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Manipulation of receptor oligomerization as a strategy to inhibit signaling by TNF superfamily members

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Abstract

Signaling by receptor activator of nuclear factor- κ B (RANK) in response to its ligand RANKL, which is a member of the tumor necrosis factor (TNF) superfamily of cytokines, stimulates osteoclast formation and bone resorption. Thus, this ligand-receptor pair is a therapeutic target for various disorders, such as osteoporosis and skeletal metastasis. RANKL exists as a physiological homotrimer with each monomer recognizing a single molecule of RANK or the anti-osteoclastogenic decoy receptor osteoprotegerin (OPG). We engineered a RANKL protein in which all three monomers of RANKL were encoded as a single polypeptide chain, which enabled us to independently control receptor-binding at each binding interface. To generate an effective RANK inhibitor, we used an unbiased forward genetic approach to identify mutations in RANKL that had a 500-fold increased affinity for RANK, but had decreased affinity for the natural decoy receptor for RANKL, osteoprotegerin. Incorporating receptor-blocking mutations into this high-affinity RANKL variant generated a mutant RANKL that completely inhibited wild-type RANKL-induced osteoclastogenesis in vitro and bone resorption in mice. Our approach may be generalized to enable the inhibition of other TNF receptor signaling systems, which are implicated in wide range of pathological conditions.

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Introduction

Excessive activation of receptors for members of the tumor necrosis factor (TNF) superfamily of cytokines induces myriad pathological conditions (1, 2). Whereas biological agents have had positive effects on the course of these diseases, each agent carries substantial complications (3). The TNF superfamily member receptor activator of nuclear factor- κ B ligand (RANKL) is the key cytokine that stimulates the formation and activity of osteoclasts (4–7), and excess activation of its receptor RANK promotes many, if not most, forms of pathological bone loss. RANKL exists as a homotrimer in solution (8), and each of the three interfaces separating the monomers contains a binding groove that accepts a single molecule of RANK or the anti-osteoclastogenic decoy receptor osteoprotegerin (OPG) (9–11). The most common means of therapeutically inhibiting TNF receptor signaling is sequestration of the cytokine by a humanized monoclonal antibody. Similarly, the sole approved inhibitor of the RANKL pathway, is the humanized monoclonal antibody, denosumab, which targets the cytokine, but not its receptor, and its effects last for 7 to 9 months (12). Given the profound suppression of bone remodeling that accompanies cytokine removal or other anti-bone resorptive strategies, shorter acting agents are needed. We herein report generation of an inhibitor of RANKL-stimulated signaling which blocks RANK activation with a high affinity mutant and fails to recognize OPG. The capacity to block one receptor of a given cytokine, while sparing another suggests the same strategy may be effective in regulating other TNF superfamily members.

Results

We covalently linked three RANKL monomers with two short glycine-rich linkers (Fig. 1A), which were modeled after previously reported single-chain versions of other TNF superfamily members (13–16). This version of RANKL encoded as a single-chain (scRANKL) protein enabled individual modification of the binding affinities of the three binding sites for RANK and OPG. We refer to the noncovalently linked, homotrimeric version of RANKL as “htRANKL.” Two additional surface “solubility” mutations Cys²²⁰→Ser and Glu²⁴⁶→Ile (C220S/E246I), which do not affect the binding of the mutant RANKL to RANK or its function, were introduced to improve protein production (fig. S1, A and B). This version of RANKL is referred to as “WT-SM htRANKL.” Therefore, all versions of htRANKL and scRANKL incorporate these two solubility mutations. As expected, no monomeric species of scRANKL was observable on a denaturing gel (Fig. 1B), and scRANKL migrated similarly to the trimeric species of chemically cross-linked wild-type htRANKL. To avoid potential discrepancies in molecular mass when comparing the cross-linked protein to the native protein, we more precisely determined the molecular masses of htRANKL and scRANKL by multi-angle light scattering (MALS) analysis. We found that scRANKL had a molecular mass consistent with that of three covalently linked RANKL monomers (fig. S2A). Moreover, scRANKL induced bone marrow macrophages (BMMs) to undergo osteoclastogenesis as effectively as did the wild-type cytokine (Fig. 1C and fig. S2B).

To engineer scRANKL constructs that blocked receptor binding at one site (“single-block scRANKL”) or two sites (“double-block scRANKL”, Fig. 1D), we inserted short sequences

into several loops of RANKL (Fig. 2A) or introduced point mutations that disrupted salt bridge formation (fig. S3). One mutant, in which three amino acid residues (Gly-Gly-Ser) were inserted into the CD loop (residues 224–233) (CDins htRANKL), failed to bind to a recombinant fusion protein of Fc and RANK (RANK-Fc) despite undergoing proper folding, as established by its ability to bind to OPG-Fc in a dose-dependent manner (Fig. 2B). Consistent with its failure to bind to RANK, the CDins htRANKL mutant was incapable of inducing osteoclast formation or promoting RANK signaling in bone marrow macrophages (BMMs) (Fig. 2C). Next, we generated single-block and double-block scRANKL mutants by inserting CDins into one or two monomers, respectively, of the single-chain trimer. We compared the degree of receptor binding at saturation by flowing monomeric RANK, as an analyte, over a surface plasmon resonance (SPR) chip. As expected, blocking each binding site diminished receptor recognition by approximately one-third (Fig. 2D).

Having developed scRANKL variants that had an altered capacity to initiate trimeric receptor clustering, which is presumed to be required for optimal signaling, yet retained the ability to bind to RANK, we postulated that these proteins might act as inhibitors of osteoclastogenesis induced by wild-type htRANKL; however, this proved not to be the case (fig. S4). This lack of inhibitory ability likely reflected the failure of the one (or two) intact binding site(s) to overcome the avidity afforded by the three sites of wild-type htRANKL. We reasoned that we might increase the inhibitory effectiveness by compensating for the reduced avidity of single-block or double-block scRANKL through increasing the affinity for RANK at the intact site(s). This required identifying previously uncharacterized RANKL mutations that increased its affinity for RANK. To this end, we performed two generations of in vitro affinity maturation through yeast surface display (YSD) (17). The first round involved creating a library of htRANKL mutants through error-prone polymerase chain reaction (PCR) assays and sorting for clones that retained the ability to bind to RANK-Fc. Notably, OPG, the principal biological inhibitor of RANK-induced osteoclastogenesis (18–20), exerts its effects by competing with RANK for binding to RANKL. Because both RANK and OPG bind to the same groove of RANKL, it is possible that increasing the affinity of RANKL for RANK could simultaneously increase its binding to the decoy receptor. To obviate this possibility, we simultaneously sorted the library for clones with higher affinity for RANK and with decreased affinity for OPG-Fc (fig. S5A). Reversion mutagenesis yielded individual point mutations (K194N, Q236H, F269Y) in htRANKL which, when expressed in combination (KQF), substantially increased the affinity of the mutant htRANKL for RANK-Fc while decreasing its affinity for OPG-Fc (fig. S5, B and C).

In the second generation of in vitro evolution, we selected htRANKL variants with long RANK kinetic half-lives. We again constructed an htRANKL mutant library through error-prone PCR, but this time we used the high-affinity KQF htRANKL mutant as a starting template. Because of its rapid off-rate, monomeric RANK did not stain yeast-displayed wild-type htRANKL, despite their established interaction (Fig. 3A). We therefore sorted the second library with sequentially limiting amounts of monomeric RANK. This sorting strategy yielded a population of htRANKL variants that were capable of binding monomeric RANK (Fig. 3A). Next, we selected htRANKL variants that continued to bind to limiting amounts of RANK after incubation in the presence of unlabeled OPG for 5 min at room temperature (fig. S6). We identified several additional mutations at residue K194 and chose

to use the K194E mutation going forward in KQF htRANKL, because the K194N mutation introduced a potential N-linked glycosylation site that could confound results when comparing proteins produced by yeast and mammalian cells. These strategies also yielded the htRANKL point mutant (H270Y), which, when incorporated into the KQF htRANKL mutant, further increased its affinity for RANK without yielding detectable binding to OPG (Fig. 3B).

We then determined the affinities and kinetic parameters of the RANKL variant that showed the greatest binding to RANK by SPR (Fig. 3C). Although the two solubility mutations that were introduced to produce scRANKL had no effect on RANK binding, they resulted in an approximately ten-fold decrease in binding to OPG (fig. S7). The KQFH htRANKL variant bound to RANK with an approximately 500-fold greater affinity than did wild-type htRANKL; however, it exhibited substantially impaired binding to OPG (Fig 3B). These changes in affinity for RANK largely reflected a prolonged half-life of binding, changing from a relatively rapid dissociation time ($t_{1/2}=3$ s for WT htRANKL) to a dissociation time that was more than 200-times longer ($t_{1/2}=675$ s for KQFH htRANKL). The KQFH mutations cluster in two regions of RANKL that were previously implicated in binding to RANK (9) (fig. S8). To ensure that the RANK-blocking interface would also block binding to OPG, we combined CDins with the Q236H mutation, because Gln²³⁶ was identified by YSD as the amino acid residue responsible for decreased binding to OPG. Indeed, we confirmed that the variant RANKL that combined CDins with the Q236H mutation did not bind to RANK (fig. S9).

To generate an effective inhibitor of RANKL-mediated signaling, we incorporated the high-affinity KQFH variant (RANK^{high}) into our single-block and double-block constructs (Fig. 4A). Before determining the capacity of these scRANKL variants to act as inhibitors of RANKL-RANK signaling, we assessed their capacity to generate osteoclasts. Both the single-block and double-block scRANKL variants that had increased affinity for RANK (single-block, RANK^{high} and double-block, RANK^{high}) were incapable of generating osteoclasts in vitro (Fig. 4B). Concurrently, we observed a lack of associated signals induced by the single-block, RANK^{high} variant (Fig. 4C). In contrast to the failure of single-block or double-block scRANKL containing unaltered residual monomer(s) to inhibit RANK signaling and osteoclast formation, the double-block, RANK^{high} scRANKL variant effectively inhibited signaling stimulated by wild-type htRANKL with an IC₅₀ of ~10 nM (Fig. 4D). The potency of inhibition increased (resulting in an IC₅₀ of 0.2 nM) when two sites were available to bind RANK with high affinity (single-block, RANK^{high}), and this effect was not attributable to cytotoxic effects (fig. S10A). Additionally, blockade was specific for RANK-mediated signaling, because TNF receptor 1 (TNFR1)-mediated phosphorylation of inhibitor of κ B α (I κ B α) downstream of TNF- α was not blocked by the single-block, RANK^{high} variant (fig. S10B).

The inhibitory effects of the single-block, RANK^{high} variant were not limited to exogenous wild-type htRANKL, because it also dose-dependently inhibited osteoclast formation induced by htRANKL produced by osteoblasts (Fig. 4E). Lastly, we sought to test the effectiveness of the single-block, RANK^{high} variant at blocking recombinant wild-type htRANKL-induced osteoclastic bone resorption in vivo. Intraperitoneal injection of

recombinant wild-type htRANKL into 8-week old BALB/c mice increased osteoclast function as determined by measuring serum carboxy-terminal collagen crosslinks (CTX), a marker of bone resorption (Fig. 4F). This increase in bone resorption was completely abrogated by the addition of an equal amount of the single-block, RANK^{high} variant, which suggests that this inhibitor may have therapeutic potential.

Discussion

Our results suggest that scRANKL, consisting of a combination of blocked and high-affinity RANK binding sites, inhibits RANK signaling, thus acting as an effective inhibitor of RANKL-mediated osteoclast formation and function. RANKL, a member of the TNF superfamily of cytokines, binds to multiple receptors (RANK and OPG) with different biological effects. Within this superfamily, there are several examples of cytokines that demonstrate promiscuity in receptor usage (21). For example, TNF- α , which recognizes both TNFR1 and TNFR2, is central to the pathogenesis of disabling disorders, such as rheumatoid arthritis and psoriasis (22). Indeed, treatment of these diseases has been greatly facilitated by systemic blockade of TNF- α with humanized antibodies or soluble receptor (23, 24). As effective as these drugs are, however, they cause major complications, such as a predisposition to malignancy and serious infections, including tuberculosis (25, 26). Current evidence indicates that the positive effects of anti-TNF- α therapy reflects suppressed activation of TNFR1, whereas the negative consequences are a result of inhibition of the pro-immune properties of TNFR2 (27, 28). TNF and RANKL interact with their receptors in a homologous fashion (9, 10, 29, 30). Our data suggest that the strategy of combining high-affinity mutations and blocking mutations into a single-chain can be used to construct an effective inhibitor that is receptor-selective. This suggests a potential mechanism for blocking TNFR1 stimulation while sparing TNFR2, thereby reducing systemic complications. In principal, a covalently linked system offers an advantage over the use of mixed heterotrimers (31) in that the composition of each receptor-binding interface is pre-determined. Indeed, this strategy may be broadly applicable to all members of the pathologically important TNF superfamily.

Materials and Methods

Construction of an scRANKL vector and cloning of cDNAs encoding scRANKL, htRANKL, and TNF- α into a mammalian expression vector

Initially, the cDNA encoding scRANKL was cloned into the pGEX vector (GE-Healthcare) by PCR-based amplification of the coding sequence of the mouse RANKL monomer (including amino acid residues 162 to 316 of NCBI Reference Sequence NP_035743) with primer pairs that inserted the following restriction enzyme sites: 5'-SmaI-RANKL-BspEI-3', 5'-BspEI-RANKL-BamHI-3', and 5'-BamHI-RANKL-NotI-3'. The primers were designed such that the three monomers are separated by a linker sequence [(Gly-Gly-Ser-Gly) $\times$ 3]. Each insert was double-digested with the appropriate restriction enzymes (Fermentas) and ligated into the pGEX vector between the SmaI and NotI sites. The entire scRANKL cDNA was subcloned into the mammalian protein expression vector pFM (32) (gift of Dr. FilipoMancia) downstream of the signal peptide from pHLsec

(MGILPSPGMPALLSLVSLLSVLLMGCVA) (33). To aid protein recovery, a tobacco etch virus (TEV) protease cleavage site and a 6x-histidine tag were added at the C-terminus (SSGRENLYFQGHHHHHH). In brief, the construct encodes: a signal-peptide, RANKL (residues 162 to 316), a linker, RANKL (residues 162 to 316), a linker, RANKL (residues 162 to 316), a TEV cleavage site, and the 6x His tag. Expression of the construct is driven by the CMV promoter. Transfection efficiency was monitored by detection of red fluorescent protein, whose expression is initiated downstream of scRANKL at an internal ribosomal entry site. To clone the cDNA encoding htRANKL into the pFM mammalian expression vector, the cDNA sequence encoding amino acid residues 162 to 316 was amplified, digested with the restriction enzymes Bsu36I and SalI, and ligated downstream of the pHLsec signal peptide and upstream of the TEV cleavage site. To clone the cDNA encoding murine TNF- α , the cDNA sequence encoding amino acid residues 90 to 325 was amplified by overlap extension PCR to add a 5' pHLsec signal peptide site and a 3' SalI restriction site. The cDNA was digested with XbaI and SalI and ligated downstream of the CMV promoter and upstream of the TEV cleavage site.

Production of mammalian RANKL and TNF- α proteins

Suspension-adapted 293-Freestyle cells (Life Technologies) were maintained in serum-free Freestyle 293 expression medium (Life Technologies) according to the manufacturer's protocol. For transfection, DNA was prepared with an endotoxin-free maxiprep kit (Qiagen). Cells were seeded at a density of 0.5×10^6 /ml in 200 ml of medium 24 hours before transfection. On the day of transfection, DNA and polyethylenimine (33) were mixed at a ratio of 1:3 (htRANKL or TNF- α , 200 μ g:600 μ g) or 1:2 (scRANKL variants, 200 μ g: 400 μ g) in opti-mem (Life Technologies), incubated for 15 min at room temperature, and added directly to the cells. Cell culture medium was harvested four and seven days after transfection, filtered through a 0.22- μ m filter, and equilibrated by the addition of 0.1 volume of 10X phosphate-buffered saline (PBS, Gibco) and 10 mM imidazole. The protein was captured on Ni-NTA Superflow resin (Qiagen) and washed with 10 mM imidazole in PBS. Protein was eluted in steps from 25 to 500 mM imidazole. Fractions containing purified protein were identified by coomassie stained SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Protein-containing fractions were pooled and concentrated with a disposable YM30 centricon (Millipore). All proteins were sterile-filtered for use in cell culture. Only lipopolysaccharide (LPS)-free plastics and reagents were used for all purifications.

SPR measurements

All SPR experiments were performed on a Biacore T-100 (GE Healthcare) with CM5 sensor chips and HBS-EP buffer (10 mM HEPES (pH 7.4), 150 mM NaCl, 3 mM EDTA, and 0.05% v/v Surfactant P20). To confirm receptor binding to scRANKL variants, 4,000 response units (RU) of wild-type (WT) scRANKL or variant scRANKL were coupled to individual lanes, leaving one reference flow cell uncoupled. Monomeric RANK (20 μ M) was flowed over until saturation. Total RUs bound at equilibrium were calculated with BIA Evaluation software. Experiments to determine kinetic affinity constants of RANKL variants for RANK or OPG were performed and analyzed as previously described (9).

Generation of osteoclasts from primary bone marrow-derived macrophages

Long bones isolated from eight week-old mice were flushed, and the marrow was subjected to red blood cell lysis. The remainder of the whole marrow was cultured on petri dishes or bone powder as previously described (34), maintained at 37°C and 6% CO₂ in α -MEM medium containing 10% heat-inactivated fetal bovine serum (FBS), penicillin (100 U/ml), streptomycin (100 μ g/ml), (α -10 medium) supplemented with 1:10 CMG [conditioned medium supernatant containing recombinant M-CSF (macrophage colony-stimulating factor)] (35). Osteoclasts were differentiated in α -10 medium with 1:50 CMG and the relevant RANKL variant.

Detection of osteoclast formation

Cells were fixed in 4% paraformaldehyde in PBS for 15 min and stained for tartrate-resistant acid phosphatase (TRAP) with a specific kit (Sigma). Additionally, osteoclasts were quantified by a solution assay of TRAP enzyme activity. Cells were fixed and lysed in 90 mM citrate buffer (pH 5.2), 80 mM sodium tartrate, 0.1% Triton-X-100 for 10 min at room temperature. Colorimetric nitrophenyl phosphate (a substrate of TRAP) was added and visualized after 15 min by the addition of sodium hydroxide. Data were acquired with the 405-nM absorbance filter on a Bio-rad plate reader. Because of the limited range of the TRAP solution assay, a more quantitative assessment of TRAP activity was performed with the fluorescent phosphatase substrate ELF-97 (Molecular Probes). Fixed cells were incubated with 100 μ M ELF-97 in 90 mM citrate buffer (pH 4.8), 80 mM sodium tartrate for 15 min at room temperature. The reaction was stopped by the addition of sodium hydroxide, and fluorescence was visualized with the 345/530 excitation/emission filter on a Spectramax M2 plate reader.

Multi-angle light scattering (MALS) analysis

Purified RANKL proteins were applied to a Wyatt WTC-030 S5 size-exclusion column mounted on a Waters HPLC system attached to a multi-angle light scattering (MALS) device. The light detectors, a Dawn HELEOS-II 18-angle light scattering detector and an OptilabREX refractive index, were previously calibrated against monomeric bovine serum albumin. MALS was monitored during the experiments, and the resulting data were analyzed with associated software. For each experiment, 250 μ g of sample was applied at a concentration of 1 mg/ml in running buffer [25 mM hepes (pH 7.4), 150 mM NaCl, 0.01% sodium azide) at 20°C and a flow rate of 0.5 ml per minute.

Chemical cross-linking

Purified WT-RANKL protein (500 ng) was incubated with varying concentrations (0 to 500 μ M) of the chemical cross-linker bis-(sulfosuccinimidyl)-suberate (BS³, Pierce) in PBS at room temperature for 30 min, at which time the reaction was stopped by the addition of 10 mM Tris-HCl (pH 7.0). Samples were boiled under reducing conditions and loaded onto SDS-PAGE gels (10%) alongside 500 ng of scRANKL protein. Bands were stained with the coomassie derivative Imperial protein stain (Pierce) and visualized with the Odyssey scanner (Licor).

Identification of RANKL mutants that did not bind to RANK

RANKL residues forming salt bridges or hydrogen bonds with RANK were targeted for site-directed mutagenesis with PISA software based on the RANK-RANKL co-crystal structure (9). Loops at the RANK-RANKL binding interface were disrupted by amino acid insertion. Mutations were introduced into the expression construct, pGEX-GST-RANKL, by PCR with Phusion polymerase (NEB). The constructs were verified by nucleic acid sequencing. *Escherichia coli* strain BL21-CodonPlus (DE3)-RIL competent cells (Agilent Technologies) were transformed with the mutant RANKL-encoding constructs to generate proteins. Correctly folded soluble proteins were purified from cell lysates on glutathione sepharose (8).

Bio-layer interferometry (BLI)

All BLI experiments were performed on an Octet RED system (ForteBio). Glutathione-S-transferase (GST)-RANKL fusion proteins were biotinylated with NHS-PEG4-biotin (Pierce) according to the manufacturer's protocol, and excess biotin was removed by desalting over Zeba Spin Columns (7-kD molecular mass cutoff, Pierce). Biotinylated proteins were adsorbed onto super-streptavidin sensor pins (ForteBio). Binding of RANK-Fc or OPG-Fc was measured in HBS-EP containing 1% BSA. Because of the dimeric nature of Fc-tagged receptors, only apparent K_D values were observed.

Quantitative real-time PCR analysis

To quantitate the abundances of mRNAs for markers of osteoclast formation, total RNA was isolated from cultured cells with the Qiagen RNeasy miniprep kit according to the manufacturer's protocol. Equal amounts of RNA were used to perform reverse transcription with Bio-rad iScript, and quantitative real-time PCR analysis was performed with a Sso Fast EvaGreen qPCR kit (Bio-Rad) with a 7500 fast machine (ABI). Cyclophilin was used as the housekeeping control gene. Data were analyzed according to the C_t method, and expressed relative to a control containing no RANKL addition (labeled BMM). The primers used were as follows: Cathepsin K (Forward: 5'-ATGTGGGTGTTCAAGTTTCTGC-3', Reverse: 5'-CCACAAGATTCTGGGGACTC-3'); NFATc1 (Forward: 5'-CCCGTCACATTCTGGTCCAT-3'; Reverse: 5'-CAAGTAACCGTGTAGCTGCACAA-3'), TRAP (Forward: 5'-CAGCTCCCTAGAAGATGGATTTCAT-3', Reverse: 5'-GTCAGGAGTGGGAGCCATATG), $\beta 3$ (Forward: 5'-TTCGACTACGGCCAGATGATT-3', Reverse: 5'-GGAGAAAGACAGGTCCATCAAGT-3') and Cyclophilin (Forward: 5'-AGCATACAGGTCCTGGCATC-3', Reverse: 5'-TTCACCTTCCCAAAGACCAC-3').

Western blotting

Cells were washed three times in ice-cold PBS and lysed with RIPA buffer (Millipore) supplemented with protease and phosphatase inhibitor cocktail (Pierce). After 10 min of incubation on ice, cell lysates were cleared of debris by centrifugation for 15 min at 21,000 *g*. Forty to fifty micrograms of protein were resolved by 10% SDS-PAGE, transferred onto polyvinylidenedifluoride (PVDF) membranes, and incubated with primary antibody

overnight. After extensive washing and incubation with near-infrared-labeled secondary antibody, membranes were visualized with the Odyssey scanner (Licor). Primary antibodies to detect phosphorylated or total NF- κ B or p38 MAPK proteins were obtained from Cell Signaling; antibody against actin was from Sigma; and fluorescently labeled secondary antibodies were obtained from Rockland.

YSD of RANKL and flow cytometric staining with monomeric RANK or OPG

The cDNA encoding WT-SM RANKL was subcloned into the pYD1 yeast display vector (Life Technologies) at the NheI and XhoI restriction sites to generate the yeast mating protein Aga2p fused to the RANKL N-terminus and having a V5-epitope tag at the C-terminus. EBY100 yeast cells were transformed with the pYD1-RANKL construct using the lithium acetate/single-stranded DNA method as described (36) and colonies were selected in tryptophan-deficient, glucose-based medium at 30°C. Display of RANKL protein was induced by inoculating into galactose-based selective medium and incubating at 30°C with shaking for 24 to 48 hours. Surface expression of RANKL was detected with a fluorescein isothiocyanate (FITC)-conjugated anti-V5 antibody (Invitrogen). After incubation with RANK-Fc or OPG-Fc for 10 min at room temperature and washing with ice-cold PBS, receptor binding was detected with an allophycocyanin (APC)-conjugated anti-human Fc antibody (Molecular Probes). All experiments were performed with LSR II or Canto II flow cytometers (BD Biosciences) and data were analyzed with the FlowJo software package (Tree Star, Inc.). Alternatively, RANK-6x His or OPG-6x His proteins were detected with APC-labeled anti-6x His antibody (MBL International).

Generation of RANKL library and selection

Primers annealing immediately 5' or 3' to the cDNA sequence encoding RANKL in the pYD1 vector were designed and used in error-prone PCR amplification (Gene Morph II, Agilent). Lower and higher mutation rates were accomplished by manipulating the amount of starting template and the number of amplification cycles. The resulting product was further amplified with the high-fidelity Phusion polymerase (Finnzymes). Simultaneously, the pYD1 vector backbone was amplified with primers that extended outward from the regions surrounding RANKL-V5, leaving 24 base pairs of overlap between the vector backbone and the amplified mutant RANKL-V5 insert. These purified PCR products were used in the transformation of EBY100 cells according to established protocols (36), which yielded a library of approximately 1×10^6 transformants. Selections were made with magnetic assisted cell sorting (MACS, Miltenyi). Approximately 1×10^7 cells were induced from either the low or high mutation rate libraries, and both were first sorted for the expression of the V5 C-terminal tag, which indicated proper folding of the full-length protein. This was performed by incubating the cells with FITC-labeled anti-V5 antibody and selecting cells with anti-FITC microbeads for cell separation. After growth of the selected clones, cells were again induced to display RANKL protein, incubated with OPG-Fc, and washed, and those clones that did not bind to OPG were collected as the flow-through on a Protein A magnetic bead column. These cells were then labeled with RANK-Fc, and this time those clones that retained binding to the Protein A column were collected. After sorting, cells were allowed to multiply in selective medium, and this strategy was repeated twice to yield clones termed "LM3S" and "HM3S". Approximately 200 individual colonies

were isolated from the libraries and stained with OPG-Fc. Those clones with little to no detectable staining were then assessed for their ability to bind to RANK-Fc. DNA was extracted from the top scoring clones of interest with a yeast miniprep kit (Zymoprep) and used to transform chemically competent DH5 α *E. coli* (Invitrogen) for sequencing. Individual point mutations were then added to the cDNA encoding RANKL by site-directed mutagenesis, and subsequent combinations were cloned in a similar fashion. A second round of error-prone PCR used the identical primers and protocol described earlier, but used as the starting template either of the F164Y/Q236H/F269Y or K194N/Q236H/F269Y triple mutants. Clones were selected over three rounds of sorting with three sequentially lower amounts of monomeric RANK-6x His and anti-6x His microbeads. Finally, the resulting library was incubated with RANK-6x His at room temperature for 10 min and then tested for the ability to outcompete the presence of unlabeled OPG at room temperature for 5 min. Residual RANK binding was detected with APC-labeled anti-6x His antibody. In the course of sorting, the K194E mutation was identified and used to replace the K194N mutation, which introduced a potential N-linked glycosylation site at the interface with RANK or OPG.

Assessment of cell viability with the MTT assay

Cells were seeded in 96-well plates either in α MEM alone, WT htRANKL (200 ng/ml) alone or in combination with single-block, RANK^{high} (2,000 ng/ml), or only single-block, RANK^{high}. After 3 days, a 1:10 dilution of MTT reagent [3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide, Sigma-Aldrich) was added (final concentration of 0.5 mg/ml) to the culture medium and incubated at 37°C. After 3 hours, 150 μ l of 0.04 N HCl in isopropanol was added to each well to stop the reaction, and MTT absorbance was determined at an OD of 595 nm.

Co-culture assays

Calvarial osteoblasts were isolated and expanded in number as previously described (37). Briefly, osteoblasts were isolated from the calvaria of three day-old pups by 3 $\times$ 20-min treatments with collagenase. Cells were expanded in number in α -MEM medium, and then were lifted and plated together with bone marrow-derived macrophages in a 96-well plate. Cells were co-cultured in the presence of 10 nM 1,25-Vitamin D3 in α -MEM medium with the indicated concentrations of single-block, RANK^{high} scRANKL. On day 7, cells were treated with 0.1% collagenase for 15 min to remove osteoblasts, and then were fixed for TRAP staining.

Intraperitoneal injection of mice with RANKL

Eight week-old female Balb/c mice were purchased from NCI Frederick, housed in the animal facility at Washington University School of Medicine, and maintained according to the guidelines set by the Association for Assessment and Accreditation of Laboratory Animal Care. All animal studies were approved by the Animal Studies Committee of Washington University School of Medicine. PBS, WT-SM RANKL (0.5 mg/kg), or WT-SM RANKL and single-block, RANK^{high} scRANKL (0.5 mg/kg) were injected intraperitoneally into the mice at 0, 24, and 48 hours, as previously described (38). Mice were sacrificed 1.5 hours after the third injection, and serum was collected by cardiac puncture. Serum

concentrations of CTx were determined by ELISA according to the manufacturer's protocol (Immunodiagnosics Systems).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Construction and validation of single-chain RANKL

(A) Wild-type (WT) homotrimeric RANKL (htRANKL) is assembled from three individual polypeptides (monomers), whereas WT single-chain RANKL (scRANKL) is a single polypeptide containing RANKL monomers that are joined by two [GGSG] $\times$ 3 amino acid linkers. (B) Coomassie-stained SDS-PAGE gel of WT htRANKL chemically cross-linked by increasing concentrations of BS3. A sample of scRANKL without crosslinking is also shown. 500 ng of each protein was used. $n=3$ independent experiments. (C) BMMs were incubated in the presence of recombinant WT htRANKL or WT scRANKL as described in the Materials and Methods and the osteoclasts generated were identified by TRAP staining. $n=5$ independent experiments. (D) Individual monomers of single-block scRANKL or double-block scRANKL were mutated to inhibit their binding to RANK. Thus, single-block scRANKL contains two intact receptor-binding sites, whereas the double-block scRANKL contains one.

Fig. 2.

Design of scRANKL variants from mutants that do not bind to RANK. **(A)** The three labeled loops of RANKL form one side of the RANK-binding interface in the RANK-RANKL co-crystal structure. **(B)** The binding of the indicated concentrations of WT htRANKL, CDins htRANKL, or GST (a negative control) to RANK-Fc (top) or OPG-Fc (bottom) after incubation for the indicated times was assessed by bi-layer interferometry. Curves from triplicate experiments are shown. **(C)** Top: BMMs were incubated with WT htRANKL or CDins htRANKL (both at 200 ng/ml) and osteoclastogenesis was assessed by TRAP staining. Bottom: BMMs were incubated with WT htRANKL or CDins htRANKL (both at 200 ng/ml) for the indicated times. Cell lysates were then analyzed by Western blotting with antibodies against the indicated proteins to assess I κ B α phosphorylation. Images and Western blots are representative of three independent experiments. **(D)** SPR analysis was used to measure the binding of equal amounts of the indicated scRANKL variants coupled to SPR chips to the analyte RANK. Binding curves were generated from triplicate measurements. The average number of RUs for each binding curve at saturation is displayed.

Fig. 3. Generation of high-affinity RANKL mutants by YSD

(A) Histograms showing flow cytometric analysis of the staining of clones with 1 μ M monomeric RANK at each phase of library sorting. To select for clones with increased binding to RANK, three rounds of sorting were initiated with a concentration of RANKL that was 10-fold greater than the K_D of RANK (Sort#1) and terminated with a concentration of RANKL that was 10-fold less (Sort#3). (B) Point mutants of htRANKL generated by YSD. K_D values were estimated by titrating different amounts of RANK with YSD htRANKL and fitting the median fluorescence intensity (MFI) values for RANK binding to a one-site binding model. Values represent the averages of three independent experiments.

ND, not enough data points to fit K_D values, despite having low amounts of detectable staining at the highest concentrations. The asterisk indicates that the reported affinity of htRANKL for monomeric RANK is 1 μ M (9). (C) Kinetic and equilibrium affinities of WT htRANKL and KQFH htRANKL for RANK and OPG were determined by SPR measurements. Variants of htRANKL were coupled to a sensor chip and monomeric fragments of RANK or OPG served as analytes. Curve fits of triplicate runs are shown as black lines and values represent means $\pm$ SD. k_a (on-rate), k_d (off-rate), K_D (equilibrium dissociation constant).

Fig. 4. Development of a competitive antagonist scRANKL

(A) Schematic displaying the mutations incorporated into the single-block, RANK^{high} and double-block, RANK^{high} variants. “X” indicates RANKL variant that does not bind RANK while “↑” indicates RANKL variant with increased affinity for RANK. (B) The relative abilities of WT scRANKL, single-block, RANK^{high} and double-block, RANK^{high} proteins to induce osteoclastogenesis in vitro was determined and quantified by a fluorescent TRAP activity assay. The curve shown is representative of three independent experiments. (C) BMMs were incubated with WT htRANKL or single-block, RANK^{high} for the indicated times. Cell lysates were then analyzed by Western blotting with antibodies against the indicated proteins to determine the phosphorylation of IκBα and p38 MAPK. Western blots are representative of three independent experiments, with IκBα and p38 run in parallel on separate gels. (D) BMMs were incubated with WT htRANKL (200 ng/ml) alone (open diamond) or in the presence of the indicated concentrations of single-block, RANK^{high} (orange squares) or double-block, RANK^{high} (green triangles). The extent of osteoclast formation was determined by measurement of TRAP activity. The curve shown is representative of our independent experiments. (E) BMMs and osteoblasts were co-cultured alone or in the presence of the indicated concentrations of single-block, RANK^{high}. Osteoclast numbers were determined by manually counting TRAP-positive multi-nucleated cells. Data are means ± SD from three independent experiments. (F) Mice were injected with PBS as a negative control or with WT htRANKL (0.5 mg/kg) alone or together with single-block, RANK^{high} (0.5 mg/kg). Two days later, serum concentrations of CTx were measured by ELISA. Data are means ± SD from eight to ten mice per group performed in cohorts of 4–5 mice per group in two independent experiments. Data were analyzed by one-way ANOVA.