Supporting Information

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SI Materials and Methods

Animals. Female C57BL6/J WT and CD40L$^{-/-}$ mice were purchased from The Jackson Laboratory. They underwent either sham surgery or ovx at 12 wk of age and were killed at 14 wk of age. This age was selected because 12-wk-old C57BL6 mice are sexually mature and their BV/TV is close to peak. At sacrifice, one femur was excised for histomorphometric analysis and one was excised for μCT analysis. BM was harvested from the remaining bones. Uterine weight was determined at death to verify successful ovx. All mice were maintained under specific pathogen-free conditions. Animals were fed sterilized food and autoclaved water.

WT mice were depleted of T cells via i.p. injection of bioreactor-generated mAbs GK1.5 and YTS169.4 (American Type Culture Collection), which are directed against CD4$^+$ and CD8$^+$ cells, respectively, or irrelevant isotype-matched control mAbs. Each Ab was injected at a dose of 500 μg twice a week for 4 wk starting 2 wk before surgery.

Bone Histology and Quantitative Bone Histomorphometry. Longitudinal sections (5 μm thick) were cut from methyl methacrylate plastic-embedded blocks along the frontal plane using a Leica 2265 microtome and were stained with Goldner's trichrome stain for the static measurements. Histomorphometry was done using the Bioquant Image Analysis System (R&M Biometrics). The measurements, terminology, and units used for histomorphometric analysis were those recommended by the Nomenclature Committee of the American Society of Bone and Mineral Research (1). Measurements were obtained in an area of cancellous bone that measured ≈2.5 mm^2 and contained only secondary spongiosa, which was located 0.5–2.5 mm proximal to the epiphyseal growth cartilage of the femurs.

In Vitro OC Formation. Whole BM or a mixture of BMMs, SCs, and T cells was cultured for 7 d in the presence of either 15 ng/mL RANKL (kindly provided by X. Feng, University of Alabama at Birmingham) and 10 ng/mL M-CSF or human PTH 1–34 (1 nM) to induce OC formation as described (2, 3). The T cells and BMMs used for these assays were purified from the spleen by positive immunomagnetic selection using mouse MACS Microbeads (Miltenyi Biotec) coupled to anti-CD90 for T cells and anti-CD11b for BMMs as described (46). The cultures were then fixed and stained for tartrate-resistant acid phosphatase (TRAP). TRAP-positive cells with three or more nuclei were scored as OCs.

SC Purification and SC and T-Cell Coculture. BM was collected from long bones by centrifugation and cultured for 7 d to allow the proliferation of SCs and their differentiation into cells of the osteoblastic lineage. After discarding nonadherent cells, adherent macrophages were eliminated by positive immunoselection using MACS Microbeads coupled to anti-CD11c Ab. This marker is expressed on nonadherent dendritic cells and adherent monocytes and macrophages. The remaining adherent cells were defined as SCs because they express ALP and are nonspecific esterase-negative. For some experiments, SCs and an equal number of T cells were cocultured for 7 d in the presence of irrelevant Ab or the CD40L Ab MR-1. The T cells were then removed by washing. The SCs were collected after a further 48-h incubation, and the mRNA levels of RANKL, OPG, and M-CSF were measured using real-time PCR.

CFU-ALP Assay. Colony-forming assays were carried out to determine the number of BM SCs with osteogenic potential, as previously described (70). Briefly, BM was cultured in α-MEM medium containing 10 mM β-glycerophosphate and 50 μg/mL ascorbic acid at a density of 2×10^6 cells/cm^2. After 7 d, the cells were fixed and stained for ALP and the number of colonies positive for ALP was counted.

Thymidine Incorporation Assay. The rate of proliferation of SCs was measured by [^{3}H]thymidine incorporation assay. SCs were purified and pulsed with [^{3}H]thymidine (0.5 μCi per 20,000 cells) for 18 h and were harvested using a Cell Harvestor (Skatron, Inc.). Incorporation of [^{3}H]thymidine was determined by an LS 6000 IC Liquid Scintillation Counter (Beckman Coulter, Inc.).

Flow Cytometry. BM cells were labeled with antigen-presenting cell (APC) anti-mouse CD4, APC anti-mouse CD8, FITC anti-mouse CD69, phycoerythrin (PE) anti-mouse TNF, or PE anti-mouse CD40L (BioLegend). Cells were analyzed by flow cytometry on a C6 flow cytometer (Accuri Cytometers, Inc.).

Real-Time RT-PCR and Primers. The expression levels of mRNA of bone sialoprotein, type I collagen (Col1), osteocalcin (Ocn), osterix (Osx), and runx2 (Runx2) in SCs were quantified by real-time RT-PCR as previously described (70). All the primers used were designed by Primer Express Express Software v2.0 (PE Biosystems). Changes in relative gene expression between sham-operated and ovx groups were calculated using the $2^{-\Delta\Delta CT}$ method with normalization to 18S rRNA. The primers used were as follows: for 18S rRNA, 5′-ATTCGAACGTCTGCCCTATCA-3′ (forward) and 5′-GTCACCCGTGGTCACCATG-3′; for Ocn, 5′-GCCTTCATGTCCAAGCAGGA-3′ (forward) and 5′-GCGCCGGAGTCTGTTCACTA-3′ (reverse); for Osx, 5′-GTGTTAGTAACCTGGCCGGG-3′ (forward) and 5′- CATTGGACTTCCCCCTTCTTG -3′ (reverse); for Runx2, 5′-CTGTGGTTACCGTCATGGCC-3′ (forward) and 5′-GGAGCTCGGCGGAGTAGTTC-3′ (reverse); and for Col1, 5′-CCCTACTCAGCCGTCTGTGC-3′ (forward) and 5′-GGGTTCGGGCTGATGTACC-3′ (reverse).

Statistical Analysis. Two-way ANOVA was applied that included the main effects for animal strain (WT vs. CD40L$^{-/-}$ mice) or T-cell status (T cell-replete vs. T cell-depleted mice) and type of surgery plus the statistical interaction between animal type and surgical procedure. When the statistical interaction between animal strain and surgery group was not significant ($P > 0.05$) or suggestive of an important interaction ($P > 0.10$), P values for the main effects tests were reported. When the statistical interaction was significant or suggestive of an important interaction, t tests were used to compare the differences between the surgical treatment means for each animal type and those between animal type for each surgical treatment, applying the Bonferroni correction for multiple comparisons. Data from the MR-1 experiments were analyzed by one-way ANOVA and Fisher protected least significant difference tests.

1. Parfitt AM, et al. (1987) Bone histomorphometry: standardization of nomenclature, symbols, and units. Report of the ASBMR Histomorphometry Nomenclature Committee. *J Bone Miner Res* 2:595–610.
2. Gao Y, et al. (2008) T cells potentiate PTH-induced cortical bone loss through CD40L signaling. *Cell metabolism* 8:132–145.
3. Terauchi M, et al. (2009) T lymphocytes amplify the anabolic activity of parathyroid hormone through Wnt10b signaling. *Cell metabolism* 10:229–240.

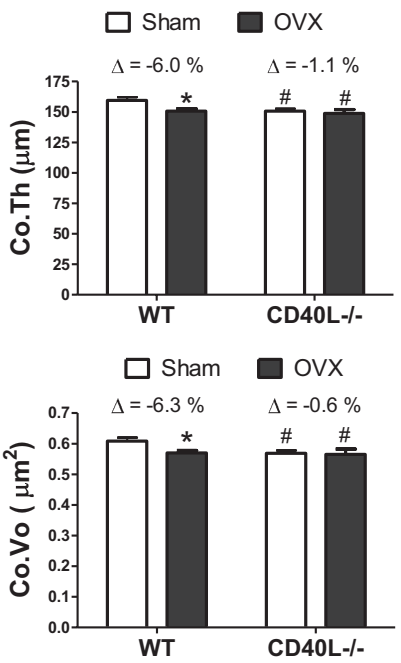

Fig. S1. Effects (mean ± SEM) of ovx on cortical thickness (Co.Th) and cortical volume (Co.Vo) in WT and CD40L$^{-/-}$ mice (n = 10 mice per group). *$P < 0.05$ compared with the corresponding sham-operated group; $^{\#}P < 0.05$ compared with WT sham-operated mice.

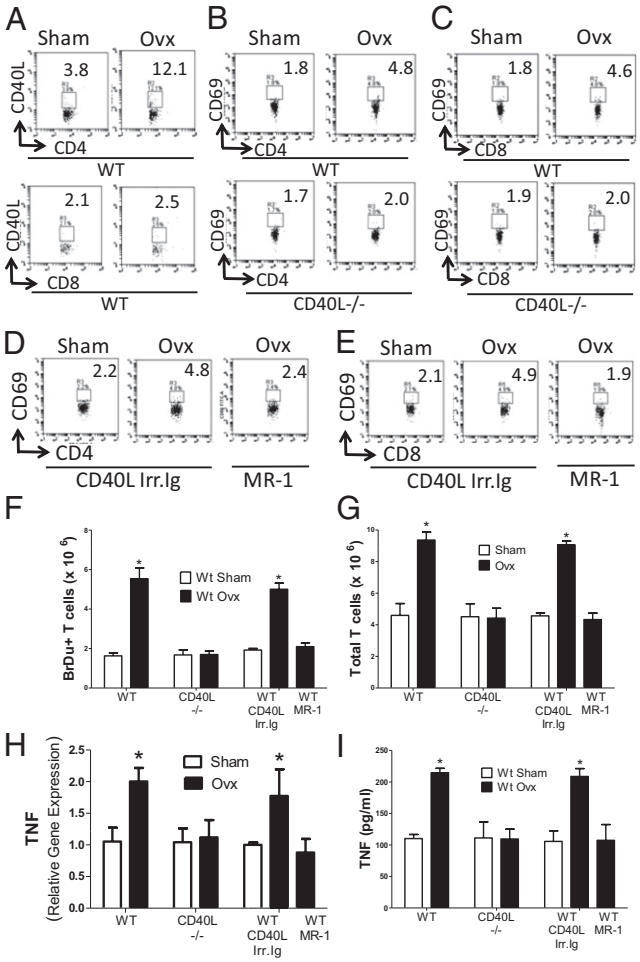

Fig. S2. Effects of ovx on T-cell number and proliferation and TNF production in WT, CD40L$^{-/-}$ mice, and MR-1–treated mice. (*A*) Expression of CD40L in WT BM CD4^{+} and CD8^{+} cells. (*B* and C) Expression of the activation marker CD69 in BM CD4^{+} and CD8^{+} cells from WT and CD40L$^{-/-}$ mice. (*D* and E) Expression of CD69 in BM CD4^{+} and CD8^{+} cells from WT mice treated with MR-1 or irrelevant isotype-matched Ab. (*F*) Proliferation of T cells in WT, CD40L$^{-/-}$, and MR-1–treated mice as assessed by in vivo BrdU labeling. (*G*) Number of total BM T cells in WT and CD40L$^{-/-}$ mice. (*H* and *I*) Secretion of TNF and TNF mRNA expression by T cells from WT, CD40L$^{-/-}$, and MR-1–treated mice. Data shown in *A*–*E* are from one of three experiments. Data shown in *F*–*I* are the mean ± SEM of three experiments. *$P < 0.001$ compared with the corresponding sham-operated group.

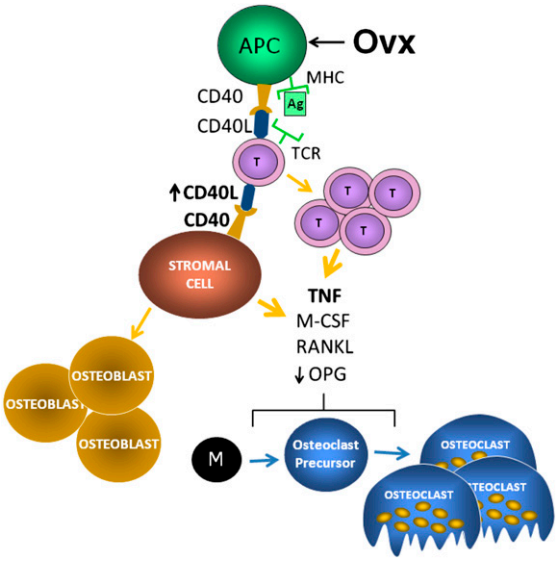

Fig. S3. Schematic representation of the role of T cells and CD40L in the mechanism by which ovx promotes osteoclastogenesis and osteoblastogenesis. Previous reports have shown that E deficiency promotes T-cell activation by increasing antigen (Ag) presentation. This process requires the interaction of MHC molecules on APCs with the T-cell receptor (TCR). The current study shows that T-cell activation also requires a costimulatory signal provided by T cell-expressed CD40L and presumably APC-expressed CD40. Activated T cells secrete TNF, which stimulates OC formation primarily by potentiating the response to RANKL. In addition, T cell-expressed CD40L increases the osteoclastogenic activity of SCs by augmenting their production of RANKL and M-CSF and blunting their secretion of OPG. An additional effect of ovx mediated by CD40L is increased SC differentiation into OBs. Therefore, in the absence of T cells and/or CD40L, ovx fails to stimulate osteoclastogenesis and osteoblastogenesis.

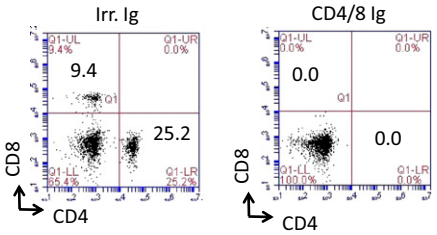

Fig. S4. Percentage of spleen $CD4^+$ and $CD8^+$ T cells in WT mice treated with irrelevant Ig (Irr. Ig) and anti-CD4/8 Ig for 4 wk. The figure shows $CD4^+$ and $CD8^+$ cells expressed as the percentage of total nucleated cells. Spleen cells were labeled with APC anti-mouse CD4 and FITC anti-mouse CD8.