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Depletion of CD4 and CD8 T Lymphocytes in Mice In Vivo Enhances 1,25-Dihydroxyvitamin D3-Stimulated Osteoclast-Like Cell Formation In Vitro by a Mechanism That Is Dependent on Prostaglandin Synthesis

Danka Grčević, Sun-Kyong Lee, Ana Marušić, and Joseph A. Lorenzo

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 D₃ (1,25-(OH)₂D₃) 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)₂D₃-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 PGE₂ concentration in the medium of 1,25-(OH)₂D₃-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)₂D₃-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)₂D₃-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 interaction between 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 or their precursors or through the responses of intermediate cells in the bone marrow, such as macrophages. These produce osteoclastogenic 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) is a key regulator of osteoclastogenesis (14), which can both activate mature osteoclasts and mediate osteoclastogenesis in the presence of M-CSF (15).
CD4 and CD8 T Lymphocyte Depletion Enhances Osteoclast-Like Cell Formation

RANK-L is highly expressed in osteoblast/stromal cells, and its expression can be up-regulated by bone-resorbing factors such as vitamin D₃, IL-11, PGE₂, 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 osteoreparative cytokines and hormones 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 D₃ (1,25-(OH)₂D₃) to stimulate OCL formation in vitro. We found that depletion of T lymphocytes increased the number of OCL that formed in the culture. 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 PGE₂ 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 Immune 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 × 129Sv 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 196.4) 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% NaN₃ 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), 10⁶ cells were incubated with anti-CD4 and anti-CD8 Abs for 30 min at 4°C in the dark, washed twice in 0.1% NaN₃ 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 (10⁶ cells/cm²) 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)₂D₃ (10⁻² 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 (10⁻⁵ 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)₂D₃ 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)₂D₃ was added at different time points (days 0, 3, 5, and 7 of culture).

In some experiments radiolabeled [³²P]salmon calcitonin (sCT; NEN/DuPont, Boston, MA) was incubated with or without an excess of cold sCT (10⁻⁵ M, 10 × 10⁶-fold excess), washed, and developed by autoradiography to demonstrate the presence of CT receptors on cells. Briefly, cells were plated on slide flasks (2 × 10⁶ cells/cm²) and incubated at the end of culture with radiolabeled [¹²⁵I]sCT (0.04 μCi, 100,000 cpm/ml in the absence of CT or 10⁻¹⁰ M CT in the presence of 10⁻⁷ M CT) for 60 min at room temperature. Afterward, the cells were 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 MgCl₂), 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 (GeneAmp PCR System 2400, Perkin-Elmer) with the following program: template denaturation at 94°C for 1 min, followed by 35 cycles of 94°C for 90 s, 55°C for 1 min, 72°C for 1.25 min, and final extension at 72°C for 7 min. In some 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 amplifier sets were designed from published cDNA sequences: murine RANK-L (antisense, 5'-GGGATTTTCAACGGTCACACCGG-3'; sense, 5’-GTGCGGGCAATTCTGAAATT-3') (17), murine OPG (antisense, 5’-TCAATTGCTTGGGACAC-3’; sense, 5’-TCTCAGCAACCGGGCCTGTC-3’) (29), murine IL-1α (antisense, 5’-CCTCAGCAACCGGGCCTGTC-3’; sense,
5'-ATGGCCAAAGTTCCTGACTTGTTT-3' (30), and β-actin (anti-sense, 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).

**PGE₂ assay**

Culture medium was collected on day 7 from cultured bone marrow cells, and PGE₂ 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. 1A), 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. 1A). 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. 1B). 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. 1C).

**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)₂D₃**

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)₂D₃ (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 [125I]sCT binding (Fig. 3).

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 [125I]sCT binding (Fig. 3).

**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 (10⁶) 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.

**FIGURE 2.** 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)₂D₃ (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).

**FIGURE 3.** 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 [125I]sCT binding (Fig. 3).

**FIGURE 4.** We next examined the reverse time course for OCL formation in 1,25-(OH)₂D₃-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)₂D₃ 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)₂D₃ showed significantly more OCL (p < 0.05) in...
than 10 OCL formed in all groups that were not treated with 1,25-(OH)₂D₃ 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)₂D₃-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)₂D₃-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 PGE₂ concentrations in the conditioned medium of bone marrow cell cultures from T lymphocyte-depleted and intact mice. PGE₂ was undetectable in cultures that were treated with indomethacin. In contrast, in cultures that were stimulated with 1,25-(OH)₂D₃ and not treated with indomethacin, PGE₂ 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)₂D₃**

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

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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)₂D₃ (10⁻⁸ M). Bone marrow cells were cultured for 7 days at a density of 2 × 10⁶ cells/well of a 24-well plate, and 1,25-(OH)₂D₃ 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)₂D₃ (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.

**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)₂D₃ (10⁻⁸ M). A and C, Brightfield 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).
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)₂D₃. 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)₂D₃-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)₂D₃ 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)₂D₃ from IL-1RI KO and wild-type mice**

IL-1α is a potent osteoclastogenic cytokine that is secreted by cells of the monocyte/macrophage lineage and can enhance PGE₂ synthesis in mouse cell cultures (24, 26). To further investigate the effects...
CD4 and CD8 T lymphocyte depletion enhances osteoclast-like cell formation

Evidence that T lymphocytes play a critical role in the regulation of osteoclastogenesis comes from studies using genetically immunodeficient mice and rats. Bone marrow cultures from these animals do not show alterations in bone resorption, whereas bone marrow cultures from intact mice do. The differences in the number of osteoclast-like cells (OCL) that formed in bone marrow cultures from intact and CD4/CD8 T lymphocyte-depleted mice were 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 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 as well as up-regulation of RANK-L expression.

**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)2D3-stimulated bone marrow cell cultures from CD4/CD8 T lymphocyte-depleted mice compared with nondepleted mice. Nevertheless, studies of athermic 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 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 suggest 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 D3. 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 nonactivated (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.

**Table I. PGE2 concentration in 7-day bone marrow culture medium**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>1,25-(OH)2D3</th>
<th>Indomethacin</th>
<th>PGE2 (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control intact mouse</td>
<td>+</td>
<td>+</td>
<td>6.39 ± 1.02*</td>
</tr>
<tr>
<td>CD4/CD8-depleted mouse</td>
<td>+</td>
<td>+</td>
<td>1.40 ± 0.32†</td>
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</tbody>
</table>

* Cultures from CD4/CD8 T lymphocyte depleted or intact mice were stimulated with 1,25-(OH)2D3 (10–8 M) and treated with or without indomethacin (10–6 M). Bone marrow cultures were plated at a density of 2 × 106 cells/well of a 24-well plate as quadruplicates. Media were collected at day 7 of culture, and PGE2 concentration was analyzed by RIA. ND, None detected.

†, Significant effect of 1,25-(OH)2D3 in comparison to non-1,25-(OH)2D3-treated cultures, p < 0.05.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>1,25-(OH)2D3</th>
<th>Indomethacin</th>
<th>PGE2 (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control intact mouse</td>
<td>+</td>
<td>+</td>
<td>ND</td>
</tr>
<tr>
<td>CD4/CD8-depleted mouse</td>
<td>+</td>
<td>+</td>
<td>40.97 ± 15.14*†</td>
</tr>
</tbody>
</table>

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

**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)2D3-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)2D3-stimulated bone marrow cell cultures and is not shown); C, cDNA samples of nonstimulated and 1,25-(OH)2D3-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.
T lymphocyte subsets by Ab injection, which is a widely employed method of immunosuppression (28). To avoid the possible non-specific 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)_{2}D_{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)_{2}D_{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)_{2}D_{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)_{2}D_{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)_{2}D_{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)_{2}D_{3} (data not shown). Our finding that OCL formation rates are similar in 1,25-(OH)_{2}D_{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.
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 osteoclast 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 osteoclastogenesis. Our finding that PG, RANK-L, and OPG are involved in this response and our results in stromal cell-osteoclast formation in vitro. Our finding that PG, RANK-L, and OPG are involved in this response and our results in stromal cell-osteoclast formation in vitro. Our finding that PG, RANK-L, and OPG are involved in this response and our results in stromal cell-osteoclast formation in vitro. Our finding that PG, RANK-L, and OPG are involved in this response and our results in stromal cell-osteoclast formation in vitro. 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