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TCR and cytokine receptor signaling play key roles in the complex homeostatic mechanisms that maintain a relative stable number of T cells throughout life. Despite the homeostatic mechanisms, a slow decline in naive T cells is typically observed with age. The CD3γ di-leucine-based motif controls TCR down-regulation and plays a central role in fine-tuning TCR expression and signaling in T cells. In this study, we show that the age-associated decline of naive T cells is strongly accelerated in CD3γLLAA knock-in mice homozygous for a double leucine to alanine mutation in the CD3γ di-leucine-based motif, whereas the number of memory T cells is unaffected by the mutation. This results in premature T cell population senescence with a severe dominance of memory T cells and very few naive T cells in middle-aged to old CD3γ mutant mice. The reduced number of naive T cells in CD3γ mutant mice was caused by the combination of reduced thymic output, decreased T cell apoptosis, and increased transition of naive T cells to memory T cells. Experiments with bone marrow chimeric mice confirmed that the CD3γLLAA mutation exerted a T cell intrinsic effect on T cell homeostasis that resulted in an increased transition of CD3γLLAA naive T cells to memory T cells and a survival advantage of CD3γLLAA T cells compared with wild-type T cells. The experimental observations were further supported by mathematical modeling of T cell homeostasis. Our study thus identifies an important role of CD3γ-mediated TCR down-regulation in T cell homeostasis. The Journal of Immunology, 2009, 183: 4994–5005.

To ensure survival and health, the immune system must react swiftly and efficiently against invading pathogen microorganisms. A diverse TCR repertoire of the peripheral T cells is required for optimal immune function because it allows recruitment and activation of complementary specificities upon pathogen encounter (1–3). The TCR repertoire is generated by random TCRα and β gene rearrangement in the thymocytes. The subsequent combined processes of positive and negative selection in the thymus allow a small fraction of thymocytes with low affinity to self-peptide-MHC (pMHC) complexes to survive and mature into single-positive T cells (4). Mature T cells exit the thymus and form the pool of naive T cells that continuously circulate between blood and the lymph through defined areas of the secondary lymphoid tissues in search of intruding pathogens (5). Activation by pathogen Ag/MHC ligands induces naive T cells to undergo massive expansion and to acquire effector functions required for elimination of the pathogen. Most effector cells die quickly after pathogen clearance but a small fraction of the cells survive as memory T cells (6).

Homeostatic mechanisms determine the number of cells in each organ of the body. The immune system is no exception, and the survival and composition of the T cell pool are governed by complex homeostatic mechanisms (7, 8). However, although the total number of T cells declines only modestly, the composition of the T cell pool changes with age in both mouse and humans (1–3, 9–13). In young individuals where the thymus is large, the pool of T cells consists mostly of naive T cells. As the thymus shows a continuous involution from early childhood to the end of life (14) and becomes atrophic by middle age, a shift occurs in the T cell pool with age from naive T cells to a predominance of memory T cells. This results in an age-associated increase in the memory to naive T cell ratio. The gradual loss of naive T cells diminishes the diversity of the TCR repertoire and contributes to the impaired effectiveness of the immune system to react against new Ags/pathogens in old individuals (1–3, 9–13). This is reflected in the higher susceptibility to infectious diseases and the reduced efficiency of vaccination in aged individuals (15, 16). In addition to export of newly generated T cells from the thymus, the size of the naive T cell pool is regulated by 1) the survival/apoptosis rate of the naive T cells, 2) the homeostatic proliferation rate of the naive T cells, and 3) the transition rate of the naive T cells to memory T cells (17). Both survival and homeostatic proliferation of naive T cells are dependent on TCR signaling from interaction with self-pMHC ligands plus IL-7R signaling from interaction with IL-7 (7, 8). Transition of naive T cells to memory T cells also depends on TCR signaling, either following interaction with foreign Ag/MHC ligands leading to Ag-specific memory T cells or following interaction with self-pMHC leading to memory-phenotype T cells. Thus, the TCR is a key receptor in T cell homeostasis.

The TCR is a multichain receptor composed of the pMHC-recognition αβ heterodimer and the tightly associated signal-transducing CD3γε, CD3δε, and CD3ζζ dimers (18, 19). Like many other cell surface receptors, the TCR is down-regulated following
ligand triggering. At least two distinct pathways exist for TCR down-regulation (20, 21). One pathway is dependent on protein tyrosine kinase activity and leads to TCR ubiquitination and degradation (22, 23). The other pathway is dependent on protein kinase C-mediated activation of the di-leucine-based (dil) motif found in the CD3γ chain of the TCR and leads to TCR recycling. The CD3γ dil motif plays a unique role in TCR trafficking and the motif has been characterized in details at the molecular and cellular levels in previous studies (24–32). To study the physiological roles of CD3γ dil motif-mediated TCR down-regulation, we generated CD3γLLAA knock-in mice homozygous for a double leucine to alanine mutation in the CD3γ dil motif. We have recently described that the TCR expression levels on resting T cells from CD3γLLAA mice are increased by ~10% compared with T cells from wild-type (WT) mice and that TCR down-regulation is severely impaired following TCR triggering. Accordingly, CD3γLLAA T cells express ~40–50% more TCR than WT T cells following stimulation with specific pMHC. The increased TCR expression results in stronger TCR signaling and increased activation-induced cell death in CD3γLLAA T cells compared with WT T cells during acute virus infections (32).

The aim of the present study was to determine the role of the CD3γ dil motif in T cell homeostasis.

Materials and Methods

Mice

Generation of the CD3γLLAA knock-in and P14LLAA mice strains has recently been described (32). The CD3γLLAA strain was backcrossed for 10 generations to C57BL/6 mice. C57BL/6 B6.SJL and P14.SJL mice were used as WT controls. The mice were kept under specific pathogen-free conditions. All animal experiments were approved by the Animals Ethics Inspectorate, the Danish Ministry of Justice (approval no. 2007/561–1357).

Abs and flow cytometry

Thymocytes, spleen, and lymph node cells were collected using standard protocols. To analyze expression of various surface markers, cells were incubated with fluorochrome-conjugated anti-TCRvβ2 (B20.1), anti-TCRβ (H57-597), anti-CD3 (145.2C11), anti-CD4 (RM4-5), anti-CD8a (53-6.7), anti-CD24 (M1/69), anti-CD44 (IM7), anti-CD45.1 (A20), anti-CD62L (MEL-14), anti-CD122 (TMG1), anti-CD127 (B12-1) mAb or the mouse Vβ TCR screening panel, all from BD Biosciences. The CD4+ and CD8+ T cell populations were subdivided into two subpopulations: naive cells defined as CD44‡CD62Lhigh cells and memory cells defined as cells that were not CD44lowCD62Lhigh. To determine apoptotic cells, 1 × 10⁶ cells were stained with fluorochrome-conjugated Abs, washed twice in cold PBS, and resuspended in 1× annexin V binding buffer (BD Biosciences). Annexin V (BD Biosciences) was added and the cells were vortexed and incubated for 15 min at room temperature in the dark. Subsequently, 400 μl of 1× annexin V binding buffer was added and the samples were analyzed by flow cytometry within 1 h. Before analysis, propidium iodide (PE; BD Biosciences) was added to the cell samples. For intracellular Bel-2 staining, 1 × 10⁶ cells were fixed by incubation with 1% paraformaldehyde for 10 min on ice. The cells were subsequently washed, permeabilized by treatment with 0.5% saponin for 10 min at room temperature, and then resuspended in 20 μl of PE-conjugated anti-Bel-2 (BD Biosciences). Cells were analyzed on a FACScalibur or a LSRII flow cytometer (BD Biosciences). FlowJo software (Tree Star) was used for data analysis.

Intrathymic injections

Intrathymic FITC injections were performed as previously described (33, 34). Briefly, 4- to 6-wk-old mice were anesthetized and a 1-cm midline incision was made in the skin of the thorax from the base of the neck. Ten microliters of 1 mg/ml FITC isomer I (Sigma–Aldrich) in PBS was injected directly into each thymic lobe. The skin was closed with sterile sutures and the organs were harvested and analyzed 72 h after injection.

BrdU labeling

Mice were treated with drinking water containing 0.8 mg/ml BrdU and 2% sucrose. BrdU containing drinking water was protected from light and changed daily. After 14 days, the organs were harvested and the cells were stained with anti-BrdU mAbs as described by the manufacturer (BD Biosciences) and analyzed by flow cytometry.

Cell sorting and quantitative RT-PCR

CD4+ TCRhigh and CD8+ TCRhigh mature single-positive thymocytes were isolated on a FACSaria II (BD Biosciences) cell sorter. RNA was purified from the isolated subpopulations using a RNAeasy kit (Qiagen) and cDNA was produced using a RevertAid First Strand cDNA synthesis kit (Fermentas). Quantitative RT-PCR was performed using the TaqMan assay with AmpliTaq gold DNA polymerase (Applied Biosystems) using an Mx3000P real-time thermal cycler (Stratagene). All primers and probes were used at the concentration of 300 nM. Kruppel-like factor 2 (KLF2) forward primer AGGCTCATTTGGCCGCTTCT, KLF2 reverse primer CCAACACGTTGGTTAGTGTCCCT, KLF2 probe CCCTGTCGGCGGA AATGAAC, sphinogline-1-phosphate receptor 1 (S1P1) forward primer GTGTAGACCCAGAGTCTCCGG, S1P1 reverse primer AGCCTTTCTCT TGCTGGAGAG, S1P1 probe CGGCTTGAGAGGGCTGCTGT, β-actin forwardprimer ACGGCCCAGTCTCATACTATG, β-actin reverse primer CAAGAAAGGAAGGCTGGGAAAAG, and β-actin probe CAACGAGCGGTTCCAGATGCC were all from TAGC. The amounts of KLF2 and S1P1 mRNAs were calculated relative to the amount of β-actin mRNA and normalized to WT by using the 2⁻ΔΔCt method as previously described (35).

Homeostatic proliferation and mixed bone marrow chimeras

For studies of homeostatic proliferation, equal numbers of CFSE (Molecular Probes)-labeled congenic P14.SJL (CD45.1+) and P14LLAA (CD45.2+) cells were injected into sublethally irradiated (6 Gy) B6.SJL mice. Seven days after injection, the mice were killed and the TCR-transgenic Vα2CD8+ T cells were analyzed. Bone marrow chimeric mice were produced as previously described (32). Briefly, lethally irradiated (12 Gy) C57BL/6 mice were transplanted with bone marrow from congenic WT and CD3γLLAA mice in a 1:1 ratio. T cell subpopulations in the reconstituted mice were determined by FACS analyses of blood samples or spleen cells obtained from 2 to 15 mo after bone marrow transfer.

Statistical analysis

All statistical analyses were performed using Student’s t test or the non-parametric Mann-Whitney U test when appropriate, with a 5% significance level, unpaired observations, and equal variance. In the analysis of the intrathymic FITC data, we used a paired t test because the FITC stock varied greatly between experiments.

Results

Accelerated age-associated decline of naive T cells in CD3γLLAA mice

We recently showed that T cells use the CD3γ dil motif to fine-tune TCR expression levels and TCR signaling (32). By applying a panel of Vβ-specific mAb, we confirmed that TCR expression levels are increased on both naive and memory CD4+ and CD8+ T cells from CD3γLLAA mice compared with T cells from WT mice in the present study (supplemental Fig. 1a). Because TCR signaling plays a key role in T cell homeostasis (7, 8), we hypothesized that T cell homeostasis is affected in CD3γLLAA mice with increased TCR expression and altered TCR signaling. To test this hypothesis, we analyzed T cell subsets from 2- to 24-mo-old CD3γLLAA and WT mice. We grouped the mice into three groups according to their age; young mice from 2 to 3 mo old, middle-aged mice from 12 to 14 mo old, and old mice from 18 to 24 mo old. In CD3γLLAA mice, we found a minor reduction in the total number of CD4+ cells that accelerated with age. Thus, young CD3γLLAA mice had a 6% reduction, middle-aged mice a 12% reduction, and old mice a 38% reduction in the total numbers of CD4+ cells compared with age-matched WT mice (Fig. 1A). The CD8+ T cells were even more affected than the CD4+ T cells by the mutation. Thus, the total number of CD8+ T cells was reduced by ~16% in young, 37% in middle-aged, and 57% in old mice.

The online version of this article contains supplemental material.
CD3γLLAA mice compared with age-matched WT mice (Fig. 1A). The greater reduction in CD8+ compared with CD4+ T cells resulted in an increased CD4+/CD8+ ratio in CD3γLLAA mice. Further analyses of naive and memory T cell subpopulations demonstrated that the reduction in T cell numbers in CD3γLLAA mice was exclusively caused by a reduction in the numbers of naive T cells, whereas the numbers of memory T cells were unaffected (Fig. 1, B–E). In young CD3γLLAA mice, the memory:naive ratio of CD4+ and CD8+ T cells was only modestly affected, but it became significantly skewed with age. In 24-mo-old CD3γLLAA mice, the memory:naive ratio of CD4+ T cells had increased to 6.5, whereas it was 2.5 in age-matched WT mice (Fig. 1F). In parallel, the memory:naive ratio of CD8+ T cells in 24-mo-old CD3γLLAA mice was 4.5, whereas it was only 1.5 in age-matched
WT mice (Fig. 1G). Analyses of T cell subpopulations from the lymph nodes gave similar results (supplemental Fig. 2).

Taken together, these results demonstrate that the CD3γ dim motif plays an important role in T cell homeostasis. Thus, CD3γ mutant mice had reduced numbers of naive T cells and an accelerated age-associated decline of naive T cells. In contrast, the absolute numbers of memory T cells were unaffected by the mutation. Collectively, this resulted in a skewed composition of the T cell subpopulations and a significantly increased memory:naive T cell ratio with severe depletion of naive T cells in old CD3γLLAA mice.

Reduced numbers of thymocytes in CD3γLLAA mice

The maintenance of the naive T cell pool is dependent on the thymus function (8, 36–39). To assess the development of T cells, we determined the total numbers of thymocytes from 8- to 12-wk-old WT and CD3γLLAA mice and analyzed them for surface expression of CD4 and CD8 by flow cytometry. Thymi from CD3γLLAA mice contained reduced numbers of thymocytes (7.4 ± 0.8 × 10⁷ for CD3γLLAA mice vs 9.2 ± 0.8 × 10⁷ for WT mice, p = 0.04) but similar proportions of double-negative, double-positive, and single-positive subpopulations compared with their WT littermates (Fig. 2A). The total number of single-positive CD4 and CD8 cells was reduced by ~20% in CD3γLLAA mice (Fig. 2B). Mature single-positive thymocytes that are ready for export express high levels of the TCR. To further study how the CD3γLLAA mutation affected T cell development, we determined the absolute numbers and percentages of TCRhigh single-positive thymocytes. We found that the absolute number of mature TCRhigh single-positive CD4 and CD8 cells was reduced by ~20% in CD3γLLAA mice compared with WT mice (Fig. 2C). Furthermore, as reflected in the peripheral pool of T cells, we found a slightly increased CD4:CD8 ratio of the TCRhigh single-positive thymocytes from CD3γLLAA mice (Fig. 2D).

Decreased thymic output in CD3γLLAA mice

Several studies have indicated that the thymic output of mature T cells is proportional to the thymic mass (8, 33, 37, 40). However, another study has suggested the existence of compensatory mechanisms that partially maintain the output of mature T cells when the number of T cell precursors is reduced in the thymus (38). To determine whether the thymic output was affected by the reduced numbers of thymocytes or whether compensatory mechanisms ensured normal thymic output in CD3γLLAA mice, we injected FITC directly into the thymic lobes of WT and CD3γLLAA mice. This method gives random labeling of the thymus population (Fig. 3A) and can be used to determine the extent of the export of mature T cells from the thymus to the periphery (33). We harvested the inguinal lymph nodes 72 h after the FITC injections and determined the percentage and absolute number of FITC⁺ T cells. We found a 20–30% reduction of FITC⁺ T cells in CD3γLLAA lymph nodes compared with WT lymph nodes (Fig. 3, A and B). To verify that the FITC⁺ T cells in the periphery represented recent thymic emigrants, we determined the expression levels of CD24/heat-stable Ag on the lymph node T cells. CD24 is highly expressed on immature thymocytes but becomes rapidly downregulated on mature T cells during export from the thymus. This implies that in the peripheral pool of T cells only recent thymic emigrants express evident levels of CD24. We found that the FITC⁺ T cells in the lymph nodes expressed CD24, confirming that the FITC⁺ T cells represented recent thymic emigrants (Fig. 3C). To evaluate the thymic output without exposing the mice to surgical approaches, we then treated mice for 14 days with the DNA precursor BrdU in their drinking water. Because thymocytes have a rapid turnover, >98% of the thymocytes incorporated BrdU during the treatment (inset in Fig. 3D). In contrast, naive T cells have a very slow turnover and hardly incorporate any BrdU. Consequently, following treatment for 14 days with BrdU, the majority of BrdU-positive naive T cells found in the peripheral pool of T

![FIGURE 2. Reduced numbers of thymocytes in CD3γLLAA mice. A. Thymocytes of 8- to 12-wk-old WT and CD3γLLAA mice were enumerated and analyzed by flow cytometry for the expression of CD4 and CD8. The total cell numbers are shown above the corresponding dot plots as mean ± SEM. The mean percentage of cells within the single-positive (SP) CD4 and CD8 gate is indicated. The CD4:CD8 ratio of single-positive thymocytes is given in the lower left corner of each dot plot. B. Absolute numbers of single-positive CD4 and CD8 cells in the thymus from WT and CD3γLLAA mice. C. Absolute numbers of TCRhigh single-positive CD4 and CD8 cells in the thymus from WT and CD3γLLAA mice. D. TCRhigh thymocytes were analyzed by flow cytometry for the expression of CD4 and CD8. The mean percentage of cells within the single-positive CD4 and CD8 gate is indicated. The CD4:CD8 ratio of single-positive TCRhigh thymocytes is given in the lower left corner of each dot plot. A–D. The data show values obtained from four independent experiments with 12 mice in each group.](http://www.jimmunol.org/)
FIGURE 3. Decreased thymic output in CD3γLLAA mice. A, Thymocytes (upper row) and lymph node cells (lower row) from WT and CD3γLLAA mice were analyzed by flow cytometry for FITC staining and expression of CD3 72 h after intrathymic injections of FITC. The percentage of FITC+CD3+ T cells in the lymph nodes is given in the upper right quadrant. Data are representative of five independent experiments. B, Absolute numbers of FITC+ T cells in the inguinal lymph nodes from WT and CD3γLLAA mice 72 h after intrathymic injections of FITC. The data show mean values ± SEM obtained from five independent experiments. C, FITC+ and FITC− lymph node T cells and thymocytes were analyzed by flow cytometry for BrdU staining. The percentage of BrdU-positive naive T cells was reduced by 20–30% in CD3γ mice compared with WT mice (Fig. 3E). We also observed slightly increased intracellular levels of the anti-apoptotic molecule Bcl-2 in naive T cells from CD3γLLAA mice compared with WT mice (Fig. 3E). This suggests that the reduced thymic export in CD3γLLAA mice was a direct result of the reduced numbers of thymocytes.

Survival and homeostatic proliferation of naive T cells in CD3γLLAA mice

In addition to export of mature T cells from the thymus, the size of the pool of naive T cells is regulated by the survival/apoptosis rate of the naive T cells, the homeostatic proliferation rate of the naive T cells, and the transition rate of the naive T cells to memory T cells (7, 8, 17). To investigate whether the survival/apoptosis rate of the naive T cells was affected in CD3γLLAA mice, we isolated spleen cells and stained them with annexin V and PI. We found that the fraction of apoptotic naive CD4+ and CD8+ T cells was reduced by ~20% in CD3γLLAA compared with WT mice (Fig. 4, A and B). We also observed slightly increased intracellular levels of the anti-apoptotic molecule Bcl-2 in naive T cells from CD3γLLAA mice compared with WT mice (Fig. 4C).

The homeostatic proliferation rate of naive T cells in euthymic mice is very low and BrdU incorporation in naive T cells is normally taken as a measure of recent thymic emigrants rather than of homeostatic proliferation (41, 42). However, naive T cells begin to proliferate in syngeneic lymphopenic hosts because of the increased availability of specific self-pMHC ligands and IL-7, i.e., the same factors required for homeostatic proliferation of naive T cells (7, 8), and this model has been used to evaluate the homeostatic proliferation potential. To evaluate whether homeostatic proliferation was affected by the CD3γLLAA mutation, we therefore studied proliferation of naive TCR-transgenic T cells from P14 and P14LLAA mice in the same lymphopenic host. P14 TCR-transgenic mice express a TCR (Vα2* Vβ8*) specific for lymphocytic choriomeningitis virus glycoprotein (gp33–41) bound to H-2Dβ (46, 47). To exclude effects that could be caused by a
were stained with annexin V and PI and analyzed by flow cytometry. The percentages of annexin V+PI+ and annexin V+PI− cells are given in the upper left and right quadrants, respectively. B. The fraction of apoptotic annexin V+ naive CD4+ and CD8+ T cells from the spleen of WT and CD3yLLAA mice. Data are given as mean values ± SEM from four independent experiments with 12 mice in each group. C. Bcl-2 expression in naive CD4+ and CD8+ T cells from the spleen of WT and CD3yLLAA mice. The data represent mean values ± SEM obtained from two independent experiments with six mice in each group. D and E, CD127 (D) and CD122 (E) expression on naive WT (bold line) and CD3yLLAA (dotted line) CD4+ and CD8+ T cells.

Collectively, these results indicate that the survival rate of naive T cells was slightly increased in CD3yLLAA mice compared with WT mice, whereas the homeostatic proliferation potential was unaffected by the CD3y mutation. The increased survival could either be caused by increased TCR signaling, by increased cytokine signaling, or by a combination of the two. We found that naive CD3yLLAA T cells express higher levels of TCR at their cell surface than naive WT T cells (supplemental Fig. 1), but express equal levels of receptors for the key homeostatic cytokines IL-7 and IL-15. This argues for the possibility that the increased survival of naive CD3yLLAA T cells was caused by increased TCR signaling; however, at this stage, we could not rule out the possibility that the CD3y mutant mice had higher levels of IL-7 and IL-15 than WT mice.

Decreased apoptosis of memory T cells in CD3yLLAA mice

The increased memory-naive T cell ratio in CD3yLLAA mice suggests that the rate of transition of naive T cells to memory T cells was increased in CD3yLLAA T cells. In addition, the higher ratio could be caused by increased survival and/or homeostatic proliferation of the memory T cells. To investigate whether the CD3y diL motif affected the survival/apoptosis rate of memory T cells, we isolated spleen cells of WT and CD3yLLAA mice and stained them with annexin V and PI. We found that the fraction of apoptotic memory T cells was reduced by ~15% in CD3yLLAA compared with WT mice (Fig. 5, A and B), indicating that the survival rate of memory T cells was increased in CD3yLLAA mice compared with WT mice. Furthermore, we found that CD4+ memory T cells from CD3yLLAA mice expressed increased levels of the anti-apoptotic molecule Bcl-2 compared with CD4+ memory T cells from WT mice (Fig. 5C). Memory T cells from WT and CD3yLLAA mice expressed equal levels of CD122 and CD127 (Fig. 5D and data not shown).

To investigate whether the CD3y diL motif affected homeostatic proliferation of the memory T cells, we treated CD3yLLAA and WT mice with BrdU in the drinking water for 14 days and subsequently determined the uptake of BrdU in memory T cells. In agreement with previous studies (42), we found that a significant fraction of the memory T cells was dividing. Thus, ~40% of the CD4+ memory T cells in both WT and CD3yLLAA mice incorporated BrdU during 14 days of BrdU treatment. For CD8+ memory T cells, BrdU incorporation was reduced in CD3yLLAA mice compared with WT mice (Fig. 5E).

In conclusion, the increased memory:naive T cell ratio in CD3yLLAA mice was not caused by an increased proliferation of the memory T cells but most probably by an enhanced transition of naive to memory T cells in combination with increased survival of the memory T cells.

The CD3yLLAA mutation exerts a T cell-intrinsic effect on T cell homeostasis

Collectively, the data described above suggested that the CD3yLLAA mutation gave a developmental disadvantage to thymocytes, a survival advantage to both naive and memory T cells and an enhanced transition of naive T cells to memory T cells. This resulted in a disturbed T cell homeostasis with an accelerated loss
of naive T cells and an increased memory:naive T cell ratio in CD3γLLAA mice. To study whether the perturbed T cell homeostasis in the CD3γLLAA mice was an intrinsic property of CD3γLLAA T cells or whether it was caused by T cell extrinsic factors in the host, we generated mixed bone marrow chimeras. We reconstituted lethally irradiated C57BL/6 mice with bone marrow from congenic WT and CD3γLLAA mice in a 1:1 ratio. From 2.5 to 9 mo after bone marrow transfer, we regularly determined the percentages of naive and memory CD4+ and CD8+ WT and CD3γLLAA T cells in blood samples from the chimeric mice (exemplified for 2.5 and 7.5 mo after bone marrow transfer in Fig. 6, A and B). We then calculated the memory:naive ratio of CD4+ and CD8+ WT and CD3γLLAA T cells separately for each of the chimeric mice. As for the nonchimeric mice, we found a significantly increased memory:naive ratio for CD3γLLAA T cells compared with WT T cells (Fig. 6C). Because the CD3γLLAA and WT cells developed in the same mouse, the increased memory:naive CD3γLLAA T cell ratio must be caused by a T cell-intrinsic property.

To study the relative survival advantages between WT and CD3γLLAA T cells, we then determined the ratio of 1) CD3γLLAA to WT naive CD4+ T cells, 2) CD3γLLAA to WT naive CD8+ T cells, 3) CD3γLLAA to WT memory CD4+ T cells, and 4) CD3γLLAA to WT memory CD8+ T cells separately for each of the chimeric mice (exemplified for 2.5 and 7.5 mo after bone marrow transfer in Fig. 6D). Up to ~3 and 7 mo after bone marrow transfer, the mean CD3γLLAA:WT ratio of naive CD4+ and CD8+ T cells, respectively, was below 1, supporting the existence of a developmental disadvantage of CD3γLLAA thymocytes as observed in the nonchimeric mice (Fig. 6, D and E). However, the ratio gradually increased with age and surmounted 1 by 3 and 7 mo for naive CD4+ and CD8+ T cells, respectively. Thus, although produced in lower numbers, naive CD3γLLAA T cells gradually equaled or surpassed naive WT T cells in numbers, indicating that once produced, the naive CD3γLLAA T cells had a small survival advantage compared with naive WT T cells. For memory T cells, the mean CD3γLLAA:WT ratio of both CD4+ and CD8+ T cells was significantly increased compared with the correspondent ratio for naive T cells already 2.5 mo after bone marrow transfer (Fig. 6F). The CD3γLLAA:WT ratio of CD4+ and CD8+ memory T cells rose from ~1.2 at 2.5 mo after bone marrow transfer to 2.3 for memory CD4+ cells and 1.8 for memory CD8+ cells 9 mo after bone marrow transfer (Fig. 6E). This supported that the transition of naive CD3γLLAA T cells to memory cells was increased and that CD3γLLAA memory T cells had a survival advantage over WT T cells.

Eleven months after bone marrow transfer, we determined the absolute numbers and percentages of naive and memory CD4+ and CD8+ WT and CD3γLLAA T cells in the spleen and lymph nodes from half of the chimeric mice. In accordance with the results obtained by analyses of the blood samples, we found an almost equal number of naive WT and CD3γLLAA cells but a significantly increased number of CD3γLLAA memory T cells compared with WT memory T cells (Fig. 6F). This resulted in an increased memory:naive ratio for CD3γLLAA T cells compared with WT T cells. Thus, the memory:naive ratio for CD3γLLAA CD4+ cells was 5.4, whereas the corresponding ratio for WT CD4+ cells was 3.5. Likewise, the memory:naive ratio for CD3γLLAA CD8+ cells was 1.5, whereas the corresponding ratio for WT CD8+ cells was 0.5 (Fig. 6G). The CD3γLLAA:WT ratio of naive CD4+ and CD8+ T cells was still close to 1, whereas the CD3γLLAA:WT ratio of memory CD4+ and CD8+ T cells was significantly increased to between 2 and 3 (Fig. 6H). At 15 mo after bone marrow transfer, the rest of the mice were analyzed.
FIGURE 6. The CD3γLAA mutation exerts a T cell-intrinsic effect on T cell homeostasis. A and B, Blood samples were taken regularly from lethally irradiated recipient mice from 2.5 to 9 mo after transfer of a 1:1 ratio of WT and CD3γLAA bone marrow. The percentages of naive and memory CD4+ (A) and CD8+ (B) WT (upper row) and CD3γLAA (lower row) T cells were determined by flow cytometry. Naive T cells were defined as CD44lowCD62Lhigh and memory T cells as not CD44lowCD62Lhigh as in Fig. 1, B and C. The data are representative of 12
CD3γLLAA CD4+ and CD8+ memory T cells now completely dominated the T cell population (Fig. 6f). The memory:naive ratio for CD3γLLAA CD4+ cells had dramatically increased to 22.6, whereas the corresponding ratio for WT CD4+ cells was 3.2. Likewise, the memory:naive ratio for CD3γLLAA CD8+ cells was increased to 18.5, whereas the corresponding ratio for WT CD8+ cells was 0.9 (Fig. 6f). The CD3γLLAA:WT ratio of naive CD4+ and CD8+ T cells was still close to 1, whereas the CD3γLLAA:WT ratio of memory T cells had greatly increased to 8.4 for CD4+ and 26.2 for CD8+ T cells (Fig. 6f).

Collectively, these results demonstrate that the CD3γLLAA mutation exerted a T cell-intrinsic effect on T cell homeostasis that resulted in an increased transition of CD3γLLAA naive T cells to memory T cells and a significant survival advantage of CD3γLLAA memory T cells compared with WT memory T cells.

Mathematical modeling of T cell homeostasis

To this point, our results indicated that the reduced number of naive T cells in the CD3γLLAA mice was caused by the combination of reduced thymic output, decreased T cell apoptosis, and increased transition of naive T cells to memory T cells. At first sight it might be expected that an increase in the transition rate of naive T cells to memory T cells in combination with a decrease in the apoptosis rate of the memory T cells would lead to an increased memory T cell population in CD3γLLAA mice compared with WT mice. However, we found an unaffected number of memory T cells in the CD3γLLAA mice. Therefore, we next tested how our results fitted into a mathematical model of T cell homeostasis. Fig. 7A shows the essential parameters of our kinetic model for T cell homeostasis where \( [T]^n \), \( [T]^m \), and \( [T]^m_{\text{lat}} \) denote the numbers of thymocytes, naive T cells, and memory T cells, respectively; \( k_x \) denotes the rate constant for thymic export, \( k_{am} \) denotes the rate constant for transition of naive T cells to memory T cells, \( k_p \) and \( k_{nm} \) denote the proliferation rate constant for naive and memory T cells, respectively; and \( k_{an} \) and \( k_{nm} \) denote the apoptosis rate constant for naive and memory T cells, respectively. From Fig. 7A we got the following equation for changes in \( [T]^m_{\text{lat}} \):

\[
\frac{d[T]^m_{\text{lat}}}{dt} = k_{an}[T]^m_t - (k_{an} + k_{nm} - k_p)[T]^m
\]

(1)

and the following equation for changes in \( [T]^m \):

\[
\frac{d[T]^m}{dt} = k_{am}[T]^m_t - (k_{an} - k_{nm})[T]^m
\]

(2)

At steady state, the left-hand sides in Equations 1 and 2 are equal to 0 and we got the following equation for the number naive T cells:

\[
[T]^n = \frac{k_{am}[T]^m_t}{(k_{an} + k_{nm} - k_p)}
\]

(3)

and the following equation for the number memory T cells:

\[
[T]^m = \frac{k_{am}[T]^m_t}{(k_{an} - k_{nm})}
\]

(4)

We then compared two scenarios for T cell homeostasis with the experimentally obtained data for T cells in middle-aged mice. In scenario 1, all parameters were held constant between WT and CD3γLLAA mice except for the numbers of thymocytes \( [T]^n \) and thereby the thymic output. Scenario 2 represented a situation where CD3γLLAA mice had a combination of reduced thymic output \( [T]^n \), decreased T cell apoptosis \( k_{an} \), and increased transition of naive T cells to memory T cell \( k_{nm} \), compared with WT mice (Fig. 7B). In scenario 1, the mathematical model predicted that a reduction in thymocyte numbers resulted in a proportional reduction of both naive and memory T cells in the CD3γLLAA mice (Fig. 7, B and C). However, by decreasing the apoptosis rate constants 10% and increasing the transition rate constant by 50% in scenario 2, the mathematical model predicted a 45% reduction in naive T cells simultaneously with unaffected numbers of memory T cells as seen for the experimentally obtained data (Fig. 7, B and C).

Discussion

In this study, we identified a previously unknown role of the CD3γ diL motif in regulating T cell homeostasis. We found that CD3γLLAA knock-in mice had reduced numbers of naive CD4+ and CD8+ T cells compared with WT mice. A decline in naive T cells and a simultaneous increase in the memory:naive T cell ratio are characteristics of T cell population senescence. CD3γLLAA mice showed signs of premature T cell population senescence with an accelerated age-associated decline in naive T cells. Together with an unaffected number of memory T cells, this resulted in a significantly increased memory:naive T cell ratio with a severe dominance of memory T cells and very few naive T cells in middle-aged to old CD3γLLAA mice.

The CD3γ diL motif of the TCR has been carefully characterized at the molecular level and its role in TCR trafficking in T cell lines has been previously described (24–31). Furthermore, we have recently established the critical role of the CD3γ diL motif for constitutive TCR endocytosis and activation-induced TCR down-regulation in primary T cells (32). Constitutive TCR endocytosis was severely reduced in naive T cells from CD3γLLAA mice and, in accordance, TCR expression levels on naive individual bone marrow chimeric mice. C. The memory:naive ratio of CD4+ (upper panel) and CD8+ (lower panel) WT (●) and CD3γLLAA (▼) T cells in the blood samples of bone marrow chimeric mice from 2.5 to 9 mo after bone marrow transfer. Linear regression lines were fitted to the data obtained from WT (solid line) and CD3γLLAA (dashed line) T cells. D. The CD3γLLAA:WT ratio of naive and memory CD4+ and CD8+ in blood samples of bone marrow chimeric mice 2.5 and 7.5 mo after bone marrow transfer. The data points represent individual values obtained from each of the 12 chimeric mice and the bars represent the mean value for each of the T cell subpopulations. E. The mean CD3γLLAA:WT ratio (as calculated in D) of naive (left panel) and memory (right panel) CD4+ (●) and CD8+ (▼) T cells in blood samples from bone marrow chimeric mice from 2.5 to 9 mo after bone marrow transfer. The data points represent individual values obtained from each of the 12 chimeric mice and the bars represent the mean value for each of the T cell subpopulations. F. Absolute numbers of WT (●) and CD3γLLAA (▼) naive and memory CD4+ and CD8+ T cells in the spleen of bone marrow chimeric mice 11 mo after bone marrow transfer. G. The memory to naive ratio of CD4+ and CD8+ WT (●) and CD3γLLAA (▼) T cells in the spleen of bone marrow chimeric mice 11 mo after bone marrow transfer (note that the y-axis is in a logarithmic scale). H. The CD3γLLAA:WT ratio of naive and memory CD4+ and CD8+ in the spleen of bone marrow chimeric mice 11 mo after bone marrow transfer. The data points represent individual values obtained from five chimeric mice and the bars represent the mean value for each of the T cell subpopulations. I. FACS profiles of the distribution of naive and memory CD4+ (first column) and CD8+ (second column) WT (upper row) and CD3γLLAA (lower row) T cells in the spleen of bone marrow chimeric mice 15 mo after bone marrow transfer. J. The memory:naive ratio of CD4+ and CD8+ WT (●) and CD3γLLAA (▼) T cells in the spleen of bone marrow chimeric mice 15 mo after bone marrow transfer. K. The CD3γLLAA:WT ratio of naive and memory CD4+ and CD8+ in the spleen of bone marrow chimeric mice 15 mo after bone marrow transfer. L–K. The data represent values obtained from pooled spleen cells from three chimeric mice. Note that the y-axis is in a logarithmic scale in J and K.
CD3\textsuperscript{y}LLAA T cells were increased by \(~10\%) compared with TCR expression on WT T cells. Likewise, TCR down-regulation was severely impaired following TCR triggering, and CD3\textsuperscript{y}LLAA T cells expressed 40–50\% more TCR than WT T cells following stimulation with specific pMHC. The increased TCR expression results in stronger TCR signaling in CD3\textsuperscript{y}LLAA T cells compared with WT T cells (32).

We found that the total numbers of thymocytes were reduced by \(~20\%) in CD3\textsuperscript{y}LLAA mice and in accordance with other studies, we found a proportionally diminished export of mature T cells from the thymus of CD3\textsuperscript{y}LLAA mice (8, 33, 37, 40). We have not fully determined the thymus defect in CD3\textsuperscript{y}LLAA mice, but our preliminary studies indicate that an increased fraction of CD3\textsuperscript{y}LLAA thymocytes undergoes apoptosis during the proliferative burst that takes place during the transition from the double-negative to the double-positive stage (our manuscript in preparation). This observation is in accordance with previous studies that found that a reduction in the number of double-negative precursor cells leads to a proportional reduction in the double-positive and single-positive thymocyte subpopulations (38). It was previously shown that the fraction of recent thymus emigrants is constant at 1–2\% of thymocytes per day, independently of the number of thymocytes and peripheral T cells (8, 33, 37, 40). In agreement with this, we found a proportional reduction in the number of thymocytes and recent thymus emigrants in the CD3\textsuperscript{y}LLAA mice. Furthermore, we found that the number of naive T cells was more affected than the number of memory T cells by the CD3\textsuperscript{y}LLAA mutation. This is in accordance with previous studies that found that a reduction in the number of single-positive mature T cells in the thymus mainly causes a reduction in the number of naive T cells (38, 39). We have recently shown that TCR signaling is increased in CD3\textsuperscript{y}LLAA T cells (32). In the present study, we found an increased CD4:CD8 ratio of mature single-positive thymocytes and peripheral T cells in the CD3\textsuperscript{y}LLAA mice. This is in good agreement with several studies that have demonstrated that enhanced TCR signaling strength in developing thymocytes favors

![Mathematical model of T cell homeostasis.](image)
the development of the CD4 lineage, whereas reduced TCR signaling strength favors development of the CD8 lineage (48–50).

In addition to interaction with IL-7 and to a lesser extent IL-15, prolonged survival of naive T cells is dependent on TCR signaling from interaction with self-pMHC complexes (51–55). We found a 20% reduction in the fraction of apoptotic naive T cells in CD3γLLAA mice compared with WT mice. This suggested that naive CD3γLLAA T cells had a survival advantage compared with naive WT T cells. This suggestion was supported by studies of bone marrow chimeric mice containing WT and CD3γLLAA T cells in which the numbers of naive CD3γLLAA T cells equalized or surpassed naive WT T cells with age even though CD3γLLAA T cells were produced in lower numbers. The increased survival of CD3γLLAA T cells could not be explained by an altered interaction with IL-7 or IL-15 as CD3γLLAA and WT T cells expressed equal levels of CD122 and CD127 and as CD3γLLAA and WT T cells were exposed to identical external circumstances in the chimeric mice. Thus, only a T cell-intrinsic effect caused by the CD3γ mutation could explain the improved survival of the CD3γLLAA T cells. We propose that enhanced TCR signaling caused by the increased TCR expression level in CD3γLLAA T cells results in better survival of CD3γLLAA naive T cells. This is analogous to the reduced survival observed in T cells with weakened TCR signaling (53, 54).

As for naive T cells, we found a reduced fraction of apoptotic memory T cells in CD3γLLAA mice compared with WT mice. Analyses of the bone marrow chimeric mice confirmed that CD3γLLAA memory T cells had a survival advantage compared with WT memory T cells. Already 2.5 mo after bone marrow transfer, the numbers of CD3γLLAA memory T cells had surpassed the numbers of WT memory T cells, and 15 mo after bone marrow transfer CD3γLLAA memory T cells completely dominated the T cell population. Although the role of TCR signaling in homeostasis of memory T cells is not as clearly defined as for naive T cells (7, 8, 56, 57), our study demonstrates that the CD3γ diL motif plays a critical role for memory T cell homeostasis. In accordance, molecules that control signaling through the TCR can have evident influence on memory T cell survival. Thus, experiments with bone marrow chimeric mice, where T cells deficient of the negative regulator of TCR signaling BTLA (B and T lymphocyte attenuator) and WT T cells develop in the same mouse, have demonstrated a major survival advantage of BTLA-deficient memory T cells compared with WT memory T cells (58).

The exact mechanisms behind the gradual transition of naive to memory phenotype T cells are not fully known, but it has been suggested that the combined strength of the signaling from the TCR and the IL-7R determines the propensity of naive T cells to differentiate into memory T cells (7, 8). It seems likely that increased TCR signaling causes a higher rate of transition of naive to memory T cells in CD3γLLAA mice. In accordance, BTLA-deficient mice with enhanced TCR signaling are more efficient than WT mice in generating memory CD8+ T cells and have a higher memory:naive CD8+ T cell ratio than do WT mice (58). An enhanced transition of naive to memory T cells in CD3γLLAA mice is also in agreement with our recent studies on Ag-specific T cell responses following virus infection, where we found a more efficient transition of virus-specific effector to memory CD8+ T cells in CD3γLLAA mice compared with WT mice (32). To firmly establish that the perturbed transition of naive CD3γLLAA T cells to memory T cells was caused by T cell-intrinsic mechanisms and not by an altered cytokine milieu or other unknown host factors, we studied T cell transition in bone marrow chimeric mice containing WT and CD3γLLAA T cells. As for the nonchimeric mice, we found a highly increased ratio of memory:naive CD3γLLAA T cells compared with WT cells in the same host. This indicates that the CD3γ diL motif-mediated TCR down-regulation normally affects the equilibrium between naive and memory T cells in favor of the maintenance of naive T cells.

It could be speculated whether the CD3γ LLAA T cell phenotype simply could be explained by an isolated reduction in thymic output, or whether it was explained by the combined effects of reduced thymic output, decreased apoptosis, and increased transition of naive T cells to memory T cells. We believe that the bone marrow chimeric experiments conclusively excluded the possibility that the CD3γLLAA phenotype could be explained solely by a decreased thymic output. In that case, we would not observe equal numbers of naive CD3γLLAA and WT T cells and significantly increased numbers of memory CD3γLLAA T cells compared with memory WT T cells in the bone marrow chimeric mice. Furthermore, our mathematical model of T cell homeostasis predicted that an isolated thymic reduction would lead to a proportional reduction in both naive and memory T cells given that all other parameters were held constant between the WT and CD3γLLAA mice. In contrast, the mathematical model supported that the CD3γLLAA T cell phenotype was explained by the combination of reduced thymic output, decreased apoptosis, and increased transition of naive T cells to memory T cells (Fig. 7).

Taking into consideration that our model is a knock-in mouse and that the only difference between CD3γLLAA and WT mice is a subtle mutation of the CD3γ diL motif, we found that our results convincingly demonstrate an important role of the CD3γ diL motif in T cell homeostasis. The significant role of the CD3γ diL motif in T cell biology is further supported by the observation that this motif has been conserved for > 350 million years of evolution and is found even in the common ancestor of the CD3γ and δ-chains in amphibians (59).

In conclusion, we propose that the CD3γ diL motif serves to regulate TCR expression and thereby to fine-tune TCR signaling and regulate T cell homeostasis to support the maintenance of naive T cells even into old age and postpone T cell population senescence. Furthermore, our results point to the potential of the CD3γ diL motif as a target to manipulate immune responses. One of the central goals of vaccination is to induce the formation of long-lived, Ag-specific memory T cells. Our data suggest that blockade of the CD3γ diL motif might enhance Ag-specific memory T cell formation under appropriate immunization conditions. We are presently investigating this possibility.

Disclosures
The authors have no financial conflict of interest.

References