectDT plasmid (Lexicon Genetics) with the following primer pairs: 5′ DTA: 5′-tgtgagctgctgctgcattg-3′ and 3′ DTA: 5′-ctcgattgctgatagtgc-3′. The amplified DTA was then subcloned into SacI/XhoI digest into the pBEG plasmid behind the loxP-flanked neomycin resistance cassette and in front of the bovine growth hormone poly(A) sequence (17). The modified pBEG plasmid was cotransfected with PacI/AeI and cloned into the PacI/AeI-digested PROSA26-1 plasmid (http://www.fhrc.org/labs/soriano/ vectors/pROSA26-1.html) to generate the final targeting vector. Targeting vector (30 μg) was linearized with KpnI and electroporated into E14 ES cells (129/Sv background). Southern blots were performed with EcoRV-digested DNA using a probe (337 bp) generated by NotI digest of the PROSA26 promoter plasmid (http://www.fhrc.org/ labs/soriano/vectors/pROSA26promoter.html). Eighty ES cell colonies showed homologous recombination. Mice were generated by blastocyst injection in the transgenic core facility at the University of California at San Francisco (UCSF).

CD4Cre mice (C57BL/6NTac-Tg(N)18) were purchased from Taconic (New York) and bred to R-DTA mice on a mixed 129/Sm × C57Bl/6 background. Thy1.1 mice (B6.PL-Thy1.1) and Ly5.1 mice (B6.SJL-129S7/SvImJ) were purchased from The Jackson Laboratory. Mice were housed under specific pathogen-free conditions according to institutional guidelines.

Retroviral transduction of T cells

CD4<sup>+</sup> T cells from R-DTA mice (Thy1.2) and control Thy1.1 mice were isolated from spleen and mesenteric lymph node by negative selection using a CD4<sup>+</sup> T cell isolation kit (Miltenyi Biotech), stimulated for 2 days with plate-bound anti-TCR (H57) and anti-CD28 Ab (BD Pharmingen), and transduced by spin-infection with a murine stem cell virus-based retrovirus containing the neomycin-resistance cassette (kindly provided by Kevin Shannon, UCSF).

Flow cytometry and cell sorting

Single-cell suspensions were incubated with anti-CD16/CD32 blocking Ab (2.4G2) for 5 min at room temperature and stained with the corresponding Ab mixtures. The following mAbs were purchased from Caltag/Invitrogen unless otherwise indicated: APC-labeled anti-CD8, PE-Alexa Fluor 700-labeled anti-CD4, FITC-labeled anti-CD28, and PE-labeled anti-CD44 Abs, fixed, and permeabilized. Then genomic DNA was fragmented with DNase I (Sigma-Aldrich) and stained with FITC-labeled anti-CD45.1 (Southern Biotechnology), biotinylated anti-CD5 (BD Pharmingen), PE-labeled anti-sialic acid-binding Ig-like lectin (Siglec-F) (BD Pharmingen), biotinylated anti-IgE (Southern Biotech), PE-labeled anti-Thy1.1 (eBioscience), biotinylated anti-killer cell lectin-like receptor G1 (KLRG1) (eBioscience), PE-labeled streptavidin (SouthernBiotech), and APC-labeled streptavidin (SouthernBiotech). Intracellular staining for Foxp3 was performed with the anti-mouse/rat Foxp3 staining set (eBioscience). Intracellular cytokine staining was performed with FITC-labeled anti-IFN-γ (XMG1.2, eBioscience), PE-labeled anti-IL-4 (BV6D6-24G2, Invitrogen), and Alexa Fluor 647-labeled anti-IL-17A (eBioTC11–18H10.1, eBioscience) after cells had been stimulated for 4 h with 1 μg/ml ionomycin and 20 ng/ml PMA, with brefeldin A having been added at 5 μg/ml for the last 2 h. Naïve CD4<sup>+</sup> T cells (CD62L<sup>+</sup>CD25<sup>−</sup>) were sorted from spleen and lymph node of Thy1.1 mice using a FACSAria high speed cell sorter (BD Biosciences) with a purity of >98%. CFSE labeling was done by incubation of cell suspensions with 0.5 μM CFSE at 5 × 10<sup>5</sup> cells/ml for 10 min at 37°C. Cells were analyzed on a FACSCalibur instrument (BD Biosciences).

In vitro T cell polarization

Untouched CD4<sup>+</sup> T cells were purified from spleen and lymph nodes by MACS technology (Miltenyi Biotech) and cultured for 5 days under Th1 (5 ng/ml IL-12, 20 μg/ml anti-IL-4 (11B11)), or Th2 (20 ng/ml IL-4, 20 μg/ml anti-IFN-γ (XMG1.2)) conditions with plate-bound anti-αβTCR (H57, 0.2 μg/ml) and anti-CD28 (0.2 μg/ml) in the presence of 20 ng/ml IL-2. On day 5 cells were restimulated for 4 h with 1 μg/ml ionomycin and 40 ng/ml PMA and subjected to quantitative RT-PCR analysis.

Mixed bone marrow chimeras

Bone marrow from CD4Cre/R-DTA mice (Ly5.2<sup>+</sup>Thy1.1<sup>+</sup>) was mixed at a 10:1 ratio with bone marrow from wild-type mice (Ly5.2<sup>+</sup>Thy1.2<sup>+</sup>) and 2 × 10<sup>6</sup> cells were injected into lethally irradiated (2 × 600 rad) wild-type Ly5.1<sup>+</sup> mice. Chimeras were kept with antibiotics containing drinking water (2 g/L neomycin sulfate and 100 mg/L polymixin B). Mice were analyzed at the indicated time points after reconstitution.

Histology

Frozen tissue from mesenteric lymph nodes and spleen was cut in 5-μm-thick sections and stained with biotinylated anti-Thy1.2 (Caltag/Invitrogen) and Alexa Fluor 647-labeled anti-B220 (Caltag/Invitrogen). Images were acquired on a Leica DM RXA microscope. Original magnification was ×80.

PCR analysis

For semi quantitative RT-PCR analysis, CD4<sup>+</sup> and CD8<sup>+</sup> T cells were sorted on a FACSaria cell sorter with >96% purity. RNA was isolated using a total RNA isolation kit (Fluka) and transcribed in cDNA with SuperScript II reverse transcriptase (Invitrogen). Cre expression was determined on serial dilutions of cDNA samples using the following primer pairs: Cre1, 5′-agatagctgctgctgatcagtcg-3′ and Cre2, 5′-ctcgattgctgatagtgc-3′. Hypoxanthine phosphoribosyltransferase (Hprt) expression was analyzed with Hprt1 (5′-tgggtgccagtgttgctcagt-3′) and Hprt2 (5′-tgggatgtgctgctgatagtgc-3′) primers.

For genomic PCR analysis, DNA was isolated from sorted CD4<sup>+</sup> and CD8<sup>+</sup> T cells by proteinase K digest and isopropanol precipitation. The primers for amplification of the neo cassette were neo1 (5′-ctcattgatagctggctggtggcagatgg-3′) and neo2 (5′-ctcattgatagctggctggtggcagatgg-3′). All PCR reactions were performed with 35 cycles, 56°C annealing temperature, and 60 s extension time at 72°C.

To determine the expression of cytokines in T cell cultures, quantitative RT-PCR was performed using the following primer pairs: IL-4 forward, 5′-actcgattgctgctgct-3′ and IL-4 reverse, 5′-ctcgattgctgctgctgcttta-3′; IFN-γ forward, 5′-ctcattgctgctgctgctgcttta-3′ and IFN-γ reverse, 5′-ctcgattgctgctgctgctgcttta-3′; and IFN-γ forward, 5′-ctcattgctgctgctgctgcttta-3′ and IFN-γ reverse, 5′-ctcgattgctgctgctgctgcttta-3′.

BrDU analysis

Mice were given 0.8 mg/ml BrdU in the drinking water for 7 days. Single-cell suspensions of the spleen were labeled with anti-CD4, anti-CD8, and anti-CD44 Abs, fixed, and permeabilized. Then genomic DNA was fragmented with DNase I (Sigma-Aldrich) and stained with FITC-labeled anti-BrDU Ab (BD Pharmingen).

Figure 1A

Serum ELISA

Serum ELISA levels were determined by standard ELISA technique using the mAb R35–72 (BD Pharmingen) for coating and the biotinylated mAb R35–118 (BD Pharmingen) for detection.
FIGURE 1. Generation of R-DTA mice and selective T cell ablation in vitro. 

**A**, Schematic representation of the targeting construct used to generate R-DTA mice, which express the DTA subunit under control of a loxP-flanked stop cassette in the ubiquitously expressed ROSA26 locus. Cre activity removes the stop cassette and allows DTA expression. 

**B**, Southern blot analysis of tail DNA from heterozygous (Het) offspring and wild-type (WT) mice. The wild-type allele generates a band at 11 kb and the targeted allele at 3.8 kb. 

**C**, Efficient Cre-mediated killing of T cells from R-DTA mice in vitro. A mixed T cell culture with CD4⁺ T cells from control mice (Thy1.1⁺) and R-DTA mice (Thy1.2⁺) was stimulated for 2 days with plate-bound anti-TCR Ab and then transduced with a retrovirus expressing a GFP-Cre fusion protein. Thirty-six and 60 h after transduction the ratio of Thy1.1/Thy1.2 cells was analyzed among the transduced cells (GFP⁺) and nontransduced cells (GFP⁻) by flow cytometry.
N. brasiliensis infection

Third-stage larvae of *N. brasiliensis* were recovered from the cultured feces of infected rats, washed extensively in 0.9% saline (37°C), and injected (500 organisms) into mice subcutaneously at the base of the tail. Mice were treated with antibiotic-containing water (2 g/L neomycin sulfate, 100 mg/L polymyxin B sulfate; Sigma-Aldrich) for the first 5 days after infection. Worm expulsion was determined by counting adult worms in the small intestine on day 9 after infection.

Statistical analysis

The *p* values were determined with Student’s *t* test using SigmaPlot software (SPSS) (*p* < 0.05 was considered to be statistically significant).

Results

Conditional DTA expression in T cells induces cell death without bystander toxicity

T cell development and homeostasis were analyzed using a newly generated mouse strain (R-DTA) with conditional expression of the DTA gene. R-DTA mice were constructed by homologous recombination of a loxP-flanked neomycin resistance gene followed by DTA into the ubiquitously expressed ROSA26 locus (19) (Fig. 1, A and B). The loxP-flanked cassette prevents DTA expression in the absence of Cre activity. Mice carrying the targeted allele were born at the expected Mendelian frequency, were healthy and fertile, and could be bred to homozygosity, demonstrating that toxin expression was under tight control. To analyze the efficiency of T cell deletion and potential toxicity to bystander T cells, a coculture was set up with CD4⁺ T cells from wild-type (Thy1.1⁺) mice and R-DTA (Thy1.2⁺) mice, which were stimulated for 2 days with plate-bound anti-TCR Ab and then transduced with a retrovirus expressing a GFP-Cre fusion protein. At 36 h after transduction most GFP⁺ cells from R-DTA mice had died (with a further decrease at 60 h), while GFP⁺ cells from control mice remained alive (Fig. 1C). The ratio of GFP⁺ T cells from R-DTA and wild-type mice remained stable. This demonstrates that cell death occurs fast after toxin expression and that DTA expression in T cells from R-DTA mice is not toxic to bystander T cells.

T cell ablation in CD4Cre/R-DTA mice

R-DTA mice were crossed to CD4Cre mice to analyze T cell deletion in vivo. The regulatory elements used to generate CD4Cre transgenic mice faithfully reflect expression of the endogenous CD4 gene, which starts at the double-negative (DN) 3 stage and continues through the double-positive (DP) and CD4 single-positive (SP) stage but is shut off in CD8 SP cells (20, 21). Flow cytometric analysis of the thymus in CD4Cre/R-DTA mice revealed that total numbers of DP thymocytes were reduced by ~50% and CD4 SP cells were almost absent (Fig. 2). In contrast, only partial deletion of CD8 SP cells was observed. The CD8 SP cells had an immature phenotype (CD5lowCD24high), indicating incomplete thymic maturation (Fig. 2B). Subsets of the DN population were not different as compared with control mice (data not shown). Therefore, the toxin starts to be effective at the DP stage,
and continued expression in the CD4 lineage leads to efficient ablation of CD4 SP thymocytes.

Next, T cell subsets were analyzed in peripheral lymphoid organs. Efficient, although incomplete, deletion of both CD4⁺ and CD8⁺ T cells was observed in lymph nodes, spleen, and blood (Fig. 3, A and B). The reduced number of CD8⁺ T cells was not due to leaky Cre expression in these cells because no Cre mRNA could be detected in CD8⁺ T cells of CD4Cre mice by RT-PCR (Fig. 3C). The remaining CD4⁺ T cells in CD4Cre/R-DTA mice expressed the Cre recombinase; however, they did not recombine the stop cassette in the ROSA26 locus and therefore remained neo positive by genomic PCR analysis (Fig. 3, C and D). These results confirm a previous report using CD4Cre mice in combination with conditional diphtheria toxin receptor expression from the ROSA26 locus, where a small fraction of peripheral T cells also retained the stop cassette (22). The reason for this resistance to Cre recombination is currently unclear and requires further investigation.

**CD4Cre/R-DTA mice contain a normal-sized pool of T<sub>SP<sub> cells and remain lymphopenic over time**

Interestingly, the remaining CD4⁺ T cells in CD4Cre/R-DTA mice lost expression of CD62L, whereas most CD8⁺ T cells were

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**FIGURE 3.** Low numbers of peripheral T cells in CD4Cre/R-DTA mice. A, Mesenteric lymph node cells from CD4Cre/R-DTA and CD4Cre control mice were stained with anti-CD4, anti-CD8, and anti-CD62L Abs and analyzed by flow cytometry. B, Blood, spleen, and mesenteric lymph nodes of three individual mice were analyzed for the frequency of CD4⁺ and CD8⁺ T cells among total cells (left panels) and the frequency of CD62L⁺ T cells among CD4⁺ and CD8⁺ T cells (right panels). Results are representative of several independent experiments. C, Semiquantitative RT-PCR analysis for Cre and Hprt expression of sorted CD4⁺ and CD8⁺ T cells from CD4Cre mice (left) and sorted CD4⁺ T cells from CD4Cre/R-DTA mice (right). D, Genomic PCR of sorted CD4⁺ and CD8⁺ T cells from CD4Cre and CD4Cre/R-DTA mice to determine the presence or absence of the loxP-flanked neomycin resistance gene.
CD62L+/H11001 (Fig. 3, A and B). To analyze whether the CD62L+/H11001 CD8+ T cells belong to the naive T cell pool or to the T_Mp cell pool, cell suspensions were stained for CD44, which is expressed at increased levels on activated T cells and T_Mp cells but not on naive T cells. Both CD4+ and CD8+ T cells were mainly CD44high, indicating that they had acquired a memory T cell phenotype probably due to lymphopenia-induced proliferation in these mice (Fig. 4A). CD62L expression is usually lost after activation but can be reexpressed on memory CD8+ T cells and marks them as central memory T cells with the capacity to enter lymph nodes via high endothelial venules (23). Thus, most remaining CD8+ T cells appeared to have a central memory T cell phenotype, whereas most...
FIGURE 5. Remaining T cells in CD4Cre/R-DTA mice are functional and show normal homeostatic proliferation. A. Turnover of T<sub>SM</sub> cells was analyzed by BrdU labeling in the drinking water for 7 days and staining for CD4, CD8, CD44, and BrdU. The graph shows the percentage of BrdU<sup>+</sup> cells among CD44<sup>high</sup> gated CD4<sup>+</sup> or CD8<sup>+</sup> T cells from 3 R-DTA mice (open bars) and four CD4Cre/R-DTA mice (filled bars). B. KLRG1 expression was analyzed on splenic CD4<sup>+</sup> and CD8<sup>+</sup> T cells of CD4Cre/R-DTA mice (upper panels) or R-DTA mice (lower panels) by staining for CD62L, KLRG1, and CD4 or CD8. Percentages indicate the frequency of KLRG1<sup>+</sup> cells among CD62L<sup>+</sup> cells. C. Expansion of T cells from CD4Cre/R-DTA or control mice in vitro. Single-cell suspensions of spleen and mesenteric lymph nodes were cultured in vitro for 5 days in the presence of 20 ng/ml IL-2. The total numbers of CD4<sup>+</sup> and CD8<sup>+</sup> T cells were determined on indicated days after setup of the culture. The results show the mean from 2 individual mice per group. D, TCR-mediated in vitro proliferation. Single-cell suspensions of spleen and mesenteric lymph nodes were labeled with CFSE, incubated with 0.2 μg/ml anti-TCR (H57) and 0.2 μg/ml anti-CD28 Ab in the presence of 20 ng/ml IL-2, and analyzed 3 days later. The experiment has been repeated with comparable results. E, Spontaneous in vivo proliferation. Single-cell suspensions from spleen and mesenteric lymph nodes of CD4Cre/R-DTA or control mice were labeled with CFSE and transferred into 600-rad irradiated recipient mice. Seven days later mice were analyzed for spontaneous proliferation of transferred T cells. F, Quantitative RT-PCR to determine the expression of IL-4 and IFN-γ in polarized T cell cultures from CD4Cre/R-DTA (filled bars) and control mice (open bars). Error bars show SD of triplicate samples.
CD4+ T cells differentiated to T cells with an effector memory phenotype (Fig. 4A).

Analysis of natural T regulatory (Treg) cells (CD4+Foxp3+ T cells) revealed that thymic and peripheral Treg cells were reduced but not completely absent in CD4Cre/R-DTA mice compared with control mice (Fig. 4B). CD4Cre/R-DTA mice further showed selective loss of the CD4+ subset of NKT cells in the liver (Fig. 4C). Histological analysis of spleen and lymph nodes showed smaller T cell zones as compared with wild-type mice, but the overall architecture with defined T and B cell zones remained intact, which indicates that naive T cells are not required to establish these structures (Fig. 4D). Strikingly, the size of the Tmp cell pool in CD4Cre/R-DTA mice was comparable to control mice, despite the drastic reduction of total peripheral T cell numbers (Fig. 4E). The available space in CD4Cre/R-DTA mice was not filled, and they remained lymphopenic over time (Fig. 4F). Even the few naive CD8+ T cells (CD44lowCD62L+) did not increase, although it has been shown that mixed bone marrow chimeras with limited thymic output could reconstitute peripheral T cells in Rag2-deficient hosts and that naive CD8+ T cells could expand using the niche of naive CD4+ T cells, and vice versa (9, 24).

**Turnover, survival, and functionality of Tmp cells in CD4Cre/R-DTA mice**

To compare the turnover of Tmp cells in CD4Cre/R-DTA mice and control mice, BrdU-containing drinking water was administered for 7 days. BrdU incorporation was determined in CD44high T cells in both groups of mice to directly compare the turnover within the Tmp cell pool. CD4+CD44high T cells from CD4Cre/R-DTA mice showed a slightly higher turnover in comparison to CD4+CD44high T cells from wild-type mice, whereas no significant difference was observed for the CD8+ CD44high T cell populations (Fig. 5A). We further compared the replicative history of T cell subsets in both mice by staining for KLRG1, a marker for replicative senescent T cells that is expressed after numerous rounds of replication (25). KLRG1 was expressed with comparable frequency among CD62L− effector/memory phenotype T cells of both CD4Cre/R-DTA and control mice, indicating a similar replicative history (Fig. 5B). This illustrates that homeostatic proliferation and turnover of T cells within the memory phenotype pool is comparable in lymphopenic CD4Cre/R-DTA mice and normal control mice. When T cells from CD4Cre/R-DTA mice were cultured in vitro for 5 days in the presence of 20 ng/ml IL-2, they increased in total numbers (Fig. 5C), which further demonstrates that T cells that escaped Cre recombination during development were not deleted at later stages. Furthermore, T cells from CD4Cre/R-DTA mice responded with vigorous proliferation upon in vitro stimulation for 3 days with plate-bound anti-TCR Abs, demonstrating their responsiveness to TCR-mediated stimulation (Fig. 5D). CFSE-labeled T cells from CD4Cre/R-DTA mice underwent homeostatic proliferation after transfer into irradiated recipient mice, which indicates that they survived and proliferated in vivo (Fig. 5E). Finally, T cells from CD4Cre/R-DTA mice could be polarized to Th1 and Th2 cells, demonstrating that TML cells from these mice can become functional effector T cells (Fig. 5F).

The TCR repertoire in CD4Cre/R-DTA mice is oligoclonal and allows spontaneous proliferation of adoptively transferred T cells

It has been shown that an established pool of Tmp cells generated by spontaneous proliferation of adoptively transferred naive T cells into Rag-deficient mice blocks spontaneous proliferation of a second wave of transferred naive T cells but does not inhibit the establishment of a naive T cell pool by endogenously generated thymic emigrants (14, 16, 26). Spontaneous proliferation after adoptive transfer of naive CD4+ T cells was only observed when the repertoire of TCR specificities from the host was incomplete (16). To determine the TCR repertoire in CD4Cre/R-DTA mice, the pattern of the Vβ-chain usage was analyzed by flow cytometry of peripheral blood samples. As indicated in Fig. 6A, CD4+ and CD8+ T cells from CD4Cre/R-DTA mice showed biased Vβ-chain usage as compared with their negative littermates, indicating an incomplete repertoire of TCR specificities. To analyze whether this incomplete TCR repertoire allows spontaneous proliferation of adoptively transferred CD4+ T cells, purified naive wild-type CD4+ T cells were labeled with CFSE and transferred into nonirradiated R-DTA or CD4Cre/R-DTA mice. Seven days later mice were analyzed for proliferation of transferred CD4+ T cells, which only occurs in hosts with an incomplete TCR repertoire.

**FIGURE 6.** Biased Vβ repertoire indicates TCR repertoire incompleteness. A. The peripheral blood of 2 CD4Cre/R-DTA mice (filled bars) and two age- and sex-matched control R-DTA mice (open bars) was analyzed by staining for CD4, CD8, and individual TCR-Vβ domains. The relative increase/decrease of the frequency of certain Vβ domains in CD4Cre/R-DTA mice indicates a biased TCR repertoire. The experiment has been repeated with similar results. B. Sorted naive CD4+ T cells from wild-type Thy1.1 mice were labeled with CFSE and transferred into nonirradiated R-DTA or CD4Cre/R-DTA mice. Seven days later mice were analyzed for proliferation of transferred CD4+ T cells, which only occurs in hosts with an incomplete TCR repertoire.
cell depletion, the remaining CD4+ T cells in CD4Cre/R-DTA mice were depleted with anti-CD4 mAb, and the phenotype of newly generated thymic emigrants in the peripheral blood of CD4Cre/R-DTA mice was analyzed by flow cytometry (Fig. 7A). Interestingly, naive phenotype T cells could be observed during an early phase between days 5 and 7 after Ab administration before the size of the TMP cell pool was reestablished (Fig. 7B). This transient population of naive T cells most likely represents recent thymic emigrants. Due to the lymphopenic environment, these cells underwent spontaneous proliferation and converted to a memory phenotype (CD62LlowCD44high). However, thymic output was too low to fill the naive T cell pool. To further substantiate these findings, mixed bone marrow chimeras were generated with bone marrow from wild-type (Ly5.2+/H11001Thy1.2+/H11001) and CD4Cre/R-DTA mice (Ly5.2+/H11001Thy1.1+/H11001) and transferred at a ratio of 1:10 into lethally irradiated wild-type (Ly5.1+/H11001) recipient mice. The first detectable peripheral CD4+ T cells at 4 wk after reconstitution were derived from bone marrow of CD4Cre/R-DTA mice reflecting the higher inoculum of bone marrow cells from CD4Cre/R-DTA mice. However, from week 6 to week 8 after reconstitution, the frequency of T cells derived from cotransferred wild-type bone marrow increased and outcompeted the T cells derived from bone marrow of CD4Cre/R-DTA mice (Fig. 7C). Thymocyte development at 10 wk after reconstitution clearly showed that the vast majority of CD4 SP thymocytes were derived from the relatively small inoculum of wild-type bone marrow cells, further supporting the notion that CD4+ T cells are deleted during transition from the DP to CD4 SP stage of thymic development without causing bystander toxicity (Fig. 7D). In peripheral lymph nodes of these mixed chimeras, 10–17% of donor-derived CD4+ T cells were of CD4Cre/R-DTA origin, and this population consisted of >50% of naive T cells.
(CD44low) T cells, which is comparable to the frequency of
CD44low T cells of wild-type origin and is several fold higher
than in CD4Cre/R-DTA mice (Fig. 7, D and E). Therefore, we
conclude that wild-type T cells established a peripheral T\textsubscript{MP}
cell pool with a complete repertoire of TCR specificities that
prevented further conversion of naive T cells to T\textsubscript{MP} cells and
therefore allowed recent thymic emigrants of CD4Cre/R-DTA
origin to remain in the naive T cell pool.

FIGURE 8. Infection of CD4Cre/R-DTA mice with \textit{N. brasiliensis} reveals the functionally impaired immune response. \textbf{A} and \textbf{B}, Frequency of
basophils (IgE\textsuperscript{+}B220\textsuperscript{+}) and eosinophils (Siglec-F\textsuperscript{+}SSChigh) in lung and peripheral blood on day 9 after \textit{N. brasiliensis} infection. Dot plots are gated
on CD4\textsuperscript{+} cells and are representative of four independent experiments. \textbf{C}, Total numbers of eosinophils, basophils, and CD4 T
cells in the lung of R-DTA (n = 5) and CD4Cre/R-DTA (n = 6) mice on day 9 after \textit{N. brasiliensis} infection. The graph shows pooled results from
three independent experiments. Error bars indicate SD. Differences between groups were statistically highly significant (p < 0.01, Student’s t test).
\textbf{D}, Serum IgE levels of \textit{N. brasiliensis}-infected R-DTA (n = 7) and CD4Cre/R-DTA (n = 8) mice from four independent experiments. The difference
is not significant (p = 0.13, Student’s t test). \textbf{E}, Intracellular cytokine staining of CD4\textsuperscript{+} T cells isolated from mesenteric lymph nodes of indicated
mice on day 9 after \textit{N. brasiliensis} infection. The experiment was repeated with comparable results. \textbf{F}, The number of adult worms in the small
intestine was determined on day 9 after infection of R-DTA (n = 6) or CD4Cre/R-DTA (n = 8) mice. The graph shows pooled results from three
independent experiments.
Infection of CD4Cre/R-DTA mice with N. brasiliensis results in impaired effector cell recruitment and worm expulsion

To address the functional consequences of an immune system with an oligoclonal T cell repertoire, we infected CD4Cre/R-DTA mice and R-DTA control mice with the helminth parasite N. brasiliensis. In this model, worm expulsion from the intestine and recruitment of eosinophils and basophils to the lung are strictly dependent on conventional CD4+ T cells (27, 28). On day 9 after infection, CD4Cre/R-DTA mice showed reduced frequencies of basophils (IgE+ B220+) and eosinophils (Siglec-F+ SSCmed) in the blood and lung compared with R-DTA mice (Fig. 8A and B). Total numbers of eosinophils, basophils, and CD4+ T cells in the lung were 5–10-fold lower (Fig. 8C). Interestingly, the serum IgE levels were comparable between CD4Cre/R-DTA and R-DTA mice (Fig. 8D), which indicates that Tmp, cells could recognize N. brasiliensis Ag and induced a strong humoral immune response. Intracellular cytokine staining of CD4+ T cells isolated from the mesenteric lymph nodes of infected mice revealed that CD4+ T cells from wild-type mice had preferentially differentiated toward Th2 cells whereas T cells from CD4Cre/R-DTA mice were polarized toward Th1 and Th17 cells (Fig. 8E). This inappropriate T cell response might explain why worm expulsion from the small intestine was significantly impaired with 4-fold higher worm counts in CD4Cre/R-DTA mice compared with R-DTA mice (Fig. 8F).

Taken together, DTA expression in T cells resulted in efficient elimination of naive CD4+ and CD8+ T cells. Low thymic output and lymphopenia-induced proliferation led to rapid conversion of recent thymic emigrants to TSM cells with an incomplete TCR repertoire. The size and turnover of this T cell pool was comparable to the normal TSM cell pool found in wild-type mice, indicating that homeostasis of the TSM cell pool is regulated independently of the available immunological space. Infection with N. brasiliensis resulted in diminished effector cell recruitment and worm expulsion, demonstrating the insufficiency of TSM cells to orchestrate an effective primary type 2 immune response.

Discussion

The TSM cell pool is filled shortly after birth by expansion of the first thymic emigrants, which enter a lymphopenic environment, proliferate, and convert to TSM cells (29). The size of the TSM cell pool is remarkably stable throughout life, and Ag-experienced memory T cells are thought to use the same survival niches as TSM cells. In fact, transient lymphopenia, a common phenomenon associated with viral or bacterial infections, often results in attrition of the numbers of Ag-experienced memory T cells due to competition with newly generated TSM cells, which proliferate under lymphopenic conditions (30). Furthermore, competition among TSM cells results in oligoclonal T cell expansion, reduces the diversity of the T cell repertoire, limits the responsiveness of the immune system against newly encountered Ags, and is thought to be a major cause of morbidity and mortality associated with infectious diseases in patients with such alterations of the T cell compartment and in the elderly population (31). Lymphopenia can result in autoimmunity, since autoimmune clones that escaped negative selection in the thymus might expand and/or survive better than T cells that are tolerant to self-Ags (10, 32). This fatal situation might be triggered by high levels of IL-21, which is thought to costimulate proliferation of autoreactive T cells (10). Lymphopenia-induced T cell expansion has further been shown to be a barrier for solid organ transplantation since lymphopenia-induced TSM cells cannot be tolerized by Abs against costimulatory molecules (33). In contrast, lymphopenia has been shown to be beneficial for tumor surveillance, probably due to better priming or expansion of tumor-specific T cells under such conditions (34, 35). These examples illustrate that a better understanding of TSM cells with regard to their turnover, effector function, and in vivo migration is critically needed.

Using a newly generated mouse model, we show herein that the turnover of endogenously generated TSM cells is comparable to TSM cells in normal mice. Our results clearly demonstrate that the size of the TSM cell pool is regulated independently of the naive T cell pool and remains constant even in the complete absence of naive T cells. One elegant study described T cell homeostasis in chimeric mice that were generated by reconstitution of lethally irradiated Rag2-deficient mice with bone marrow from wild-type and T cell-deficient mice mixed at different ratios (15). Using this approach, it could be demonstrated that the size of peripheral T cell pools are maintained even when the thymic output falls below 10% of normal. However, an even lower thymic output resulted in lymphopenia and was associated with a strong bias toward T cells with a memory phenotype and an oligoclonal TCR repertoire. Therefore, efficient continuous thymic output seems to be required but might not be sufficient to establish and maintain normal numbers of naive peripheral T cells (15, 26). Interestingly, when the remaining peripheral CD4+ T cells in CD4Cre/R-DTA mice were depleted with a mAb, a transient wave of naive phenotype T cells was observed at day 7 after depletion, indicating that new thymic migrants were detected that had not yet converted to a memory phenotype. The low thymic output in CD4Cre/R-DTA mice is not sufficient to establish a peripheral pool of naive T cells. However, mixed chimeras with bone marrow from wild-type and CD4Cre/R-DTA mice revealed that peripheral T cells with a naive phenotype could be generated from CD4Cre/R-DTA mice when the peripheral T cell pools had been filled with T cells derived from wild-type bone marrow. Interestingly, spontaneous proliferation of adoptively transferred naive CD4+ T cells in lymphopenic hosts could be blocked by preexisting TSM cells (14, 16). However, proliferation could still occur when the TCR repertoire of the TSM cell population was oligoclonal (16). This indicates that the repertoire diversity and not the size of the TSM cell pool controls spontaneous proliferation of naive T cells. The biased TCR repertoire in CD4Cre/R-DTA mice suggests oligoclonal expansion of T cells that might be specific for autoantigens or commensal Ags. Further studies including the generation of gnotobiotic mice are required to distinguish between both possibilities.

The behavior of T lymphocytes under lymphopenic conditions has been studied mainly by adoptive cell transfers into irradiated or genetically lymphopenic mice (7, 36). The spontaneous lymphopenic mouse model described here avoids ex vivo handling of T cells, which has been shown to change the expression level of ~200 genes (13). CD4Cre/R-DTA mice might help to complete our understanding of the functionality of lymphopenic immune systems in fighting tumors and infections. As we show herein, a lymphopenic immune system with a strong bias toward TSM cells cannot mount an efficient primary immune response against N. brasiliensis, although TSM cells could be polarized to Th1 and Th2 cells in vitro. The inefficient immune response in CD4Cre/R-DTA mice could be due to an insufficient precursor frequency of N. brasiliensis-specific CD4+ T cells, which is indicated by the reduced TCR diversity and the low total number of T cells. It is also possible that T cell activation under lymphopenic conditions results mainly in Th1/Th17 polarization and prevents differentiation toward Th2 cells. Additional experiments are required to address this point.

It will be interesting to study CD8+ T cell responses against viral infections and tumors in a lymphopenic environment with a
special focus on the homeostasis of Ag-specific memory T cell populations that have to compete with T\textsubscript{EM} cells for survival in the T\textsubscript{MP} cell pool. These studies might help to develop novel vaccination strategies for elderly people as well as for patients with chronic viral infections or cancer patients recovering from chemotherapy who have in common that their peripheral T cell pool consists mainly of T\textsubscript{MP} cells with an oligoclonal TCR repertoire.

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Disclosures

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References