RANKL-targeted combination therapy with osteoprotegerin variant devoid of TRAIL binding exerts biphasic effects on skeletal remodeling and antitumor immunity

SUPPLEMENTARY METHODS

Development of 4T1.2 cell line, constitutively expressing firefly luciferase

Approximately, $2x10^5$ 4T1.2 cells were plated in 6-well tissue culture dish; next day, the cells were transduced with a recombinant lentiviral vector expressing luciferase and puromycin N -acetyl-transferase as a selection marker (Neuroscience NINDS Vector and Virus Core, UAB) in the presence of 10 μg/ml polybrene. Puromycin (3 μg/ml) was added to cells 48 hrs post infection and medium was changed every 2-3 days. Resistant clones were selected and expanded. Luciferase expression from stable clones was measured in cell lysates using Luciferase Assay Systems kit (Promega, E1500) in Tube Luminometer-Junior LB 9509 (Berthold Technologies). To establish that selected luciferase-positive 4T1.2 cells ($4T1.2^{luc}$) retained marker gene expression *in vivo* with identical growth characteristics to that of the parental cell line, equal amount of 4T1.2 and $4T1.2^{luc}$ clones were subcutaneously injected into female BALB/c mice and luciferase expression were monitored by non-invasive IVIS imaging. Tumor growth kinetics was measured using digital caliper and tumor volume was calculated as (length x $width^2$)/2.

Cloning, packaging, and purification of recombinant AAV expressing OPG

For recombinant adeno-associated viral (AAV) vector production, coding sequences of OPG wild type (OPG^{WT}) and Y49R mutant (OPG^{Y49R}) were amplified by polymerase chain reaction (PCR) using primers: forward 5'-TAAGCAGGATCCATGAACAAGTGGCTGTGC-3' and reverse 5'-CCGTGAAAATAAGCTGCTTATAAAAGCTTTAAGCA-3' and subcloned into pAAV-MCS vector between CMV-CBA promoter and polyA region. Expression and extracellular secretion of $OPG^{WT/Y49R}$ was confirmed by transfecting the AAV plasmids into HEK 293T cells following which rAAV-$OPG^{WT/Y49R}$ plasmids were packaged into mature virions using AAV serotype-4 capsid helper plasmid and titers of the purified vectors were determined by qPCR.

Western blot analysis

Cells were harvested for protein isolation using Pierce RIPA buffer (Thermo scientific, 89900) containing protease and phosphatase inhibitors (Thermo scientific, 88666 and 78428) and protein concentrations were measured by Pierce BCA protein assay kit (Thermo scientific, 23227). Denatured protein samples (20–40 μg) were resolved on 10–15% SDS-PAGE gels, transferred onto nitrocellulose membrane (Bio-Rad, 1620115). The membranes were blocked in 5% nonfat milk in TBS with 0.1% Tween 20 (TBST) and probed with appropriate primary antibodies. Following overnight incubation with primary antibodies at 4°C and subsequent washes (3x10 min) with TBST, an appropriate HRP-conjugated secondary antibody was applied for 1 hr at room temperature and then washed with TBST (3 × 10 min). Blots were then incubated with

chemiluminescence reagent and the images were obtained with PXi gel imaging system (Syngene). Quantitation of bands was performed using ImageJ and normalized to β-actin, GAPDH, or total form of respective signaling protein.

Site-directed mutagenesis

Murine OPG^{Y49R} variant was generated using Quickchange XL Site-directed Mutagenesis Kit (Agilent Technologies, 200516) as per the manufacturer's instruction. The sequences of primers used were: Forward, 5'-CCTTGCCCTGACCACTCTCGTACGGACAGCTGGCACACC-3', and Reverse 5'-GGTGTGCCAGCTGTCCGTACGAGAGTGGTCAGGGCAAGG-3'. Following PCR amplification, the reaction mix was digested with restriction enzyme DpnI to remove the template DNA. Two microliters of the reaction mix was used to transform α-Select Chemically Competent bacteria (Bioline, Bio-85027) and plated onto agar containing 100 μg/mL ampicillin. Clones were screened for the identification of respective mutations by DNA sequencing. Expression of mutant forms of OPG was confirmed by immunoblotting, as described above.

Quantitative real-time RT-PCR

Total RNA from cells was isolated using NucleoSpin® RNA kit according to the manufacture's protocol (Macherey Nagel, 740955). cDNA was synthesized from total RNA using iScript cDNA synthesis kit (Bio-Rad, 1708891) flowing manufacturer's instructions. Real-time PCR was performed using Fast SYBR Green Master Mix (Applied Biosystems™, 4385612) under the following protocol: 95°C for 3 min, following 95°C for 3 sec, and 60°C for 30 sec for 40 cycles. The sequences of primers used were: *Arginase 1*, Forward, 5'-CATTGGCTTGCGAGACGTAGAC-3', Reverse, 5'-GCTGAAGGTCTCTTCCATCACC-3'; *CCL17*, Forward, 5'-CGAGAGTGCTGCCTGGATTACT-3', and Reverse, 5'-GGTCTGCACAGATGAGCTTGCC-3'; *GAPDH*, Forward, 5'-TGCACCACCAACTGCTTA-3', Reverse, 5'-GGATGCAGGGATGATGT-3'. Experiments were performed in triplicate and *GAPDH* was used as internal control to normalize respective gene transcripts. Data was analyzed using ΔΔCt method.

TRAIL-induced apoptosis

Human osteolytic cell line, PC3, was cultured in 60-mm dishes (2×10^5 cells per dish) and allowed to adhere for 24 hrs. Then, 100 ng/ml of recombinant TRAIL was added either alone or in combination with conditioned media containing OPG^{WT} or OPG^{Y49R} and cultured for additional 5 hours. The cells were then harvested and lysed in RIPA buffer, with protease inhibitors. Degree

of apoptosis induction was analyzed by Western blot using caspase-3 and cleaved caspase-3 antibodies. From duplicate cultures, PC3 cells were fixed after 24 hrs using 100% methanol and stained with 0.05% Crystal violet. Images were taken using Leica light microscope and cells were enumerated using ImageJ software.

Osteoclast differentiation assay

RAW 264.7 cells were cultured in 24-well plates (1×10^4 cells/well) and allowed to adhere for 24 hrs. Then, 100 ng/ml of recombinant RANKL was added either alone or in combination with conditioned media containing either OPG^{WT} or OPG^{Y49R} mutant and cultured for 10 days. The medium was changed every 2 day, following which tartrate-resistant acid phosphatase (TRAP) staining was performed using Leukocyte Acid Phosphatase kit, as per manufacturer's instructions (MilliporeSigma, 387A-1KT) for multinucleated TRAP-positive osteoclasts. Images were taken using Leica light microscope.

Cell proliferation assay

$4T1.2^{Luc}$ cells were cultured in 6-well plates (6×10^4 cells/well) and allowed to adhere for 24 hrs. Then, cells were serum-starved for 1 hr, following with 100 ng/ml of recombinant RANKL was added with/without 100 ng/ml of recombinant OPG and cultured for additional 2 days in the medium containing 2% FBS. Later, cells were fixed using 100% methanol and stained with 0.05% Crystal violet. Images were taken using Leica light microscope and cell proliferation was enumerated using ImageJ software.

Analysis of cytokine and chemokine signals in the TME

Primary tumors from untreated and OPG^{Y49R} treated mice were explanted and soluble proteins were extracted using RIPA buffer containing protease and phosphatase inhibitors and protein concentrations were measured by BCA assay. Equal amount of protein (40 μg) from individual samples (N=5) were pooled together, then analyzed using Proteome Profiler Mouse Cytokine Array Kit (R&D Systems, ARY006) flowing manufacturer's instructions. Briefly, samples were diluted and mixed with a cocktail of biotinylated detection antibodies for 1 hr and then incubated with Mouse Cytokine Array membrane for overnight. After washing, Streptavidin-Horseradish Peroxidase was added and incubated for 30 minutes, following chemiluminescent detection using PXi gel imaging system (Syngene). Array data was analyzed by densitometry for Integral optical density using HLImage++ (Western Vision Software).

Flow cytometry analysis and cell sorting

For flow cytometry analysis, single cell suspensions were prepared from spleens or tumors from tumor-challenged mice that were untreated or treated with OPG^{Y49R}. Mouse Tumor Dissociation Kit (Milteni Biotec, 130-096-730) was used for isolating cells from explanted tumor tissues following the manufacturer's instructions. Next, cells were treated with ACK buffer (Lonza, 10-548E) to lyse red blood cells, then resuspended in fluorescence-activated cell sorter (FACS) buffer (PBS + 3% FBS). For analyzing cells from tissue culture, cells were detached from culture plates, then resuspended in FACS buffer. For all experiments, cells were divided into individual tubes with 100 µl FACS buffer and stained with appropriate antibodies. For intracellular staining, cells were fixed using 2% paraformaldehyde following staining with appropriate antibodies prepared in eBiosciences permeabilization buffer (ThermoFisher Scientific, 00-8333-56). Flow cytometry data was acquired in a BD LSR II instrument (BD Biosciences) and analyzed using FlowJo v.10 software (FlowJo). Sterile sorting of specific subsets of immune cells was performed using a BD FACSAria instrument (BD Biosciences).

Non-invasive imaging

For bioluminescent imaging, tumor-challenged mice were injected intraperitoneally (IP) with 100 µl of D-Luciferin (15 mg/ml), then anesthetized with 3% isoflurane, and tomographic images were taken 10 minutes post luciferin injection using IVIS Illumina III *in vivo* imaging system (PerkinElmer).

Micro-CT and histology of bone tissue

For determination of the 3-dimensional (3D) architecture of the trabecular bones, mice were sacrificed, tibiae were harvested and scanned using the Scanco µCT40 desktop cone-beam micro-CT scanner. Histomorphometric parameters including bone volume, trabecular thickness, trabecular numbers, and trabecular separation were evaluated from micro-CT data. Upon acquisition of micro-CT scans, bone tissues were decalcified and embedded in paraffin. For histology, 5 µM sections of the bones were prepared and stained with hematoxylin and eosin (H&E) to determine tumor growth in the bone. Goldner’s trichrome staining was performed to determine area of bone damage. To determine osteoclast activity in bone sections, TRAP staining was performed using Leukocyte Acid Phosphatase kit. In adjacent slides, Briefly, the slides were deparaffinized and rehydrated, then incubated in pre-warmed TRAP Staining Solution Mix at 37°C

for 1 hr. Slides were subsequently rinsed in running tap water, then mounted using Permount™ mounting medium (Fisher scientific, SP15-100).