OX40L blockade is therapeutic in arthritis, despite promoting osteoclastogenesis

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An immune response is essential for protection against infection, but, in many individuals, aberrant responses against self tissues cause autoimmune diseases such as rheumatoid arthritis (RA). How to diminish the autoimmune response while not augmenting infectious risk is a challenge. Modern targeted therapies such as anti-TNF or anti-CD20 antibodies ameliorate disease, but at the cost of some increase in infectious risk. Approaches that might specifically reduce autoimmunity and tissue damage without infectious risk would be important. Here we describe that TNF superfamily member OX40 ligand (OX40L; CD252), which is expressed predominantly on antigen-presenting cells, and its receptor OX40 (on activated T cells), are restricted to the inflamed joint in arthritis in mice with collagen-induced arthritis and humans with RA. Blockade of this pathway in arthritic mice reduced inflammation and restored tissue integrity predominantly by inhibiting inflammatory cytokine production by OX40L-expressing macrophages. Furthermore, we identify a previously unknown role for OX40L in steady-state bone homeostasis. This work shows that more targeted approaches may augment the “therapeutic window” and increase the benefit/risk in RA, and possibly other autoimmune diseases, and are thus worth testing in humans.

The cytokine-dependent pathophysiology of the destructive process in rheumatoid arthritis (RA) implied that manipulation of the immune system may help to restore bone physiology and prevent bone damage. This was exemplified by the success of concomitant biologic TNF inhibition and methotrexate treatment and the inhibiting of joint damage in RA (1). However, a minority of patients do not show a response to such treatment, and the beneficial effects reduce with time. For such patients, alternative strategies are effective in some, and include rituximab (that targets CD20 and lyses B cells), abatacept (a CTLA-4 Ig fusion protein that competes with T-cell–expressed CD28), and tocilizumab (an IL-6 receptor inhibitor). All these strategies modify large components of immunity that may leave the patient more susceptible to infection (reviewed in ref. 2). Thus, safer immune modulators with more selective mechanisms of action, such as those only targeting a subpopulation of a particular immune cell type or their function, are required. However, their presence in human disease, the efficacy of their manipulation in animal models of arthritis and any function in the noninflamed joint or other tissues need to be investigated.

Targeting autoreactive T cells is an attractive possibility, but only if they can be distinguished from those not involved in the disease process. The TNF family member CD40L (CD154) has received much attention as it is selectively expressed on activated T cells, its overexpression correlates with higher disease activity (3), and agonistic anti-CD40 Abs exacerbate disease in mice (4). Conversely, administration of blocking anti-CD40L antibodies ameliorates the disease (5). Clinical trials of anti-CD40L in systemic lupus erythematosus unfortunately revealed significant toxicity (6). However, there are alternatives that have yet to be tested.

The TNF superfamily member OX40 (CD134) is induced 24 to 48 h after T-cell activation and binds the equally inducible OX40 ligand (OX40L) on antigen-presenting cells (APCs), causing a bi-directional activating signal to both cells. OX40 signaling promotes T-cell survival and their division and cytokine production, and, in APCs, OX40L signaling causes maturation and the release of inflammatory mediators (7), or, in the case of B cells, increased IgG production (8). T cells expressing OX40 accumulate in the synovial fluid of patients with RA (9), suggesting that they may be involved in disease pathogenesis, although a direct contribution has not previously been demonstrated. The narrow window of expression of OX40 on T cells and OX40L on APCs makes them ideal for therapeutic intervention because they would influence only those cells directly involved in the ongoing inflammatory process. However, the mechanism of therapeutic manipulation of such paired activating receptors must be interpreted with care because any manipulation may affect T-cell and/or APC function.

We identify roles for OX40/OX40L in the joint in the steady state and during inflammation. In the steady state, members of the TNF/TNF receptor superfamily play pivotal roles in multiple processes from lymphoid architecture to T-cell survival and bone homeostasis. RANK-L is a TNF family member produced by activated T cells (10, 11), osteoblasts (12, 13), keratinocytes (14), and B lymphocytes (15, 16), among other cell types (reviewed in ref. 17), and, with macrophage colony-stimulating factor (M-CSF), drives osteoclast commitment from bone marrow precursors (18, 19), leading to bone resorption. The receptor for RANK-L, RANK, is a TNF receptor family member found in dendritic cells, and reveals an unexpected role for an immune receptor–ligand pair in bone homeostasis.

Significance

Current therapies to alleviate autoimmune conditions use global strategies that affect large compartments of the immune response. These strategies mop up the excesses of disease without slowing disease progression and carry a significant risk of infection. This article describes the selective inhibition of autogressive T cells with the ability to regress established arthritis and reveals an unexpected role for an immune receptor–ligand pair in bone homeostasis.


The authors declare no conflict of interest.

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bone, skeletal muscle, thymus, liver, colon, small intestine, and adrenal gland. The balance between bone resorption by osteoclasts and bone matrix deposition by osteoblasts is mediated by the relative abundance of factors that drive each phenotype, but also the secretion of a decoy soluble receptor for RANK-L called osteoprotegerin, which inhibits osteoclastogenesis (reviewed in ref. 17). We show that signaling through OX40L inhibits osteoclastogenesis, even when osteoclastogenic factors are present, and that an absence of OX40L leads to smaller bone indices.

Bone loss is a feature of many diseases and occurs, for example, during chronic infection, autoimmune RA, and menopause, and in leukemia. Infiltrating and resident synovial cells, such as T cells, monocytes, and synovial fibroblasts, act as sources of osteoclast differentiation signals, and may be dysregulated by external stimuli (trauma, infection). These initiating events cause the production of inflammatory cytokines (M-CSF, RANK-L, TNF, IL-1, and IL-17) that play a dominant role in the pathogenesis of arthritis-associated bone loss. We show that, during inflammation, OX40L plays a detrimental role in that, despite inhibiting osteoclastogenesis, it instead skews myeloid precursors into inflammatory macrophages that in turn cause joint pathologic conditions. Therapeutic inhibition of OX40L ameliorates collagen-induced arthritis (CIA), and this is efficacious even when the signal through OX40 is intact. Inhibition of OX40:OX40L therefore represents a better focused therapeutic for inflammatory joint disease.

Results

Specific Expression of OX40 and OX40L in the Inflamed Joint. The abundance of OX40 and OX40L in cells from synovial fluid from patients with arthritis, and also from mice >15 d after injection of collagen to induce CIA, was examined. PCR analysis confirmed that OX40 and OX40L mRNA was as much as sixfold higher than the levels in peripheral blood (Fig. 1A). Flow cytometry revealed that the majority of OX40 expression was on CD4+ T cells in the synovial fluid from patients with RA and mice with CIA (Fig. 1B). In mice, no T cells were recovered from the noninflamed joints and very few were present in the peripheral blood of patients or mice with or without arthritis (Fig. 1B). The number of CD8+ T cells present was too low in synovial samples to accurately comment on OX40 expression. For ethical reasons, synovial samples from control patients were not available.

To examine the effect of preventing the interaction between OX40 and OX40L, mice immunized with bovine collagen to induce arthritis were treated at the same time with a pegylated blocking antibody to OX40, anti-OX40 Fab′PEG. In blocking OX40, this reagent also prevents reverse signaling through OX40 and OX40L, mice immunized with bovine collagen to induce CIA (Fig. 1, C, Left). Anti-OX40 Fab′PEG treatment reduced all signs of joint inflammation and degradation of bone and cartilage (Fig. 1C, Middle). However, the use of this blocking antibody does not clarify whether the improvement in disease is a result of OX40 or OX40L blockade. We therefore used a soluble OX40L:lg fusion protein that binds and signals through OX40 but competitively blocks OX40L. Because of the ability of this reagent to promote OX40-expressing activated T cells, we expected a worsening of joint inflammation, whereas treatment actually abolished all signs of disease in the CIA model (Fig. 1C, Right). This implies that signaling via OX40L promotes pathogenic joint inflammation, whereas promotion of OX40-expressing T cells does not. Treatment with the anti-OX40 Fab′PEG blocking antibody and the OX40L:lg fusion protein delayed the time of onset of arthritis and reduced the overall clinical score (Fig. 1D and E, respectively). This improvement was also clearly demonstrated by a reduction in paw swelling in anti-OX40 Fab′PEG-treated mice (Fig. S1).

B cells are known to express OX40L, and so the improvement in arthritic mice by treating with a soluble competitor of OX40L (OX40L:lg) or blocking its interaction with OX40 (by using anti-OX40 Fab′PEG) may be a result of reduced anti-collagen type II antibody production. However, neither of these treatments altered antibody titers (Fig. S1). Therapeutic administration of anti-OX40 Fab′PEG antibody (once the mice had achieved a clinical score of greater than 0.5 for 2 d) regressed paw inflammation from its starting point (Fig. 1F). This improvement occurred despite an increase in the concentration of the collagen C-terminal telopeptide (CTX) that indicates elevated activity of osteoclasts (Fig. 1G). Thus, the tradeoff between reducing inflammation, but enhancing osteoclast activity, seems to be beneficial in this context.
Signaling Through OX40L Inhibits Osteoclastogenesis. Because macrophages and osteoclasts develop from the same precursor cell population, we evaluated whether agonizing OX40L affected their development. Initially, RAW 264 cells, a murine macrophage like cell line, were cultured in the presence or absence of M-CSF and RANK-L to drive osteoclastogenesis. The inclusion of OX40L (to activate OX40L signaling) led to a dose-dependent decrease in the development of osteoclasts (Fig. 2A), as shown by the reduction in tartrate-resistant acid phosphatase (TRAP)-positive multinucleated giant cells and reduced resorption lacunae on dentine slices (Fig. 2B and C). This effect was not a result of cell death, but a functional switch causing an up-regulation of MHC class II and CD86 (Fig. 2D and E); the production of IL-12, TNF, and IL-10 (Fig. 2F–H); and a reduction in Triggering receptor expressed on myeloid cells (TREM)-2 from 90 ± 2.3% to 30 ± 1.8% on RAW macrophages (Discussion). In these experiments, pretreatment with IFN-γ was necessary to up-regulate OX40L on the surface of the cells.

A similar functional switch was observed when OX40L was included in cultures of human peripheral blood mononuclear cells (PBMCs) with RANK-L and CSF, with OX40L causing a dose-dependent inhibition of TRAP+ osteoclasts, that is, also reflected in their ability to resorb bone (Fig. 3A). In these same cultures, the levels of IL-10 and IL-8, cytokines known to inhibit osteoclastogenesis, appear earlier and increase with time and the dose of OX40L (Fig. 3B and C, respectively).

Signaling through OX40L seems to provide a functional switch in precursor cells that determines their differentiation into subtypes with very different functions; inflammatory in its presence and bone resorbing osteoclasts in its absence. To test this hypothesis further, we examined the ability to generate osteoclasts from the bone marrow of OX40L-deficient mice. More osteoclasts developed per field of view from the same amount of total bone marrow of the ox40l−/− compared with WT mice (Fig. 4A), leading to more TRAP+ multinucleated giant cells requiring lower doses of RANK-L (Fig. 4B) and an increase in the resorption of dentine (Fig. 4C and D).

Although signaling through OX40L causes the preferential development of inflammatory macrophages and its blockade increases the number osteoclasts when osteoclastogenic factors are present, its role in bone homeostasis in the steady state is not known. We therefore looked in more detail in ox40l−/− mice.

The Role of OX40L in Bone Homeostasis. MicroCT has become an important tool for the analysis of bone morphology and has previously been used in the CIA murine model (20). MicroCT analysis of the long bones from ox40l−/− mice revealed basal bone turnover defects (Fig. 5A) that were reflected in a significant reduction in tibia and tail length and tibia width (Fig. 5B–D). Furthermore, bone volume (BV), trabecular (Tb) BV fraction [BV/Tb volume (TV)] Tibial Tb thickness (Tb.Th), Tb thickness (Tb.Th), Tb number (Tb.N), and mineralized bone surface were significantly reduced in ox40l−/− mice, whereas Tb separation (Tb.Sp) was increased (Table S1). This alteration of bone architecture was associated with higher osteoclast numbers (Fig. 5E) and an enhanced concentration of the collagen C-terminal peptide CTX that indicates increased activation of osteoclasts (Fig. 5F). An absence of OX40L at birth therefore causes a progressive accumulation of osteoclasts that affects basal bone integrity. However, it would appear that its blockade during arthritis removes the more damaging events of inflammation. To prove this dichotomy more directly, we administered an OX40L fusion protein during induction of CIA and showed that, unlike anti-OX40 Fab PEG or OX40L-Ig treatment, joint inflammation and bone degradation was significantly worse (Fig. 5G). Thus, during development, OX40L is required to prevent the generation of too many osteoclasts in the steady state, but, in joint inflammation, the inflammatory microenvironment, together with the presence of OX40-expressing T cells, pushes the development of a self-perpetuating innate immune response.

Discussion

OX40 and OX40L are inducible and hence are of interest as potential therapeutic targets. OX40L clearly functions in specific microenvironments to direct myeloid cell precursors into an appropriate cell type. The noninflamed joint is devoid of OX40-expressing T cells, pushes the development of a self-perpetuating innate immune response.

Fig. 2. OX40L signaling prevents osteoclastogenesis. (A) Mouse bone marrow-derived monocyte/macrophage precursors were stimulated with RANK-L and M-CSF together with the indicated concentration of OX40L. At 7 d, the number of TRAP+ osteoclasts per field of view (FOV) was enumerated. Results show the mean and SD of six repeats. (B) Dentine slices showing lacunar pit formation following 14 d of culture in the presence of different concentrations of OX40L and (C) representative images of the extent of TRAP+ osteoclast development (D–H). RAW macrophages were stimulated with 100 ng/mL IFN-γ for 24 h to induce OX40L expression, washed, and then incubated in medium alone (black bars) or 5 μg/mL OX40L (open bars). At the hours indicated, cells were stained for MHC class II (D) and CD86 (E) and analyzed by flow cytometry. At 24 h after OX40L addition, supernatants from the same experiment were analyzed by ELISA for IL-12 (F), TNF (G), and IL-10 (H). Results represent mean ± SD of four replicates assayed in triplicate.
function to prevent further calcified tissue destruction in sites of inflammation.

The link between OX40L and osteoclast development is strengthened by our studies in \textit{ox40l} \textsuperscript{−/−} mice, which have a decrease in all indices of bone integrity and an enhanced osteoclastogenic capacity. A similar decrease in bone density is observed in vitro is enhanced by T-cell depletion (30); removing T cells would also remove any OX40 expressed by them. CD8\textsuperscript{T} cells are also a source of RANK-L, mice, causes accumulation with time.

The absence of OX40L does not immediately cause an overshoot in osteoclasts but, like T-cell-deficient mice, causes accumulation with time.

An absence of OX40L would not lead to the development of osteoclasts in inappropriate areas because they also require local production of RANK-L. T cells are also a source of RANK-L, but not the major source (18, 24), and we expect the OX40\textsuperscript{+} T-cell inhibitory effect on osteoclastogenesis develops in the more complex microenvironment of inflammation. IFN-γ, for example, enhances the ubiquitin–proteome system that degrades TRAF-6 ubiquitinated by RANK-L signaling (25); TRAF-6 is important for osteoclastogenesis (26, 27). In the absence of inflammation, therefore, TRAF-6 degradation is slower. The binding of T-cell–expressed OX40 to OX40L drives the production of IFN-γ and TNF by both participating cells (28, 29), which may override the osteoclastogenic activity of RANK-L. It is also possible that reverse signaling by OX40L may directly activate the ubiquitin–proteasome system or TRAF-6 ubiquitination without the requirement for inflammatory cytokines. Indirect evidence in the literature also supports our conclusions that activated T cells inhibit osteoclastogenesis, and the present study provides a potential mechanism. For example, vitamin D3 stimulation of osteoclasts in vitro is enhanced by T-cell depletion (30); removing T cells would also remove any OX40 expressed by them. CD8\textsuperscript{T} cells are also

antiosteoclastogenic (15, 31) and express high levels of OX40 during infections (32–34) associated with arthritic disease flare (35).

The decrease of TREM-2 upon OX40L ligation of myeloid precursors is interesting in terms of mechanism. TREM-2, like signal-regulatory protein-β1, is an ITAM-containing DAP12-associated receptor (36). IL-10, which is increased upon OX40L ligation, at the transition to osteoclast precursors, suppresses RANK-L signaling and human osteoclastogenesis, in part by inhibiting transcription of TREM-2. It is noteworthy that loss-of-function mutations in TREM-2 cause Nasu-Hakola disease, characterized by defective osteoclastogenesis and abnormal bone growth (37).

As a therapeutic strategy to reduce joint inflammation, inhibition of OX40L signaling is complex, as it would limit the development of inflammatory macrophages, but at the same time might promote osteoclastogenesis. However, empirically, this was not found to be the case, as joint bone architecture improved considerably when OX40L was inhibited. Its likely that autologous T cells expressing OX40 divert the enhanced numbers of recruited myeloid precursors to an inflammatory macrophage via OX40L, but that inhibition of OX40L reduces inflammation and returns myeloid precursor recruitment and bone homeostasis back to the steady state. This concept is supported by the administration of OX40-expressing liposomes early in the adjuvant-induced arthritis murine model that ameliorates disease (38); however, the link with innate inflammation and osteoclastogenesis was not examined.

T-cell activation is an important part of immune surveillance for malignancy and infection. Treatment of a chronic disease by blocking OX40L (directly or by using antibodies that prevent OX40 binding to OX40L), however, does not prevent the initiation of an immune response, as CD28 and ICOS paired signaling is still intact. Furthermore, redundancy in such late costimulators that act at the same stage as OX40/OX40L (such as 4-1BB/4-1BBL) would provide the signals required for maintenance of sufficient inflammation and the development of immunological memory (32, 34, 39).

\begin{itemize}
  \item [A] Human peripheral blood monocytes were incubated with RANK-L and M-CSF and indicated concentrations of OX40L. After 7 d, the number of TRAP\textsuperscript{+} multinucleated cells was enumerated per field of view (FOV). Supernatants from the experiment in A were assayed by ELISA for IL-10 (B) and IL-8 (C). Mean ± SD of six replicates is shown.
  \item [B] Bone marrow-derived monocyte/macrophage precursors from WT (open bars) or \textit{ox40l} \textsuperscript{−/−} (closed bars) mice were incubated with M-CSF and the indicated concentration of OX40L. Mean ± SD of five replicates is shown.
  \item [C] Resorption capability of bone marrow precursors from WT (open bars) or \textit{ox40l} \textsuperscript{−/−} (closed bars) were assayed by ELISA for IL-10 (B) and IL-8 (C). Mean ± SD of five replicates is shown.
  \item [D] Representative dentine slices incubated with osteoclasts derived from WT (Upper) or \textit{ox40l} \textsuperscript{−/−} (Lower) mice.
\end{itemize}

Fig. 3. OX40L signaling impairs human osteoclast formation. (A) Human peripheral blood monocytes were incubated with RANK-L and M-CSF and indicated concentrations of OX40L. After 7 d, the number of TRAP\textsuperscript{+} multinucleated cells was enumerated per field of view (FOV). Supernatants from the experiment in A were assayed by ELISA for IL-10 (B) and IL-8 (C). Mean ± SD of six replicates is shown.

\begin{itemize}
  \item [A] Bone marrow-derived monocyte/macrophage precursors from WT (open bars) or \textit{ox40l} \textsuperscript{−/−} (closed bars) were incubated with M-CSF and the indicated concentration of OX40L. Mean ± SD of five replicates is shown.
  \item [B] Bone marrow-derived monocyte/macrophage precursors from WT (open bars) or \textit{ox40l} \textsuperscript{−/−} (closed bars) were incubated with M-CSF and the indicated concentration of OX40L. Mean ± SD of five replicates is shown.
  \item [C] Bone marrow-derived monocyte/macrophage precursors from WT (open bars) or \textit{ox40l} \textsuperscript{−/−} (closed bars) were incubated with M-CSF and the indicated concentration of OX40L. Mean ± SD of five replicates is shown.
  \item [D] Bone marrow-derived monocyte/macrophage precursors from WT (open bars) or \textit{ox40l} \textsuperscript{−/−} (closed bars) were incubated with M-CSF and the indicated concentration of OX40L. Mean ± SD of five replicates is shown.
\end{itemize}

Fig. 4. \textit{ox40l} \textsuperscript{−/−} mice develop more osteoclasts. (A) Bone marrow-derived monocyte/macrophage precursors from WT (open bars) or \textit{ox40l} \textsuperscript{−/−} (closed bars) were incubated with M-CSF and the indicated concentration of OX40L. Mean ± SD of five replicates is shown. (B) Representative TRAP-stained monolayers of osteoclasts from WT (Upper) and \textit{ox40l} \textsuperscript{−/−} (Lower) mice. (C) Resorption capability of bone marrow precursors from WT or \textit{ox40l} \textsuperscript{−/−} mice after culture for 7 d in M-CSF plus 25 ng/mL (open bars) or 50 ng/mL (closed bars) RANK-L. Mean ± SD of five replicates is shown. (D) Representative dentine slices incubated with osteoclasts derived from WT (Upper) or \textit{ox40l} \textsuperscript{−/−} (Lower) mice.
and ox40l from 11 patients with RA (patients fulfilling American College of Rheumatology criteria for RA), but before any treatment (three men, eight women; age range, 45–77 y). Approval was obtained from the Riverside Research Ethics Committee, and informed patient consent was obtained.

Induction of CIA. Mice were injected s.c. at the base of the tail with 100 μL of bovine type II collagen (2 mg/mL) emulsified in complete Freund adjuvant (BD Biosciences), as described previously (44). Arthritis typically developed 14 to 28 d later. Inflammation in each paw was measured by using calipers and scored for clinical signs of illness, whereby 0 indicated normal, 1 indicated slight swelling and/or erythema, 2 indicated pronounced swelling, and 3 indicated joint rigidity. A maximum score of 12 was therefore available per mouse. In some experiments, the hind paws were removed, fixed, decalcified, and stained with H&E. Sections were scored as follows by light microscopy: 0 indicated no damage, 1 indicated mild inflammation, 2 indicated moderate inflammation with some joint erosion, and 3 indicated severe inflammation and loss of joint architecture.

Reagents. An anti-mouse OX40 Fab′ was conjugated with PEG2MAL40K. This PEGylated anti-mouse OX40 Fab′ (anti-OX40 Fab PEG) blocked the binding of OX40L to OX40-expressing cells without evidence of costimulation. A PEGylated anti-human TNF Fab′ was used as a negative control (control Fab′ PEG).

The murine OX40:mlgG1 fusion protein, OX40-lg, and OX40L:mlgG1, OX40L-lg, were obtained from Xenova Research and were constructed by using a chimeric cDNA that contained the extracellular domain of OX40 or OX40L fused to the constant region of murine IgG1. These constructs were used to transfect clonal CHO cells, and fusion proteins were purified from the culture supernatant by using protein G Sepharose.

OX40/OX40L Human and Mouse mRNA by RT-PCR. RT-PCR analysis of OX40/OX40L human and mouse mRNA is described in SI Text.

MicroCT Analysis. The tibia metaphyses were analyzed in vivo on a microCT device (no. 1076; SkyScan) with a high-resolution low-dose X-ray scanner. Bone marrow density, a 3D bone characteristic parameter, was analyzed in 50 consecutive slices. Tb BV was measured by using the software developed for bone histomorphometry (CT-analyzer software; SkyScan). Serum CTX analysis in WT and ox40l−/− mice with arthritis and WT mice treated with anti-OX40 Fab′ PEG was analyzed by using the Mouse C-telopeptide of type II collagen CLIA Kit (Wuhan ElabAb Science) according to the manufacturer’s instructions.

Osteoclastogenesis Assays. Mouse bone marrow cells were isolated by flushing femurs of 12-wk-old C57BL/6 and ox40l−/− mice. Cells (5 × 104) were plated in 96-well plates in the presence of recombinant murine M-CSF (25 ng/mL) and RANK-L (25 ng/mL and 50 ng/mL; R&D Systems), in the presence or absence of OX40-lg (5–5,000 ng/mL), at three wells per treatment. Human monocytes from buffy coat PBMC preparations from healthy donors were seeded (1 × 106 cells per milliliter) into 24-well plates containing M-CSF (100 ng/mL). After 3 d, the cells were scraped and seeded (2 × 105 cells per well) into 96-well tissue culture plates. The cells were cultured in medium containing M-CSF (25 ng/mL) and RANK-L (20 ng/mL) in the presence or absence of OX40-lg (5–5,000 ng/mL), at three wells per treatment. Supernatant from the osteoclastogenesis cultures was collected and stored for ELISA at −20 °C. Cultures were replenished with fresh media and treatment factors every 3 or 4 d. On day 7 of culture, TRAP histochemical staining of the cultures was then performed as previously described (45), and TRAP positive multinucleated cells (greater than three nuclei) were counted by light microscopy. Functional evidence of osteoclast differentiation and activation was determined by lacunar resorption of dentine discs (n = 3 dentines per treatment). Murine cultures were incubated for 14 d, and human cultures for 21 d. Cells were removed; dentine was rinsed in water and stained with toluidine blue. Resorption pits were assessed by light microscopy, and the average area of three fields of view on three dentines per treatment were measured by using Image J image analysis software.

In Vitro Assays to Analyze the Function of OX40L. RAW 264.7 cells were incubated at 1 × 106 cells per well in 24-well plates in DMEM plus 10% FCS. Following adherence, cells were treated with 100 ng/mL IFN-γ to up-regulate surface OX40L (expression checked by flow cytometry), washed, and then incubated with or without 5 μg/mL OX40-lg at 37 °C. At the indicated time points, supernatants were removed for analysis of cytokines by ELISA, and the cells stained and analyzed by flow cytometry for MHC class II, CD86, and TREM-1 and -2 (BD Biosciences). Data were acquired on an LSR II flow cytometer.
cytometer, and 30,000 events analyzed with CellQuest Pro software (BD Biosciences). ELISAs for the detection of IL-12, TNF, IL-8, and IL-10 were performed according to their instructions.


Statistics. Statistical significance was calculated by using an unpaired Mann-Whitney test and Prism software. P values of 0.05 or lower were considered significant and are referred to as such in the text.
Supporting Information

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OX40/OX40 Ligand Human and Mouse mRNA by Real Time-PCR

Total RNA was extracted by using TRIzol (Invitrogen Life Technologies) and reverse-transcribed by using oligo(dT)12–18 (SuperScript II; Invitrogen Life Technologies). Quantitative real time (RT)-PCR was performed by using SYBR Green I dye and a Stratagene MX4000 instrument. The levels of mRNA were normalized to beta actin. Primers were as follows: human, OX40 5'-TCAGAAGTGGGAGTGAGCGGAAG-3' (forward) and 5'-GCAGAGAGCCGGAGGCAGCCATCGGC-3' (reverse); OX40 ligand (OX40L), 5'-TGCTTCACCTACATCTGCTGCA-3' (forward) and 5'-CTAGTAGGCTCAAGGCAATCTTG-3' (reverse); mouse, forward, GGGCAGGGAACACAGTCAAC; reverse, CAGAATTGCACACTACTCAG; OX40L, 5'-CCGCTCGAGATTGTGAAGATGG-3' (forward) and 5'-GCTCTAGAGCCCCTCAAAGGACAC-3' (reverse); and β-actin, 5'-GTATGCCTCTGGTGTGACCAGGCAT-3' (forward) and 5'-ACTCATCTGACTCTGCTTGATCC-3' (reverse).

Fig. S1. DBA/1 mice were injected with 100 μg of bovine collagen in complete Freund adjuvant at the base of the tail and 250 μg anti-OX40 Fab PEG (A and B) or 100 μg OX40L:Ig (B) i.p. on day 0. Injections were given every 3 d. The change in paw depth vs. baseline was recorded in a cumulative fashion per group of mice (A). Animals were killed on day 35 and serum collected for analysis of type II collagen antibodies by ELISA (B).
Table S1.  MicroCT-determined changes in tibia trabecular morphological parameters of 12- and 20-wk-old WT and Ox40l<sup>−/−</sup> male mice

<table>
<thead>
<tr>
<th>Parameter</th>
<th>WT</th>
<th>Ox40l&lt;sup&gt;−/−&lt;/sup&gt;</th>
<th>Difference of ox40l&lt;sup&gt;−/−&lt;/sup&gt; vs. WT, %</th>
<th>P value</th>
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<td><strong>12-wk males (n = 8)</strong></td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>TV, mm&lt;sup&gt;3&lt;/sup&gt;</td>
<td>2.35 ± 0.12</td>
<td>2.22 ± 0.14</td>
<td>5.53</td>
<td>0.12</td>
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<tr>
<td>BV, mm&lt;sup&gt;3&lt;/sup&gt;</td>
<td>0.42 ± 0.02</td>
<td>0.24 ± 0.06</td>
<td>42.86</td>
<td>&lt;0.001***</td>
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<tr>
<td>BV/TV</td>
<td>17.96 ± 1.23</td>
<td>10.69 ± 2.38</td>
<td>40.48</td>
<td>&lt;0.001***</td>
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<tr>
<td>TS, mm&lt;sup&gt;2&lt;/sup&gt;</td>
<td>13.68 ± 0.54</td>
<td>13.02 ± 0.50</td>
<td>4.82</td>
<td>0.04*</td>
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<tr>
<td>BS, mm&lt;sup&gt;2&lt;/sup&gt;</td>
<td>31.46 ± 2.52</td>
<td>22.46 ± 3.79</td>
<td>28.61</td>
<td>&lt;0.001***</td>
</tr>
<tr>
<td>BS/TV, 1/mm</td>
<td>13.42 ± 0.79</td>
<td>10.05 ± 1.13</td>
<td>37.11</td>
<td>&lt;0.001***</td>
</tr>
<tr>
<td>Tb.Th, mm</td>
<td>0.05 ± 0.004</td>
<td>0.04 ± 0.003</td>
<td>21.21</td>
<td>0.01**</td>
</tr>
<tr>
<td>Tb.Sp, mm</td>
<td>0.16 ± 0.02</td>
<td>0.18 ± 0.05</td>
<td>28.57</td>
<td>0.004**</td>
</tr>
<tr>
<td>Tb.N, 1/mm</td>
<td>3.62 ± 0.29</td>
<td>2.58 ± 0.36</td>
<td>28.73</td>
<td>0.001***</td>
</tr>
<tr>
<td>Tb.Pf</td>
<td>19.29 ± 1.97</td>
<td>30.12 ± 4.72</td>
<td>56.61</td>
<td>0.001***</td>
</tr>
<tr>
<td><strong>20-wk males (n = 8)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TV, mm&lt;sup&gt;3&lt;/sup&gt;</td>
<td>2.08 ± 0.21</td>
<td>2.02 ± 0.14</td>
<td>0.79</td>
<td>0.23</td>
</tr>
<tr>
<td>BV, mm&lt;sup&gt;3&lt;/sup&gt;</td>
<td>0.33 ± 0.01</td>
<td>0.22 ± 0.06</td>
<td>21.21</td>
<td>0.01**</td>
</tr>
<tr>
<td>BV/TV</td>
<td>15.96 ± 3.46</td>
<td>11.09 ± 3.14</td>
<td>38.59</td>
<td>&lt;0.001***</td>
</tr>
<tr>
<td>TS, mm&lt;sup&gt;2&lt;/sup&gt;</td>
<td>12.24 ± 0.78</td>
<td>12.43 ± 0.84</td>
<td>1.52</td>
<td>0.82*</td>
</tr>
<tr>
<td>BS, mm&lt;sup&gt;2&lt;/sup&gt;</td>
<td>26.67 ± 6.16</td>
<td>18.66 ± 2.02</td>
<td>28.57</td>
<td>0.004**</td>
</tr>
<tr>
<td>BS/TV, 1/mm</td>
<td>12.74 ± 2.26</td>
<td>9.28 ± 0.99</td>
<td>25.11</td>
<td>0.001***</td>
</tr>
<tr>
<td>Tb.Th, mm</td>
<td>0.05 ± 0.06</td>
<td>0.05 ± 0.007</td>
<td>0.09</td>
<td>0.61</td>
</tr>
<tr>
<td>Tb.Sp, mm</td>
<td>0.17 ± 0.03</td>
<td>0.19 ± 0.08</td>
<td>11.76</td>
<td>0.03*</td>
</tr>
<tr>
<td>Tb.N, 1/mm</td>
<td>3.36 ± 0.67</td>
<td>2.39 ± 0.32</td>
<td>22.62</td>
<td>0.004***</td>
</tr>
<tr>
<td>Tb.Pf, 1/mm</td>
<td>20.49 ± 5.17</td>
<td>27.15 ± 6.54</td>
<td>32.86</td>
<td>0.028*</td>
</tr>
</tbody>
</table>

BS, bone surface; BV, bone volume; Tb.N, trabecular number; Tb.Pf, trabecular pattern factor; Tb.Th, trabecular thickness; Tb.Sp, trabecular separation; TV, trabecular volume.

*P = 0.05, **P = 0.01, and ***P = 0.001, by Student t test.