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Protection against Osteoporosis by Active Immunization with TRANCE/RANKL Displayed on Virus-Like Particles

Gunther Spohn,1,* Katrin Schwarz,* Patrik Maurer,* Harald Illges,2†‡ Narendra Rajasekaran,‡ Yongwon Choi,§ Gary T. Jennings,* and Martin F. Bachmann*

TNF-related activation-induced cytokine (TRANCE), also known as receptor activator of NF-κB ligand (RANKL), is the key molecule responsible for the bone loss observed in osteoporosis. Passive administration of osteoprotegerin, the soluble decoy receptor of TRANCE/RANKL, is efficient in blocking disease progression, but may not find widespread clinical use due to patient compliance problems and the expected high costs. In this study, we describe an efficient, safe, and potentially cost-effective active immunization strategy against TRANCE/RANKL. We show in mice that immunization with TRANCE/RANKL covalently linked to virus-like particles can overcome the natural tolerance of the immune system toward self proteins and produce high levels of specific Abs without the addition of any adjuvant. Serum Abs of immunized mice neutralized TRANCE/RANKL activity in vitro and were highly active in preventing bone loss in a mouse model of osteoporosis. Active immunization against TRANCE/RANKL was essentially reversible and did not produce any measurable immunosuppressive side effects, underscoring its potential as a new therapeutic approach to the treatment of human bone-degenerative disorders. The Journal of Immunology, 2005, 175: 6211–6218.

Osteoporosis is a metabolic disease, which affects ~30% of postmenopausal women and 10% of elderly men (1). Fractures resulting from decreased bone mass are characteristic of the disease and a major health problem, resulting in significant economic losses to society. Current therapies for the treatment of osteoporosis include mainly bisphosphonates and selective estrogen modulators, which are efficient in reducing bone resorption but carry the risk of considerable gastrointestinal and circulatory side effects. In addition, dosing regimens are sometimes difficult because of the poor bioavailability of some drugs, and patient compliance can be a major hurdle for efficient long-term treatment (2). A strong medical need for highly efficient antiresorptive agents with good safety and tolerability profiles therefore still exists.

Novel therapeutic approaches focus on specifically blocking the molecular interactions at the basis of the disease process. It has been shown that estrogen deficiency and the associated increase in the differentiation and activation of bone-resorbing osteoclasts with respect to bone-forming osteoblasts is the cause of osteoporosis in postmenopausal women. Osteoclastogenesis is initiated by the interaction of TNF-related activation-induced cytokine (TRANCE)3/receptor activator of NF-κB ligand (RANKL) (3, 4) expressed on stromal cells and RANK (4) on the surface of osteoclast precursors. In the presence of permissive concentrations of M-CSF, this receptor-ligand interaction triggers the activation of NF-κB in precursor cells, which leads to their differentiation into mature osteoclasts (for review, see Refs. 5 and 6). A soluble decoy receptor of TRANCE/RANKL called osteoprotegerin (OPG) has been shown to inhibit this process by preventing the binding of TRANCE/RANKL to RANK (7, 8). Administration of recombinant OPG has proven efficient in reducing osteoclast formation and bone resorption, thereby inhibiting bone loss in a variety of animal models (9–11). A genetically engineered OPG-Fc fusion protein has already proven efficient in reducing bone turnover in postmenopausal women (12) and multiple myeloma patients (13). Although attractive as a drug candidate for the treatment of osteoporosis, the expected need for frequent administration and the high cost of goods may preclude the use of OPG in a large fraction of the population. In addition OPG might not be optimal for long-term therapy because of possible inactivating Ab responses and because of its affinity to TRAIL (14), which is an important apoptotic factor for tumor cells (15) and thymocytes (16).

Active induction of specific Abs in the host against TRANCE/RANKL might be an alternative to the administration of OPG or mAbs. However, several obstacles must first be overcome. 1) The immune system is usually tolerant to its own proteins. This tolerance has to be broken or circumvented by immunization technologies that can be used in humans. 2) The Abs should neutralize the target protein, therefore, it is usually important to immunize against native full-length proteins to successfully target conformational epitopes. 3) For safety reasons, the Ab response should be essentially reversible.

Highly repetitive and organized Ags present on viruses or virus-like particles (VLPs) are potent inducers of Ab responses in the absence of adjuvants (17). Moreover, self-Ags displayed in such a fashion are able to break B cell unresponsiveness (18–21). In this study, we have used a vaccine consisting of the extracellular part of cAg, hepatitis B core Ag; HBcAgp33, hepatitis B core Ag fused to peptide p33 from LCMV glycoprotein.
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of TRANCE/RANKL covalently coupled to a VLP to induce autoantibodies that block the TRANCE/RANKL-RANK interaction in vivo. Immunization of mice resulted in high levels of specific Abs that neutralized TRANCE/RANKL in vitro and potently inhibited bone destruction in a murine model of osteoporosis.

Materials and Methods

Mice

Female C57BL/6 and BALB/c mice were purchased from Charles River Laboratories. All mice were maintained under specific pathogen-free conditions and used for experimentation according to protocols approved by the Swiss Federal Veterinary Office.

Cloning, expression and purification of murine TRANCE/RANKL

The nucleotide sequence encoding aa 33–212 of mature murine RANK was amplified by PCR using the oligonucleotide pair RANKL-up/RANKL-down (5′-CTGGCAAGGCGGGGTTGCG CCGTGGGCATCAACACCATACACAGGGCTTCCAGGAAG-3′/ 5′-CCGGCTTGAGATGTCACTGTCCATGAA-3′; underlined nucleotides indicate Apad and Xhol restriction sites, respectively) and cloned into the eukaryotic expression vector pCEP-SP-Xa-Fc (from P. Saudan, Cytos Biotechnology, Schlieren, Switzerland). The resulting plasmid encodes a fusion protein consisting of the extracellular part of murine RANK and the Fc part of a human IgG1 heavy chain. This plasmid was transfected into EBNA-293 cells and the supernatant of transfected cells was loaded on a protein A Sepharose column (Amersham Biosciences) for binding of the secreted RANK-Fc fusion protein. After washing with PBS, the bound RANK-Fc fusion protein was eluted with 50 mM citric acid, 150 mM NaCl (pH 3.0), and neutralized immediately by addition of 1 M Tris Cl (pH 9.0) (100 μl/ml eluate). The purified fusion protein was then dialysed against PBS and stored further under ~80°C.

Immunizations and ELISA analysis of Ab induction

Q8-C-TRANCE (50 μg) or Q8 VLPs alone (50 μg) were diluted in PBS to 200 μl and injected s.c. (100 μl on two ventral sides) in the absence of adjuvants. VLPs derived from the hepatitis B core Ag (HBAg) fused to a 14 kDa peptide p33 from lymphocytic choriomeningitis virus (LCMV) glycoprotein (HBcAg33, 50 μg) (23) were mixed with 20 nmol phosphothioate-modified CpG (5′-GGGTTCAACGTTGAGGGG-3′; Microsynth), diluted with PBS to 200 μl and injected in the same way. Sera from immunized mice were serially diluted in PBS containing 0.05% H₂O₂ (pH 5.0), 2% BSA and applied to ELISA plates (Nunc) which had been coated with 10 μg/ml recombinant C-TRANCE, TRAIL (BIOMOL), or HBcAg (23). Reactivity of serum Abs with the target protein was determined using HRP-conjugated goat anti-mouse IgG secondary Ab (Jackson ImmunoResearch Laboratories) at a dilution of 1/10,000 in PBS/0.05% Tween 20/2% BSA. After development with 1,2-phenylenediamine dihydrochloride (0.4 mg/ml in 0.066 M Na₂HPO₄, 0.035 M citric acid, 0.01% H₂O₂, (pH 5.0), OD₄₅₀ were determined using an ELISA reader (Bio-Rad). Titers were expressed as those serum dilutions which lead to half-maximal OD₄₅₀ (OD₅₀%).

Neutralization assay

Sera from immunized mice were serially diluted in PBS/0.05% Tween 20 containing a constant amount of 16 ng/ml recombinant murine RANK-Fc fusion protein and applied to ELISA plates that had been coated with 10 μg/ml C-TRANCE. Bound receptor was detected with HRP-conjugated goat anti-human IgG secondary Ab (Jackson ImmunoResearch Laboratories) at a dilution of 1/10,000 in PBS/0.05% Tween 20/2% BSA. After development with 1,2-phenylenediamine dihydrochloride (0.4 mg/ml in 0.066 M Na₂HPO₄, 0.035 M citric acid, 0.01% H₂O₂, (pH 5.0), OD₄₅₀ were determined using an ELISA reader (Bio-Rad). Titers were expressed as those serum dilutions which lead to half-maximal OD₄₅₀ (OD₅₀%).

Osteoclast assay

Bone marrow cells from a naïve C57BL/6 mouse were incubated overnight with recombinant mouse M-CSF (5 ng/ml) in α-MEM/10% FCS. Floating cells were collected and further cultured for 6–7 days in α-MEM/10% FCS supplemented with 50 μg/ml mouse M-CSF, 1 μg/ml PGE₂ and different concentrations of C-TRANCE, Q8-C-TRANCE, RANK-Fc, or OPG (Peprotech) as indicated. At day 3, the culture was changed with fresh media. Cells were fixed and stained for tartrate resistant acid phosphate using a leukocyte acid phosphatase kit (Sigma Diagnostics).

Ovariectomy-induced osteoporosis

In experiment 1, 12-wk-old female C57BL/6 mice (n = 22) were randomized into three different groups. One group was immunized with Q8-C-TRANCE at days 0, 14, 21, and 42, while the other two groups remained immunized without a complete break of the experiment program. At day 35 after the first immunization, the immunized group and one of the nonimmunized groups were subjected to complete ovariectomy, while the second nonimmunized group was subjected to sham operation. In experiment 2, 9-wk-old female C57BL/6 mice (n = 32) were randomized in four different groups. Two groups were immunized with Q8 carrier at days 0, 14, and 21 whereas the other two groups were injected with 200 μl of PBS at the same time points. At day 28 one Q8-immunized group and one PBS-treated group were subjected to complete ovariectomy, while the remaining groups were sham operated. All animals were sacrificed 35 days after the operation and the...
femurs were excised, cleaned from musculature, and postfixed for 1 day in fixative. The left femur of each mouse was used for the measurement of various bone parameters with a Stratec XCT-Research M pQCT apparatus (Stratec Biomedical Systems) 1.9 and 4 mm proximally from the distal end of the femur. The right femur of animals of experiment 1 was decalcified and matrix parameters of the area of secondary spongiosa were analyzed by histomorphometry according to standard procedures. Serum osteocalcin and deoxypyridinoline cross-links were measured with a mouse Osteocalcin IRMA kit (Immuno) and a DPD EIA kit (Quidel), respectively, according to the manufacturer’s instructions.

**FACS analyses**

C57BL/6 mice (n = 3) were immunized with Qβ-C-TRANCE at days 0 and 14. These mice as well as age-matched control mice (n = 3) were injected at day 44 with HBcAgp33/CpG. Eight days after injection of HBcAgp33/CpG peripheral blood from HBcAgp33- and Qβ-C-TRANCE/HBcAgp33-immunized mice, respectively, as well as from unimmunized control mice (n = 3) was collected in PBS, 2% FCS, 5 mM EDTA (pH 8.0), and cells were stained with the following Abs (BD Biosciences): rat anti-mouse CD4-allophycocyanin (clone RM4-5), rat anti-mouse CD8-PTTC (clone 53-6.7), rat anti-mouse CD44-PE (clone IM-7), rat anti-mouse CD69-PE (clone H1.2F3), anti-mouse CD8-allophycocyanin (clone 53-6.7), hamster anti-mouse CD69-PE (clone H1.2F3), anti-mouse CD8-allophycocyanin (clone 53-6.7), H2-D^d-p33-tetramer-PE was obtained from ProImmuno. After lysing the erythrocytes for 10 min in FACS lysing solution (BD Biosciences), cells were analyzed by flow cytometry on a FACSCalibur using CellQuest software (BD Biosciences).

**Vaccinia virus challenge**

C57BL/6 mice (n = 3) were immunized with Qβ-C-TRANCE at days 0 and 14. These mice as well as age-matched control mice (n = 3) were injected at day 44 with HBcAgp33/CpG. Immunized mice as well as naive mice (n = 3) were then challenged at day 52 by an i.p. injection of 2 × 10⁷ PFPUs of recombinant vaccinia virus expressing LCMV glycoprotein. Five days later ovaries were collected and smashed in MEM containing 2% FCS, and confluent BSC40 cells in 24-well plates were infected with serial dilutions of virus containing material. After a 1-h incubation at 37°C, the medium was changed to MEM containing 5% FCS and cells were incubated for 12–36 h at 37°C. Monolayers were stained with crystal violet and plaques were counted.

**Results**

**Production of a VLP-based autologous TRANCE/RANKL vaccine**

We recently reported the development of a modular assembly system that allows for the production of highly ordered Ag arrays on the surface of VLPs (20, 24). In this system the Ag of interest is modified to contain an N- or C-terminal cysteine residue, which can be covalently attached to a lysine residue on the surface of a VLP via a chemical cross-linker. The use of specific attachment sites and heterobifunctional cross-linkers ensures that the Ag is presented in an oriented and repetitive fashion, which promotes efficient cross-linking of B cell receptors and, consequently, the induction of a strong and long-lasting B cell response. We designed and produced a VLP-based vaccine consisting of murine TRANCE/RANKL covalently linked to VLPs derived from the bacteriophage Qβ. These VLPs form capsids with a diameter of 30–35 nm, which contain 180 monomers. The extracellular domain of murine TRANCE/RANKL spanning aa 158–316 was engineered to contain a cysteine at its C terminus and expressed in E. coli. This soluble recombinant TRANCE/RANKL derivative (C-TRANCE) was purified to homogeneity and shown to be biologically active as judged by its ability to induce osteoclast formation from bone marrow cells at a concentration of 1 μg/ml (data not shown). The osteoclastogenic activity of C-TRANCE was completely reversed by addition of 10 μg/ml of either OPG or a soluble RANK-Fc fusion protein, further confirming identity and activity of the purified protein (data not shown). Recombinant C-TRANCE was rendered highly repetitive by chemical coupling to Qβ VLPs.

The product of the coupling reaction was analyzed by SDS-PAGE and Western blot using Abs specific for either murine TRANCE/RANKL or Qβ. The Coomassie-stained SDS polyacrylamide gel demonstrated the presence of several bands in the coupling reaction with molecular masses corresponding to C-TRANCE molecules covalently linked to one or more Qβ monomers. Western blot analysis with either TRANCE/RANKL- or Qβ-specific Abs showed immunoreactive bands of the same size, confirming the successful covalent attachment of C-TRANCE to Qβ (Fig. 1A).

We calculated the degree of coupling to 14%, indicating that about one of seven Qβ monomers is covalently attached to a C-TRANCE monomer. As TRANCE/RANKL forms a trimer, it can be assumed that ~25 C-TRANCE trimers are displayed per VLP. Because of the larger size of the C-TRANCE trimer (63 kDa) compared with the Qβ monomer (14 kDa) we estimate that about half of the VLP surface is covered by C-TRANCE. Qβ-C-TRANCE was able to efficiently induce osteoclast formation from bone marrow cells at a concentration of 1 μg/ml, indicating that chemical cross-linking did not affect the biological activity of C-TRANCE (Fig. 1B).

**FIGURE 1.** Production of C-TRANCE-VLPs. A. Chemical cross-linking of C-TRANCE to Qβ-VLPs. VLPs of the bacteriophage Qβ were cross-linked via the bifunctional chemical cross-linker succinimidyl-6-(β-maleimidopropionamido)hexa noate to a modified TRANCE/RANKL protein containing an N-terminal cysteine residue (C-TRANCE). Derivatized Qβ (dQβ), C-TRANCE, and the coupled vaccine (Qβ-C-TRANCE) were analyzed by reducing SDS-PAGE (left panel), as well as TRANCE/ RANKL and Qβ-specific immunoblot (middle and right panels, respectively). Chemical cross-linking yields several high molecular mass bands (arrows) which react with both TRANCE/RANKL- and Qβ-specific Abs, indicating successful coupling of TRANCE/RANKL to the VLP subunits. B. Induction of osteoclastogenesis by C-TRANCE coupled to Qβ-VLPs. Freshly isolated bone marrow cells were incubated with M-CSF, PGE_2_, and 1 μg/ml of either Qβ-VLPs alone (left panel) or Qβ-C-TRANCE VLPs (right panel) and then fixed and stained for tartrate-resistant acid phosphatase. Osteoclasts appear as large, intensely red stained cells.
adjuvants. The immunization elicited TRANCE/RANKL-specific antibodies in mice by immunization with Q\(\beta\)C-TRANCE-VLPs. A. Induction of TRANCE/RANKL-specific autoantibodies in mice by immunization with Q\(\beta\)C-TRANCE. BALB/c mice (\(n = 4\)) were immunized at days 0, 16, and 64 (arrows) with Q\(\beta\)C-TRANCE in the absence of adjuvant and sera from the indicated time points were analyzed for TRANCE/RANKL-specific Abs in an ELISA. Average titers are given with SEs of the mean. B. Neutralizing activity of Abs induced by immunization with Q\(\beta\)C-TRANCE. C57BL/6 mice (\(n = 9\)) were immunized at days 0 and 14 with Q\(\beta\)C-TRANCE, and sera were collected at day 21. ELISA plates coated with C-TRANCE were incubated with a constant amount of RANK-Fc, a soluble version of the cognate receptor of TRANCE/RANKL, which was premixed with different dilutions of mouse sera. Bound receptor was then detected with an anti-Fc Ab (for immune sera, averages of nine mice are given with SDs).

FIGURE 2. Breaking of immunological tolerance and production of neutralizing Abs by TRANCE/RANKL coupled to VLPs. A. Induction of TRANCE/RANKL-specific autoantibodies in mice by immunization with Q\(\beta\)C-TRANCE. BALB/c mice (\(n = 4\)) were immunized at days 0, 16, and 64 (arrows) with Q\(\beta\)C-TRANCE in the absence of adjuvant and sera from the indicated time points were analyzed for TRANCE/RANKL-specific Abs in an ELISA. Average titers are given with SEs of the mean. B. Neutralizing activity of Abs induced by immunization with Q\(\beta\)C-TRANCE. C57BL/6 mice (\(n = 9\)) were immunized at days 0 and 14 with Q\(\beta\)C-TRANCE, and sera were collected at day 21. ELISA plates coated with C-TRANCE were incubated with a constant amount of RANK-Fc, a soluble version of the cognate receptor of TRANCE/RANKL, which was premixed with different dilutions of mouse sera. Bound receptor was then detected with an anti-Fc Ab (for immune sera, averages of nine mice are given with SDs).

Breaking of B cell unresponsiveness by immunization with Q\(\beta\)C-TRANCE

To investigate whether Q\(\beta\)C-TRANCE has the ability to overcome the natural tolerance of the immune system to endogenous proteins and induce an autoantibody response against TRANCE/RANKL, we immunized mice s.c. with 25 \(\mu\)g of Q\(\beta\)C-TRANCE in the absence of adjuvants. The immunization elicited TRANCE/RANKL-specific IgG responses with ELISA titers (OD50%) of \(~1/2,600\) before and \(1/8,400\) after a boost on day 16 (Fig. 2A). A further injection at day 64 was able to boost the Ab titers to \(1/19,000\). In the absence of further injections the anti-TRANCE/RANKL titers slowly declined over time with a half-life of roughly 2–3 mo (Fig. 2A). Taken together these data demonstrate that active immunization with Q\(\beta\)C-TRANCE could bypass immunological tolerance and yield a robust, yet reversible, anti-TRANCE/RANKL Ab response without the requirement of any adjuvant.

TRANCE/RANKL-specific Abs are neutralizing in vitro

Sera from immunized mice were analyzed in vitro for their ability to block the TRANCE/RANKL-RANK interaction. We performed a competition ELISA to test the ability of the immune sera to block binding of TRANCE/RANKL to recombinant RANK. Half-maximal inhibition was reached at serum dilutions of \(~1/30\) (Fig. 2B). Addition of preimmune serum or serum from Q\(\beta\)-immunized mice (data not shown) did not result in any inhibition of the TRANCE/RANKL-RANK interaction. We also attempted to measure the ability of the immune sera to inhibit osteoclast formation in vitro in a dose-dependent manner, but failed to yield reproducible results, possibly due to nonspecific interference of the sera with osteoclastogenesis.

Based on the data from the competition ELISA we conclude that immunization with Q\(\beta\)C-TRANCE yielded Abs which specifically neutralize the TRANCE/RANKL-RANK interaction in vitro.

Immunization with Q\(\beta\)C-TRANCE protects from osteoporosis

Next we assessed the ability of anti-TRANCE/RANKL immunization to protect from bone loss in a murine model of osteoporosis. Mice were immunized three times with Q\(\beta\)C-TRANCE and after TRANCE/RANKL-specific IgG titers of \(~1/4400\) (day 28) had been reached, mice were ovariectomized to induce estrogen depletion and initiate bone resorption. At the same time two unimmunized age-matched control groups were either ovariectomized or subjected to sham operation. Q\(\beta\)C-TRANCE-immunized mice were immunized once more after ovariectomy, which lead to a further increase of TRANCE/RANKL-specific IgG titers to \(~1/9700\) (day 62) which then stayed high until the end of the experiment (1/8400, day 69). Osteoblast and osteoclast activities were assessed 5 wk after ovariectomy by measuring serum levels of osteocalcin and collagen degradation products. Deoxypyridinoline cross-links were significantly increased in ovariectomized control mice with respect to sham operated mice, indicating an increased osteoclastic resorption of bone matrix (Table I). This increase was not observed in Q\(\beta\)C-TRANCE-immunized ovariectomized mice, suggesting that osteoclast activity in these mice was reduced by the immunization. The level of the bone formation marker osteocalcin was also significantly increased in ovariectomized control mice, suggesting that the increased osteoclast activity in these mice had

Table I. Effect of immunization with C-TRANCE-VLPs on ovariectomy-induced changes in biochemical markers of bone resorption and bone formation

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Sham ((n = 8))</th>
<th>Ovx ((n = 9))</th>
<th>Ovx/Q(\beta)C-TRANCE ((n = 5))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Deoxypyridinoline cross-links (nM)</td>
<td>5.89 ± 0.37*</td>
<td>7.51 ± 0.61</td>
<td>5.28 ± 0.31*</td>
</tr>
<tr>
<td>Osteocalcin (ng/ml)</td>
<td>194.94 ± 24.56*</td>
<td>253.15 ± 11.99</td>
<td>214.50 ± 9.95*</td>
</tr>
</tbody>
</table>

* Serum deoxypyridinoline cross-links and osteocalcin were measured in Q\(\beta\)C-TRANCE-immunized ovariectomized mice (Ovx/Q\(\beta\)C-TRANCE) as well as in ovariectomized (Ovx) and sham-operated (Sham) control mice 1 day before sacrifice. Averages of five to nine mice are given with SE of the mean. Two-tailed Student’s \(t\) test was used to calculate statistical significance (*, \(p < 0.05\) vs Ovx group).
Table II. Immunization with C-TRANCE-VLPs protects from ovariectomy-induced bone loss

<table>
<thead>
<tr>
<th>Experiment 1</th>
<th>Ovx/Q</th>
<th>Sham/PBS</th>
<th>Ovx/PBS</th>
<th>Sham/Q</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bone Parameter</td>
<td>n (9)</td>
<td>/H11005</td>
<td>n (9)</td>
<td>/H11005</td>
</tr>
<tr>
<td>Cross-sectional bone mineral density (mg/mm³)</td>
<td>626 ± 208</td>
<td>528 ± 211</td>
<td>521 ± 20</td>
<td>521 ± 20</td>
</tr>
<tr>
<td>Cortical thickness (mm)</td>
<td>0.13 ± 0.016</td>
<td>0.13 ± 0.012</td>
<td>0.13 ± 0.012</td>
<td>0.13 ± 0.012</td>
</tr>
<tr>
<td>Trabecular density (mg/mm³)</td>
<td>6.59 ± 0.23</td>
<td>6.59 ± 0.23</td>
<td>6.59 ± 0.23</td>
<td>6.59 ± 0.23</td>
</tr>
<tr>
<td>Trabecular thickness (mm)</td>
<td>0.18 ± 0.009</td>
<td>0.18 ± 0.009</td>
<td>0.18 ± 0.009</td>
<td>0.18 ± 0.009</td>
</tr>
</tbody>
</table>

Bone mass density in these mice, which had been detected by the pQCT measurements. In Qβ-C-TRANCE-immunized mice were therefore solely due to the induced anti-TRANCE/RANKL Abs.

The influence of anti-TRANCE/RANKL Abs on the trabecular structure in the area of secondary spongiosa was investigated further by histomorphometry. As shown in Table III, ovariectomized control mice showed a decrease in trabecular bone volume, number, and thickness, accompanied by an increase in the mean trabecular separation. This confirms the overall decrease in trabecular bone mass density in these mice, which had been detected by the pQCT measurements. In Qβ-C-TRANCE-immunized ovariectomized mice, trabecular bone volume, number, and separation were similar to sham-operated control mice, and trabecular thickness was higher than in ovariectomized control mice, indicating that in these mice the ovariectomy-induced trabecular bone loss was efficiently inhibited by the induced TRANCE/RANKL-specific Abs.

Taken together these results strongly suggest that immunization with Qβ-C-TRANCE specifically inhibits the osteoclast-mediated bone resorption in a mouse model of osteoporosis.

*Immunized mice are not immunocompromised*
with VLPs derived from HBcAg fused to peptide p33, the major MHC class I epitope of the LCMV glycoprotein in the C57BL/6 background (23). Different parameters of the immune response were then analyzed with results summarized in Table IV.

Because TRANCE/RANKL is expressed on activated T cells we first investigated whether activated T cells in Qβ-C-TRANCE-immunized mice were depleted due to the action of TRANCE-specific Abs. We measured the total numbers of CD4^+ and CD8^+ cells and the frequencies of activated CD4^+ and CD8^+ cells by assessing for expression of CD69, CD25, and CD44. No significant difference in the number of CD4^+ and CD8^+ cells and in the ratio of CD4^+ to CD8^+ cells could be observed between naive, Qβ-C-TRANCE/HBcAgp33-immunized and HBcAgp33-immunized mice. The frequencies of CD69, CD25, and CD44, expressing CD4^+ and CD8^+ cells were also not significantly affected by preimmunization with Qβ-C-TRANCE (Table IV).

The generation of CTLs specific for p33 was assessed next. By tetramer staining no significant differences in the frequencies of p33-specific CD8^+ cells could be observed between Qβ-C-TRANCE/HBcAgp33-immunized and HBcAgp33-immunized mice. Moreover, both Qβ-C-TRANCE/HBcAgp33-immunized and control mice were protected from challenge infections with recombinant vaccinia virus expressing LCMV glycoprotein. This confirmed that activated and control mice were not depleted and demonstrated that effector CD8^+ T cells were able to protect from infection with recombinant vaccinia virus in anti-TRANCE/RANKL-immunized mice.

HBcAg induces a Th cell-dependent IgG response in mice. Thus, HBcAg-specific IgG titers were used as a readout of Th help. Again, no significant difference between Qβ-C-TRANCE-immunized and control mice could be observed, indicating that B and Th cell responses were unaltered. Taken together, these data suggest, that immunization with Qβ-C-TRANCE does not influence B and/or T cell responsiveness. This confirms the notion that TRANCE is not an essential factor for the regulation of an immune response (6, 25).

### Absence of immunopathology in immunized animals and their offspring

TRANCE/RANKL is essential for normal lymph node formation (26–28) and for mammary gland development (29). It was therefore possible that immunization of female mice with Qβ-C-TRANCE interferes with mammary gland function or influences lymph node development in offspring. To address this question, we immunized female mice twice at days 0 and 14 against TRANCE/RANKL and initiated breeding with naive male mice at day 21. Immunized mice reached an ELISA titer (OD50%) of TRANCE/RANKL-specific IgG of 1/1300 at day 21, which then declined slowly during the period of breeding, reaching a level of 1/400 at day 109. To determine whether these Abs were transmitted to the offspring, sera from three pups (4 wk of age) were assayed for the presence of TRANCE/RANKL-specific IgG in an ELISA. Although no OD50% titer could be determined, the absorption measured at a serum dilution of 1/50 was significantly higher for these sera (0.511 ± 0.03) than in preimmune sera (0.179 ± 0.02), indicating that transmission of TRANCE/RANKL-specific IgG had occurred. Offsprings from immunized and nonimmunized mice were necropsied at 4 wk of age and lymph node development assessed macroscopically. No difference in development of peripheral lymph nodes was obvious between offspring from immunized females and control mice (data not shown).

### Table III. Immunization with C-TRANCE-VLPs protects from ovariectomy-induced weakening of the trabecular architecture

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Sham (n = 8)</th>
<th>Ovx (n = 9)</th>
<th>Ovx/Qβ-C-TRANCE (n = 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trabecular bone volume (%)</td>
<td>9.24 ± 1.19*</td>
<td>6.26 ± 0.83</td>
<td>9.53 ± 1.66**</td>
</tr>
<tr>
<td>Trabecular number (μm^3)</td>
<td>1.92 ± 0.16**</td>
<td>1.55 ± 0.12</td>
<td>2.22 ± 0.32*</td>
</tr>
<tr>
<td>Trabecular thickness (μm)</td>
<td>39.19 ± 3.46**</td>
<td>32.59 ± 2.28</td>
<td>35.15 ± 2.04</td>
</tr>
<tr>
<td>Mean trabecular separation (μm)</td>
<td>505.88 ± 44.95**</td>
<td>643.35 ± 53.12</td>
<td>462.92 ± 83.98**</td>
</tr>
</tbody>
</table>

* Femurs of ovariectomized or sham-operated mice were analyzed by histomorphometry as described in Materials and Methods. Averages of five to nine mice are given with SE of the mean. Two-tailed Student’s t test was used to calculate statistical significance (*, p < 0.05, **, p ≤ 0.05 vs Ovx group).

### Table IV. Qβ-C-TRANCE-immunized mice are not immunocompromised

<table>
<thead>
<tr>
<th>Immunization</th>
<th>–</th>
<th>Qβ-C-TRANCE/HBcAgp33</th>
<th>HBcAgp33</th>
</tr>
</thead>
<tbody>
<tr>
<td>% CD4^+ cells</td>
<td>13.26 ± 1.31</td>
<td>15.68 ± 2.05</td>
<td>14.43 ± 3.28</td>
</tr>
<tr>
<td>% CD8^+ cells</td>
<td>8.14 ± 0.76</td>
<td>8.44 ± 0.78</td>
<td>9.53 ± 1.12</td>
</tr>
<tr>
<td>CD4^+ /CD8^+ ratio</td>
<td>1.63 ± 0.09</td>
<td>1.87 ± 0.32</td>
<td>1.50 ± 0.16</td>
</tr>
<tr>
<td>% CD25^+ cells of total CD4^+ cells</td>
<td>13.46 ± 0.58</td>
<td>15.02 ± 1.57</td>
<td>12.69 ± 2.51</td>
</tr>
<tr>
<td>% CD25^+ cells of total CD8^+ cells</td>
<td>2.79 ± 0.69</td>
<td>7.36 ± 8.31</td>
<td>1.93 ± 0.55</td>
</tr>
<tr>
<td>% CD69^+ cells of total CD8^+ cells</td>
<td>6.13 ± 2.80</td>
<td>8.95 ± 7.01</td>
<td>3.00 ± 0.79</td>
</tr>
<tr>
<td>% CD44^high expressing cells of total CD4^+ cells</td>
<td>29.08 ± 4.50</td>
<td>39.32 ± 2.69</td>
<td>28.08 ± 3.98</td>
</tr>
<tr>
<td>% CD44^high expressing cells of total CD8^+ cells</td>
<td>30.57 ± 4.21</td>
<td>33.88 ± 3.33</td>
<td>29.51 ± 1.58</td>
</tr>
<tr>
<td>% p33-specific tetramers</td>
<td>0.36 ± 0.08</td>
<td>3.63 ± 1.45</td>
<td>3.48 ± 1.77</td>
</tr>
<tr>
<td>Vaccinia virus titers in ovaries</td>
<td>(9.2 ± 10) × 10^6</td>
<td>&lt;50</td>
<td>&lt;50</td>
</tr>
<tr>
<td>HBcAg-specific IgG</td>
<td>6388 ± 3079</td>
<td>3344 ± 2565</td>
<td></td>
</tr>
</tbody>
</table>

* Naive and Qβ-C-TRANCE-preimmunized C57BL/6 mice were injected with HBcAgp33, and T cell frequencies, activation markers, p33-specific tetramers and protection from infection by vaccinia virus expressing LCMV glycoprotein was assessed as described in Materials and Methods. For determination of HBcAg-specific Ab responses C57BL/6 mice (n = 6) were immunized on days 0 and 14 with Qβ-C-TRANCE, and these mice as well as age-matched control mice (n = 6) were immunized on day 44 with HBcAgp33/CpG. Sera were analyzed for HBcAg-specific IgG on day 52. Averages are given with SE of the mean. Two-tailed Student’s t test was used for statistical analysis.
shown). Moreover litter size was comparable between immunized and control mice and offsprings were raised normally, indicating that mammary gland function was not impaired in immunized female mice. The possibility of immune complex disease arising from the interaction of TRANCE/RANKL-specific Abs with soluble or membrane-bound TRANCE/RANKL was also investigated. Histological examination of kidneys from Qβ-C-TRANCE-immunized mothers showed no detectable signs of inflammation or other pathology, suggesting that immune complex deposition did not occur in these mice (data not shown).

Discussion
Currently marketed osteoporosis therapies including bisphosphonates and selective estrogen modulators are efficacious but inconvenient for the patient because of the need for frequent administration and the risk of adverse side effects. OPG, which is currently being developed for clinical use, could overcome some of these problems, but may suffer from other drawbacks including the expected high price and the limited specificity, which may render its use problematic for long-term treatment. The active immunization strategy against the OPG ligand TRANCE/RANKL described here may offer a safe, efficient and cost-effective new therapeutic option for the treatment of osteoporosis.

Our approach is based on the notion that highly repetitive Ag arrays, as presented on microbial pathogens like viruses or certain bacterial components, can efficiently cross-link B cell receptors and elicit rapid and potent Ab responses. When presented in such an ordered fashion both foreign and self Ags can induce activation of B cells, as the natural ability of the immune system to distinguish between these types of Ags is lost in this context (17–21). We used chemical cross-linking to couple a recombinant TRANCE/RANKL molecule (C-TRANCE) to VLPs of the bacteriophage Qβ, and yielded C-TRANCE-VLPs which displayed ~25 C-TRANCE trimers in an oriented fashion on their surface. These C-TRANCE molecules retained their tertiary structure and biological activity, as judged by their ability to induce osteoclast formation from bone marrow cells (Fig. 1B). Immunization of mice with C-TRANCE-VLPs resulted in the generation of high titers of TRANCE/RANKL-specific Abs, indicating that the natural unresponsiveness of the immune system to self proteins could be efficiently overcome (Fig. 2A). These Abs were highly efficacious in inhibiting the TRANCE/RANKL-RANK interaction in vitro (Fig. 2B) and in preventing ovariectomy-induced bone loss in a mouse model of osteoporosis. Moreover, in contrast to OPG, they showed only very limited cross-reactivity to the homologous TRAIL (data not shown).

In contrast to other active immunization regimens which have been proposed before for the treatment of chronic diseases (30–32), our approach is not dependent on the use of strong adjuvants like CFA to overcome B cell tolerance. In addition to a good tolerability profile, such an adjuvant-free system has several advantages with regard to the safety of the immune reaction. Strong adjuvants, such as CFA, not only help to generate self-specific Abs but also facilitate induction of self-specific T cell responses. However, specific T cell responses directed against self-molecules are highly undesirable, because such T cells may cause immunopathology, as is suggested to be the case in a recent clinical trial in Alzheimer’s patients (33–35). Despite the ability of VLPs to trigger high-titered IgG responses, induction of CTLs (23) and inflammatory Th cells (data not shown) is limited in the absence of adjuvants.

Another safety concern associated with autologous immunization approaches regards the possible adverse side effects associated with the persistence of high titers of self-specific Abs. In the present study we could not observe any obvious adverse effects related to the immunomodulatory function of TRANCE/RANKL or to the possibility of immune complex formation and associated inflammatory reactions. Moreover, the anti-TRANCE/RANKL Ab response was essentially reversible, displaying a half-life of about 2–3 mo in the absence of booster injections. Because no adjuvants were used, no long-term Ag depots were created which would prolong the exposure of the immune system to the Ag. Although it is not completely understood at present time how maintenance of Ab levels is regulated (36–39), it is possible that such Ag depots would increase the duration of the Ab response and limit its reversibility (for review see Ref. 40).

In summary, the present study demonstrates that immunization against TRANCE/RANKL using C-TRANCE-VLPs is feasible and induces high titers of neutralizing Abs in the absence of adjuvants. C-TRANCE-VLPs may therefore be an attractive therapeutic agent for the treatment of osteoporosis and other diseases with pathological bone resorption, potentially overcoming issues of patient compliance and cost of good of mAbs or recombinant soluble receptors.

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Disclosures
G. Spohn, P. Maurer, K. Schwarz, G. T. Jennings, and M. F. Bachmann are employees of, and hold stock or stock options with, Cytos Biotechnology. In conjunction with Cytos, M. F. Bachmann, P. Maurer, and G. Spohn are the authors of patent application no. US2004321A1.

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