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Depletion of CD4 and CD8 T Lymphocytes in Mice In Vivo Enhances 1,25-Dihydroxyvitamin D_3-Stimulated Osteoclast-Like Cell Formation In Vitro by a Mechanism That Is Dependent on Prostaglandin Synthesis[1]

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To investigate the role of T lymphocytes in osteoclastogenesis, we performed in vivo depletion of CD4 and/or CD8 T lymphocyte subsets and evaluated in vitro osteoclast-like cell (OCL) formation. T lymphocyte depletion (TLD) with mAbs was confirmed 24 h later by flow cytometry. OCL formation was stimulated with 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$) in bone marrow and with recombinant mouse (rm) receptor activator of NF-κB ligand (RANK-L) and rmM-CSF in bone marrow and spleen cell cultures. OCL formation was up to 2-fold greater in 1,25-$(OH)_2D_3$-stimulated bone marrow cultures from TLD mice than in those from intact mice. In contrast, TLD did not alter OCL formation in bone marrow or spleen cell cultures that were stimulated with rmRANK-L and rmM-CSF. The effects of TLD seemed to be mediated by enhanced PG synthesis, because the PGE_2 concentration in the medium of 1,25-$(OH)_2D_3$-stimulated bone marrow cultures from TLD mice was 5-fold higher than that in cultures from intact mice, and indomethacin treatment abolished the stimulatory effect of TLD on OCL formation. There was a 2-fold increase in RANK-L expression and an almost complete suppression of osteoprotegerin expression in 1,25-$(OH)_2D_3$-stimulated bone marrow cultures from TLD mice compared with those from intact mice. Although there was a small (20%) increase in IL-1α expression in 1,25-$(OH)_2D_3$-stimulated bone marrow cultures from TLD mice, TLD in mice lacking type I IL-1R and wild-type mice produced similar effects on OCL formation. Our data demonstrate that TLD up-regulates OCL formation in vitro by increasing PG production, which, in turn, produces reciprocal changes in RANK-L and osteoprotegerin expression. These results suggest that T lymphocytes influence osteoclastogenesis by altering bone marrow stromal cell function. ***The Journal of Immunology,*** **2000, 165: 4231–4238.**

The regulation of bone resorption involves complex interactions between osteoclasts, the principle bone-resorbing cells, their precursor cells, and other cells in the bone marrow (marrow stromal cells, osteoblasts, hemopoietic cells, and lymphocytes) (1, 2). The most important cells influencing osteoclast differentiation and activation are those of the osteoblast lineage, which regulate osteoclast formation and function by a contact-dependent mechanism (3). Stromal cells, which give rise to osteoblasts, can also support osteoclastogenesis (4). In addition, nonstromal hemopoietic cells and lymphocytes may affect bone cell function (1, 2). Pathologic function of hemopoietic or lymphocytic cells in the bone marrow microenvironment may arise from changes in the production of hormones, cytokines, or growth factors and lead to the development of skeletal abnormalities (1).

Within the bone marrow microenvironment there is a close interdependence of the bone and immune systems. Both subsets of T lymphocytes ($CD4^+$, which are associated with helper/inducer function, and $CD8^+$, which are associated with suppressor/cytotoxic function) (5) may be involved in the maintenance of normal bone homeostasis via the production of inhibitory or stimulatory factors that regulate osteoclast differentiation. A number of factors that influence osteoclast formation are known products of T lymphocytes. IFN-γ, IL-4, IL-10, and IL-13 inhibit osteoclastogenesis (6); TNF-α, TNF-β, and IL-6 stimulate it (6, 7); whereas TGF-β and GM-CSF have both stimulatory and inhibitory effects (6–9).

Activated T lymphocytes are important regulators of bone resorption in acute inflammatory states. These effects are probably mediated by cytokines, which act either directly on osteoclasts and their precursors or through the responses of intermediate cells in the bone marrow, such as macrophages. These produce osteoresorptive cytokines, such as IL-1 and TNF, that mediate some effects of periodontal disease, osteomyelitis, rheumatoid arthritis, and certain malignancies of bone (10–12). T lymphocytes may also have clinically important roles in normal bone homeostasis, because glucocorticoids and cyclosporin A, which inhibit T lymphocyte functions, contribute to the rapid bone loss that occurs after organ transplantation (13).

A recently described TNF-related family of ligands and receptors appears to be critical regulators of osteoclastogenesis. Receptor activator of NF-κB ligand (RANK-L)[3] is a key regulator of osteoclastogenesis (14), which can both activate mature osteoclasts and mediate osteoclastogenesis in the presence of M-CSF (15).

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[3] Abbreviations used in this paper: RANK-L, receptor activator of NF-κB ligand; OPG, osteoprotegerin; OCL, osteoclast-like cells; 1,25-$(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; IL-1RI KO mice, type I IL-1R knockout mice; FC, flow cytometry; rm, recombinant mouse; TRAP, tartrate-resistant acid phosphatase; sCT, salmon calcitonin; CD4/CD8 depletion, depletion of CD4 and CD8 T lymphocyte subsets.

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RANK-L is highly expressed in osteoblast/stromal cells, and its expression can be up-regulated by bone-resorbing factors such as vitamin D_3, IL-11, PGE_2, and parathyroid hormone (16). RANK is the cellular receptor for RANK-L (17). It is a member of the TNF receptor family and is expressed on dendritic cells, T lymphocytes, and hemopoietic precursors. Binding of RANK-L to RANK regulates dendritic cell function and T lymphocyte activation in the immune system (18) as well as osteoclast differentiation (19). Activated T lymphocytes may induce bone loss and joint destruction in adjuvant arthritis by increasing RANK-L production (20). Osteoprotegerin (OPG) is a soluble molecule that also belongs to the TNF receptor family, acts as a decoy receptor for RANK-L, and inhibits formation of osteoclast-like cells (OCL) and bone resorption in vivo and in vitro (21, 22). OPG is expressed on B cells, dendritic cells, and follicular dendritic cells, which implicates its involvement in immune responses (23). PGs are produced in bone by many cells (24–26) and can enhance the ability of RANK-L to stimulate OCL formation in vitro (25). In addition, several osteoresorptive hormones and cytokines stimulate PG production through induction of the inducible prostaglandin G/H synthase (26).

To investigate the role of T lymphocytes in osteoclast differentiation, we depleted CD4 and/or CD8 subsets of T lymphocytes in vivo in mice, extracted bone marrow cells 24 h later, and cultured them with or without 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$) to stimulate OCL formation in vitro. We found that depletion of T lymphocytes increased the number of OCL that formed in the cultures. This effect appeared to be dependent on PG synthesis, as it was inhibited by indomethacin, an inhibitor of PG synthase, and to be associated with increased PGE_2 concentration in the culture media.

Materials and Methods

Mice

C57BL/6 mice were purchased from Charles River Farms (Wilmington, MA) and housed at the Center for Laboratory Animal Care at the University of Connecticut Health Center (Farmington, CT). Mice lacking type I IL-1R (IL-1RI KO mice) were produced at Immunex Research and Development Corp. (Seattle, WA). They were prepared by gene targeting, using a previously described method (27). The original strain of IL-1RI KO mice was on a C57BL/6 × 129/Sv genetic background. Controls for IL-1RI KO mice were F_2 generation mice from The Jackson Laboratory (Bar Harbor, ME) of a similar genetic background. The animal care committee of the University of Connecticut Health Center approved all animal protocols. Animals were fed and watered ad libitum. All experiments used males that were 10–12 wk old.

T lymphocyte depletion

mAbs were used for in vivo depletion of CD4 or/and CD8 T lymphocyte subsets. Depletion was performed by single i.p. injection of 500 μg of purified monoclonal Abs. Rat anti-mouse Abs (clone YTS 191.1) were used for CD4 depletion, and rat anti-mouse Abs (clone YTS 169.4) were used for CD8 depletion (28). Control (nondepleted) mice were treated with a single i.p. injection of 500 μg of nonimmune rat IgG (ICN Pharmaceuticals, Aurora, OH). The depletion was assessed 24 h after the treatment with mAbs by flow cytometric (FC) analysis of lymph node, spleen, and bone marrow cells.

FC analysis

The depletion of T lymphocyte subsets was confirmed by FC. For FC analysis we used mAbs directed against epitopes of CD4 and CD8 molecules different from the injected Abs. Lymph nodes and spleens were dissected out, mashed in a homogenizer, resuspended in α-MEM, and passed through a 40-μm pore size nylon cell strainer. Bone marrow cells were obtained from mouse femurs and tibias by flushing the bones with α-MEM through a 23-gauge needle. Harvested cells were washed with 0.1% NaN_3 and 1% BSA in PBS and were analyzed by FC using FACScalibur (Becton Dickinson Immunocytometry Systems, San Jose, CA). Two-color FC analysis was performed by staining the cells with PE-anti-CD4 Abs (clone H129.19; PharMingen, San Diego, CA) and FITC-anti-CD8 Abs (clone 53-6.7; PharMingen). After erythrocyte lysis with Tris-ammonium chloride buffer (pH 7.4), 10^6 cells were incubated with anti-CD4 and anti-CD8 Abs for 30 min at 4°C in the dark, washed twice in 0.1% NaN_3 and 1% BSA in PBS, and immediately analyzed using CellQuest software (Becton Dickinson Immunocytometry Systems).

Bone marrow and spleen cell cultures

Mouse spleen or bone marrow cells were extracted 24 h after injection of mAbs, washed twice with α-MEM, and cultured (10^6 cells/cm^2) in α-MEM supplemented with 10% heat-inactivated FCS for 7 days (for OCL quantitation assay) or 5 days (for RT-PCR assay). Cultures were fed every 3 days with fresh medium. For studies of OCL differentiation in the absence of bone marrow stromal cells, spleen cells were cultured with recombinant mouse (rm) M-CSF (30 ng/ml; R&D Systems, Minneapolis, MN) and rmRANK-L (30 ng/ml; a gift from Dr. Dirk Anderson, Immunex), added on day 0 and with each medium change. For OCL formation in bone marrow cultures, bone marrow cells were cultured with 1,25-$(OH)_2D_3$ (10^{-8} M; added on day 0 and with each medium change) or with rmM-CSF and rmRANK-L (30 ng/ml for both, added during the last 3 days of culture). In all experiments unstimulated cultures contained <10 OCL/well. In some experiments cells were treated with indomethacin (10^{-6} M; Sigma, St. Louis, MO), which was added on day 0 and with each medium change.

In some experiments bone marrow cell cultures were stimulated with 1,25-$(OH)_2D_3$ in a reverse time-course manner. Bone marrow cells were cultured for 7 days as in other bone marrow cell culture experiments, while 1,25-$(OH)_2D_3$ was added at different time points (days 0, 3, 5, and 7 of culture).

Osteoclast-like cell quantitation

Cells were cultured in either 24-well (spleen cell cultures that were stimulated with rmM-CSF and rmRANK-L and bone marrow cell cultures that were stimulated with 1, 25-$(OH)_2D_3$) or 48-well (bone marrow cell cultures that were stimulated with rmM-CSF and rmRANK-L) culture plates, fixed on day 7 with 2.5% glutaraldehyde in PBS for 30 min at room temperature, and then stained for tartrate-resistant acid phosphatase (TRAP). Enzyme histochemistry for TRAP was performed using a commercial kit (Sigma). TRAP-positive multinucleated giant cells with more than four nuclei per cell were considered OCL and were counted per well using an inverted microscope at ×100 magnification. In all experiments, four wells per group were counted.

In some experiments radiolabeled [^{125}I]salmon calcitonin (sCT; NEN/DuPont, Boston, MA) was incubated with or without an excess of cold sCT (10^{-7} M, 10×10^6-fold excess), washed, and developed by autoradiography to demonstrate the presence of CT receptors on cells. Briefly, cells were plated on slide flasks (2×10^6 cells/cm^2) and incubated at the end of culture with radiolabeled [^{125}I]sCT (0.04 μCi, 100,000 cpm/ml) in the absence or the presence of cold sCT (10^{-7} M; Bachem, Torrance, CA) at room temperature for 2 h. They were then washed twice with PBS to remove nonspecific radioactivity and fixed with 2.5% glutaraldehyde in PBS. Slides were dipped in LM-1 photographic emulsion (1/1 dilution with 1.7% glycerol; Amersham, Arlington Heights, IL) for autoradiography and then developed and stained with Giemsa.

PCR amplification

Total RNA was extracted from cultured bone marrow cells using a commercial kit (Tri-Reagent, Molecular Research Center, Cincinnati, OH). RNA was converted to cDNA by reverse transcriptase (SuperScript II, Life Technologies, Grand Island, NY). An initial RT mixture of total RNA (10 μg), random hexamer, and RNase inhibitor was incubated at 70°C and then quenched on ice before addition of the RT buffer (50 mmol/L Tris-HCl (pH 8.3), 75 mmol/L KCl, and 3 mmol/L $MgCl_2$), dNTPs, DTT, and reverse transcriptase. The final mixture was incubated for 1 h at 37°C, pulsed with reverse transcriptase, and incubated for another 1 h at 37°C. The first-strand cDNA was resuspended in sterile water. The amount of cDNA corresponding to 0.5 μg of the reverse transcribed RNA was amplified by PCR. The PCR mixture without enzyme was overlaid with mineral oil and heated to 94°C for 5 min. During the last minute, Taq polymerase (AmpliTaq, Perkin-Elmer, Norwalk, CT) was added to the PCR mixture according to a hot start procedure. PCR was performed in a thermal cycler (Perkin-Elmer) using the following cycles: denaturation at 94°C for 1 min, primer annealing at 65°C for 2 min, and extension at 72°C for 3 min for 10 cycles. In subsequent cycles, the primer annealing temperature was decreased stepwise down to 45°C by increments of 5°C. After the last cycle the mixture was incubated at 72°C for 7 min. Specific amplimer sets were designed from published cDNA sequences: murine RANK-L (antisense, 5′-GGGAATTACAAAGTGCACCAG-3′; sense, 5′-GGTCGGGCAATTCTGAATT-3′) (17), murine OPG (antisense, 5′-TCAAGTGCTTGAGGGCATAC-3′; sense, 5′-TGGAGATCGAATTCTGCTTG-3′) (29), murine IL-1α (antisense, 5′-CCTTCAGCAACACGGGCTGGTC-3′; sense,

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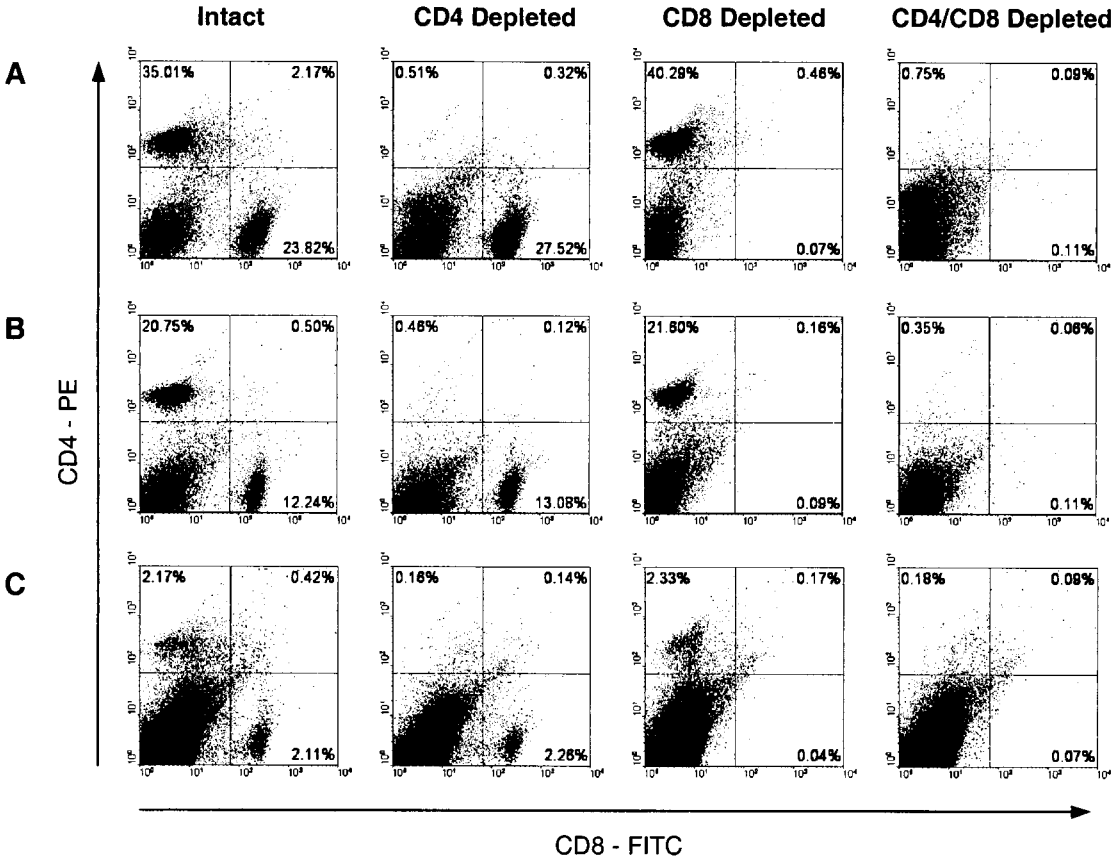

FIGURE 1. Confirmation of T lymphocyte depletion using two-color FC analysis of cells from intact, CD4-depleted, CD8-depleted, and CD4/CD8-depleted mice. Cells (10^6) from lymph nodes (*A*), spleen (*B*), or bone marrow (*C*) were stained with FITC-anti-CD8 Abs and PE-anti-CD4 Abs 24 h after in vivo depletion. Results are shown on dot plots with a logarithmic scale.

5′-ATGGCCAAAGTTCCTGACTTGTTT-3′) (30), and β-actin (antisense, 5′-CTCTTTGATGTCACGCACGATTTC-3′; sense, 5′-GTGGGC CGCTCTAGGCACCAA-3′) (31). For each amplimer set, we performed the amplification over a range of 21–33 cycles to generate amplification curves and determine the PCR conditions that produced the linear range of PCR amplification, as previously described (32). We used PCR amplification of 33 cycles for OPG, 30 cycles for IL-1α, and 27 cycles for β-actin and RANK-L for further semiquantitative analyses, because those conditions were verified to be in the midlinear range of each PCR amplification. The amplified products were run in a 1.5% agarose gel, stained with ethidium bromide, and photographed under UV illumination. Images were captured by a FOTO/Analyst Archiver Electronic Documentation system (Fotodyne, Hartland, WI), and OD was determined using a digital image processing and analysis program (Scion Image, Scion, Frederick, MD).

PGE_2 assay

Culture medium was collected on day 7 from cultured bone marrow cells, and PGE_2 accumulation was measured by RIA as described previously (26).

Statistics

Statistical analysis was performed using one-way ANOVA and the Bonferroni post-hoc test when ANOVA showed significant differences ($p < 0.05$). All experiments were repeated at least twice.

Results

Efficiency of in vivo T lymphocyte depletion

Depletion of CD4 or/and CD8 T lymphocyte subsets from mouse lymph node, spleen, and bone marrow cell populations in vivo was confirmed 24 h after treatment by FC analysis (Fig. 1). Lymph nodes contain mainly T lymphocytes (35.01% $CD4^+$, 23.82% $CD8^+$, and 2.17% $CD4^+CD8^+$ cells; Fig. 1*A*), and the degree of T lymphocyte depletion was most obvious in these organs (reduction to 0.75% $CD4^+$, 0.11% $CD8^+$, and 0.09% $CD4^+CD8^+$ cells by anti-CD4/CD8 Ab treatment; Fig. 1*A*). In spleen cells, anti-CD4/CD8 Ab treatment reduced $CD4^+$ cells from 20.75 to 0.35%, $CD8^+$ cells from 12.24 to 0.11%, and $CD4^+CD8^+$ cells from 0.50 to 0.06% (Fig. 1*B*). Mouse bone marrow contains relatively few mature $CD4^+$ and $CD8^+$ T lymphocytes (33). However, we found that $CD4^+$ cells decreased from 2.17 to 0.18%, $CD8^+$ cells from 2.11 to 0.07% and $CD4^+CD8^+$ cells from 0.42 to 0.09% after anti-CD4/CD8 Ab treatment (Fig. 1*C*).

Effect of depletion of T lymphocyte subsets in vivo on OCL differentiation in vitro in mouse bone marrow cell cultures that were treated with 1,25-$(OH)_2D_3$

Pooled data from three experiments (Fig. 2) showed that the depletion of each subset of T lymphocytes significantly increased the number of OCL formed in the bone marrow cell cultures stimulated with 1,25-$(OH)_2D_3$ (10^{-8} M; from 106.2 ± 8.5 in cultures from intact mice to 198.3 ± 15.7 in cultures from CD4-depleted mice and 140.1 ± 12.2 in cultures from CD8-depleted mice; $p < 0.05$ for both). Depletion of both subsets in vivo produced an additive effect on the number of OCL that formed in culture (264.0 ± 18.3 in cultures from CD4/CD8-depleted mice; $p < 0.05$; Fig. 2). In addition, >90% of OCL that formed in cultures of either intact or CD4/CD8-depleted mice expressed high levels of CT receptor, a marker of the osteoclast phenotype, as assessed by specific [^{125}I]sCT binding (Fig. 3).

We next examined the reverse time course for OCL formation in 1,25-$(OH)_2D_3$-stimulated bone marrow cultures from intact and CD4/CD8 T lymphocyte-depleted mice (Fig. 4). The highest number of OCL was observed in cultures in which 1,25-$(OH)_2D_3$ was present during the last 5 days of culture for both intact (393.3 ± 17.9) and CD4/CD8-depleted mice (516.3 ± 17.5). Comparison of the number of OCL in cultures that were treated with 1,25-$(OH)_2D_3$ showed significantly more OCL ($p < 0.05$) in

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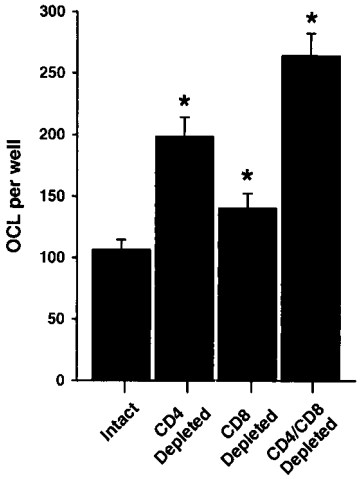

FIGURE 2. Effects of CD4 and/or CD8 T lymphocyte depletion on the number of TRAP-positive OCL that formed in bone marrow cell cultures that were treated with 1,25-$(OH)_2D_3$ (10^{-8} M). Bone marrow cells were cultured for 7 days at a density of 2×10^6 cells/well of a 24-well plate, and 1,25-$(OH)_2D_3$ was added on day 0 and with each medium change. Less than 10 OCL formed in all groups that were not treated with 1,25-$(OH)_2D_3$ (data not shown). Values are the mean ± SEM for four replicates per group. Data were pooled from three independent experiments. *, Significant difference from intact mice, $p < 0.05$.

cultures from CD4/CD8 T lymphocyte-depleted mice than in those from intact mice at 3, 5, and 7 days of 1,25-$(OH)_2D_3$ treatment (Fig. 4).

Effect of depletion of T lymphocyte subsets in vivo on OCL differentiation in vitro in mouse bone marrow and spleen cell cultures that were treated with rmM-CSF and rmRANK-L

To determine whether T lymphocyte depletion affected the number of osteoclast progenitor cells, we performed in vivo depletion and then examined the number of OCL that formed in spleen and bone marrow cell cultures. Spleen cells contain few stromal support cells and do not form OCL with 1,25-$(OH)_2D_3$ stimulation. However, they do form maximal numbers of OCL in the presence of rmM-CSF and rmRANK-L (30 ng/ml for both) (15). In contrast, bone marrow contains significant numbers of stromal cells, which produce M-CSF, RANK-L, and OPG in a manner that allows enhanced generation of OCL after treatment with 1,25-$(OH)_2D_3$ as well as after treatment with rmM-CSF and rmRANK-L (Figs. 2 and 5). We previously determined the culture conditions and concentrations of rmM-CSF and rmRANK-L that produced maximal OCL formation in these cultures (30 ng/ml for both). There was no difference in the number of OCL that formed in bone marrow (Fig. 5) and spleen cell cultures (Fig. 6) from CD4 and/or CD8 T lymphocyte-depleted mice compared with intact mice. These results imply that the number of osteoclast progenitors that respond to M-CSF and RANK-L was similar in CD4/CD8 T lymphocyte-depleted and intact mice.

Effect of indomethacin treatment on in vitro OCL formation in bone marrow cell cultures from T lymphocyte-depleted and intact mice

To determine whether PG synthesis was involved in the effect of T lymphocyte depletion on 1,25-$(OH)_2D_3$-stimulated in vitro osteoclastogenesis, we added indomethacin (10^{-6} M), an inhibitor of PG synthesis, to bone marrow cell cultures from intact and CD4/CD8 T lymphocyte-depleted mice. Indomethacin abrogated the enhancing effect of T lymphocyte depletion on the number of OCL that formed in 1,25-$(OH)_2D_3$-stimulated cultures (183.8 ± 4.9 without indomethacin compared with 86.5 ± 7.2 with indomethacin treatment in cultures from CD4/CD8 T lymphocyte-depleted mice; $p < 0.05$; Fig. 7). The number of OCL in indomethacin-treated cultures from T lymphocyte-depleted mice (86.5 ± 7.2) was comparable to that in cultures from intact mice that were treated with (90.0 ± 6.6) or without indomethacin (93.5 ± 10.9; Fig. 7).

We also measured PGE_2 concentrations in the conditioned medium of bone marrow cell cultures from T lymphocyte-depleted and intact mice. PGE_2 was undetectable in cultures that were treated with indomethacin. In contrast, in cultures that were stimulated with 1,25-$(OH)_2D_3$ and not treated with indomethacin, PGE_2 concentrations were significantly greater (by >5-fold) in the medium of cultures from CD4/CD8 T lymphocyte-depleted mice compared with those in the medium of cultures from intact mice (Table I).

RANK-L, OPG, and IL-1α mRNA expression in mouse bone marrow cell cultures that were treated with 1,25-$(OH)_2D_3$

To investigate the underlying mechanism responsible for the stimulatory effect of T lymphocyte depletion on osteoclastogenesis, we performed RT-PCR analysis of bone marrow cells on day 5 of culture. Initially, we performed the amplification over a variety of

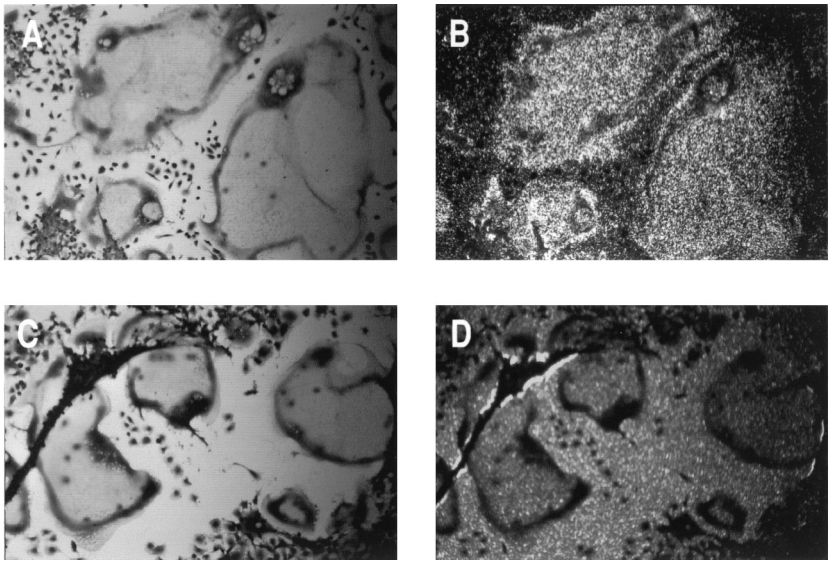

FIGURE 3. [^{125}I]sCT binding assay. Light photomicrographs of 7-day bone marrow cell cultures from CD4/CD8 T lymphocyte-depleted mice that were treated with 1,25-$(OH)_2D_3$ (10^{-8} M). *A* and *C*, Bright-field images (silver grains appear as black spots). *B* and *D*, Darkfield images (silver grains appear as white spots). To demonstrate the specificity of [^{125}I]sCT binding, some cultures (*C* and *D*) were treated with excess cold sCT (10^{-7} M). *A* and *B*, Specific localization of silver grains on OCL after radiolabeled sCT binding. Original magnification, ×200. Similar results were obtained in bone marrow cell cultures from intact mice (data not shown).

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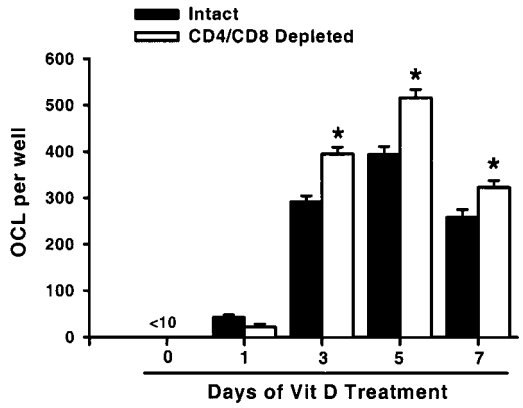

FIGURE 4. Reverse time course for the effect of CD4/CD8 T lymphocyte depletion on the number of TRAP-positive OCL that formed in bone marrow cell cultures that were treated with 1,25-$(OH)_2D_3$ (vit D; 10^{-8} M). Bone marrow cells were cultured for 7 days at a density of 2×10^6 cells/well of a 24-well plate. 1,25-$(OH)_2D_3$ was added during the last 1, 3, 5, or 7 days of culture. Less than 10 OCL formed in both groups (cultures from intact and CD4/CD8 T lymphocyte-depleted mice) that were not treated with 1,25-$(OH)_2D_3$ (day 0 of vit D treatment). Values are the mean ± SEM for four replicates per group. *, Significant difference from intact mice, $p < 0.05$.

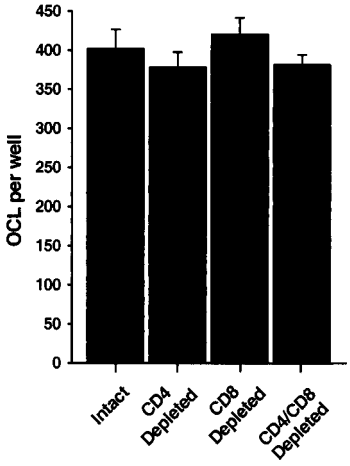

FIGURE 6. Effects of CD4 and/or CD8 T lymphocyte depletion on the number of TRAP-positive OCL that formed in spleen cell cultures that were treated with rmM-CSF and rmRANK-L (30 ng/ml for both). Bone marrow cells were cultured for 7 days at a density of 2×10^6 cells/well of a 24-well plate, and rmM-CSF and rmRANK-L were added on day 0 and with each medium change. Less than 10 OCL formed in all groups that were not treated with rmRANK-L and rmM-CSF (data not shown). Values are the mean ± SEM for four replicates per group.

PCR cycles (21–33) to determine the linear range of amplification for each amplimer set (Fig. 8). cDNA samples were from cells of either CD4/CD8 T lymphocyte-depleted or intact mice that were cultured with or without 1,25-$(OH)_2D_3$. Semiquantitative analysis of mRNA levels was performed by examining relative band intensities of PCR products in the linear range of amplification for each amplimer set after normalizing the OD of the respective band to the OD of β-actin (Fig. 9). In cultures from both intact and T lymphocyte-depleted mice RANK-L and IL-1α mRNA levels were always higher in 1,25-$(OH)_2D_3$-stimulated cultures than in control nonstimulated cultures (Fig. 8). In addition, RANK-L and IL-1α mRNA levels were ~80 and 20% higher, respectively, in cultures from T lymphocyte-depleted mice than in similar cultures from intact mice (Fig. 9). OPG mRNA levels were greater in unstimulated cultures from T lymphocyte-depleted mice than in similar cultures from intact mice (Figs. 8 and 9). However, treatment of both intact and CD4/CD8 T lymphocyte-depleted bone marrow cell cultures with 1,25-$(OH)_2D_3$ markedly decreased OPG mRNA expression to barely detectable levels (Fig. 9).

Effect of depletion of T lymphocyte subsets in vivo on OCL differentiation in vitro in bone marrow cell cultures that were treated with 1,25-$(OH)_2D_3$ from IL-1RI KO and wild-type mice

IL-1α is a potent osteoresorptive cytokine that is secreted by cells of the monocyte/macrophage lineage and can enhance PGE_2 synthesis in mouse cell cultures (24, 26). To further investigate the

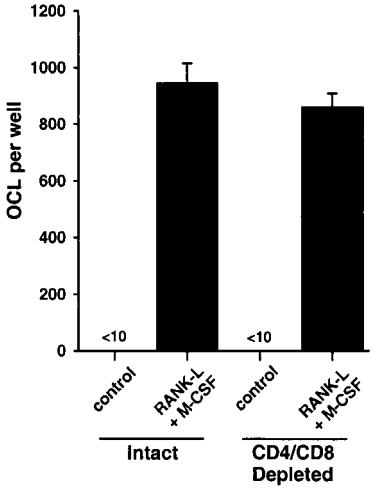

FIGURE 5. Effect of CD4/CD8 T lymphocyte depletion on the number of TRAP-positive OCL that formed in bone marrow cell cultures that were treated with rmM-CSF and rmRANK-L (30 ng/ml for both). Bone marrow cells were cultured for 7 days at a density of 10^6 cells/well of a 48-well plate, and rmM-CSF and rmRANK-L were added during the last 3 days of culture. Less than 10 OCL formed in all groups that were not treated with rmRANK-L and rmM-CSF. Values are the mean ± SEM for four replicates per group.

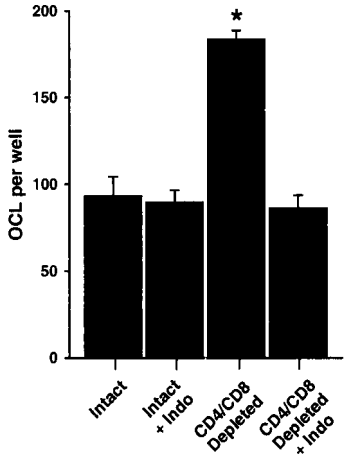

FIGURE 7. Effect of indomethacin (Indo; 10^{-6} M) on the number of TRAP-positive OCL that formed in bone marrow cell cultures from intact and CD4/CD8 T lymphocyte-depleted mice. Cultures were treated with 1,25-$(OH)_2D_3$ (10^{-8} M). Bone marrow cells were cultured for 7 days at a density of 2×10^6 cells/well of a 24-well plate, while 1,25-$(OH)_2D_3$ was added on day 0 and with each medium change. Less than 10 OCL formed in all groups that were not treated with 1,25-$(OH)_2D_3$ (data not shown). Values are the mean ± SEM for four replicates per group. *, Significant difference from intact mice and indomethacin-treated cultures, $p < 0.05$.

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Table I. *PGE_2 concentration in 7-day bone marrow culture medium*[a]

Group	Treatment		PGE_2 (nM)
	1,25-$(OH)_2D_3$	Indomethacin	
Control intact mouse	−	−	ND
	+	−	6.39 ± 1.02*
	−	+	ND
	+	+	ND
CD4/CD8-depleted mouse	−	−	1.40 ± 0.32†
	+	−	40.97 ± 15.14*†
	−	+	ND
	+	+	ND

[a] Cultures from CD4/CD8 T lymphocyte depleted or intact mice were stimulated with 1,25-$(OH)_2D_3$ (10^{-8} M) and treated with or without indomethacin (10^{-6} M). Bone marrow cells were plated at a density of 2×10^6 cells/well of a 24-well plate as quadruplicates. Media were collected at day 7 of culture, and PGE_2 concentration was analyzed by RIA. ND, None detected.

*, Significant effect of 1,25-$(OH)_2D_3$ in comparison to non-1,25-$(OH)_2D_3$-treated cultures, $p < 0.05$.

†, Significant effect of CD4/CD8 T lymphocyte depletion in comparison to control intact mouse, $p < 0.05$.

possible role of IL-1α in our model, we examined the effects of T lymphocyte depletion on bone marrow cell cultures from wild-type and IL-1RI KO mice. IL-1RI KO mice lack the biologically active receptor for IL-1, and hence, cells from these mice cannot respond to IL-1 (27). Bone marrow cells from IL-1RI KO and wild-type mice were cultured for 7 days with or without 1,25-$(OH)_2D_3$. We found no differences in the number of OCL that formed in bone marrow cell cultures from IL-1RI KO and wild-type mice that were either intact or T lymphocyte depleted (Fig. 10). These results demonstrate that IL-1 is not essential for the stimulatory effect of in vivo T lymphocyte depletion on in vitro osteoclastogenesis.

Discussion

It has been shown that immune cells may exert profound effects on bone cell function (1). We found a 2-fold increase in the number of OCL that formed in 1,25-$(OH)_2D_3$-stimulated bone marrow cell cultures from CD4/CD8 T lymphocyte-depleted mice compared with nondepleted mice. Nevertheless, studies of athymic and genetically immunodeficient mice and rats do not show alterations in bone turnover (34–36). However, experiments in rats suggested that T lymphocytes play a critical role in the cyclosporin A-induced in vivo increase in osteoclast number (37). It appears that acute inhibition of T lymphocytes by cyclosporin A or their acute removal, as occurs in the current experiments, enhances osteoclast formation. Clinical studies in patients with postmenopausal osteoporosis support a role of T lymphocytes in the regulation of bone mass, because they showed a significant negative correlation between bone mineral density and the number of $CD3^+$ $CD56^+$ T lymphocytes (38) or the CD4/CD8 T lymphocyte ratio (39, 40).

Our results suggest that T lymphocytes are negative regulators of osteoclast formation in bone marrow cell cultures that are stimulated with vitamin D_3. In contrast, other authors have found that T lymphocytes enhance bone resorption in inflammatory conditions (10, 11). This discrepancy may be explained by the fact that other studies used activated T lymphocytes to investigate the role of T lymphocytes in the regulation of osteoclast activation and bone resorption. Activation of T lymphocytes with bacterial Ags or Abs leads to specific patterns of inflammatory cytokine production, increased expression of cell adhesion molecules, and other changes characteristic of local inflammation (6, 10, 41) as well as up-regulation of RANK-L expression (20). Many of these responses are known to increase osteoclast formation and enhance bone resorption (11, 20, 42–44). In contrast, our model examined the role of naive (nonactivated) T lymphocytes in the bone microenvironment, where there are few mature T lymphocytes (33, 45). A previous study also demonstrated an inhibitory effect of T lymphocytes on osteoclast differentiation (46). These authors performed in vitro depletion of T lymphocytes from mouse bone marrow and spleen cells and used a coculture method of in vitro OCL formation (3). They found that CD8 T lymphocyte subset depletion had more potent stimulatory effects on OCL formation in the culture. However, the extent of T lymphocyte depletion in bone marrow was not assessed in that study, and it is possible that the depletion of bone marrow was incomplete. The differences between the results of these authors and our findings may also be explained by the fact that the cytokine and growth factor network is probably different in bone marrow cells that are cultured alone compared with that in cocultures of bone marrow and stromal/osteoblastic support cells. In this study we performed acute in vivo depletion of

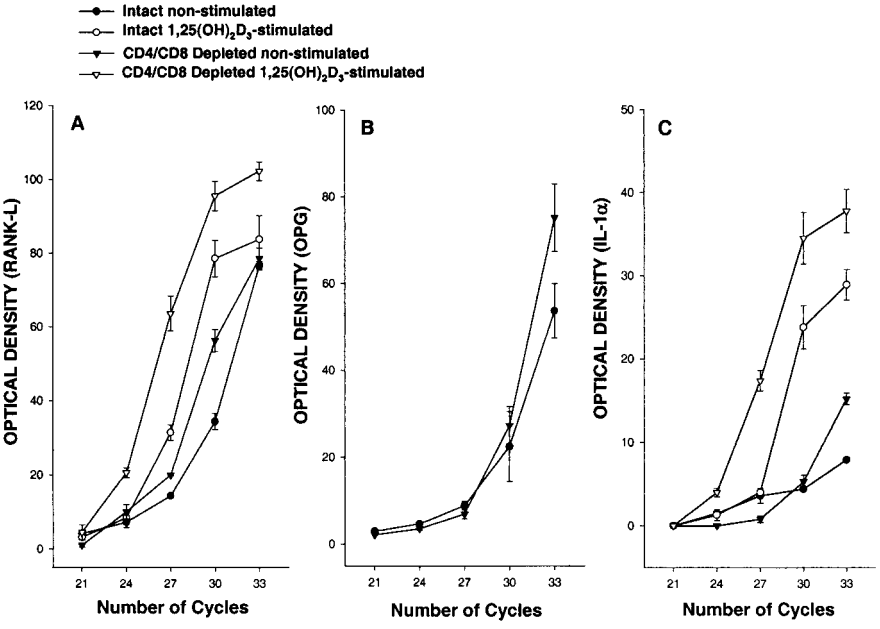

FIGURE 8. PCR amplification curves for RANK-L, OPG, and IL-1α. PCR amplification was performed over the range of 21–33 cycles. *A*, cDNA samples of nonstimulated and 1,25-$(OH)_2D_3$-stimulated 5-day bone marrow cell cultures from intact and CD4/CD8 T lymphocyte-depleted mice amplified for RANK-L; *B*, cDNA samples of nonstimulated 5-day bone marrow cell cultures from intact and CD4/CD8 T lymphocyte-depleted mice amplified for OPG (OPG expression was barely detectable in 1,25-$(OH)_2D_3$-stimulated bone marrow cell cultures and is not shown); *C*, cDNA samples of nonstimulated and 1,25-$(OH)_2D_3$-stimulated 5-day bone marrow cell cultures from intact and CD4/CD8 T lymphocyte-depleted mice amplified for IL-1α. All PCR amplifications were performed in triplicate. Values are the mean ± SEM.

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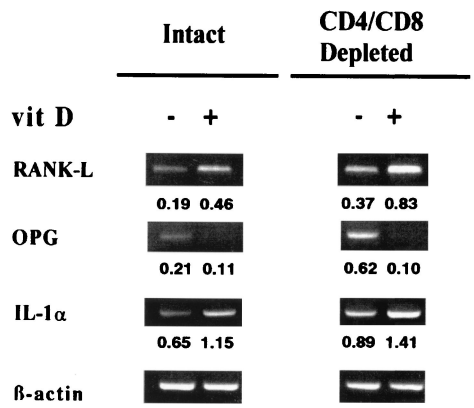

FIGURE 9. Patterns of mRNA expression for RANK-L, OPG, IL-1α, and β-actin in bone marrow cell cultures from intact and CD4/CD8 T lymphocyte-depleted mice assessed by RT-PCR. Bone marrow cells were cultured for 5 days with or without 1,25-$(OH)_2D_3$ (vit D; 10^{-8} M). Photographs are the results of PCR analysis from a representative experiment. Numbers below each band represent the ratio of the OD of the respective band normalized to the OD of β-actin. RANK-L and β-actin were amplified for 27 cycles, OPG was amplified for 33 cycles, and IL-1α was amplified for 30 cycles.

T lymphocyte subsets by Ab injection, which is a widely employed method of immunosuppression (28). To avoid the possible nonspecific effect of Abs we used highly purified mAbs that had been tested for specificity and efficiency as well as nonimmune Abs for the control (nondepleted) group. FC confirmation of in vivo depletion was performed with mAbs that were directed against epitopes of CD4 and CD8, which differed from the injected Abs that were used for in vivo depletion. This was done to demonstrate that the CD4 and CD8 molecules were not simply blocked, but, rather, that the specific cell populations that expressed these Ags were eliminated. Our results demonstrated that depletion of CD4 T lymphocytes had a greater enhancing effect on 1,25-$(OH)_2D_3$-stimulated OCL formation than did depletion of CD8 T lymphocytes, and depletion of both (CD4 and CD8) subsets had an additive effect.

We found no difference in the number of OCL that formed in bone marrow cell cultures from CD4/CD8 T lymphocyte-depleted and intact mice that were stimulated with optimal concentrations of rmM-CSF and rmRANK-L. Similarly, T lymphocyte depletion did not alter the number of OCL in mouse spleen cell cultures that were stimulated with rmM-CSF and rmRANK-L. These results suggest that acute T lymphocyte depletion did not alter the number of osteoclast progenitors in mice, but, instead, influenced the ability of stromal cells to support osteoclastogenesis. Our findings that T lymphocyte depletion increased RANK-L mRNA levels by about 2-fold in both basal and 1,25-$(OH)_2D_3$-stimulated cultures and increased basal OPG levels support this hypothesis, because RANK-L and OPG are stromal cell products that are critical regulators of osteoclast formation (47, 48). Because the production of OCL in bone marrow cultures is tightly correlated with the reciprocal regulation of RANK-L and OPG mRNA expression (32), it is likely that the increased production of OCL in T lymphocyte-depleted cultures after 1,25-$(OH)_2D_3$ treatment is mediated by increased expression of RANK-L and almost complete suppression of OPG. In basal conditions few (<10) OCL form in these bone marrow cell cultures. Because RANK-L mRNA is expressed in unstimulated bone marrow cell cultures, it is likely that high level expression of OPG prevents RANK-L from activating OCL formation in unstimulated cultures. In contrast, in 1,25-$(OH)_2D_3$-stimulated bone marrow cell cultures, OPG production was almost completely inhibited, and therefore, the 2-fold increase in RANK-L expression that was seen in cultures from T lymphocyte-depleted mice was the likely cause of the increase in OCL formation.

PGs are mediators of the biologic responses of bone cells to many stimulators, and synthesis of PGs is blocked by indomethacin (24, 26). In addition, PGs stimulate RANK-L production in bone cells (25). Our finding that treatment of bone marrow cell cultures with indomethacin abolished the stimulatory effect of T lymphocyte depletion on OCL formation suggests that increased PGE_2 production is an intermediary in the effects of T lymphocyte depletion on osteoclastogenesis in our model. The demonstration that the PGE_2 concentration in the conditioned medium increased 5- to 6-fold in cultures from T lymphocyte-depleted mice supports this hypothesis. It is well known that certain osteoresorptive cytokines, such as IL-1α and TNF-α, can exert their osteoresorptive effect by stimulating inducible PG synthase (24, 26, 49).

We found no effect of T lymphocyte depletion on TNF-α mRNA expression by RT-PCR analysis of cultured bone marrow cells (data not shown) and only a weak (20%) increase in IL-1α mRNA expression in 1,25-$(OH)_2D_3$-stimulated cultures from CD4/CD8 T lymphocyte-depleted mice compared with cultures from intact mice. We also examined whether in vivo T lymphocyte depletion down-regulated the production of factors that are known to inhibit osteoclastogenesis in bone marrow cell cultures (6, 7, 9). By RT-PCR analysis we failed to find expression of IFN-γ or GM-CSF mRNA in bone marrow cell cultures from intact and T lymphocyte-depleted mice that were either unstimulated or treated with 1,25-$(OH)_2D_3$ (data not shown). Our finding that OCL formation rates are similar in 1,25-$(OH)_2D_3$-stimulated bone marrow cultures from CD4/CD8 T lymphocyte-depleted wild-type and IL-1RI KO mice demonstrates that IL-1 is unlikely to be involved in the effects of T lymphocyte depletion on OCL formation.

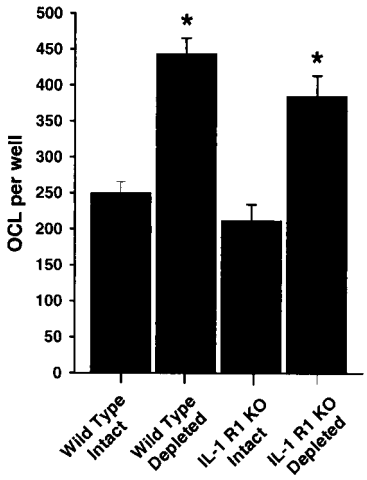

FIGURE 10. Effect of CD4/CD8 T lymphocyte depletion on the number of TRAP-positive OCL that formed in bone marrow cell cultures from wild-type and IL-1RI-deficient (KO) mice that were treated with 1,25-$(OH)_2D_3$ (10^{-8} M). Bone marrow cells were cultured for 7 days at a density of 2×10^6 cells/well of a 24-well plate, while 1,25-$(OH)_2D_3$ was added on day 0 and with each medium change. Less than 10 OCL formed in all groups that were not treated with 1,25-$(OH)_2D_3$ (data not shown). Values are the mean $\pm$ SEM for four replicates per group. Groups were cultures from wild-type intact, wild-type CD4/CD8 T lymphocyte-depleted, IL-1RI KO intact, and IL-1RI KO CD4/CD8 T lymphocyte-depleted mice. *, Significant difference from intact mice, $p < 0.05$.

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The current studies demonstrated that T lymphocytes influence osteoclast formation in vitro. Our finding that PG, RANK-L, and OPG are involved in this response and our results in stromal cell-poor spleen cell cultures suggest that T lymphocytes influence stromal cell function by regulating their ability to support osteoclastogenesis. This may occur either through the production of factors by naive T lymphocytes that inhibit PG synthesis and osteoclast formation or by effects of in vivo T lymphocyte depletion on the type of stromal cells that populates the bone marrow.

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