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Supplemental information

Mice lacking DKK1 in T cells exhibit high bone mass and are protected from estrogen-deficiency-induced bone loss

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Transparent Methods

STAR★Methods

Lead Contact and Materials Availability

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Martina Rauner (martina.rauner@ukdd.de). This study did not generate new unique reagents.

Experimental Model and Subject Details

For global *Dkk1* deletion tamoxifen-inducible global *Dkk1* knock-out mice (*Dkk1*$^{fl/fl}$*;Rosa26-CreERT2*) were generated 28 (Colditz et al., 2018). At the age of seven weeks male and female *Dkk1*$^{fl/fl}$*;Rosa26-CreERT2*-positive and -negative control mice were injected with 100 µl tamoxifen (10 g/l, Sigma, Germany) for 5 consecutive days to induce global deletion of *Dkk1*. For cell-specific deletion of *Dkk1* in osteogenic cells, doxycycline-repressible *Dkk1*$^{fl/fl}$*;Osx-Cre* (osteoprogenitor-specific) and constitutive *Dkk1*$^{fl/fl}$*;Dmp1-Cre* (osteocyte-specific) transgenic mouse lines were generated (Colditz et al., 2018). *Dkk1*$^{fl/fl}$*;Osx-Cre* breeding pairs received doxycycline in their drinking water (10 mg/ml in a 3% sucrose solution) *ad libitum* to repress Cre activity during embryogenesis. *Dkk1*$^{fl/fl}$*;Osx-Cre* offspring received doxycycline-drinking water until the age of five weeks. *Dkk1*$^{fl/fl}$ mice (*Dkk1*$^{fl/fl}$) 27(Pietilä et al., 2011) in which exon 1 and 2 are flanked by *loxP* sites were crossed with *Lck-Cre* mice 86 (Baschant et al., 2011) to generate T cell-specific *Dkk1* knock-out mice (*Dkk1*$^{fl/fl}$*;Lck-Cre*). Respective Cre-negative littermates were used as controls. Ly5.1 mice (Jackson Laboratory, USA) were used for T cell transfer experiments. All mice were routinely genotyped and recombination of *Dkk1* deletion in different organs was investigated. Breeding of the mouse lines was approved by the Landesdirektion Sachsen.

Method Details

In vivo experiments

All animal procedures were approved by the institutional animal care committee of the TU Dresden and the Landesdirektion Sachsen. All mice were fed a standard diet with water *ad libitum* and were kept in groups of 4 animals per cage. Mice were exposed to a 12 h light/dark cycle and an air-conditioned room at 23°C (no specific pathogen-free room). Enrichment was provided in forms of cardboard houses and bedding material. Mice were randomly assigned to treatment groups and the subsequent analyses were performed in a blinded-fashion.

Bone phenotyping: Phenotype analysis of male and female *Dkk1$^{fl/fl}$;Lck-Cre* mice was performed at an age of 12 weeks . Each group consisted of 8-11 mice.

Ovariectomy: Female 10-week-old *Dkk1$^{fl/fl}$;Rosa26-CreERT2*, *Dkk1$^{fl/fl}$;Osx-Cre*, *Dkk1$^{fl/fl}$;Dmp1-Cre*, and *Dkk1$^{fl/fl}$;Lck-Cre* mice and their respective Cre- controls were bilaterally ovariectomized or sham operated. After four weeks, mice were sacrificed for further analyses. Each group consisted of 8-16 mice.

T cell transfer: T cells were isolated from the spleen of 12-week-old Ly5.1 mice using the Pan T Cell Isolation Kit II (Miltenyi Biotec, Germany). Five or ten million T cells were transplanted via retro-orbital injection into *Dkk1$^{fl/fl}$;Lck-Cre* and littermate control mice. After three weeks bone parameters were assessed.

Micro-CT

Bone microarchitecture was analyzed *ex vivo* using the vivaCT40 (Scanco Medical, Switzerland). The femur and the fourth lumbar vertebra were imaged at a resolution of 10.5 µm with a X-ray energy of 70 kVp, 114 mA, and an integration time of 200 ms. The trabecular bone in the femur was assessed in the metaphysis 20 slices below the growth plate using 100 slices. In the vertebral bone, 100 slices were measured between both growth plates. The

cortical bone was determined in the femoral midshaft (100 slices). Pre-defined scripts from Scanco were used for the evaluation.

Bone histomorphometry

Mice were injected with 20 mg/kg calcein (Sigma-Aldrich, Germany) five and two days before sacrifice. Dynamic bone histomorphometry was performed as described previously 87 (Rauner et al.). Briefly, the third lumbar vertebra and the tibia were fixed in 4% PBS-buffered paraformaldehyde and dehydrated in an ascending ethanol series. Subsequently, bones were embedded in methacrylate and cut into 7 µm sections to assess the fluorescent calcein labels. Unstained sections were analyzed using fluorescence microscopy to determine the mineralized surface/bone surface (MS/BS), the mineral apposition rate (MAR), and the bone formation rate/bone surface (BFR/BS).

For tartrate-resistant acid phosphatase (TRAP) staining the femur and fourth lumbar vertebra were decalcified for one week using Osteosoft (Merck, Germany), dehydrated, and embedded into paraffin. TRAP staining was performed on 4 µm sections to assess the number of osteoblasts per bone perimeter (N.Ob/B.Pm), the number of osteoclasts per bone perimeter (N.Oc/B.Pm), and the number of osteocytes per bone area (N.Ot/B.Ar). Bone sections were analyzed using the Osteomeasure software (Osteometrics, USA) following international standards 88 (Dempster et al., 2013).

Cell culture

T cells were isolated from the spleen of 12-week-old WT, *Dkk1$^{fl/fl}$;Rosa26-CreERT2* or control mice using the Pan T Cell Isolation Kit II (Miltenyi Biotec, Germany). At the age of seven weeks, Dkk1 deletion was induced in *Dkk1$^{fl/fl}$;Rosa26-CreERT2*-positive and -negative mice using tamoxifen. T cells were cultured at 1x10^6 cells/ml in 24-well plates coated with murine anti-CD3 (1 µg/ml, clone 145-2C11, 1XE, Invitrogen, Germany) for activation, or cultured on uncoated wells for 3 days (inactive T cells). To obtain conditioned medium (CM), cell suspensions were harvested, centrifuged, and the supernatant was collected for storage at -

80 °C until use. To assess *Dkk1* expression in inactive and active T cells, RNA was isolated after 3 days of culture.

Co-culture of osteoblasts and T cell-derived CM was started when osteoblasts reached 70% confluence. Primary osteoblasts were differentiated from the bone marrow of WT mice using standard osteogenic medium in DMEM with 10% FCS, 1% penicillin/streptomycin (Life Technologies, Germany), 100 µM ascorbate phosphate, and 5 mM β-glycerol phosphate. The T cell CM was added to the osteoblasts at different concentrations (12.5–75%) and fresh CM was added at each medium change (two independent experiments with 3 n each). In osteogenic differentiation medium, the concentration of osteogenic stimuli was corrected for the total volume of medium. After 7 days RNA from primary osteoblasts was isolated. For DKK1 neutralizing experiments, 10 µg/ml human/mouse DKK-1 neutralizing antibody and isotype control antibody (both R&D Systems, Germany) were added to the medium and cells were differentiated for 7 days.

To obtain osteoclasts, bone marrow cells from wild-type C57BL/6 mice were isolated. Briefly femora and tibiae of each mouse were flushed through a 100 µm Nylon cell strainer with approximately 8 ml Modified Minimum Essential Medium (Alpha Medium) supplemented with 10% FCS and 1% penicillin/streptomycin, and 1 mM L-alanyl-L-glutamine (basal medium) using a 26G syringe. Cells were centrifuged for 5 min at 500 g and cell pellet was resuspended in 1 ml medium. Afterwards cells were counted and $1x10^6$ cells per cm^2 were seeded on 6-well plates. For the first 48 h basal medium was supplemented with 25 ng M-CSF. Afterwards osteoclast differentiation was induced by simultaneously treating the cells with 25 ng M-CSF and 50 ng RANKL for 7 days. After 7 days of culture, RNA was isolated using the High Pure RNA isolation kit (Promega, Madison, WI, USA).

To obtain primary macrophages, femora and tibiae of wild-type C57BL/6 mice were flushed using Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% FCS, 2 mM L-alanyl-L-glutamine and 1% penicillin/streptomycin (lymphocyte medium) as described above. After centrifugation for 5 min at 500 g, cells were resuspended in 1 ml lymphocyte medium supplemented with 30% L929 conditioned media (macrophage medium). For cell

culture $1x10^6$ cells per cm^2 were seeded on 6-well plates. After 24 h 1 ml fresh macrophage medium was added. At day 4 of cell culture, cells were washed twice with PBS and fresh macrophage medium was added. This step was repeated on day 6. After 7 days of culture, RNA was isolated using the peqGOLD TriFast™ isolation kit.

B cells were isolated from the spleen of 12-week-old WT mice. After the cell number was determined, B cells were sorted using Dynabeads (Invitrogen, Germany). For that, cells were stained with anti-CD45R-biotin (1:100) for 20 min at 4°C in the dark. After incubation cells were washed and the suspension was transferred into a new 2 ml reaction tube. Then 50 µl of Anti-Biotin Dynabeads per 10^7 total cells were added to the cell suspension and incubated for 30 min at 4°C with gentle rotation. Tubes were placed in a magnet for 2 min and the supernatant was carefully aspirated. After removing the tubes from the magnet, cells were resuspended in 1 ml MACS buffer, and put back in the magnet for another 2 min. Once supernatant was removed, cell pellet was stored at -80°C until RNA isolation. After 7 days of culture, RNA was isolated using the peqGOLD TriFast™ isolation kit (Promega, Madison, WI, USA)).

Flow cytometry

The right femur and the spleen were dissected and cells were isolated and filtered through a 100 µm cell strainer. Total cell number was counted, cells were blocked with anti-rat IgG and CD16/CD32, and subsequently stained with anti-CD11b-PE (1:500), anti-CD45-PE-Cy7 (1:200), and anti-Gr1-A700 (1:100) for bone marrow cells or with anti-CD45R-FITC (1:500), anti-CD3-APC (1:200), anti-CD4-PE-Cy7 (1:200), anti-CD8-A700 (1:200) and anti-CD69-PECy5 (1:100) for splenic cells for 40 minutes at 4°C in the dark. For analysis of naïve T cells, splenic cells were stained with anti-CD44-PE (1:200), anti-CD62L-APC (1:200), anti-TCRβ-APC (1:200) and anti-CD4-eF450 (1:200). Regulatory T cells were analyzed by staining for the expression of surface markers with anti-CD4-FITC (1:100), anti-CD25-PE (1:300) and, after fixation and permeabilization with intra-nuclear staining for FoxP3 using anti-FoxP3-APC (1:400) and the FOXP3 Fix/Perm Buffer Set (eBiosciences, Germany).

Thereafter, cells were washed twice with PBS and analyzed with the BD LSR II flow cytometer (BD Biosciences, San Jose, CA, USA) and the FlowJo vX software (Tree Star Inc., Ashland, OR, USA). Thymic cells were analyzed by dissecting the thymus and isolating the cells through a 100 µM cell strainer. Subsequently, total cell number was counted, cells were blocked with anti CD16/CD32 and stained with anti-CD4-PerCPCy5.5 (1:500) and anti-CD8-APC (1:300) for 40 minutes at 4°C in the dark.

For intracellular staining of DKK1, the FOXP3 Fix/Perm Buffer Set (Biolegend, Germany) was used according to protocol. After cells were fixed and permeabilized, cells were stained with the primary DKK1 biotinylated antibody (BAM1765, R&D Systems, Germany; 1:100) for 40 min at 4°C. Cells were washed twice and then stained with the secondary antibody streptavidine-Fluor450 (1:200) for 20 min at 4°C. After washing the cells they were analyzed using the LSR II and BD Diva software (BD Bioscience).

Luciferase-based reporter assays

A TCF/LEF-reporter assay (Qiagen) was done using the murine myoblast C2C12 cell line, which is commonly used to study BMP and Wnt signaling. These cells were seeded at a concentration of 1.5×10^4 cells per well in 48-well plates and were cultured in DMEM medium with 10% fetal bovine serum 1% Penicillin/Streptomycin, and transfected with the Signal TCF/LEF Reporter (CCS-018L, Qiagen, Hilden, Germany) to assess the activation of the TCF/LCF Wnt promotor. Briefly, 123 ng/cm^2 of the promotor construct was transfected using the FuGENE HD Transfection Reagent (Promega, Madison, WI, USA) according to the manufacturer's protocols. After 24 h, C2C12 cells were treated with or without Wnt3a-containing L-cell medium and/or CM from inactive or active T cells. Luciferase activity was assayed 48 h post treatment using the Dual Luciferase Reporter Assay kit (Promega, Madison, WI, USA) as instructed by the manufacturer.

Serum analysis

Blood was taken via heart punctuation of anaesthetized mice and serum was collected after

10 minutes centrifugation at 400 g. DKK1, C-terminal telopeptide (CTX), tartrate-resistant acid phosphatase form 5b (TRAP5b) and type 1 procollagen amino-terminal-propeptide (P1NP) were measured using an immunoassay kit (DKK1: R&D Systems, USA; CTX, TRAP5b and P1NP: Immundiagnostik Systems, Germany) according to the manufacturer's protocols. Samples were diluted according to protocol.

RNA isolation, RT, and real-time PCR

RNA from cell cultures was isolated using the High Pure RNA Isolation Kit (Roche) and RNA from the bones of mice was isolated by crushing flushed bones (femur and tibia) in liquid nitrogen and collecting the bone powder in Trifast (Peqlab, Germany). Five-hundred ng RNA were reverse transcribed using Superscript II (Invitrogen, Germany) und subsequently used for SYBR green-based real-time PCR analysis using a standard protocol (Life Technologies). The primer sequences were: β-actin s: GATCTGGCACCACACCTTCT, β-actin as: GGGGTGTTGAAGGTCTCAAA; Alp s: CTACTTGTGTGGCGTGAAGG, Alp as: CTGGTGGCATCTCGTTATCC; Axin2 s: GCAGTGATGGAGGAAAATGC, Axin2 as: ATTCAAGGTGGGGAGGTAGC; Dkk1 s: GCCTCCGATCATCAGACGGT, Dkk1 as: GCAGGTGTGGAGCCTAGAAG; Lef1 s: CAAATAAAGTGCCCGTGGTG, Lef1 as: TCGTCGCTGTAGGTGATGAG; Ocn s: GCGCTCTGTCTCTCTGACCT, Ocn as: ACCTTATTGCCCTCCTGCTT; Opg s: CCTTGCCCTGACCACTCTTA, Opg as: ACACTGGGCTGCAATACACA; Osx s: CTTCCCAATCCTATTTGCCGTTT, Osx as: CGGCCAGGTTACTAACACCAATCT; Rankl s: CCGAGACTACGGCAAGTACC, Rankl as: GCGCTCGAAAGTACAGGAAC; Runx2 s: CCCAGCCACCTTTACCTACA, Runx2 as: TATGGAGTGCTGCTGGTCTG. The results were calculated using the ΔΔCT method and are presented in x-fold increase relative to ***β-actin*** mRNA levels.

<u>Quantification and Statistical Analysis</u>

Data are presented as mean ± standard deviation (SD) and P values of < 0.05 were considered statistically significant. Graphs and statistics were prepared using GraphPad

Prism 7.0 software. For examination of the effect of *Dkk1* deletion and estrogen deficiency and to discover two-way interactions, a two-way factorial Analysis of variance (ANOVA) was carried out. To investigate the dose-dependency in our T cell co-culture one-way ANOVA was performed. For examination of the effect of *Dkk1* deletion on osteoblast differentiation a two-way factorial ANOVA was carried out. To analyze the effect of T cell specific *Dkk1* deletion on bone a Student's t-test was performed. Furthermore, the *Kolmogorov-Smirnov normality test* was used to examine if variables are normally distributed.

Supplementary Figures

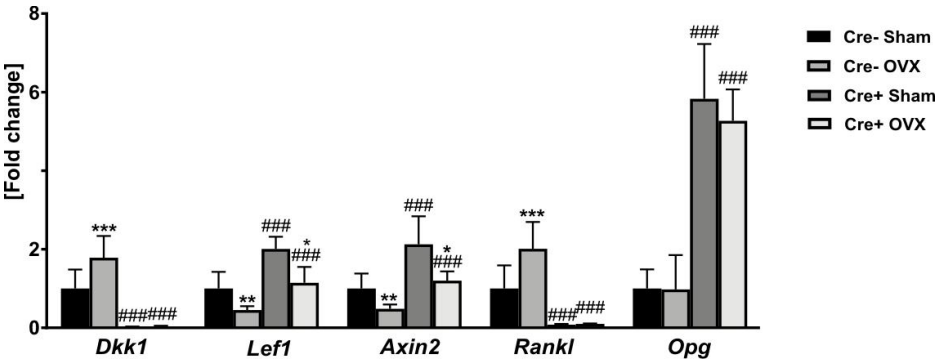

Figure S1. Global *Dkk1* cKO mice show altered Wnt signaling as well as *Rankl/Opg* ratio after estrogen withdrawal (related to main Figure 1). Gene expression analysis of dickkopf-1 (*Dkk1*), lymphoid enhancer-binding factor 1 (*Lef1*), axin-related protein 2 (*Axin2*), receptor Activator of NF-κB Ligand (*Rankl*), and osteoprotegerin (*Opg*) in femoral bone tissue of Sham vs. OVX operated 14-week-old *Dkk1$^{fl/fl}$;Rosa26-CreERT2* and control mice was performed using Real-time PCR. Cre- Sham, n = 12; Cre- OVX, n = 12; Cre+ Sham, n = 10; Cre+ OVX, n = 12. Gene expression levels were normalized to *β-Actin.* Data represent the mean ± SD, Statistical analysis was performed using Two-way-ANOVA. *p<0.05, **p<0.01, ***p<0.001 vs. Sham or #p<0.05, ##p<0.01, ###p<0.001 vs. respective Cre-negative control.

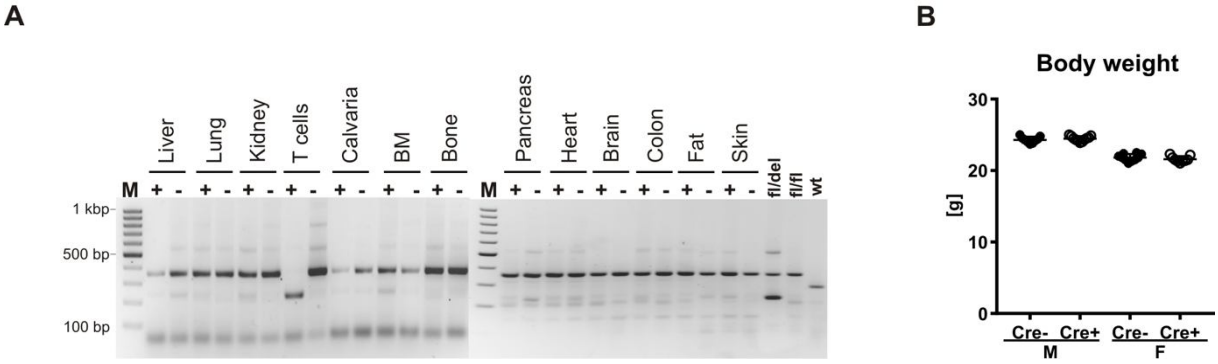

Figure S2. ***Dkk1$^{fl/fl}$;Lck-Cre*** **mice exhibit T cell-specific *Dkk1* deletion (related to main Figure 3).** (A) Recombinatory efficiency of Lck-Cre was analyzed. Representative examples of tissue-specific Cre recombination analyzed by PCR of *Dkk1$^{fl/fl}$;Lck-Cre* and control mice. Controls represent *Dkk1* deletion (fl/del: 228bp), floxed *Dkk*1 (fl/fl: 358bp), and wild-type (wt: 288 bp). (B) Body weight of *Dkk1$^{fl/fl}$;Lck-Cre* and control mice was determined. Statistical analysis was performed by the Student's t-test.

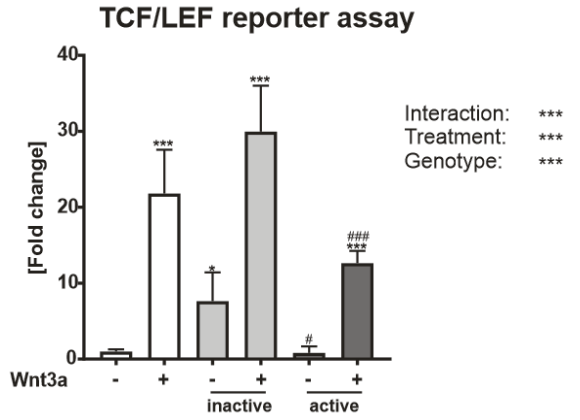

Figure S3. **CM from activated T cells suppressed Wnt3a-induced TCF/LEF Wnt promoter activity (related to main Figure 5).** TCF/LEF reporter assay of C2C12 cells treated with CM from activated or inactivated T cells for 48h. n = 6 biological replicates per group. Data represent the mean ± SD. Statistical analysis was performed by the Two-Way-ANOVA *p<0.05, **p<0.01, ***p<0.01 vs. untreated or #p<0.05, ##p<0.01, ###p<0.001 vs. respective inactive control.

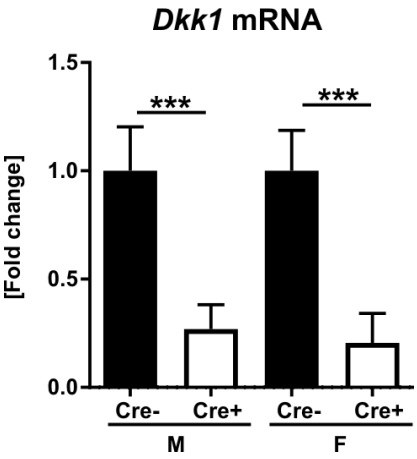

Figure S4. Global *Dkk1* deletion successfully reduced *Dkk1* expression in T cells (related to main Figure 5). *Dkk1* mRNA expression in T cells of 12-week old *Dkk1*$^{fl/fl}$*;Lck-Cre* and Cre-negative control mice was analyzed using Real-time PCR. n = 8 animals group. Gene expression levels were normalized to *β-Actin.* Data represent the mean ± SD. Statistical analysis was performed by the Student's *t*-test.*p<0.05, **p<0.01, ***p<0.001.

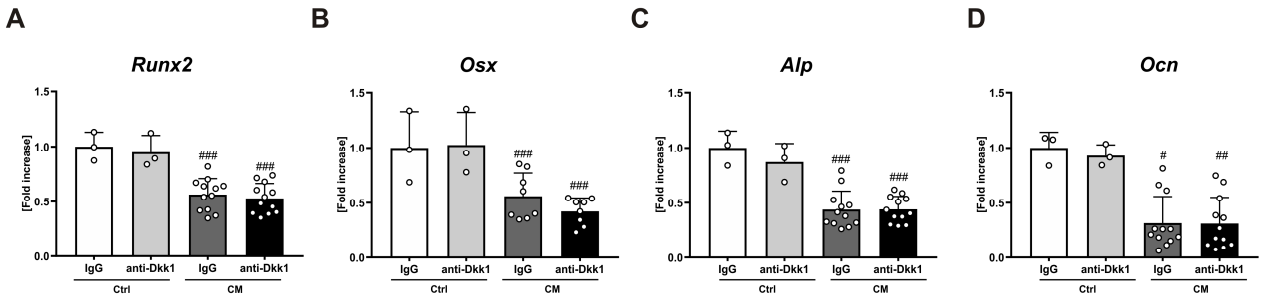

Figure S5. Neutralization of DKK1 does not restore the downregulation of osteoblastic marker genes (related to main Figure 5). Primary osteoblasts from 12-week-old wild-type mice were differentiated and treated with CM from activated WT T cells in the presence of a DKK1 antibody or an isotype control for 7 days. Gene expression analysis of the osteogenic genes (A) Runt-related transcription factor 2 (*Runx2*), (B) osterix (*Osx*), (C) alkaline phosphatase (*Alp*), and (D) and osteocalcin (*Ocn*) was performed using real-time PCR. Gene expression levels were normalized to *β-Actin.* Data represent the mean ± SD. n = 3 individual mice per group (plus combinations of T cell CM from up to 4 individual mice). Statistical analysis was performed using Two-way-ANOVA. *p<0.05, **p<0.01, ***p<0.01 vs. inactive or #p<0.05, ##p<0.01, ###p<0.001 vs. respective isotype control.

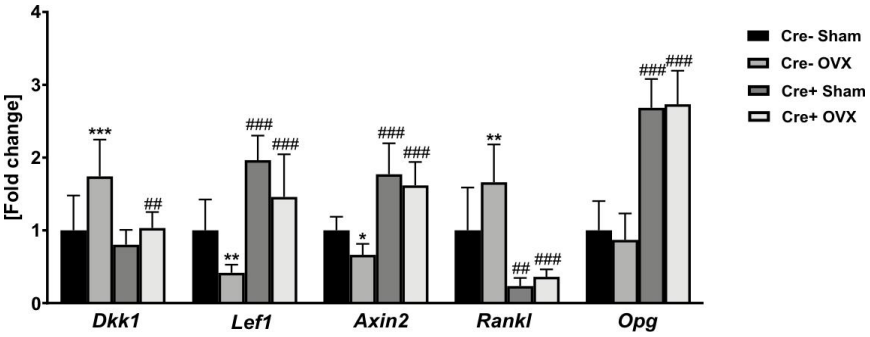

Figure S6. T cell-specific *Dkk1* cKO mice exhibit altered Wnt signaling as well as *Rankl/Opg* ratio after estrogen withdrawal (related to main Figure 6). Gene expression analysis of dickkopf-1 (*Dkk1*), lymphoid enhancer-binding factor 1 (*Lef1*), axin-related protein 2 (*Axin2*), receptor Activator of NF-κB Ligand (*Rankl*), and osteoprotegerin (*Opg*) in femoral bone tissue of Sham vs. OVX operated 14-week-old *Dkk1$^{fl/fl}$;Lck-Cre* and control mice was performed using Real-time PCR. Cre- Sham, n = 8; Cre- OVX, n = 11; Cre+ Sham, n = 9; Cre+ OVX, n = 10. Gene expression levels were normalized to *β-Actin.* Data represent the mean ± SD, Statistical analysis was performed using Two-way-ANOVA. *p<0.05, **p<0.01, ***p<0.001 vs. Sham or #p<0.05, ##p<0.01, ###p<0.001 vs. respective Cre-negative control.

Supplementary Table 1 (related to main Figure 2). Total number (*10^6) of splenic T cell populations and bone marrow-derived myeloid suppressor cells and spleen-derived B cells in sham vs. ovariectomized (OVX) *Dkk1$^{fl/fl}$;Rosa26-CreERT2*, *Dkk1$^{fl/fl}$;Osx-Cre* and *Dkk1$^{fl/fl}$;Dmp1-Cre* mice and their respective controls.

		Cre-			Cre+	
Cell populations	**Sham**	**OVX**	**P value and % change**	**Sham**	**OVX**	**P value and % change**
Dkk1$^{fl/fl}$;Rosa26-CreERT2	**n=13**	**n=15**		**n=13**	**n=10**	
$CD3^+$	17.2 ± 1.32	21.9 ± 3.42	<0.05 +27%	19.7 ± 3.85	23.4 ± 2.18	<0.05 +19%
$CD11b^+GR1^+$	1.75 ± 0.41	1.65 ± 0.25	>0.999 -6%	1.78 ± 0.39	1.79 ± 0.43	>0.999 +1%
$CD45R^+$	36.0 ± 5.43	38.2 ± 4.55	0.954 +6%	33.7 ± 6.75	36.4 ± 6.02	0.898 +8%
Dkk1$^{fl/fl}$;Osx-Cre	**n=13**	**n=15**		**n=13**	**n=10**	
$CD3^+$	11.7 ± 2.54	15.3 ± 2.56	<0.05 +31%	11.6 ± 2.34	14.7 ± 1.69	<0.05 +27%
$CD3^+CD4^+$	4.24 ± 0.68	5.80 ± 1.00	<0.05 +37%	4.64 ± 1.08	5.95 ± 0.85	<0.05 +28%
$CD3^+CD4^+CD69^+$	0.38 ± 0.07	0.52 ± 0.08	<0.001 +37%	0.45 ± 0.05	0.55 ± 0.05	<0.01 +22%
$CD3^+CD8^+$	4.40 ± 0.68	5.57 ± 0.89	<0.05 +27%	4.58 ± 0.69	5.75 ± 0.98	<0.05 +26%
$CD3^+CD8^+CD69^+$	0.13 ± 0.03	0.18 ± 0.04	<0.05 +38%	0.13 ± 0.02	0.18 ± 0.03	<0.05 +38%
$CD11b^+GR1^+$	1.69 ± 0.24	1.73 ± 0.25	>0.999 +2%	1.77 ± 0.18	1.80 ± 0.20	>0.999 +2%
$CD45R^+$	30.0 ± 6.84	30.9 ± 4.98	>0.999 +3%	30.7 ± 6.40	31.3 ± 4.36	>0.999 +2%
Dkk1$^{fl/fl}$;Dmp1-Cre	**n=13**	**n=15**		**n=13**	**n=10**	
$CD3^+$	12. ± 2.39	15.4 ± 1.31	<0.05 +19%	13.0 ± 2.18	15.7 ± 1.95	<0.05 +21%
$CD3^+CD4^+$	4.11 ± 0.80	5.12 ± 0.76	<0.05 +25%	3.95 ± 0.65	5.01 ± 0.99	<0.05 +27%
$CD3^+CD4^+CD69^+$	0.52 ± 0.09	0.73 ± 0.18	<0.01 +40%	0.52 ± 0.06	0.72 ± 0.15	<0.01 +38%
$CD3^+CD8^+$	3.96 ± 0.65	4.76 ± 0.58	<0.05 +20%	3.66 ± 0.72	4.53 ± 0.62	<0.05 +24%
$CD3^+CD8^+CD69^+$	0.12 ± 0.02	0.15 ± 0.02	<0.05 +25%	0.13 ± 0.02	0.16 ± 0.02	<0.05 +23%
$CD11b^+GR1^+$	1.38 ± 0.39	1.42 ± 0.18	>0.999 +3%	1.45 ± 0.36	1.51 ± 0.30	0.999 +4%
$CD45R^+$	31.2 ± 4.99	31.6 ± 3.25	>0.999 +1%	31.8 ± 3.17	31.9 ± 3.76	>0.999 +0%

Cell types were distinguished by flow cytometry according to the indicated surface markers. $CD3^+$ = T cells, $CD3^+CD4^+$ = T helper cells, $CD3^+CD4^+CD69^+$ = activated T helper cells, $CD3^+CD8^+$ = cytotoxic T cells, $CD3^+CD8^+CD69^+$ = activated cytotoxic T cells, $CD11b^+GR1^+$ = myeloid-derived suppressor cells, $CD45R^+$ = B cells. Data represent the mean ± SD. Statistical analysis was performed by the Two-Way-ANOVA. #p<0.05, ##p<0.01, ###p<0.001 vs respective Cre negative control.

Supplementary Table 2 (related to main Figure 3). Bone microstructure and histological parameters of the femora and tibiae of 12-week-old *Dkk1$^{fl/fl}$;Lck-Cre* mice.

	Male			Female		
	Cre- (n=9)	**Cre+ (n=11)**	***P* value and % change**	**Cre- (n=9)**	**Cre+ (n=8)**	***P* value and % change**
Dkk1$^{fl/fl}$;Lck-Cre	**n=9**	**n=11**		**n=9**	**n=8**	
*µCT**						
BV/TV [%]	7.76 ± 0.51	10.5 ± 0.43	<0.001 +35%	1.17 ± 0.80	2.02 ± 0.83	<0.05 71%
Tb.N [1/mm]	4.21 ± 0.33	4.75 ± 0.53	<0.05 +13%	2.31 ± 0.45	2.83 ± 0.17	<0.01 23%
Tb.Th [µm]	41. 1 ± 5.16	43.2 ± 3.64	0.306 +5%	33.4 ± 2.07	36.6 ± 3.86	0.051 10%
Tb.Sp [mm]	0.24 ± 0.02	0.22 ± 0.03	<0.05 -8%	0.40 ± 0.06	0.35 ± 0.03	<0.05 -13%
Histomorphometry#						
MS/BS [%]	21.0 ± 2.45	22.9 ± 2.51	0.125 +9%	26.0 ± 3.40	28.4 ± 2.06	0.095 +9%
MAR [µm/d]	1.72 ± 0.35	2.27 ± 0.56	<0.05 +32%	1.77 ± 0.58	2.51 ± 0.79	<0.05 42%
BFR/BS [$\mu m^3/\mu m^2/d$]	0.33 ± 0.16	0.52 ± 0.13	<0.01 +58%	0.48 ± 0.22	0.73 ± 0.16	<0.05 52%
N.Ob/B.Pm [#/mm]	26.5 ± 5.66	29.1 ± 6.27	0.352 +10%	18.9 ± 3.83	19.6 ± 3.09	0.697 +4%
N.Oc/B.Pm [#/mm]	4.27 ± 1.41	2.85 ± 1.07	<0.05 -33%	6.28 ± 1.24	4.15 ± 1.12	<0.01 -34%

BV/TV = bone volume/total volume, Tb.N = trabecular number, Tb.Th = trabecular thickness, Tb.Sp = trabecular separation, MS/BS = mineralizing surface/bone surface, MAR = mineral apposition rate, BFR/BS = bone formation rate/bone surface, N.Ob/B.Pm = number of osteoblasts, N.Oc/B.Pm = number of osteoclasts/bone perimeter. Data represent the mean ± SD. Statistical analysis was performed by the Student's t-test.*p<0.05, **p<0.01, ***p<0.001 vs. Cre-negative control. *µCT was performed at the femur. #undecalficied histology for bone formation parameters was determined at the tibia. Number of osteoblasts and osteoclasts was determined at the femur.

Supplementary Table 3 (related to main Figure 1 and 3). Total number of splenic and thymic T cell populations in *Dkk1$^{fl/fl}$;Rosa26-CreERT2 and Dkk1$^{fl/fl}$;Lck-Cre* mice and their respective controls.

Cell populations	Cre-	Cre+	P value
Dkk1$^{fl/fl}$;Rosa26-CreERT2 Spleen	**n=3**	**n=3**	
Total counts (*10^6)	19.4 ± 2.90	23.1 ± 7.32	0.463 +19%
$CD3^+CD4^+$	2.57 ± 0 .43	3.49 ± 1.40	0.338 +26%
$CD3^+CD4^+CD69^+$	0.30 ± 0.07	0.36 ± 0.09	0.478 +20%
$CD3^+CD8^+$	2.33 ± 0.41	3.02 ± 0.87	0.107 +30%
$CD3^+CD8^+CD69^+$	0.04 ± 0.01	0.05 ± 0.02	0.469 +25%
$CD4^+CD25^+FoxP_3^+$	0.26 ± 0.03	0.33 ± 0.14	0.441 27%
$CD4^+TCR\beta^+CD44^{low}CD62L^{high}$	0.23 ± 0.17	0.19 ± 0.13	0.651 -17%
Dkk1$^{fl/fl}$;Rosa26-CreERT2 Thymus	**n=3**	**n=3**	
Total counts (*10^6)	65.3 ± 13.4	44.1 ± 15.3	0.145 -32%
$CD4^+CD8^+$	53.8 ± 11.5	36.9 ± 12.8	0.164 -31%
$CD4^+$	3.42 ± 0.51	2.07 ± 0.73	0.058 -39%
$CD8^+$	1.15 ± 0.46	0.47 ± 0.09	0.064 -59%
$CD4^-CD8^-$	2.60 ± 0.46	1.22 ± 0.39	$0.017^{\#}$ -53%
***Dkk1$^{fl/fl}$;Lck-Cre* Spleen**	**n=5**	**n=3**	
Total counts (*10^6)	22.0 ± 11.2	24.3 ± 6.35	0.756 +10%
$CD3^+CD4^+$	2.67 ± 1.53	2.96 ± 0.80	0.755 +11%
$CD3^+CD4^+CD69^+$	0.17 ± 0.09	0.19 ± 0.08	0.727 +12%
$CD3^+CD8^+$	2.12 ± 1.02	2.39 ± 0.58	0.696 +13%
$CD3^+CD8^+CD69^+$	0.06 ± 0.03	0.07 ± 0.03	0.766 +17%
$CD4^+CD25^+FoxP_3^+$	0.28 ± 0.16	0.17 ± 0.03	0.301 -39%
$CD4^+TCR\beta^+CD44^{low}CD62L^{high}$	0.32 ± 0.16	0.29 ± 0.08	0.755 -9%
***Dkk1$^{fl/fl}$;Lck-Cre* Thymus**	**n=5**	**n=3**	
Total counts (*10^6)	65.3 ± 27.3	100 ± 57.2	0.278 +53%
$CD4^+CD8^+$	43.9 ± 18.1	66.5 ± 37.2	0.281 +51%
$CD4^+$	2.44 ± 1.43	5.22 ± 3.06	0.123 +114%
$CD8^+$	0.98 ± 0.79	1.77 ± 1.06	0.275 +81%
$CD4^-CD8^-$	1.10 ± 0.53	2.11 ± 1.18	0.135 +92%

Cell types were distinguished by flow cytometry according to the surface markers. Data represent the mean ± SD. Statistical analysis was performed by student`s *t*-test. #p<0.05.

Supplementary Table 4 (related to main Figure 6). Bone phenotype of third and fourth vertebral body from 14-week-old female sham vs. ovariectomized (OVX) *Dkk1$^{fl/fl}$;Lck-Cre* mice.

		Cre-			*Cre+*	
Spine	**Sham**	**OVX**	**P value and % change**	**Sham**	**OVX**	**P value and % change**
Dkk1$^{fl/fl}$;Lck-Cre	**n=9**	**n=12**		**n=9**	**n=11**	
µCT						
Tb.N [1/mm]	3.53 ± 0.25	3.14 ± 0.32	<0.05 -12%	3.96 ± 0.26$^{\#}$	3.62 ± 0.44$^{\#\#}$	0.095 -9%
Tb.Sp [mm]	0.30 ± 0.03	0.34 ± 0.03	<0.05 +8%	0.26 ± 0.02$^{\#}$	0.26 ± 0.03$^{\#\#\#}$	>0.999 +0%
Histomorphometry						
MS/BS [%]	23.7 ± 3.07	25.5 ± 3.07	>0.999 +8%	25.4 ± 2.10	26.2 ± 3.57	>0.999 +3%
MAR [µm/d]	1.63 ± 0.29	2.56 ± 0.69	<0.01 +57%	2.50 ± 0.34$^{\#}$	2.58 ± 0.57	0.406 +3%
N.Ob/B.Pm [#/mm]	18.4 ± 3.47	19.0 ± 3.97	>0.999 +3%	19.2 ± 2.87	21.2 ± 2.61	>0.999 +10%

MAR = mineral apposition rate, BFR/BS = bone formation rate/bone surface, N.Ob/B.Pm = number of osteoblasts, N.Oc/B.Pm = number of osteoclasts/bone perimeter. Data represent the mean ± SD. Statistical analysis was performed by the Two-Way-ANOVA. #p<0.05, ##p<0.01, ###p<0.001 vs respective Cre negative control.

Supplementary Table 5 (related to main Figure 6). Total number [*10^6] of splenic T cells populations and bone marrow-derived myeloid suppressor cells and spleen-derived B cells in sham operated vs. ovariectomized (OVX) *Dkk1$^{fl/fl}$;Lck-Cre* mice.

		Cre-			**Cre+**	
Cell populations	**Sham (n=6)**	**OVX (n=6)**	***P* value and % change**	**Sham (n=6)**	**OVX (n=6)**	***P* value and % change**
Dkk1$^{fl/fl}$;Lck-Cre	**n=6**	**n=6**		**n=6**	**n=6**	
$CD3^+$	21.0 ± 2.79	26.0 ± 2.14	<0.001 +24%	20.0 ± 2.71	24.3 ± 1.22	<0.05 +21%
$CD3^+CD4^+$	5.51 ± 0.73	6.99 ± 0.77	<0.05 +27%	5.65 ± 0.74	6.63 ± 0.67	0.177 +17%
$CD3^+CD4^+CD69^+$	0.72 ± 0.10	1.02 ± 0.12	<0.001 +42%	0.71 ± 0.06	0.93 ± 0.15	<0.05 +31%
$CD3^+CD8^+$	5.13 ± 0.79	6.23 ± 0.52	<0.05 +18%	5.13 ± 0.57	6.11 ± 0.53	0.071 +19%
$CD3^+CD8^+CD69^+$	0.16 ± 0.03	0.20 ± 0.03	<0.05 +22%	0.16 ± 0.02	0.18 ± 0.03	>0.999 +13%
$CD11b^+GR1^+$	1.93 ± 0.51	1.95 ± 0.15	>0.999 +1%	2.03 ± 0.28	1.95 ± 0.30	>0.999 -4%
$CD45R^+$	37.1 ± 7.05	40.0 ± 3.75	>0.999 +8%	36.8 ± 6.77	40.8 ± 5.75	>0.999 +11%

Cell types were distinguished by flow cytometry according to the indicated surface markers. $CD3^+$ = T cells, $CD3^+CD4^+$ = T helper cells, $CD3^+CD4^+CD69^+$ = activated T helper cells, $CD3^+CD8^+$ = cytotoxic T cells, $CD3^+CD8^+CD69^+$ = activated cytotoxic T cells, $CD11b^+GR1^+$ = myeloid-derived suppressor cells, $CD45R^+$ = B cells. Data represent the mean ± SD. Statistical analysis was performed by the Two-Way-ANOVA. #p<0.05, ##p<0.01, ###p<0.001 vs respective Cre negative control.

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