Electronic Supplementary Material (ESI) for Biomaterials Science.

Supplementary materials

Mitigating RANKL-induced cholesterol overload in macrophages with β-cyclodextrin-threaded polyrotaxanes suppresses osteoclastogenesis

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S1. Preparation of cholesterol/M-β-CD inclusion complex

Cholesterol (38.6 mg; Fujifilm Wako Pure Chemical, Osaka, Japan) was added to randomly methylated β-cyclodextrin (100 mM; M-β-CD; Merck, Darmstadt, Germany) solution, and the suspension was stirred for 24 h at room temperature. The solution was passed through 0.22-μm filter to remove undissolved cholesterol. The concentration of cholesterol in the solution was determined by GC-MS. The solution was diluted with PBS to adjust the concentration of cholesterol before use.

S2 Fluorescence labeling of HEE-PRX

HEE-PRX (150 mg, 4.79 μmol) and 1,1'-carbonyldiimidazole (10.4 mg, 64.2 μmol; Merck) were dissolved in dehydrated *N*,*N*-dimethylformamide (5 mL; Fujifilm Wako Pure Chemical) under a nitrogen atmosphere, and the solution was stirred for 5 h at room temperature. 4,4-Difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-propionyl ethylenediamine hydrochloride (BODIPY FL EDA; 1.78 mg, 1.68 μmol; Thermo Fisher Scientific, Waltham, MA, USA) was added to the reaction mixture, and the solution was stirred for 24 h at room temperature with protection from light. After the reaction, the resulting polymer was purified via dialysis against water using Spectra/Por 1 (molecular weight cut-off of 6,000–8,000) for 3 days. The recovered solution was freeze-dried to yield BODIPY-labeled HEE-PRX (115 mg).

S3. In vitro bone resorption assay

RAW264.7 cells were seeded into calcium phosphate-coated 24-well plates (PG Research, Tokyo, Japan) at 5×10^4 cells/well and cultured for 24 h. The cells were then treated with HEE-PRX (1 mM β-CD) or HP-β-CD (1 mM) in the presence of recombinant soluble RANKL (50 ng/mL). These reagents were exchanged on day 3. The treated cells were washed twice with Dulbecco's PBS and treated with 5% sodium hypochlorite for 5 min. After washing the plates with PBS, the plates were dried. The surfaces were observed using an IX-71 microscope (Olympus, Tokyo, Japan) equipped with a DP-80 dual CCD microscope camera. The pit areas were analyzed using Image J software.

S4. Degradation kinetics for HEE-PRX and HEE-NPRX

Acid-degradable HEE-PRX and non-degradable HEE-NPRX were dissolved in phosphate buffer solutions (10 mM NaH_2PO_4–Na_2HPO_4, 150 mM NaCl, pH 7.4) and acetate buffer solutions (10 mM CH_3COOH–CH_3COONa, 150 mM NaCl, pH 5.0) at a concentration of 5.0 mg/mL, and the solutions were incubated at 37 °C. After incubation for the prescribed time periods, an aliquot of the solutions (500 μL) was collected and combined with 50 mM $NaHCO_3$–Na_2CO_3 buffer at pH 9.0 (100 μL). The solutions were then freeze-dried. The recovered powders were dissolved in DMSO containing 10 mM

LiBr (1 mL). Size exclusion chromatography (SEC) measurements were carried out on a Prominence-i LC-2030 Plus (Shimadzu, Kyoto, Japan) with an RID-20A refractive index detector and a combination of TSK gel α-4000 and α-2500 columns (300 mm length, 7.8 mm internal diameter; Tosoh, Tokyo, Japan). The system was eluted with DMSO containing 10 mM LiBr, at a flow rate of 0.35 mL/min at 60 °C. The degradation rates of HEE-PRX and HEE-NPRX were calculated by comparing the peak areas of PRXs and dethreaded β-CDs.

S5. Immunoblotting

The treated RAW 264.7 cells were lysed with radioimmune precipitation assay buffer (Fijifilm Wako pure Chemical) containing Complete Protease Inhibitor Cocktail (Roche, Basel, Switzerland). The lysates were clarified by centrifugation at 15,000 rpm for 10 min, and the supernatant was collected. SDS-PAGE was performed on a 10% polyacrylamide gel for 50 min at 150 V. The samples were then transferred to a poly(vinylidene difluoride) membrane (Bio-Rad, Hercules, CA, USA) using a Trans-Blot Turbo transfer system (Bio-Rad). The membrane was blocked with TBST buffer (20 mM Tris-HCl, 500 mM NaCl, 0.05% Tween 20, pH 7.5) containing 5% BSA for 60 min at room temperature. The membrane was then treated overnight with mouse monoclonal anti-NFATc1 (clone: 7A6; Santa Cruz Biotechnology, Dallas, TX, USA; 1:200 dilution) and mouse monoclonal anti-β-actin (clone: 6D1; MBL, Tokyo, Japan; 1:1000 dilution) at 4 °C. The membrane was treated with horseradish peroxidase-conjugated rabbit anti-mouse IgG (Abcam, Cambridge, UK; 1:2500 dilution) for 60 min at room temperature. The membrane was finally visualized with ImmunoStar Zeta (Fujifilm Wako Pure Chemical). The chemiluminescence images were acquired on an ImageQuant LAS500 imaging system (Cytiva, Marlborough, MA), and the intensity of the bands was analyzed using Image J software.

Table S1. Primer sets used for real-time qPCR.

Gene	Accession number	Primer sequence		Product size (bp)
Rplp0	NM_007475	Forward	GGCCCTGCACTCTCGCTTTC	124
		Reverse	TGCCAGGACGCGCTTGT	
Abca1	NM_013454	Forward	CGTTTCCGGGAAGTGTCCTA	79
		Reverse	GCTAGAGATGACAAGGAGGATGGA	
Abcg1	NM_009593	Forward	GTGGATGAGGTTGAGACAGACC	145
		Reverse	CCTCGGGTACAGAGTAGGAAAG	
Aco2	NM_080633	Forward	GGCCATGAGCCATTTTGAGC	166
		Reverse	TGTCTTTCCCCGCTCGATCT	
Acp5	NM_001102405	Forward	TCCTGGCTCAAAAAGCAGTT	212
		Reverse	ACATAGCCCACACCGTTCTC	
Ctsk	NM_007802	Forward	GCGTTGTTCTTATTCCGAGC	174
		Reverse	CAGCAGAGGTGTGTACTATG	
Dcstamp	NM_029422	Forward	TCCTCCATGAACAAACAGTTCCA	149
		Reverse	AGACGTGGTTTAGGAATGCAGCTC	
Esrra	NM_007953	Forward	GTACGTCCTGCTGAAAGCTCTG	124
		Reverse	CCGGCCAGCTTCATACTCC	
Hmgcs	NM_145942	Forward	GCCGTGAACTGGGTCGAA	77
		Reverse	GCATATATAGCAATGTCTCCT	
Ldlr	NM_010700	Forward	AGGCTGTGGGCTCCATAGG	72
		Reverse	TGCGGTCCAGGGTCATCT	
Mmp9	NM_013599	Forward	GCTGACTACGATAAGGACGGCA	114
		Reverse	GCGGCCCTCAAAGATGAACGG	
Nfatc1	NM_016791	Forward	ACGCTACAGCTGTTCATTGG	130
		Reverse	CTTTGGTGTTGGACAGGATG	
Nr1h3	NM_013839	Forward	AATGCCAGGAGTGTCGACTT	102
		Reverse	CTTGCCGCTTCAGTTTCTTC	
Oscar	NM_175632	Forward	GCTGCGCTGTGATAGCACAT	64
		Reverse	ACCTGGCACCTACTGTTGCTATTAC	
Srebf2	NM_033218	Forward	TAACCCCTTGACTTCCTTGC	160
		Reverse	CACACCATTTACCAGCCACA	

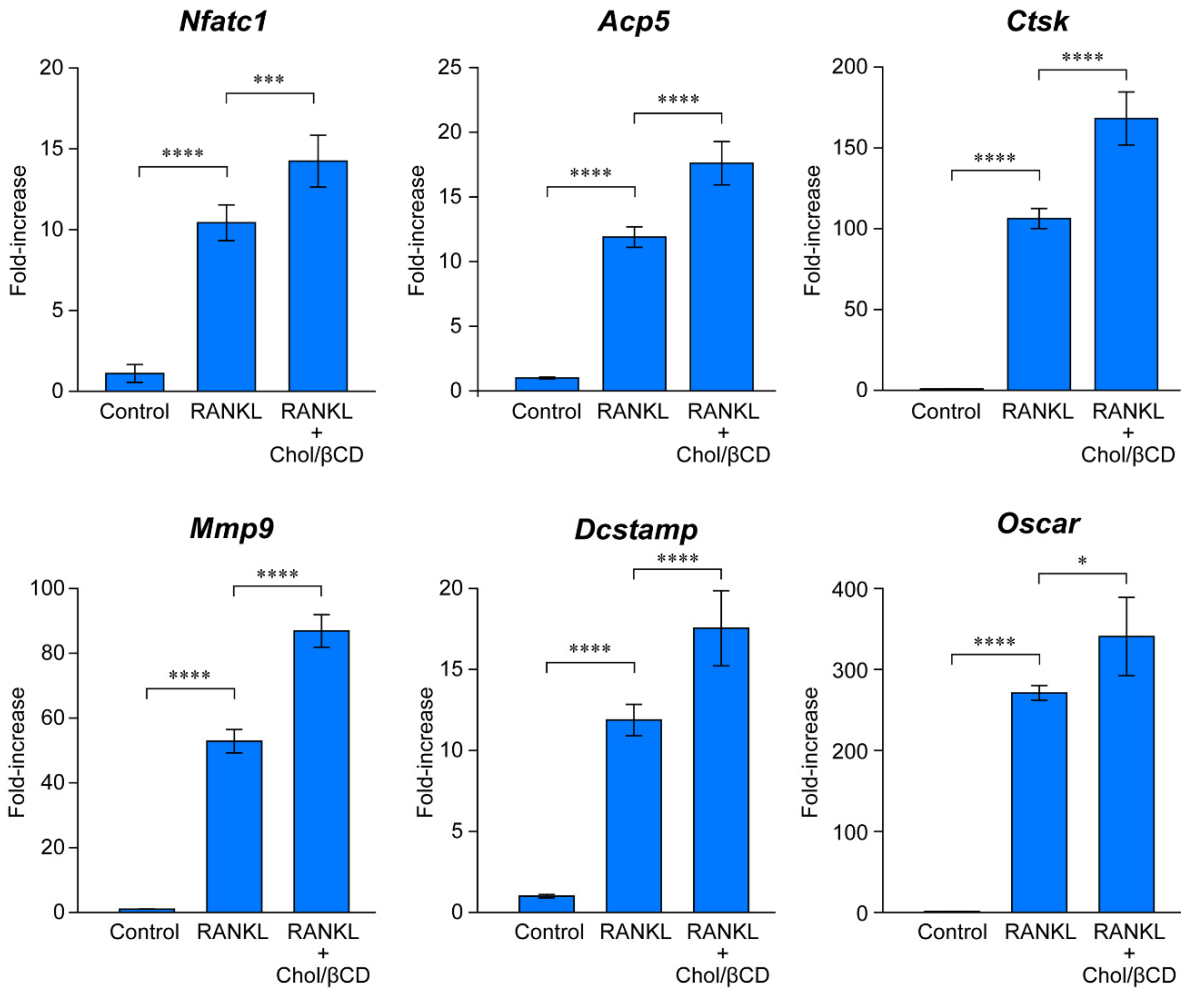

Figure S1. Expression levels of genes related to osteoclast differentiation in RAW264.7 cells treated with RANKL in combination with cholesterol/M-β-CD inclusion complex (cholesterol = 50 μM). Data are shown as the mean ± SD (n = 3–4). *$P < 0.05$, ***$P < 0.001$, ****$P < 0.0001$, by Tukey-Kramer multiple comparison test.

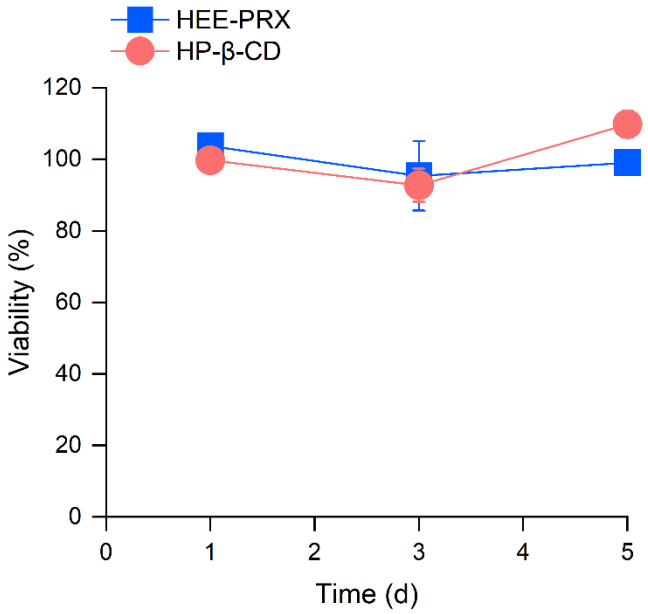

Figure S2. Time-course of the viability of RAW 264.7 cells treated with HEE-PRX (1 mM of threaded β-CD) and HP-β-CD (1 mM). Data are shown as the mean ± SD (n = 6).

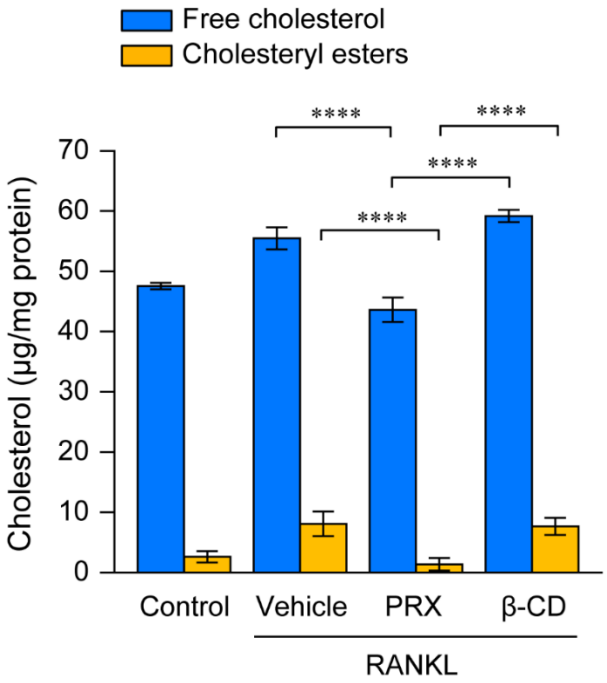

Figure S3. Amount of free cholesterol and cholesteryl esters in RAW264.7 cells treated with HEE-PRX (1 mM of threaded β-CD) and HP-β-CD (1 mM) in the presence of soluble RANKL for 5 days. Data are shown as the mean ± SD (n = 4). ****$P < 0.0001$, by Tukey's multiple comparison test.

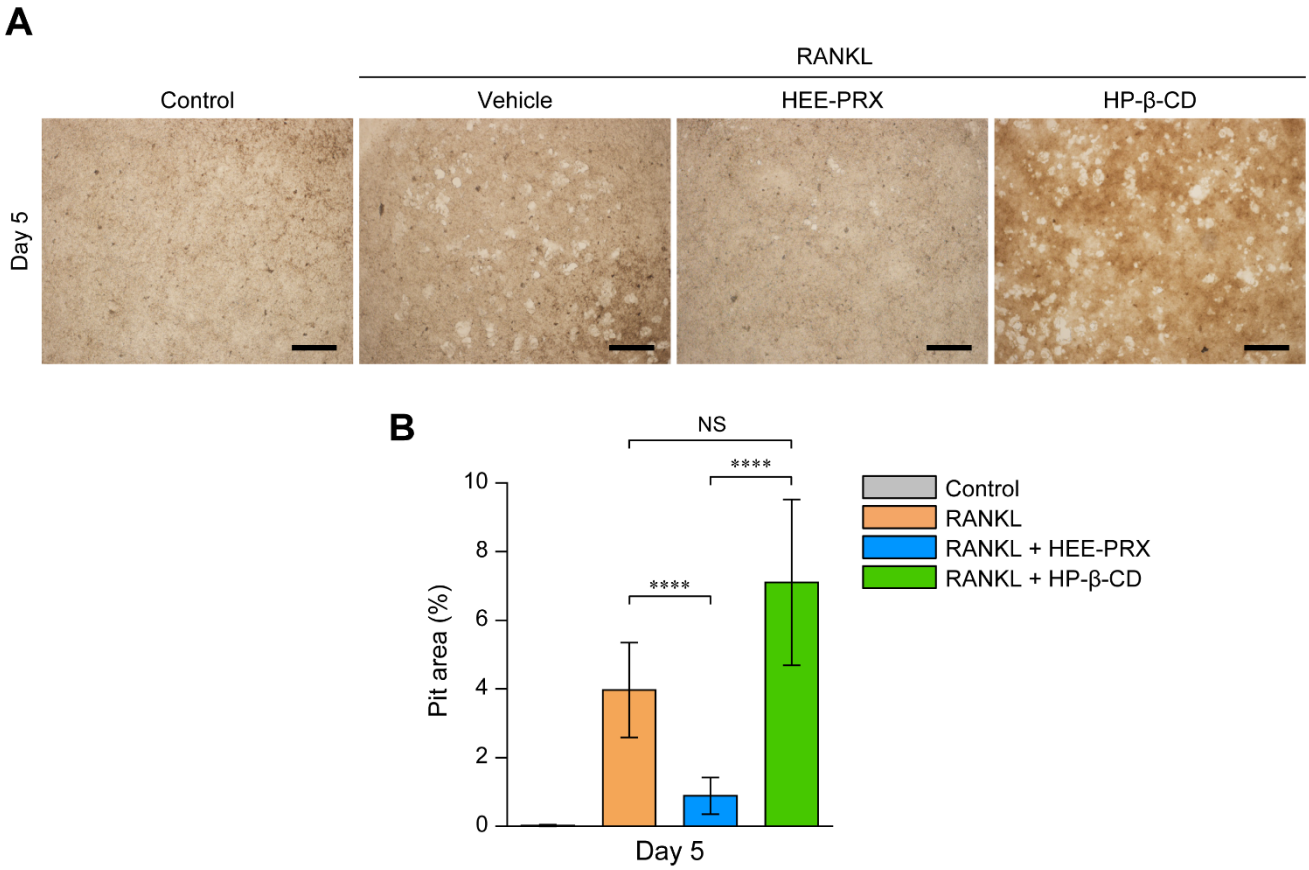

Figure S4. (A) Bright-field micrographs of calcium phosphate-coated surfaces. RAW 264.7 cells were cultured on the surfaces and treated with HEE-PRX (1 mM of threaded β-CD) and HP-β-CD (1 mM) in the presence of soluble RANKL for 5 days (scale bars = 200 μm). (B) Area of pits on calcium phosphate-coated surfaces. Data are shown as the mean ± SD (n = 3). ****$P < 0.0001$, by Tukey's multiple comparison test. NS indicates not significant.

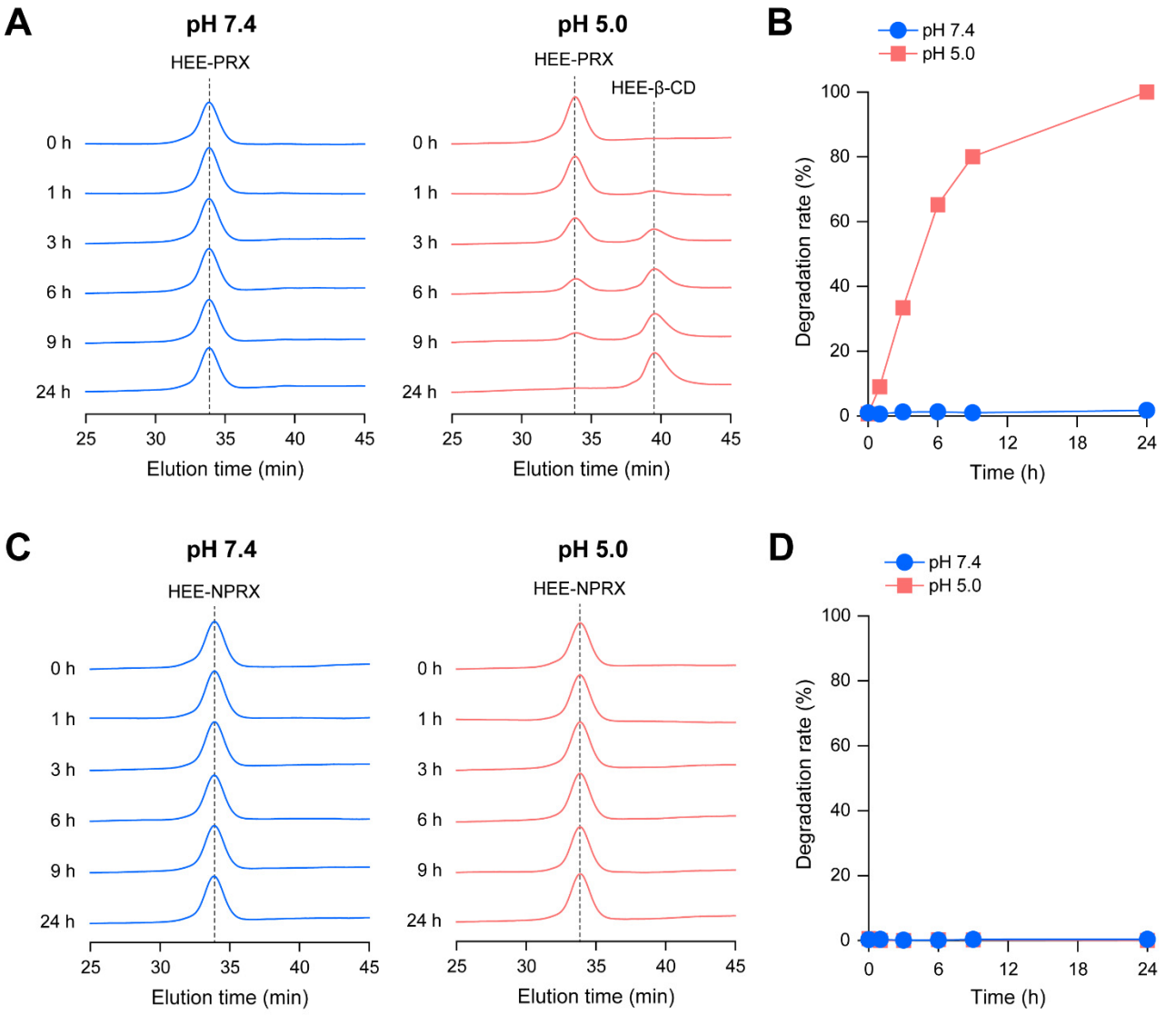

Figure S5. (A) SEC charts for acid-degradable HEE-PRX incubated at pH 7.4 and 5.0 at 37 °C. (B) Time-course of the degradation rate of HEE-PRX at pH 7.4 and 5.0 at 37 °C. (C) SEC charts for non-degradable HEE-NPRX incubated at pH 7.4 and 5.0 at 37 °C. (D) Time-course of the degradation rate of HEE-NPRX at pH 7.4 and 5.0 at 37 °C.

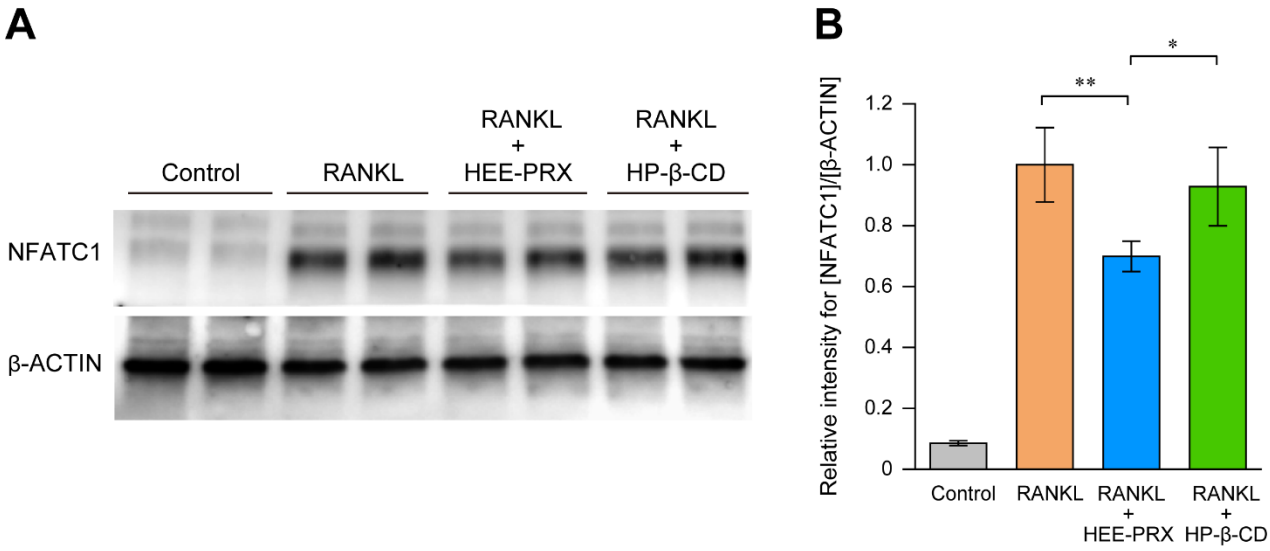

Figure S6. (A) Representative immunoblot image for NFATC1 in RAW264.7 cells treated with HEE-PRX and HP-β-CD in the presence of soluble RANKL for 2 days. (B) Relative band intensities for NFATC1/β-ACTIN. The relative intensity of NFATC1/β-ACTIN in RAW264.7 cells treated with RANKL was adjusted to 1. Data are shown as the mean ± SD (n = 4). **P* < 0.05, ***P* < 0.01, by Tukey's multiple comparison test.

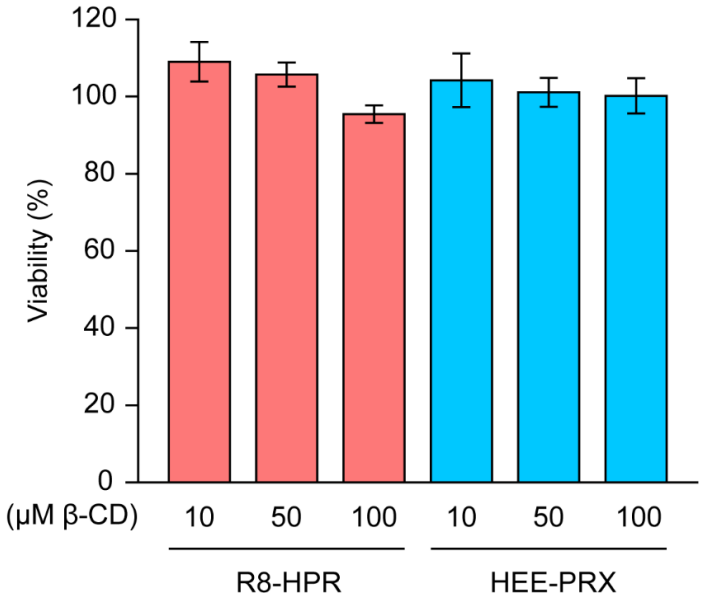

Figure S7. Viability of RAW 264.7 cells treated with R8-HPR and HEE-PRX (10, 50, and 100 μM of threaded β-CD) for 24 h. Data are shown as the mean ± SD (n = 8).