Supporting Information

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SI Materials and Methods

Reagents. Antibodies for flow cytometric analyses included anti-mouse GR1 (RB6-8C5, PE-Cy3) purchased from BD Biosciences, anti-mouse F4/80 (E131.81) from eBioscience, and anti-mouse CD163 (EDHu-1, unconjugated) obtained from Novus Biologicals. Anti-mouse CD68 (FA-11, Alexa Fluor 647) antibody and Leucoperm reagents were purchased from Abd Serotec. Clodronate (dichloromethylene bisphosphonate)-loaded liposomes (clodronate liposomes) or PBS-loaded liposomes (PBS liposomes) were purchased from ClodronateLiposomes.com.

Animal Care and Experiments. All animal experiments were performed with the approval of the University of Michigan Committee for the Use and Care of Animals. MAFIA transgenic mice (18) were purchased from Jackson Laboratories and paired for breeding. AP20187 ligand (a generous gift from ARIAD Pharmaceuticals; used for in vivo macrophage depletion) was dissolved in 4% (vol/vol) ethanol, 10% (vol/vol) polyethylene glycol 400, and 1.7% (vol/vol) Tween-20 and injected into MAFIA mice intraperitoneally. Sixteen-week-old female MAFIA mice were treated with AP20187 (10 mg/kg) for three consecutive days, followed by booster (1 mg/kg) injections every third day for 3 wk. The booster dose was reduced to 0.5 mg/kg for the next 3 wk. Six weeks of intermittent daily PTH (50 μg/kg) administrations were started after the initial three doses of AP20187 injections. MAFIA mice injected with vehicle alone were used as controls. In our second model of macrophage depletion, clodronate or PBS control liposomes were injected intraperitoneally to C57BL/6 wild-type mice (Jackson Laboratories). Sixteen-week-old female mice were treated with clodronate liposomes (10 μL/g) for three consecutive injections, then given every third day for 3 wk. For the next 1 or 3 wk, clodronate liposomes were administered at a reduced dose (6 μL/g). PBS liposomes were used as a control. Six weeks of intermittent daily PTH (50 μg/kg) administrations were started after the initial three doses of clodronate liposome injections.

Flow Cytometric Analysis. Bone marrow cells were flushed with FACS buffer [2% (vol/vol) FBS, 2 mM EDTA in PBS] from long bones and 1×10^6 cells were incubated with fluorescent-conjugated antibodies. For CD68 intracellular staining, Leucoperm Regeants were applied for fixation and permeabilization and 10 μL of CD68 was incubated with the cells for 30 min. For CD163 detection with unconjugated anti-mouse CD163 antibody, a secondary antibody conjugated with Alexa Fluor 488 was used.

Histologic Assays and TRAP Staining. Formalin-fixed, paraffin-embedded liver and spleen tissues were evaluated with H&E staining. Tibiae were fixed in 4% (wt/vol) paraformaldehyde solutions for 48 h and decalcified in 10% (wt/vol) EDTA for 21 d at 4 °C. Paraffin-embedded tibiae were cut (5 μm), stained with H&E, and histomorphometric analyses were performed using Osteomeasure (OsteoMetrics Inc.). TRAP staining on bone section was performed using a TRAP staining kit (Sigma–Aldrich).

Immunohistochemical and TUNEL Staining. Mice (16 wk old, female) were treated with intermittent PTH (50 μg/kg, daily s.c. injection) or saline for 4 wk and killed. Left tibiae were fixed in 4% (wt/vol) paraformaldehyde/PBS (4 °C, 24 h), decalcified in 10% (wt/vol) EDTA for 2 wk, and embedded in paraffin. Immunohistochemical staining was performed, using Cell & Tissue Staining Kit (HRP-DAB system; R&D systems) with rat monoclonal anti-mouse F4/80 (1:100; Abcam). To assess apoptosis in bone sections, we performed TUNEL staining using the FragEL DNA Fragmentation Detection Kit (EMD Millipore) according to the manufacturer's instructions.

Blood and Bone Marrow Serum Biochemical Assays. Serum TRAP5b and P1NP were measured using ImmunoAssay kits obtained from IDS. Total bone marrow was centrifuged from hind-limb bones into PBS containing a proteinase inhibitor mixture, and supernatant was collected. TGF-β1 levels were measured with an ELISA kit from R&D Systems.

Quantitative Real-Time PCR. The forward and reverse primer sequences used to amplify marker genes are listed in Table S1. Quantitative PCR was performed by SYBR Green PCR technology with an ABI PRISM 7900 HT sequence detection system (Applied Biosystems).

Statistical Analyses. Statistical analysis was performed by two-way ANOVA or unpaired t test using the GraphPad Instat statistical program with significance of $P < 0.05$. All data are presented as mean ± SEM.

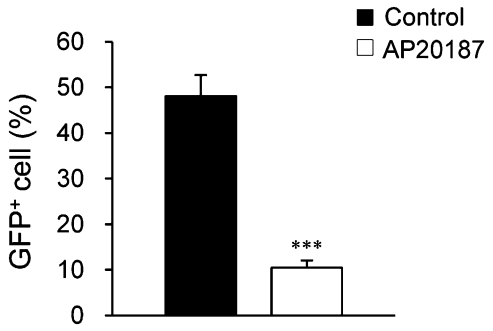

Fig. S1. Macrophage Fas-induced apoptosis (MAFIA) mice (16 wk old, female) were treated with three consecutive injections of AP20187 (10 mg/kg) and killed 24 h after last injection. Flow cytometric analyses of whole bone marrow cells were performed gating FL1 for endogenous GFP. Data are means ± SEM of three independent experiments. ***$P < 0.001$ versus control.

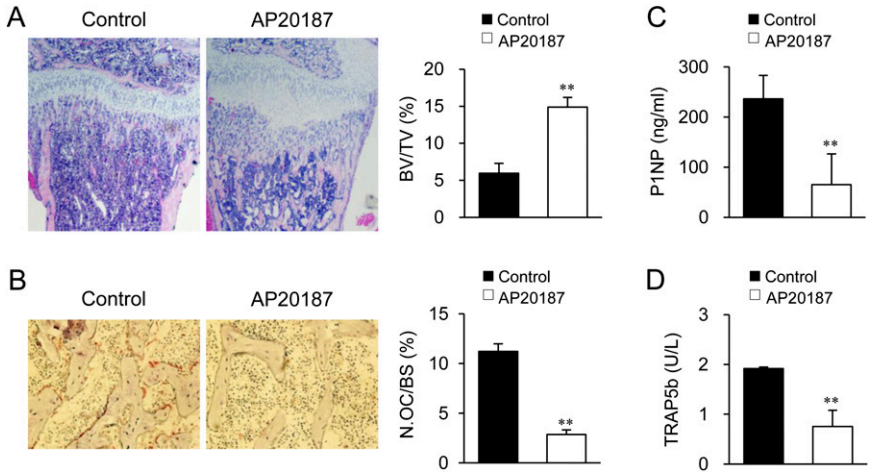

Fig. S2. Young MAFIA mice (8–29 d) were treated with three consecutive injections of AP20187 (10 mg/kg), followed by booster injections (1 mg/kg) every other day for 3 wk. (*A*) Histomorphometric analyses and (*B*) osteoclast numbers per bone volume (N.OC/BS) measured via tartrate resistant acid phosphate (TRAP) staining. (*C*) serum P1NP (nanograms per milliliter) and (*D*) TRAP5b (units per liter). All numerical data are means ± SEM of two independent experiments. $n = 12$ per group. **$P < 0.01$ versus control.

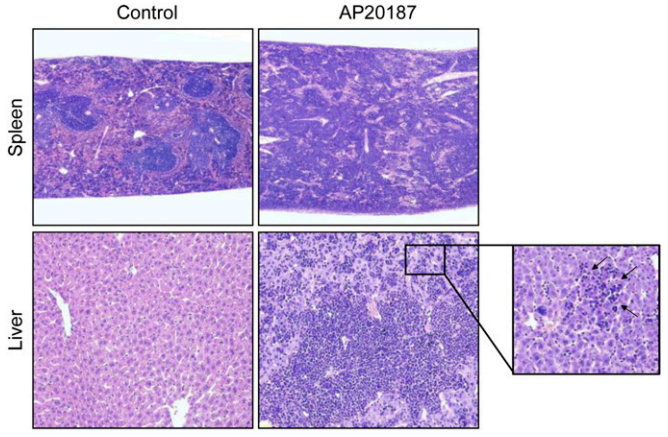

Fig. S3. MAFIA mice (16 wk old, female) were treated with three consecutive AP20187 (10 mg/kg) injections, followed by booster injections every third day (1 mg/kg for 3 wk, then 0.5 mg/kg for 3 wk). H&E staining of spleen and liver were performed after 6-wk depletions. With AP20187 treatment, spleen showed expanded interfollicular regions, as well as constricted and disrupted B-cell follicles, and liver expressed massive hematopoietic foci (black arrows) throughout the parenchyma compared with control group.

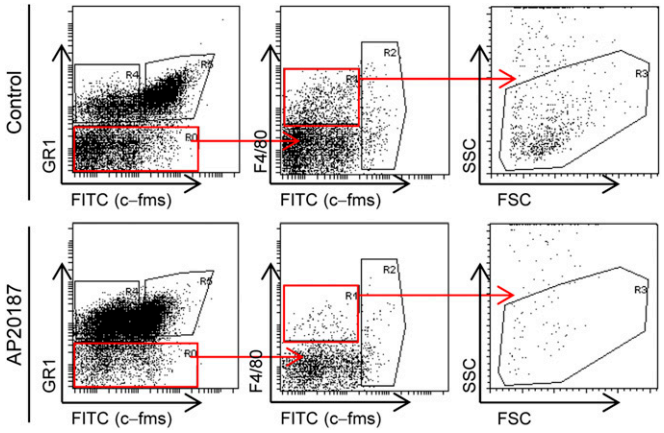

Fig. S4. MAFIA mice (16 wk old, female) were treated with three consecutive AP20187 (10 mg/kg) injections, followed by booster injections every third day (1 mg/kg for 3 wk, then 0.5 mg/kg for 3 wk). Representative dot plots gating bone marrow macrophages. The GR1^{-}c-fms^{int} cells and F4/80^{+} cells were consecutively selected and subdivided into two groups according to SSC. SSC^{hi} eosinophils were excluded and SSC$^{int/lo}$ cells were defined as macrophages.

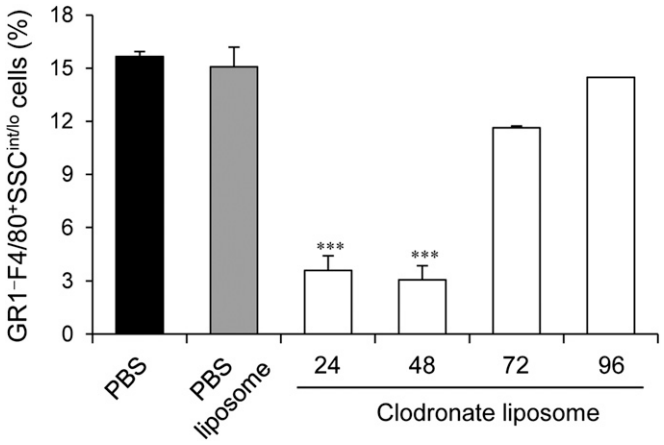

Fig. S5. Sixteen-week-old female mice were intraperitoneally injected with a single dose of PBS, PBS liposome, or clodronate liposome (10 μL/g) and killed at 24, 48, 72, or 96 h after last injection. Flow cytometric analyses of whole bone marrow cells were performed using anti-mouse GR1 and F4/80 antibodies. Graph shows quantitative analysis of GR1^{-}F4/80^{+}SSC$^{int/lo}$ cells. All numerical data are means ± SEM of three independent experiments. ***$P < 0.001$ versus PBS, or PBS liposome.

Table S1. Sequences of PCR primers used to amplify each of the genes in real-time PCR

Gene	Primer	Sequences (5′ → 3′)
MER receptor	Forward	CCCCCTCCGTGCTAACTGTT
	Reverse	TGTGGGCCTCACAACTGAAG
SR−A	Forward	GTCGGGATCTCCTGGACCTA
	Reverse	ATCCCAGCGATCATCACAGA
CD36	Forward	ATTAATGGCACAGACGCAGC
	Reverse	GCATTGGCTGGAAGAACAAA
IL−10	Forward	GCTCTTACTGACTGGCATGAG
	Reverse	CGCAGCTCTAGGAGCATGTG
MFG−E8	Forward	ATTGAATTTGGAGGAATGGT
	Reverse	CTTGAAGTACGTGTAACGTG
MRC1	Forward	TCTTTGCCTTTCCCAGTCTCC
	Reverse	TGACACCCAGCGGAATTTC
Arginase I	Forward	GAACACGGCAGTGGCTTTAAC
	Reverse	TGCTTAGCTCTGTCTGCTTTGC
IL−12	Forward	AAATGAAGCTCTGCATCCTGC
	Reverse	TCACCCTGTTGATGGTCACG
TNFα	Forward	ACGGCATGGATCTCAAAGAC
	Reverse	AGATAGCAAATCGGCTGACG
IL-1β	Forward	ATTGAATTTGGAGGAATGGT
	Reverse	CTTGAAGTACGTGTAACGTG
iNOS	Forward	CAGCTGGGCTGTACAAACCTT
	Reverse	CATTGGAAGTGAAGCGTTTCG
TGFβ1	Forward	GCGGCAGCTGTACATTGACT
	Reverse	ACTGTGTGTCCAGGCTCCAA
Wnt3a	Forward	ATTGAATTTGGAGGAATGGT
	Reverse	CTTGAAGTACGTGTAACGTG
Wnt10b	Forward	GCTGCTCTCTTTCAGCCCTT
	Reverse	AAGTAGGAACCTGAGCCCGA
β-actin	Forward	GGACCTGACAGACTACCTCA
	Reverse	TCTTTGATGTCACGCACGATTT

MFG-E8, milk fat globule glycoprotein; MRC1, mannose receptor 1; SR-A, scavenger receptor type A.