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Continuous RANKL Inhibition in Osteoprotegerin Transgenic Mice and Rats Suppresses Bone Resorption without Impairing Lymphorganogenesis or Functional Immune Responses

Marina Stolina, Denise Dwyer, Michael S. Ominsky, Timothy Corbin, Gwyneth Van, Brad Bolon, Ildiko Sarosi, James McCabe, Debra J. Zack, and Paul Kostenuik*

Receptor activator of NF-κB ligand (RANKL) is an essential mediator of osteoclast formation, function, and survival. The effects of RANKL are inhibited by a soluble decoy receptor called osteoprotegerin (OPG). Total ablation of RANKL in knockout mice leads to high bone mass, lymph node agenesis, and altered lymphocyte differentiation. In contrast, RANKL inhibition via OPG suppresses bone resorption but not inflammation in animal models of inflammatory bone loss. This suggests that the immune phenotype of RANKL knockout mice is related to total RANKL ablation. We hypothesized that prenatal RANKL inhibition via OPG overexpression would suppress bone resorption without influencing lymph node formation or subsequent immune responses. Transgenic rats were created, wherein soluble OPG was overexpressed by 100-fold vs wild type (WT) controls, by gestational day 11 (i.e., before lymph node formation). The structure of lymph nodes, spleen, and thymus of OPG-transgenic (OPG-Tg) animals were comparable to those of age-matched WT rats at gestational day 19 and in adulthood. The OPG-Tg neonates had elevated bone mass, confirming the prenatal inhibition of RANKL. Adult OPG-Tg rats and OPG-Tg mice exhibited no significant functional alterations relative to WT controls when subjected to immune challenges to test for altered innate and humoral responses (e.g., contact hypersensitivity to oxazolone, IgM response to Pneumovax, IgG response to keyhole limpet hemocyanin, or cytokine response to LPS). In summary, prenatal RANKL inhibition did not impair lymph node development, nor did continuous life-long RANKL inhibition cause obvious changes in innate or humoral immune responses in mice or rats. The Journal of Immunology, 2007, 179: 7497–7505.

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Materials and Methods

All studies were conducted in accordance with federal animal care guidelines and were approved by Amgen’s Institutional Animal Care and Use Committee.

**OPG-Tg mice and rats**

Mice (BDF1 background) overexpressing full-length native rat OPG under the control of the human apolipoprotein E (ApoE) promoter and liver-specific enhancer element were generated as previously described (7). The same transgene construct and conventional microinjection techniques were used to create OPG-Tg rats (Sprague Dawley background). For both species, OPG-Tg offspring were identified by screening for the ApoE-opg transgene in DNA prepared from ear biopsies as described (7). Mice and rats were given tap water and fed ad libium with pelleted rodent chow (Harlan Teklad) containing 1.2% calcium and 1.0% phosphorus. For necropsy, all animals were euthanized by carbon dioxide.

**Hematologic assessment of blood and spleen**

At necropsy, rats and mice were anesthetized with carbon dioxide and blood was collected by intracardiac puncture. One aliquot of whole blood was mixed with anticoagulant (sodium EDTA) and then passed through an automated hematologic analyzer (Advia 120; Bayer Corporation) to evaluate circulating populations of blood cells. Spleens were removed aseptically, disrupted, and splenic leukocyte populations were evaluated from the resulting cell suspensions (Advia 120). Additional aliquots of whole blood and splenocyte cell suspension were immunophenotyped with mAbs (BD Pharmingen) directed against epitopes on T cells (anti-CD4 and anti-CD8) and B cells (anti-CD45RA for rats, and anti-B220 for mice), followed by quantitative analysis by FACS using a standard flow cytometer (FACScan; Calibur; BD Biosciences).

**Biochemical assays of adult rodent sera**

Another whole blood aliquot was allowed to clot to produce serum. Concentrations of selected cytokines, RANKL, osteocalcin, and OPG were measured using commercially available, species-specific Luminox Ab-immobilized microbead kits (Lincos Research). Levels of tartrate-resistant acid phosphatase 5B (TRAP-5B), a bone resorption marker, were evaluated with commercially available, species-specific ELISA kits (Innologics) with a 0.1 U/liter as the lower limit of sensitivity. Major Ig classes and subclasses were examined by species-specific capture and detection Ab pairs (Bethyl Laboratories) using purified, species-specific Ig isotype controls for mouse and rat Ig standards (Southern Biotechnology Associates and BD Pharmingen).

**Biochemical assays of rat embryos**

OPG-Tg and WT rat conceptions were obtained at GD 11, GD 16, and GD 19. A small piece of tissue from each conceptus was used to identify Tg or WT genotype by PCR analysis. Each embryo was then frozen in liquid nitrogen and pulverized, after which protein was extracted with a standard digestion buffer (50 mM Tris buffer, pH 7.4, containing 0.1 M sodium chloride and 0.1% Triton X-100). Concentrations of rat OPG and RANKL were measured in embryonic protein extracts using by using Luminox Ab-im mobilized microbead kits (Lincos Research) and values were normalized to total protein concentration, which was determined using a standard kit (Bicinchoninic Acid Protein Assay; Pierce).

**Histology and immunohistochemistry**

Groups of OPG-Tg and WT rat embryos, GD 19, were fixed in zinc-formalin (Z-fix; Anatech), parsed into transverse thoracic and abdominal blocks, two of each, and then processed into paraffin. Serial 5-μm-thick cross-sections were deparaffinized, and all the odd-numbered sections were stained with H&E. The even-numbered sections were pretreated with antigen retrieval citra (Bio Genex), blocked with CAS-block (Zymed), incubated with rabbit polyclonal anti-CD3 (DakoCytomation) at 2.5 μg/ml for 30 min at room temperature, and then quenched with 3% H2O2. The location of the anti-CD3 Ab was detected by EnVision-labeled polymer HRP (DakoCytomation) followed by application of diaminobenzidine (DakoCytomation). All sections were then counterstained with H&E.

**Selected lymphoid tissues (mesenteric lymph nodes and spleen)** of 6-mo-old OPG-Tg and WT mice and rats were immersed in Z-fix, embedded in paraffin, and then sectioned serially at 5 μm. Sections were stained with H&E or anti-CD3, and the organ size and architecture were evaluated by a veterinary pathologist (B.B.) who was blinded to the genotype of the analyzed tissues.

**MicroCT imaging and bone mineral density assessments**

One-day-old OPG-Tg and WT rat pups were genotyped, as described above, then euthanized and examined with an xPlore MS MicroCT system (GE Healthcare). One neonate for each genotype was placed in the MicroCT scanner in a 60-mm-diameter acrylic tube with a density phantom. The tubes were filled with PBS and stabilized with gauze. Using volumetric conebeam technology, whole animals were scanned at 0.5° rotations for 200° (80 kVp and 80 μA) and reconstructed to yield images with a voxel size of 33 × 33 × 33 μm. Scans were examined by three-dimensional surface rendering with a common threshold, optimized using histomorphometric techniques (GE Healthcare MicroView).

Areal bone mineral density (BMD) of the left hind limb was measured in 6-mo-old OPG-Tg and WT rats by dual x-ray absorptiometry (QDR 4500a; Hologic). Volumetric BMD was measured at the proximal tibial metaphysis of 6-mo-old OPG-Tg and WT mice by peripheral quantitative computed tomography (Stratec XCT-960M; Stratec Medizintechnik).

**In vitro splenocyte culture**

Mouse or rat spleens were removed aseptically, disrupted, and incubated with ammonium chloride solution for 10 min to lyse RBC. Splenocytes from each individual animal were cultured in triplicate (1 × 106 splenocytes or 2 × 106 mouse splenocytes per ml) in 12-well plates (Falcon; BD Biosciences) in RPMI 1640 (Invitrogen Life Technologies) supplemented with 10% FBS (Stem Cell Technologies) and 1 ml of 100× penicillin-streptomycin-glutamin mixture (Invitrogen Life Technologies) per 99 ml of basal medium, yielding an iso-osmotic solution at final dilution. Cultures were maintained for 72–80 h at 37°C in an atmosphere of 5% CO2/air. At the end of each study, the number of cells in each well was evaluated by using an ELISA-based cell proliferation assay (Chemicon International).

**Splenic T and B cell proliferation**

Splenocytes were cultured in the presence or absence of anti-CD3 (BD Pharmingen) or anti-IgM (anti-mouse; Jackson ImmunoResearch Laboratories) and anti-rat; Southern Biotechnology Associates) at 1 μg/ml for 72–80 h. Cultured cells were immunophenotyped as described above, where the extent of proliferation was calculated as the fold increase of a given lymphocyte subset in the presence of T or B cell specific polyclonal activator over the subset of T or B lymphocytes in untreated splenocyte culture.

**Induction of contact hypersensitivity**

All mice were first sensitized by applying a 0.3% solution of oxazolone (Sigma-Aldrich) in acetone and olive oil onto the shaved skin of the abdomen. Seven days after sensitization, i.e., on study day 0, 1% oxazolone was applied to the right ear and vehicle (acetone/olive oil) was applied to the left ear of experimental animals. Vehicle was applied to both ears in control groups. Ear thickness was measured daily with a micrometer (Mitutoyo) starting immediately before the challenge. Results were expressed as the difference in ear thickness between right and left ears.

**Induction of the innate immune response by LPS**

Mice and rats were given a single i.v. injection of 10 or 100 μg (−0.5 ml/kg) of LPS or endotoxin, respectively (LPS was from Escherichia coli strain no. 055:B5 and catalog no. L2880; Sigma-Aldrich). For WT and OPG-Tg control groups, dexamethasone (DEX; American Pharmaceutical Partners) was given as a single s.c. injection of 100 μg or 1 mg (−5 mg/kg)
for mice or rats, respectively, 1 h before LPS treatment. Animals were bled immediately before and at 90 min, 3, and 6 h after LPS injection. Mouse and rat serum was analyzed for levels of TNF-α, IL-6, IL-1β, MCP-1, IL-8, GM-CSF, and IFN-γ using commercially available Luminex Ab-immobilized microbead kits (Lincor Research).

Induction of Ag-specific Abs in vivo and Ag-specific proliferation of splenic T lymphocytes in vitro

Mice were immunized on day 0 by s.c. injection of 100 μg of keyhole limpet hemocyanin (KLH) (Pierce) in IFA (BD Difco) or by i.p. injection of 115 μg of Pneumovax (Merck). Animals were bled immediately before and on days 7, 14, and 21 after immunization. Serum was analyzed for anti-KLH IgG and anti-Pneumovax IgM by ELISA as previously described (26). For the KLH-specific splenocyte proliferation assay, spleens were removed on day 21 post-KLH sensitization and cultured in triplicate for 4 days culture. For the KLH-dependent splenic T cell proliferation was evaluated together with immunophenotyping of T cells with anti-CD4 and anti-CD8 (BD Pharmingen) markers using FACS analysis (FACSCalibur).

Statistical analysis

Results are expressed as mean ± SEM. Comparisons between the results were made using the Student t test. A value of p = 0.05 was used to indicate significant differences between groups.

Results

OPG-Tg rat fetuses had a high bone mass phenotype but normal development of major lymphoid organs

Tg rats overexpressed a soluble rat OPG cDNA under control of the hepatic promoter ApoE. The same transgene construct was used previously in mice to demonstrate the role of OPG in suppressing bone resorption and preventing bone loss. These original OPG-Tg mice had high bone mass but no obvious differences in the development of the spleen, thymus, Peyer’s patches, or lymph nodes (7). In the present study, Tg rats were deemed preferable to mice due to their larger size, which facilitated early identification of lymphoid anlagen by histology. Total protein was extracted from whole OPG-Tg rat embryos to determine the prenatal timing of transgene overexpression. By GD 11, OPG protein levels in OPG-Tg fetuses were already ~100-fold greater than levels found in age-matched WT controls (Fig. 1a). A similarly high level of OPG overexpression was maintained until GD 19 (i.e., throughout the remainder of gestation). In rat prenatal development, formation of lymphoid organs begins between GD 13 and 15 (25). The locations (data not shown) and architecture of lymphoid nodules (lymph node precursors), spleen, and thymus in near-term GD 19 OPG-Tg fetuses were comparable to those of age-matched WT fetuses (Fig. 1, b and c). Similar T cell localization (by anti-CD3 staining) within lymphoid nodules and thymus was also apparent (Fig. 1, b and c). Prenatal inhibition of RANKL was confirmed by microcomputed tomography assessment of 1-day-old rat pups, which showed a high bone mass phenotype relative to age-matched WT littersmates (Fig. 1d).

Adult OPG-Tg rats exhibited no histologic changes in major lymphoid organs

Evaluation of immune system organs provided by a veterinary pathologist blinded to the genotype of analyzed tissues revealed that 6-mo-old OPG-Tg rats had lymphoid organs of expected size, shape, and structure. Mesenteric lymph nodes were detected in the proper locations, with similar numbers of follicles and equivalent cell distributions relative to WT controls (Fig. 2). In like manner, histologic analysis of spleen tissue showed no architectural differences between 6-mo-old OPG-Tg rats and their WT littermates. Extra-medullary hematopoiesis was apparent in the red pulp of OPG-Tg rat spleens (Fig. 2). A similar phenomenon was previously found in OPG-Tg mice and was attributed to reduced bone marrow space, secondary to chronic suppression of bone resorption (7). The appearance of thymus was also comparable for animals of both genotypes (data not shown).

Continuous RANKL inhibition increased bone density in adult mice and rats without affecting peripheral blood leukocyte composition, serum Ig levels, or serum cytokine levels

Profound RANKL inhibition in 6-mo-old OPG-Tg rats was confirmed by measuring significantly altered levels of several serum biomarkers and their corresponding anatomic impact. Levels of OPG were markedly increased (p < 0.05 compared with WT) and those of the bone resorption marker serum TRAP-5B were substantially suppressed (p < 0.05 compared with WT) in OPG-Tg rats, whereas BMD in appendicular bones was enhanced (p < 0.05 compared with WT) in OPG-Tg vs WT animals. A similarly high level of OPG overexpression was maintained until GD 19 (i.e., throughout the remainder of gestation). In rat prenatal development, formation of lymphoid organs begins between GD 13 and 15 (25). The locations (data not shown) and architecture of lymphoid nodules (lymph node precursors), spleen, and thymus in near-term GD 19 OPG-Tg fetuses were comparable to those of age-matched WT fetuses (Fig. 1, b and c). Similar T cell localization (by anti-CD3 staining) within lymphoid nodules and thymus was also apparent (Fig. 1, b and c). Prenatal inhibition of RANKL was confirmed by microcomputed tomography assessment of 1-day-old rat pups, which showed a high bone mass phenotype relative to age-matched WT littersmates (Fig. 1d).

FIGURE 1. OPG-Tg rat conceptsutes exhibit high concentrations of OPG protein throughout development leading to a high bone mass phenotype at birth but no alterations in the genesis of major lymphoid organs. a, OPG concentrations in protein extracts from rat embryos (GD 11 and GD 15) and fetuses (GD 19) measured by Luminex-based single-plex assay and adjusted to the level of total protein. Bars represent means ± SE (n = 4 per group per GD). Asterisks (*) denote a significant difference (p < 0.05) in group mean values for age-matched OPG-Tg vs WT animals. b, Near-term OPG-Tg and WT rat fetuses (GD 18–20) have similar histologic architecture of mesenteric lymph node anlagen. Immunohistochemistry was performed with anti-CD3 (H&E counterstain) to confirm lymph node location by revealing T lymphocytes. Magnification, ×20. c, Near-term OPG-Tg and WT rat fetuses (GD 18–20) have comparable histologic architecture of thymus. Immunohistochemistry was performed with anti-CD3 (H&E counterstain) to reveal T lymphocytes. Magnification, ×20. d, Neonatal OPG-Tg rats exhibit a high bone mass phenotype on postnatal day 1, as indicated by increased density of femoral bone mass in three-dimensional surface rendered MicroCT images. For both genotypes, the left panel (circular) represents a mid-diaphyseal cross section while the right panel is a frontal slice.
FIGURE 2. Adult (6-mo-old) OPG-Tg and WT rats exhibit equivalent histologic architecture of mesenteric lymph node (H&E staining 4×) and spleen (H&E staining 10×), including evidence of extra-medullary hematopoiesis in the splenic red pulp (RP). WP, white pulp (leukocyte zone).

FIGURE 3. OPG overexpression leads to a high bone mass phenotype as a consequence of diminished bone resorption in adult (6- to 8-mo-old) Tg mice and rats. a, Serum OPG concentrations were significantly elevated (*, p < 0.05) in OPG-Tg rodents relative to WT controls when measured with a Luminex-based single-plex assay. Bars represent means ± SE (n = 10–12 animals per genotype per species). b, Serum levels of the bone resorption marker TRAP-5B were significantly reduced (*, p < 0.05) in OPG-Tg rodents compared with WT littermates when quantified with species-specific enzyme activity assays. Bars represent means ± SE (n = 10–12 animals per genotype per species). c, BMD was significantly enhanced (*, p < 0.05) in the rat left hind limb (by dual x-ray absorptiometry; left panel) and the mouse proximal tibial metaphysis (by peripheral quantitative computed tomography; right panel). Bars represent means ± SE (n = 10–12 animals per genotype per species).

The high bone mass phenotype previously reported in OPG-Tg mice (7) was confirmed to result from chronic RANKL blockade to validate their use in additional experiments. As expected, 6-mo-old OPG-Tg mice showed increased serum OPG, reduced serum TRAP-5B, and increased BMD compared with age-matched WT controls (Fig. 3).

In the absence of induced immune challenges, absolute numbers and percentages of major leukocyte populations (lymphocytes, granulocytes, and monocytes) in the blood of 6-mo-old OPG Tg mice and rats were equivalent to those measured in age-matched WT controls (data not shown). Serum levels of immunoglobulins (IgM, IgG, IgA, and IgE) were similar in unchallenged 6-mo-old OPG-Tg mice and rats compared with their age-matched WT controls (Table I).

Serum concentrations of major cytokines and growth factors IL-1, IL-2, IL-6, IL-10, IL-12, TNF-α, IFN-γ, GM-CSF, IL-8, and MCP-1 were also similar in unchallenged OPG-Tg rats and mice, within a particular species, compared with their age-matched WT littermates (Table II). The only statistically significant difference noted in cytokine levels between OPG-Tg and WT animals was for serum IL-4, but the differences were modest and the direction of change was not consistent between the two rodent species.

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Adult OPG-Tg mice and rats showed no differences in splenic lymphocyte composition or in stimulated proliferation of cultured splenic lymphocytes

The absolute number and percentage of T and B lymphocytes in the spleen of 6-mo-old OPG-Tg mice and rats did not differ from WT controls (data not shown). Splenocytes isolated from OPG-Tg mice had a higher percentage of CD11b+ cells (i.e., myeloid lineage cells) compared with WT mice (18.9 ± 1.7% vs 8.9 ± 0.9%, respectively; p < 0.05). However, in rats there was no difference in the percentage of CD11b+ cells in splenocyte suspensions prepared from OPG-Tg vs WT animals (24.6 ± 1.6% vs 25.9 ± 1.8%, respectively). Since OPG-Tg mice, but not rats, had a greater splenic population of CD11b+ cells, we studied whether a higher percentage of these myeloid cells was associated with alterations in delayed contact hypersensitivity (DTH) to oxazolone in mice. OPG overexpression had no effect on DTH development, as shown by similar increases in ear thickness in oxazolone-challenged OPG-Tg mice and WT littermates (Fig. 4).

Lymphocyte function was tested in vitro by splenocyte proliferation for 72 h in the presence or absence of cell type-specific stimulators anti-CD3, a polyclonal activator for T cells (Fig. 5a), and anti-IgM, a polyclonal activator for B cells (Fig. 5b). Conventional FACS analysis of rat splenocytes exposed to anti-CD3 revealed similar 0–72 h increases in the percentage of T lymphocytes in culture from 4.7 ± 0.4% to 39.6 ± 2.3% in OPG-Tg rats and from 4.5 ± 1.2% to 36.9 ± 5.9% in age-matched WT controls (p = 0.59 between anti-CD3 stimulated OPG-Tg and WT rat splenic T cells). Cultured mouse splenocytes isolated from OPG-Tg and WT mice also showed similar and marked proliferation capacity in the presence of anti-CD3, i.e., percentage of T cells rose from 12 to 13% (±1.2%) and from 88 to 92% (±5.7%) after culture for 72 h (p = 0.85 between anti-CD3 stimulated OPG-Tg and WT mouse splenic T cells). Upon anti-IgM stimulation, both mouse and rat splenic B lymphocytes from OPG-Tg and WT groups showed similar increase in proliferation, 18–24%, over non-anti-IgM stimulated splenocytes (p = 0.73 between anti-IgM stimulated OPG-Tg and WT mouse splenic B cells; p = 0.40 between anti-IgM stimulated OPG-Tg and WT rat
both mice and rats, the peak concentration of endotoxin-induced TNF-α and IL-6 responses in both OPG-Tg and WT rodents. In both mice and rats, the peak concentration of endotoxin-induced TNF-α in sera occurred 90 min after LPS challenge (Fig. 6). In OPG-Tg and WT mice, systemic levels of TNF-α increased from 30 and 50 pg/ml (baseline, before LPS challenge) to 5.2 ± 0.95 and 4.2 ± 0.7 ng/ml, respectively (p = 0.39 between OPG-Tg and WT). A similar response was observed in rats, where LPS also caused marked increases in serum TNF-α in animals of both genotypes within 90 min (fold increases over baseline of 153 ± 28 in OPG-Tg rats vs 121 ± 22 in WT controls; p = 0.49).

DEX was used in this LPS model as a positive control for immune suppression. DEX was shown to cause similar and significant (75–90%) inhibitory effects on the peak (90 min) LPS-induced TNF-α release in OPG-Tg and WT mice and rats (p = 0.43 and p = 0.46 for mice and rats, respectively) (Fig. 6).

The IL-6 response to LPS challenge was maximal in mice and rats after 3 h, and there were no significant differences in IL-6 response between OPG-Tg mice or rats vs their WT littermate controls (Fig. 6). The inhibitory effect of DEX on the IL-6 response to LPS was relatively greater in rats, 90–98% suppression in OPG-Tg and WT rats, compared with the inhibition noted in mice, ~40% suppression in OPG-Tg and WT mice. Despite this interspecies difference in sensitivity to DEX, there was no effect of OPG overexpression on IL-6 levels in the presence or absence of LPS challenge (Fig. 6).

The responses of IL-1β, IL-8, GM-CSF, and IFN-γ to LPS challenge were maximal in mice and rats at 3–6 h, and there were no significant differences in cytokine levels between OPG-Tg mice or rats vs their WT littermate controls (data not shown).

### OPG overexpression did not affect humoral reactions

OPG-Tg mice and their age- and sex-matched WT littermates were used to study the development of humoral immune responses to either KLH (a T cell-dependent Ag) or Pneumovax (a T cell-independent Ag). Life-long RANKL inhibition by OPG overexpression did not affect production of KLH-specific IgG (Fig. 7a). Interestingly, RANKL inhibition led to a modest and transient increase in Pneumovax-specific IgM in OPG-Tg mice at day 7 (p < 0.05 compared with WT), whereas no significant differences were observed between OPG-Tg vs WT animals at day 14 or 21 (Fig. 7b). To explore whether OPG overexpression modulates the generation and function of Ag-specific memory T cells, we isolated splenocytes from mice 21 days after KLH immunization and studied splenocyte proliferation in vitro in the presence of KLH. Splenic CD4+ and CD8+ T lymphocytes from KLH-sensitized OPG-Tg and WT mice showed similar KLH dose-dependent proliferation in vitro, suggesting that RANKL inhibition in vivo had no effect on T cell priming (Fig. 8).

### Discussion

The essential role of RANKL in the formation, function, and survival of osteoclasts is well established (4, 27). This TNF family member is required for the existence of osteoclasts and for the resorption of bone, as shown by the total absence of osteoclasts in knockout mice that lack either RANKL (8) or its receptor RANK (1, 2). Analysis of these knockout animals also revealed an essential role for RANKL in the formation of lymph nodes. Genetic ablation of RANKL was associated with lymph node agenesis, although Peyer’s patches appeared normal and splenic lymphoid areas remained intact. Interestingly, T and B cells from RANKL knockout mice were able to populate WT

### Table I. Serum levels of immunoglobulins in unchallenged OPG-Tg rodents and their age- and sex-matched WT littermates

<table>
<thead>
<tr>
<th>Immunoglobulin</th>
<th>OPG-Tg</th>
<th>WT</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgG (mg/ml)</td>
<td>5.5 ± 0.8</td>
<td>3.9 ± 0.7</td>
<td>0.15</td>
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<tr>
<td>IgM (mg/ml)</td>
<td>0.3 ± 0.02</td>
<td>0.3 ± 0.02</td>
<td>0.95</td>
</tr>
<tr>
<td>IgE (μg/ml)</td>
<td>4.2 ± 0.9</td>
<td>3.2 ± 0.7</td>
<td>0.41</td>
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<tr>
<td>IgA (μg/ml)</td>
<td>470 ± 54</td>
<td>471 ± 31</td>
<td>0.99</td>
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</table>

### Table II. Serum concentrations of cytokines in unchallenged OPG-Tg rodents and their age- and sex-matched WT littermates

<table>
<thead>
<tr>
<th>Cytokine (pg/ml)</th>
<th>OPG-Tg</th>
<th>WT</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1</td>
<td>27.3 ± 4.9</td>
<td>32.2 ± 2.2</td>
<td>0.46</td>
</tr>
<tr>
<td>IL-2</td>
<td>7.0 ± 2.6</td>
<td>4.1 ± 1.5</td>
<td>0.32</td>
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<tr>
<td>IL-4</td>
<td>0.9 ± 0.1</td>
<td>1.3 ± 0.1</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>IL-6</td>
<td>4.0 ± 0.6</td>
<td>4.0 ± 0.6</td>
<td>0.99</td>
</tr>
<tr>
<td>IL-8</td>
<td>2.4 ± 0.2</td>
<td>4.8 ± 0.7</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>IL-10</td>
<td>44.6 ± 9.2</td>
<td>44.1 ± 1.6</td>
<td>0.95</td>
</tr>
<tr>
<td>IL-12</td>
<td>72.4 ± 21.0</td>
<td>122 ± 25.3</td>
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<tr>
<td>TNFα</td>
<td>27.9 ± 4.9</td>
<td>34.9 ± 2.3</td>
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<tr>
<td>IFN-γ</td>
<td>9.1 ± 0.6</td>
<td>11.3 ± 1.6</td>
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<tr>
<td>GM-CSF</td>
<td>38.9 ± 7.2</td>
<td>34.5 ± 5.4</td>
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<tr>
<td>MCP-1</td>
<td>85.5 ± 10.4</td>
<td>103 ± 9.6</td>
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</tbody>
</table>
lymph nodes in vivo and DCs appeared normal (8). These observations suggest that the immune phenotype of RANK and RANKL knockout mice might be largely restricted to lymph node agenesis and the consequences thereof. However, it is also possible that RANKL plays an ongoing role in the function of the developed immune system. RANKL inhibition is an experimental therapeutic approach for chronic bone loss conditions such as osteoporosis and rheumatoid arthritis, so it is important to characterize the potential for immunomodulatory effects during long-term RANKL inhibition. We attempted to address these issues by studying the effects of prenatal and life-long RANKL inhibition on the development of the immune system and on functional immune responses in adult animals.

RANKL activity is inhibited endogenously by OPG, a soluble decoy receptor of the TNF receptor family. OPG was discovered based on its ability to markedly increase bone mass in Tg mice that overexpressed this soluble protein (7). In contrast to the immune phenotype of RANKL knockout mice, OPG-Tg mouse develop normal lymph nodes. The lack of an immune phenotype in OPG-Tg mice suggests that a minor amount of prenatal RANKL activity is sufficient for lymph node formation. The expression of rat OPG mRNA was detected in liver of OPG-Tg mouse embryos at the one fetal time point tested, GD 12.5 (28). However, the onset of OPG-Tg protein production was not characterized in these mice and it remained possible that lymph node formation in these animals occurred before significant overexpression of OPG protein.

We generated a novel OPG-Tg rat model exhibiting profound RANKL inhibition during both prenatal development and postnatal maturation. Our OPG-Tg rats overexpressed the same full-length rat OPG transgene construct under control of the same ApoE promoter that was used in the generation of the OPG-Tg mice (7). Rats were used in favor of mice because of their larger size, which facilitates the identification of early lymphoid nodules during fetal development. Analysis of OPG protein levels in protein extracts from rat conceptuses revealed ~100-fold overexpression by GD 11, which is several days before the first appearance (GD 13–15) of lymphoid nodules during rat embryogenesis (25). By GD 19, the structure and location of the anlagen for lymph nodes, spleen, and thymus were similar in both OPG-Tg and WT rat fetuses. Consistent with prenatal inhibition of RANKL, 1-day-old OPG-Tg rat pups were shown to have a high bone mass phenotype. Adult OPG-Tg animals retained the normal location and

**FIGURE 4.** Life-long OPG overexpression did not affect contact hypersensitivity in mice. OPG-Tg mice and their sex- and age-matched WT littermates (4-to 6-mo-old females) were challenged with oxazolone on day 0 (7 days after initial sensitization with oxazolone). The change (Δ) in ear thickness was similar at all time points between OPG-Tg and WT control mice (n = 5–8 per group).

**FIGURE 5.** Proliferation of splenic lymphocytes in vitro following Ag stimulation was comparable for OPG-Tg and WT rodents. a, Splenocytes from OPG-Tg and WT mice and rats were cultured for 72 h in the presence of anti-CD3 (a super-antigen for T lymphocytes). The proliferation rate (%) was calculated by dividing the percentage of gated CD4⁺ and CD8⁺ T lymphocytes with anti-CD3 stimulation by a percentage of gated CD4⁺ and CD8⁺ T lymphocytes without anti-CD3 stimulation. Data represent group means ± SE (three culture wells per individual splenocyte suspension per culture condition, n = 5–8 animals per group). b, Splenocytes from OPG-Tg and WT mice and rats were cultured for 72 h in the presence of anti-IgM, a super-antigen for B lymphocytes. The proliferation rate was calculated for the B220⁺ mouse B lymphocyte population or CD45R⁺ rat B lymphocytes as = [percentage of gated B lymphocytes with anti-IgM stimulation/percentage of gated B lymphocytes without anti-IgM stimulation] × 100. Data represent group means ± SE (three culture wells per individual splenocyte suspension per culture condition, n = 5–8 animals per group).
and rats (b) 2 h before LPS injection (0.5 mg/kg) and at 1.5, 3, and 6 h post-LPS challenge (n = 6 animals per genotype per species). Additional animals (n = 6 animals per genotype per species) received DEX (5 mg/kg) 1 h before LPS injection. a, p < 0.05 for WT (LPS) vs WT (LPS plus DEX), and b, p < 0.05 for OPG-Tg (LPS) vs OPG-Tg (LPS plus DEX).

FIGURE 6. The production of major proinflammatory cytokines following LPS challenge is similar in adult (6-mo-old) OPG-Tg and WT rodents. Serum levels of TNF-α and IL-6 levels were measured in mice (a) and rats (b) 2 h before LPS injection (0.5 mg/kg) and at 1.5, 3, and 6 h post-LPS challenge (n = 6 animals per genotype per species). Additional animals (n = 6 animals per genotype per species) received DEX (5 mg/kg) 1 h before LPS injection. a, p < 0.05 for WT (LPS) vs WT (LPS plus DEX), and b, p < 0.05 for OPG-Tg (LPS) vs OPG-Tg (LPS plus DEX).

histologic architecture of lymphoid organs and high bone mass phenotype. These observations indicate that, in contrast to com-plete RANKL ablation via engineered null mutations to the Rankl gene, prenatal RANKL inhibition does not have an impact on the formation of spleen and thymus, and does not lead to lymph node agenesis.

Those results per se did not exclude the possibility that RANKL might play an ongoing role in the postnatal function of the developed immune system. To study that possibility, we used adult OPG-Tg rats and/or mice to evaluate the potential consequences of life-long continuous RANKL inhibition on baseline cellular, innate, and humoral immune responses in vitro and in vivo. The use of OPG-Tg mice and rats allowed us to evaluate the consistency of effects elicited by RANKL inhibition in two different species. At 6 mo of age, OPG-Tg mice and rats had serum OPG levels that were 80- to 100-fold increased relative to WT littermate controls. Overexpression of OPG in both species was associated with an expected reduction in bone resorption and a significant increase in BMD. In the absence of immune challenge, OPG-Tg rats and mice were similar to their WT controls with respect not only to histo-logic architecture of spleen and lymph nodes, but also the number and percentage of circulating lymphocytes, granulocytes and monocytes, as well as circulating levels of immunoglobulins, growth factors, and cytokines. The only statistically significant differ-ences observed with OPG overexpression (serum IL-4 levels, CD11b+ and CD4+ splenocyte percentages) were modest, and those changes were not consistent across species.

We also employed several in vivo challenge models in adult (6-mo-old) OPG-Tg rats and/or mice to test dynamic cellular, hu-moral, and innate immune responses. Cellular immune responses were evaluated in OPG-Tg mice and rats using a DTH model of contact hypersensitivity. The lack of apparent difference in the response of OPG-Tg vs WT animals suggests that the cross-talk between T cells and DCs was not significantly influenced by life-long RANKL inhibition. This conclusion is not inconsistent with in vitro data showing that the binding of RANKL from T cells to RANK on DCs can affect DC survival (10). Although RANKL is able to stimulate DCs in vitro and affect their longevity, RANKL inhibition need not result in DC suppression. The interaction be-tween CD40L and CD40, found on T cells and DCs, respectively, has been shown to comprise the major pathway for maintaining adequate cross-talk between T cells and DCs (13, 14) rather than the RANKL-RANK interaction.

T cell-independent humoral immune responses were evaluated in OPG-Tg mice by evaluating specific Ab production by B cells against Pneumovax (IgM). There was no apparent difference in this response between OPG-Tg mice and WT animals. OPG-Tg mice also showed no differences from WT controls in their T cell-dependent Ab production against KLH. These observations are consistent with previous studies in RANKL knockout mice, in which KLH immunization led to the normal formation of germinal centers despite the altered splenic microarchitecture (defects in marginal zone integrity) associated with absence of RANKL (29). Moreover, our data indicate that when splenic T cells from KLH-sensitized OPG-Tg mice were re-exposed to KLH in vitro, the proliferation of Ag specific CD4+ or CD8+ T memory cells was similar between OPG-Tg animals and WT controls. These results suggest that RANKL inhibition does not affect the initiation and maintenance of humoral immune responses in vivo, since the absence or inhibition of RANKL does not impact the proper forma-tion of B cell follicles in spleen, the generation of clonal splenic memory T cells, and the production of Ag-specific Abs in normal amounts.

The innate immune system is present at birth and changes little throughout life. Having established that RANKL was inhibited prenatally in OPG-Tg rats, we examined whether innate immune responses were influenced by continuous life-long RANKL inhibi-tion in adult OPG-Tg rats and mice. After challenge with LPS (a product of gram-negative bacteria) systemic indicators of innate immune responses to LPS, including TNF-α and IL-6, were sim-ilar between OPG-Tg animals and WT controls. These results suggest that RANKL inhibition does not affect the initiation and maintenance of humoral immune responses in vivo, since the absence or inhibition of RANKL does not impact the proper forma-tion of B cell follicles in spleen, the generation of clonal splenic memory T cells, and the production of Ag-specific Abs in normal amounts.

FIGURE 7. RANKL inhibition by lifelong OPG overexpression did not affect T cell-dependent and T cell-independent immune responses in adult (5- to 6-mo-old) mice. a, OPG-Tg animals or their WT littermates immu-nized with KLH (a T cell-dependent Ag) on day 0 had similar KLH-spe-cific IgG responses over the next 3 wk (at days 7, 14, and 21 after KLH immunization). Data represent means ± SE (n = 6–8 per genotype). b, OPG-Tg animals immunized with Pneumovax (a T cell-independent Ag) on day 0 had significantly higher serum concentrations of Pneumovax-specific IgM relative to their WT littermates 7 days after immunization (p < 0.05) but not at subsequent time points (day 14 and day 21 after Pneumovax immunization). Data represent means ± SE (n = 6–8 per genotype).
DEX in both OPG-Tg and WT controls. It was recently reported that with complete absence of RANKL (in RANKL knockout mice), an increased TNF-α and IL-6 response to LPS could be seen compared with WT controls (30). The lack of such cytokine changes in our adult OPG-Tg rat and mouse models provides another example wherein total genetic ablation of RANKL leads to structural and functional changes in the immune system that are not recapitulated by RANKL inhibition. Other examples include the lack of tooth eruption and defects in mammary gland development that are apparent in RANKL knockout mice (1, 2, 8, 31) but which have not been observed in OPG-Tg mice (7) or the OPG-Tg rats used in this study (unpublished observations).

In summary, these results suggest that the inhibition of RANKL from an early prenatal stage and throughout later life does not significantly influence the development of the rodent immune system, nor does continuous RANKL inhibition appear to impair or accentuate immune responses in adult animals. These results are consistent with the possibility that many of the immune-related changes in animals that lack RANK or RANKL may be secondary to the absence of lymph nodes and/or the lack of tooth eruption and defects in mammary gland development that are apparent in RANKL knockout mice (1, 2, 8, 31) but which have not been observed in OPG-Tg mice (7) or the OPG-Tg rats used in this study (unpublished observations).

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