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Thymic epithelial cell support of thymopoiesis does not require *Klotho*

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Abstract

Age-related thymic involution is characterized by a decrease in thymic epithelial cell (TEC) number and function parallel to a disruption in their spatial organization, resulting in defective thymocyte development and proliferation as well as peripheral T cell dysfunction. Deficiency of *Klotho*, an anti-aging gene and modifier of fibroblast growth factor signaling, causes premature aging. To investigate the role of *Klotho* in accelerated age-dependent thymic involution, we conducted a comprehensive analysis of thymopoiesis and peripheral T cell homeostasis using *Klotho*-deficient mice. At 8 weeks of age, *Klotho*-deficient mice displayed a severe reduction in the number of thymocytes (10–100 fold reduction), especially CD4 and CD8 double positive cells, and a reduction of both cortical and medullary TEC. To address a cell-autonomous role for *Klotho* in TEC biology, we implanted neonatal thymi from *Klotho*-deficient and -sufficient mice into athymic hosts. *Klotho*-deficient thymus grafts supported thymopoiesis equivalently to *Klotho*-sufficient thymus transplants, indicating that *Klotho* is not intrinsically essential for TEC support of thymopoiesis. Moreover, lethally-irradiated hosts given *Klotho*-deficient or WT bone marrow had normal thymocyte development and comparably reconstituted T cells, indicating *Klotho* is not inherently essential for peripheral T cell reconstitution. Because *Klotho*-deficient mice have higher levels of serum phosphorus, calcium, and vitamin D, we evaluated thymus function in *Klotho*-deficient mice fed with vitamin D-deprived diet. We observed that vitamin D-deprived diet abrogated thymic involution and T cell lymphopenia in 8-week-old *Klotho*-deficient mice. Taken together, our data suggests that *Klotho*-deficiency causes thymic involution via systemic effects that include high active vitamin D levels.

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Introduction

Thymic aging is a major contributing factor to immunological senescence as the thymus begins to involute with the onset of puberty, leading to a progressive in the ability to generate naïve T cells (1, 2). The primary elements of the thymic microenvironment affected by age-related involution are structural thymic epithelial cells (TECs). TECs are replaced by adipocytes and peripheral lymphocytes, resulting in decreased thymopoietic activity and T cell selection (3). Consequently, both quantity and quality of the T cell repertoire are affected, with decreased *de novo* T cell generation that is compensated by a homeostatic oligoclonal expansion of T cells in the periphery (4, 5). These dynamic changes result in an increased susceptibility to infections (6, 7), suboptimal responses to vaccines (8–12), and an increased risk to develop cancer and autoimmune diseases (13–15). The age-related decline in thymopoietic activity (1) is especially apparent in patients who have undergone chemotherapy (16) or allogeneic hematopoietic stem cell transplantation (17). The necessary preparative regimen with cytotoxic chemotherapy and/or radiation severely damages the thymus, the recovery of which is extremely limited in aged individuals (18, 19).

To study the process of aging in mice, *Klotho*-deficient (*Kl*/*Kl*) animals have been used as they grow normally up to 3 weeks of age and then begin to show premature aging phenotypes (20). *Klotho* encodes a beta-glucuronidase-related molecule in two separate isoforms, transmembrane and secreted; the transmembrane molecule serves as a co-receptor for fibroblast growth factor 23 (FGF23) by transporting this cytokine to its receptor, FGFR1c, and thereby regulating mineral metabolism (21–23). *Klotho* is expressed in the kidney and parathyroid gland and the secreted form also be found in the blood, CSF and urine (24). FGF23 suppresses phosphate reabsorption and Vitamin D synthesis in the kidney, causing negative phosphate balance due both to its phosphaturic hormone function and as a counter-regulatory hormone for Vitamin D(24). The secreted form of Klotho inhibits insulin growth factor 1 signaling and confers increased resistance to oxidative stress (25–27). Mice transgenic for *Klotho* live 20–30% longer than wild-type (WT) controls (28), while the protein’s absence results in an advanced aging syndrome resembling progeria. Multiple organs are affected in *Kl*/*Kl* mice resulting in growth retardation, pituitary abnormalities, arteriosclerosis, ectopic calcification of various organs, osteoporosis, skin atrophy, emphysema, and atrophy of both the genital organs and the thymus (20). Interestingly, mice that are FGF23 deficient or Klotho deficient have phenotypes similar to one another. These deficits can be ameliorated by reversing the effects of hyperphosphatemia either genetically or by diet, suggesting a link between aging and phosphate(24).

The *Kl*/*Kl* mouse model has provided insight into the process of aging in humans. Indeed, human KLOTHO shares 86% amino acid identity with its mouse ortholog (29). Individuals homozygous for *Klotho* variants that disrupt the molecule’s trafficking and catalytic functions experience a decreased life expectancy (29), have increased cardiovascular risk factors, such as elevated high-density lipoprotein cholesterol levels and high systolic blood pressure (30), and demonstrate an increased risk for stroke and coronary artery disease (31). Polymorphisms in *KLOTHO* (loss of function) have been associated with an increased risk for osteoporosis and spondylosis (32) and reduced KLOTHO protein expression has been noted in patients with chronic renal failure (33).

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While the effects of *Klotho*-deficiency on kidney development and function, mineral metabolism, and bone maintenance are well studied in *Kl*/*Kl* mice, the direct effect of *Klotho*-deficiency on the TECs is unknown. We therefore sought to determine whether the effects of *Klotho* on thymic aging are cell intrinsic or reflect a systemic metabolic consequence of a lack of the Klotho protein.

Methods

Mice

B6.Cg-*Foxn1*$^{nu/J}$ mice were purchased from Jackson Labs and were used at 8–12 weeks of age. *Kl/+* mice (B6-CD45.2^{+}) were generously provided by the University of California Davis mouse mutant resource center and were intercrossed (*Kl/+* by *Kl/+*) in our animal colony under the guidance of in-house veterinary staff. B6-Ly5.2/Cr (B6-CD45.1^{+}) were purchased from the National Cancer Institute and were used at 7 weeks of age. Mice were housed in a specific pathogen-free facility and used with the approval of the University of Minnesota Institutional Animal Care and Use Committee (IACUC). For vitamin D experiments, breeders were fed and pups were maintained on vitamin D-deprived diet purchased from Harlan, product TD. 89123.

Flow cytometry and Klotho expression

Mouse thymus, spleen, and lymph node (LN) were processed into single cell suspensions, and analyzed by flow cytometry. Thymic epithelial cells (TECs) were isolated as previously described (34). Dead cells were stained by a fixable viability dye conjugated to eFluor780 (eBioscience). Fixation and intracellular/intranuclear staining were performed using the eBioscience Foxp3 staining kit or BD Fixation/Permeabilization Solution Kit. The following antibodies were purchased from BD biosciences: TCRβ (H57–597), TCRγδ (GL-3) and EpCAM (G8.8). The following antibodies were purchased from eBioscience: CD3 (145–2C11), CD4 (GK1.5), CD11c (N418), CD25 (PC61.5), CD45R (B220, RA3–6B2), MHCII (M5/114) and Ki67 (SolA15). The following antibodies were purchased from BioLegend: CD8 (53–6.7), CD11b (M1/70), CD44 (IM7), CD45 (30-F11), CD62L (MEL-14), CD122 (TM-β1), Gr-1 (RB6–8C5), Ly-51 (6C3), NK-1.1 (PK136) and TER-119 (TER-119). UEA-1 was purchased from Vector Laboratories. Data were collected using a BD LSR II flow cytometer and were analyzed using FlowJo V10 (Treestar Inc.). The TaqMan Gene Expression Assay (ID: Mm00502000_m1, Thermo Fisher Scientific) for quantification of Klotho gene expression.

Neonatal Thymus Transplantation

Mice heterozygous for *Klotho* were mated overnight and then separated. At the time of harvest, neonate pups were screened for *Klotho* via PCR. WT or *Kl*/*Kl* thymi were placed under the kidney capsule of B6.Cg-Foxn1$^{nu/J}$ mice in the previously described manner (35, 36).

Bone Marrow Transplantation

B6-CD45.1^{+} recipients were lethally irradiated using 1100 cGy total body irradiation by x-ray one day before infusion. On the second day, bone marrow cells (BM) were harvested

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from *Kl/Kl* mice and littermates. Mature T-cells were removed from donor BM using anti-CD4, anti-CD8 antibodies and low-toxicity rabbit complement and given intravenously at a cell dose of 1×10^7.

Immunofluorescence staining

Thymi were harvested and snap frozen in O.C.T. compound. Frozen sections (8 μm) were cut using a CM1900 cryostat (Leica). Slides were dried for 30 min and then were immerged in acetone for 5 min at room temperature. The sections were blocked in PBS with 3% BSA (PBSB) for 1 h at room temperature and stained with the rabbit anti-mouse K5 polyclonal antibody (MBL International) and rat anti-mouse K8 monoclonal antibody (TROMA-I, Development Studies Hybridoma Bank) followed by Dylight 550 donkey anti-rabbit IgG antibody and Dylight 650 donkey anti-rat IgG (Invitrogen). ProLong Gold antifade reagent (Invitrogen) was used to prevent photobleaching. Images were obtained using a microscope (DM5500B; Leica) with a camera (DFC 340FX; Leica) operating with the Leica Application Suite Advanced Fluorescence (LAS AF; Leica) software and analyzed using ImageJ (NIH) software.

Statistical Analyses

Prism software (Graphpad) was used for statistical analysis. Data sets were compared using an unpaired Mann-Whitney test. Data are shown as mean values +/− SD. Significance was defined as $p<0.05$.

Results

Klotho-deficient mice showed profound involution of thymus at young adult age

The thymus is the primary lymphoid organ for the development of T cells by providing a unique microenvironment able to attract T cell precursors from the blood and control their T cell lineage commitment, differentiation and selection (37). The earliest stages of intrathymic T cell development are marked by the absence of CD4 and CD8 expression as well as other lineage markers (designated double negative, DN, cells). These immature DN cells are located in the cortex, where they significantly expand in number before they acquire the concomitant expression of both CD4 and CD8 (double positive, DP) and express an αβ T cell antigen receptor (TCR). DP thymocytes constitute the most abundant subpopulation of thymocytes and are subjected first to a process of positive selection which assures that thymocytes express a TCR with sufficient affinity for a peptide-MHC complex expressed on cortical thymic epithelial cells (cTECs) (38). Positively selected thymocytes differentiate into TCRβ-positive CD4 and CD8 single positive (CD4SP and CD8SP) cells, respectively, and migrate to the medulla continuing their selection and maturation (39, 40). Self-reactive thymocytes are either eliminated by negative selection (clonal deletion) or differentiate into regulatory T cells (41). Subsequently, thymocytes are selected for tolerance to tissue-restricted antigens but responsiveness to foreign antigens presented by the individual's MHC haplotype (39).

We investigated the thymic microenvironment of *Kl*/*Kl* mice from 4 weeks of age, shortly after which these mice begin to display signs of advanced aging (20). At 4 weeks of age, the

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thymi of *Kl*/*Kl* mice were comparable to that of their wild type (WT) littermates in size, cellularity and subpopulation distribution (Fig. 1A, 1B, 1C). *Kl*/*Kl* mice at 8 weeks of age (young adult) displayed a significantly reduced thymic size when compared to that of WT littermate controls (Fig. 1A) and a profound decrease of thymocytes in total cellularity (Fig. 1B). Flow cytometry revealed that the frequency of DP thymocytes was significantly decreased but that of DN, CD4SP and CD8SP thymocytes yet remained unchanged in 8-week-old *Kl*/*Kl* mice when compared to age-matched controls (Fig. 1C, Supplemental Fig. 1A-1D). All thymocyte subpopulations had, however, a significantly reduced cellularity in 8-week-old *Kl*/*Kl* mice (Fig. 1D) Semi-mature SP thymocytes were barely detected in 8-week-old *Kl*/*Kl* mice (Fig. 1E, 1F, Supplemental Fig. 1E), suggesting that *de novo* generation of SP thymocytes was severely blocked (42). Thus, the thymus of 8-week-old *Kl*/*Kl* mice was prematurely involuted to an extent only observed in older, physiologically aged mice.

Thymus involution in 8-week-old Klotho-deficient mice is associated with a severe reduction in thymic epithelial cells.

In order to assess the effect of *Klotho* on TECs, both cortical TECs (cTECs) and medullary TECs (mTECs) were evaluated. They are defined by Ly51 expression and binding capacity to the lectin, UEA-1. cTECs are critical for T cell progenitor expansion and positive selection of thymocytes whereas both cTEC and mTEC effect the negative selection of self-reactive thymocytes (43). To address whether TEC development and maintenance are impaired in the absence of Klotho, we assessed TEC cellularity and frequencies in 4 and 8 week old *Kl*/*Kl* mice. Total TEC cellularity and the frequencies of their individual subsets were comparable at 4 weeks of age with that of wildtype or heterozygote littermate controls (Fig. 2A, 2B). In contrast, 8-week-old *Kl*/*Kl* mice showed a significant reduction in the number of total TECs (Fig. 2A), affecting especially mTECs (Fig. 2B, 2C). These data suggest that a *Klotho* deficiency intrinsically or extrinsically reduced TEC cellularity in 8 week old animals.

Thymic epithelial cells do not require Klotho expression to support thymopoiesis.

In order to determine the expression of *Klotho* in TECs, we performed RT-PCR on sorted cTEC, mTEClo and mTEChi cells in both wildtype and *Kl*/*Kl* animals. *Klotho* expression was found in both mTEC compartments, but was minimal in the cTEC compartment in WT animals. As expected, there was no expression in the TECs of *Kl*/*Kl* animals. Thymic involution is the result of cell autonomous changes in cell maintenance as a function of age but can also occur as a result of systemic abnormalities via various paracrine effector mechanisms. To distinguish between these two explanations for the observed pre-senescent changes in thymic cellularity, we grafted thymic lobes from either neonatal *Kl*/*Kl* or WT mice under the kidney capsule of haploidentical athymic (i.e. nude, $Foxn1^{nu/nu}$ (44)) recipients and compared their growth and differentiation. $Foxn1^{nu/nu}$ mice are homozygously deficient for the expression of functional Foxn1, a master regulator of TEC growth, differentiation and function of TEC, and therefore athymic and T cell deficient but have otherwise intact hematopoietic stem cells. To have a chronotypic comparison with 8-week old *Kl*/*Kl* mice, thymus grafts and peripheral lymphoid tissues of grafted recipients were analyzed 8 weeks after transplantation. The size and cellularity of *Kl*/*Kl* and WT thymus grafts were comparable (Fig. 3A and 3B) and displayed identical proportions and

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cellularity of the distinct thymocyte subpopulations (Fig. 3C). The ostensibly normal thymopoietic activity of *Klotho*-deficient grafts resulted in a comparable peripheral T cell reconstitution in both groups of transplanted mice (Fig. 3E, 3F). In aggregate, these results demonstrated that non-hematopoietic thymic stromal cells including TEC do not rely on Klotho expression for the organs' thymopoietic activity.

Selective Klotho deficiency in bone marrow cells does not impair thymocyte differentiation

Although mature blood cells, including T cells, do not express Klotho, findings from the transplant experiments did not formally exclude the requirement of Klotho for progenitor cells to commit to a T cell fate and differentiate into mature thymocytes able to promote TEC maturation via thymus cross-talk. To examine this possibility, lethally irradiated H-2-matched WT mice were grafted with either *Klotho*-deficient or –proficient hematopoietic stem cells (HSCs) and analyzed 8 weeks later (Fig. 4A). Recipients rescued with *Klotho*-deficient HSCs displayed thymocyte cellularity and differentiation comparable to mice grafted with WT HSCs (Fig. 4B-4D). These results demonstrate that thymic Klotho expression in thymocytes is dispensable to repopulate the T cell lineage in irradiated hosts

Thymus involution and peripheral T-lymphopenia in 8-week-old Klotho-deficient mice is averted by vitamin D-deprivation.

Previous studies have shown that elimination of vitamin D in *Kl*/*Kl* mice diet corrected the hypervitaminosis D typically observed in these animals, and consequently improved some of their disease-related phenotype (25, 45, 46). As our transplantation data indicated that premature thymic involution in *Klotho*-deficient mice was not caused by Klotho deficiency in either hematopoietic cells or thymic stromal cells, we next investigated whether a reduction in vitamin D levels improved the thymic changes and, as a result, the peripheral T cell compartment of *Kl*/*Kl* mice. In this experiment, both breeder mice and offspring were maintained on a vitamin D-deprived diet throughout th e experiment. Eight week old *Kl*/*Kl* mice exposed since conception to low Vitamin D levels displayed a total thymus cellularity and thymocyte differentiation comparable to that of age-matched WT animals whereas *Kl*/*Kl* mice fed with a vitamin D-replete diet displayed as expected the typical hallmarks of Klotho deficiency (Fig. 5A, 5B). The thymus architecture of 8-week-old, conventionally fed *Kl/Kl* mice showed a structural disorganization, especially of the medulla (Fig. 5C), consistent with our previous observations (47). In contrast, the histological structure of the thymus in both *Kl*/*Kl* and WT mice fed with a Vitamin D-deprived diet displayed separate and well demarcated cortical and medullary compartments (Fig. 5C). These data specify that low Vitamin D levels correct the thymus phenotype of *Kl/Kl* mice and suggest that the premature thymus involution observed in *Klotho*-deficient mice is the result of high systemic levels of Vitamin D.

We considered the possibility that hypervitaminosis D in *Kl/Kl* mice may inhibit the emigration of mature thymocytes to the periphery possibly contributing to T cell proliferation and/or survival, contributing to T cell lymphopenia. We therefore probed in *Kl/Kl* and control mice the cellularity and frequency of CD4 and CD8 single positive T cells (SP4 and SP8) in spleen and lymph nodes. At 8 weeks of age, *Kl*/*Kl* fed a vitamin D-

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depleted diet had comparable percentages (Fig. 6A) but decreased numbers (Fig. 6B) of both CD4T and CD8T cells compared to controls.

An increased frequency of CD44^{hi} memory-like cells is a phenotypic hallmark of T cell lymphopenia. Interestingly, *Kl/Kl* mice also may have impaired IL-7 dependent lymphopenia-driven differentiation, expansion or survival of memory-like T cells due to decreased IL-7 production in stromal cells (e.g. BM) in *Kl/Kl* mice (48). The frequency of SP4 CD44^{hi} but not SP8 CD44^{hi} memory-like T cells was unaffected (Fig. 6C, Supplemental Fig. 2), although their cellularity were both reduced in *Kl/Kl* mice on a vitamin D depleted diet (Fig. 6D), suggesting a defect in memory-like T cell support. Moreover, the absolute cellularity of splenic SP4 and SP8 T cell lymphopenia was comparable between *Kl*/*Kl* mice fed a vitamin D-deprived diet and control animals (Fig. 6E, 6F). Overall, the data show that Klotho animals at 8 weeks of age have significant losses of both thymocytes and TECs and that these deficits can be overcome by decreasing Vitamin D levels in vivo.

Discussion

Our results demonstrate that thymus function in young *Kl*/*Kl* mice is unaffected but severe atrophy with reduced thymopoiesis is observed in these mice by 8 weeks of age. This change in thymus function was neither the consequence of a cell-intrinsic loss of Klotho expression in hematopoietic cells nor the result of a cell-autonomous deficiency in expression of this protein by thymic stromal cells. Rather, thymic hypocellularity and the loss of a regular thymic stromal architecture in *Kl*/*Kl* mice are the consequence of high Vitamin D levels, inferring that *Klotho* deficiency disrupts thymus function in an indirect, systemic fashion.

The molecular interactions of FGF23, klotho and vitamin D coordinate to regulate phosphate metabolism (24) (46, 49–51). FGF23 decreases renal tubular and phosphate reabsorption and stimulates Vitamin D3, which results in increased renal klotho synthesis (46). Klotho binds to FGFR1(IIIc) that reduces Vitamin D3 synthesis. In *Kl*/*Kl* mice, FGF23 is unable to bind the klotho transmembrane molecule precluding its transport to FGFR1c resulting in a failure to negatively regulate Vitamin D3 synthesis via FGF23 receptor mediated inhibition of 1α-hydroxylase (52).Thus, high levels of active vitamin D accumulate in *Kl*/*Kl* mice causing a state of calcium and phosphate imbalance.

To counteract the abnormal calcium and phosphorus state, vitamin D deficient diets have been tested and have been shown to ameliorate other consequences related to accelerated aging including decreased ectopic calcification of tissues, lack of skin atrophy, and increased life span (45). Feeding kl/kl mice a vitamin D deficient diet indeed improved thymic architecture, TEC differentiation, thymocyte development and maturation and peripheral T cell reconstitution. Therefore, it may be tempting to speculate that the vitamin D deficient diet had direct effects on TECs, despite the fact that the genetic deficiency of klotho in TECs would be unaffected. Similarly, the data from kidney capsule implants indicated that the TECs from kl/kl mice are able to develop and support thymopoiesis despite the klotho deficiency. Taken together, we favor the hypothesis that correction of calcium and

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phosphorus imbalance and high vitamin D levels are responsible for improvement in thymopoiesis and TEC development.

Both indirect and direct TECs effects of klotho deficiency can be envisioned. Metabolic imbalance of calcium, phosphorus and vitamin D3 may indirectly impair TEC function and development due to apoptosis and stress in the animals. High vitamin D3 levels may indirectly impair TEC development and maturation by interfering with the essential cross-talk signaling mechanisms between TECs and thymocytes needed for their mutual development (43, 54). In support of this hypothesis, thymocytes express the Vitamin D receptor according to gene expression data ((Accession Number GSE81163, http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE81163). High Vitamin D levels directly impair TEC development since cells within the thymus that express high levels of vitamin D receptor are sensitive to vitamin D-induced signaling. In the absence of *Klotho*, levels of FGF-23 are elevated (59), which may be toxic to developing thymocytes as it is to the kidney (60) or exert yet unknown effects independent of vitamin D levels. Klotho suppresses NF-κB translocation and therefore suppresses inflammatory cytokine production (57) and conversely, Klotho deficiency increases proinflammatory cytokines such as IL-6 or TNFα in monocytes (58), which could be driving thymocyte death leading to insufficient support of TECs.

Medullary thymocytes also express the vitamin D receptor and vitamin D signaling inhibits mitogenic stimulation of these cells (53, 55) which may negatively impact upon peripheral T-lineage reconstitution. We observed a reduction of regulatory T cells (Tregs) in the thymus and in the periphery of 8-week-old Kl/Kl mice (data not shown). Thymic Tregs express vitamin D receptor, suggesting that their development could be affected by vitamin D levels in a manner similar to conventional thymocytes (56). Additional potential mechanisms that might lead to thymic involution and T cell lymphopenia seen in *Kl*/*Kl* mice include decreased expression of positive cell cycle regulators, such as Cyclin D1 and c-Myc that may be lacking in developing thymocytes, resulting in low proliferative rates. Previously, we reported that keratinocyte growth factor (fibroblast growth factor-7) administration, known to stimulate the proliferation of TECs and other epithelial cells, can improve IL-7 production and thymopoiesis in 2-week but not 6-week old *Kl*/*Kl* mice that have substantially defective IL-7 production and thymopoiesis (47). Although it is not yet clear whether reduction of IL-7 production directly associates with hypervitaminosis D, mice given a vitamin D-deficient diet did not have evidence of poor T cell content in the periphery nor manifestations of IL-7 deficiency in the thymus. Further studies are needed to identify the molecular mechanisms downstream of reduced Vitamin D levels that account for the preservation of regular thymopoiesis in *Kl/Kl* mice.

In conclusion, our data suggest that *Klotho* is not cell-autonomously needed for differentiation, maintenance or function of the thymic microenvironment. However, under conditions that stress the thymus and developing T cells then *Klotho* is required to maintain normal levels of thymopoiesis. In this way, *Klotho* may be important to opposing the process of thymic involution, which naturally occurs with age.

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Supplementary Material

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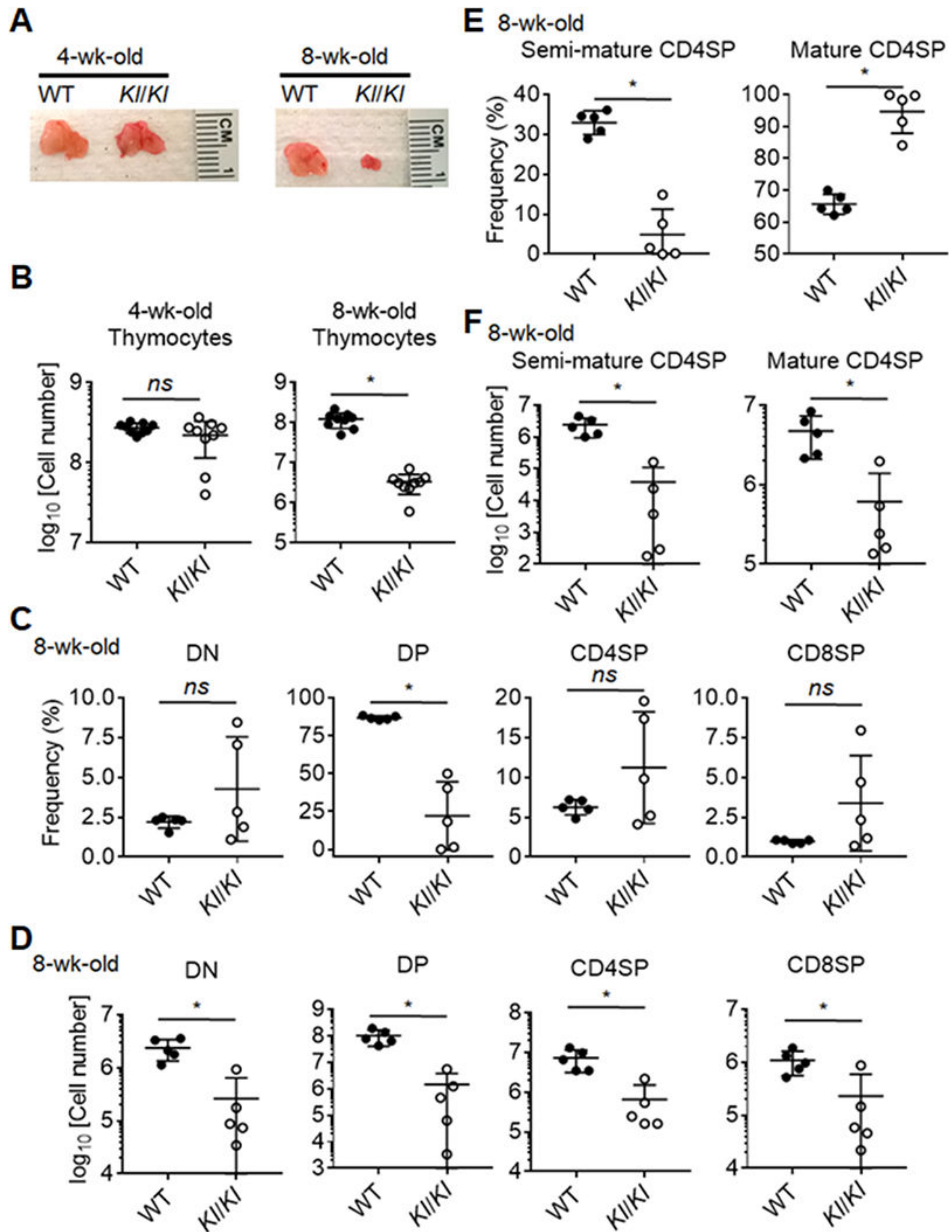

Figure 1.

Profound thymus involution in *Klotho*-deficient (*Kl*/*Kl*) mice at 8 weeks of age.

(**A**) A representative image of the thymus from *Kl*/*Kl* and littermate control mice at 4 and 8 weeks of age. (**B**) Quantification of total thymocytes in *Kl*/*Kl* mice and littermate controls. (**C**) A summary of percentages of thymocyte subpopulations, including DN (Lineage$^-$CD4$^-$CD8$^-$), DP (CD4$^+$CD8$^+$), CD4SP (TCRβ$^+$CD4$^+$CD8$^-$) and CD8SP (TCRβ$^+$CD4$^-$CD8$^+$) cells. (**D**) A graph shows quantification data of absolute numbers of thymocyte subpopulations. (**E**) Frequencies of semi-mature (CD69$^+$MHC-I$^-$) and mature

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(CD69$^{+/-}$MHC-I^{+}) subpopulations in CD4SP cells. (**F**) Quantification of absolute numbers of semi-mature and mature CD4SP in the thymi. Each symbol in the graphs represents an individual mouse (n 5); small horizontal lines indicate the group mean (± s.d.). ns, not significant (P 0.05); *, P < 0.05.

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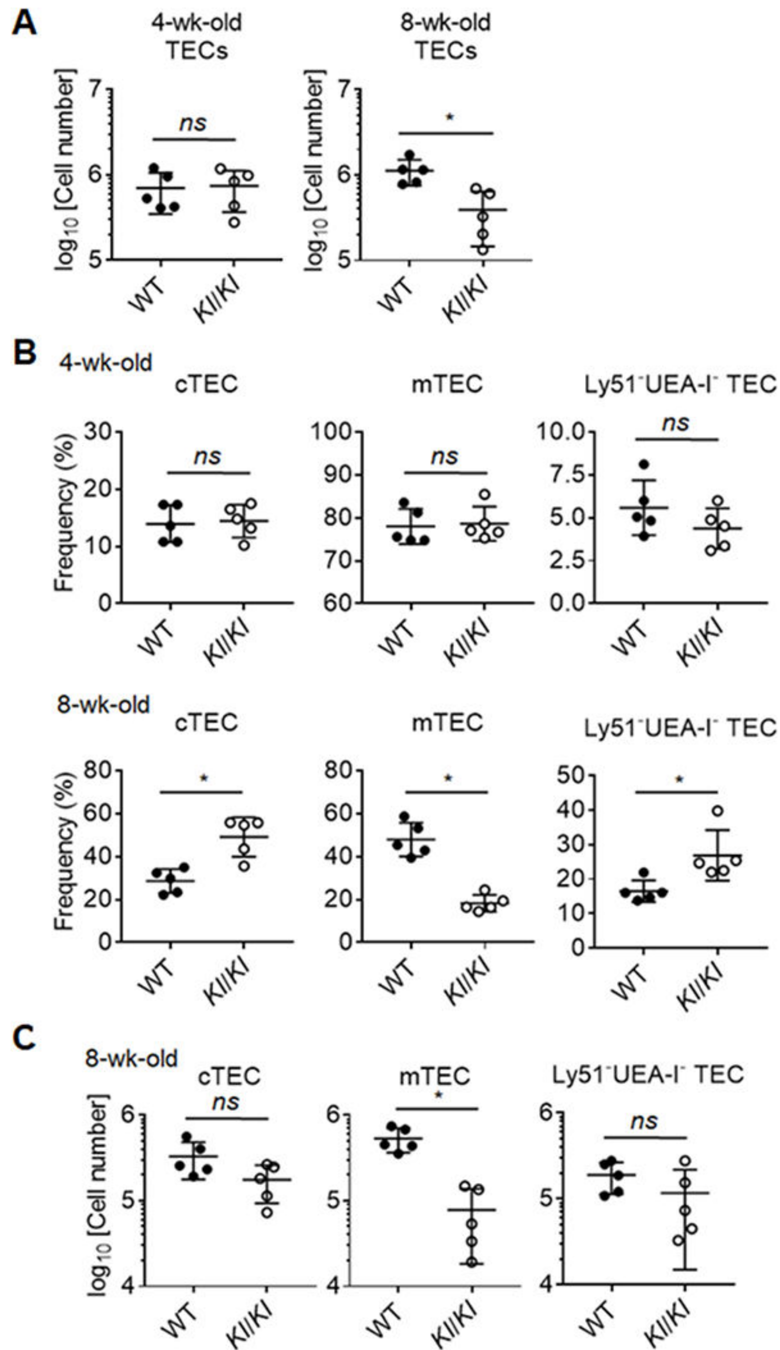

Figure 2.

Reduction of thymic epithelial cells (TECs) in 8-wk-old *Klotho*-deficient mice.

(**A**) A graph shows the absolute number of TECs in the thymus of *Kl*/*Kl* and control mice at 4 and 8 weeks of age. (**B**) Frequencies of TEC Subpopulations, cTEC (UEA-I$^-$Ly51$^+$) and mTEC (UEA-I$^+$Ly51$^-$). (**C**) Quantification of cTECs and mTECs in *Kl*/*Kl* mice and littermate control at 8-week of age. Each symbol in the graphs represents an individual mouse (n 5); small horizontal lines indicate the group mean (± s.d.). ns, not significant (P 0.05); *P < 0.05.

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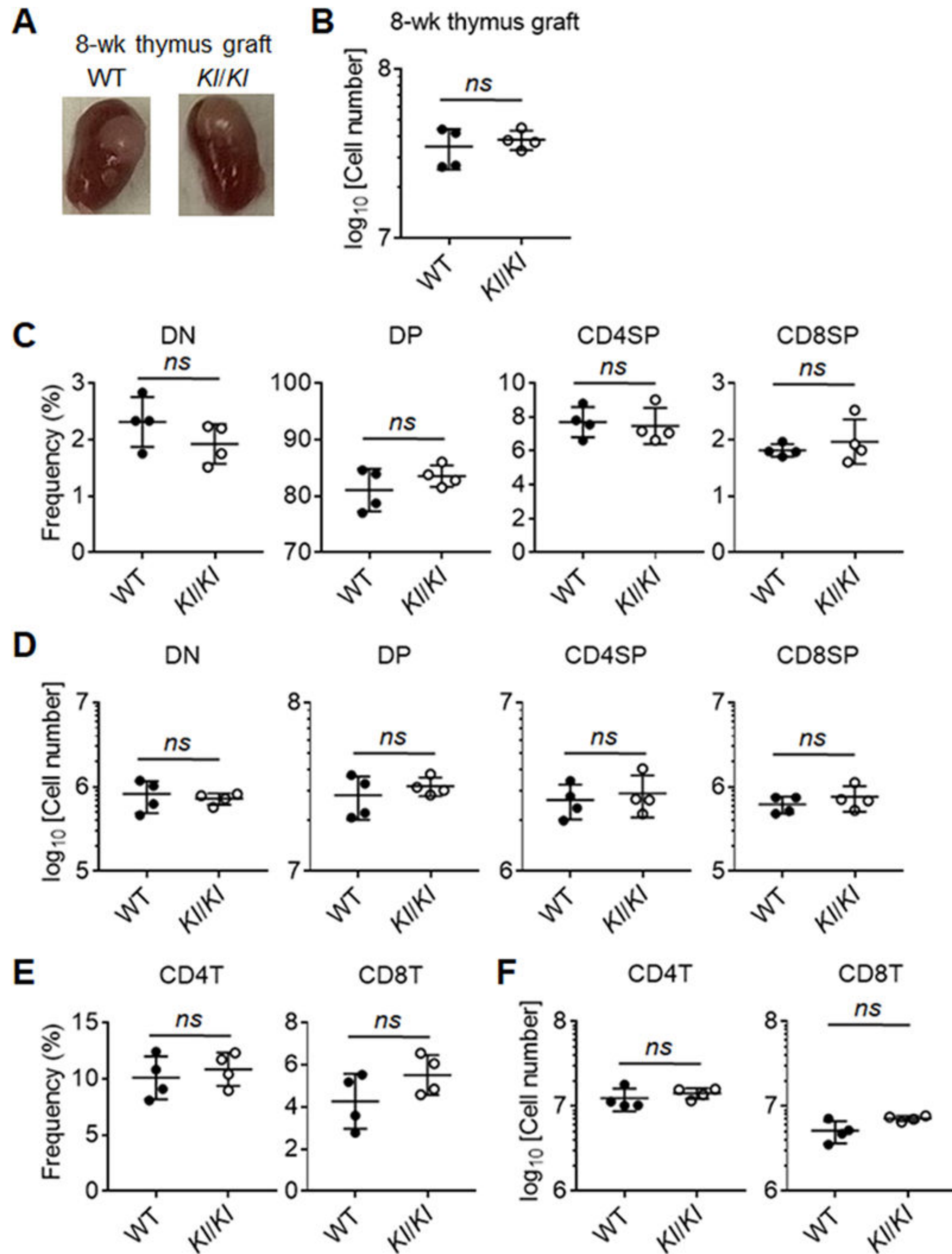

Figure 3.

Stromal cells from *Klotho*-deficient and -sufficient thymus showed comparable ability to support T cell development after thymus transplantation.

(**A**) Representative images of thymus grafts under kidney capsule in athymic host mice after 8 weeks of transplantation. (**B**) Quantification of total thymocytes of *Klotho*-deficient and -sufficient thymus grafts in the recipients after 8 weeks of transplantation. (**C**) Frequencies and cellularity (**D**) of thymocyte subpopulations in *Klotho*-deficient and -sufficient thymus grafts after 8 weeks of transplantation. (**E**) Frequencies and cellularity (**F**) of T cell subsets

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in the spleen from athymic recipients with *Klotho*-deficient and -sufficient thymus grafts after 8 weeks of transplantation. Each symbol in the graphs represents an individual mouse (n 4); small horizontal lines indicate the group mean (± s.d.). ns, not significant (P 0.05); *, P < 0.05.

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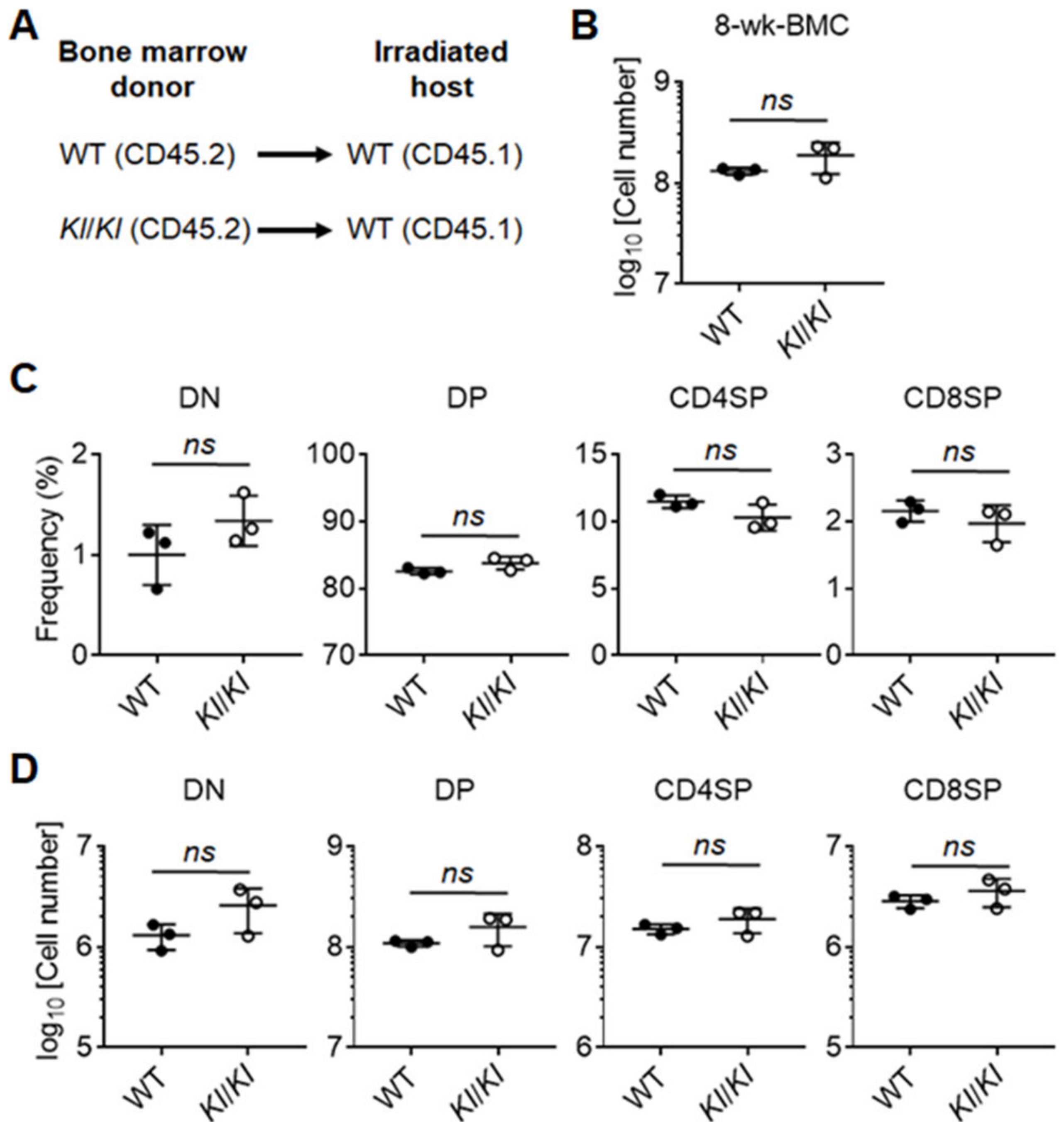

Figure 4.

Klotho-deficient and -sufficient bone marrow cells showed equivalent capability to generate T cells in the bone marrow chimeras.

(**A**) An experimental schema of making bone marrow chimeras. (**B**) Quantification of absolute cell numbers of thymocytes in recipients 8 weeks after bone marrow transplantation. (**C**) A summary of percentages of thymocyte subpopulations in the thymi from the hosts. (**D**) Quantification of absolute numbers of thymocyte subpopulations in the

hosts. Each symbol in the graphs represents an individual mouse (n = 3); small horizontal lines indicate the group mean (± s.d.). ns, not significant (P 0.05); *, P < 0.05.

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Figure 5.

Thymus involution in 8-wk-old *Klotho*-deficient mice was abrogated through feeding vitamin D-deprived diet.

(**A**) Cellularity of total thymocytes in the thymi from 8-wk-old *Kl*/*Kl* mice and littermate controls fed with vitamin D-deprived diet, and *Kl*/*Kl* mice fed with vitamin D-replete diet at. (**B**) Quantification of frequencies of thymocyte subpopulations in the indicated mice. Each symbol in the graphs represents an individual mouse (n 3); small horizontal lines indicate the group mean (± s.d.). ns, not significant (P 0.05); *P < 0.05. (**C**) Immunofluorescence

staining of thymus sections using anti-K5, anti-K8 antibodies and DAPI (4’,6-diamidino-2-phenylindole).

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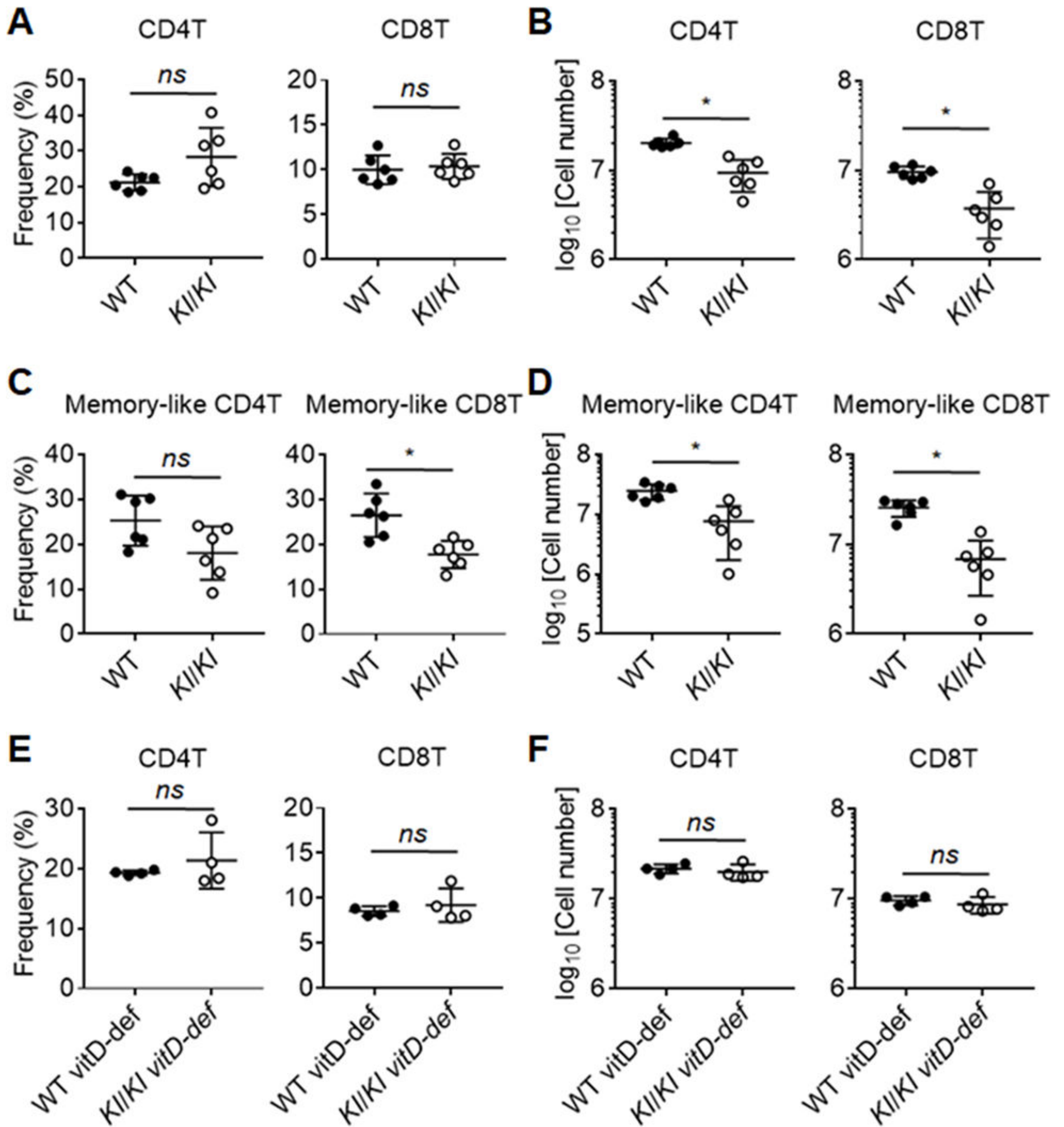

Figure 6.

Reduction of T cells was found in the periphery in 8-week-old *Klotho*-deficient mice that can be prevented by deprivation of vitamin D.

(**A**) Frequency and (**B**) cellularity of CD4T and CD8T cells in the spleen from *Kl*/*Kl* mice and littermate controls at 8 weeks of age. (**C**) Frequency and (**D**) cellularity of $CD44^{hi}$ memory-like T cells subpopulations in the spleen from *Kl*/*Kl* mice and littermate controls at 8 weeks of age. (**E**) Frequency and (**F**) cellularity of CD4T and CD8T cells in the spleen from 8-wk-old *Kl*/*Kl* mice and littermate controls fed with vitamin D-deprived diet. Each

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symbol in the graphs represents an individual mouse (n 4); small horizontal lines indicate the group mean (± s.d.). ns, not significant (P 0.05); *, P < 0.05.