

# Britanin inhibits titanium wear particle-induced osteolysis and osteoclastogenesis

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**Abstract.** Wear particle-induced osteolysis is a serious complication that occurs in individuals with titanium (Ti)-based implants following long-term usage due to loosening of the implants. The control of excessive osteoclast differentiation and inflammation is essential for protecting against wear particle-induced osteolysis. The present study evaluated the effect of britanin, a pseudoguaianolide sesquiterpene isolated from *Inula japonica*, on osteoclastogenesis *in vitro* and Ti particle-induced osteolysis *in vivo*. The effect of britanin was examined in the osteoclastogenesis of mouse bone marrow-derived macrophages (BMMs) using TRAP staining, RT-PCR, western blotting and immunocytochemistry. The protective effect of britanin was examined in a mouse calvarial osteolysis model and evaluated using micro-CT and histomorphometry. Britanin inhibited osteoclast differentiation and F-actin ring formation in the presence of macrophage colony-stimulating factor and receptor activator of nuclear factor  $\kappa$ B ligand in BMMs. The expression of osteoclast-specific marker genes, including tartrate-resistant acid phosphatase, cathepsin K, dendritic cell-specific transmembrane protein,

matrix metalloproteinase 9 and nuclear factor of activated T-cells cytoplasmic 1, in the BMMs was significantly reduced by britanin. In addition, britanin reduced the expression of B lymphocyte-induced maturation protein-1, which is a transcriptional repressor of negative osteoclastogenesis regulators, including interferon regulatory factor-8 and B-cell lymphoma 6. Conversely, britanin increased the expression levels of anti-oxidative stress genes, namely nuclear factor erythroid-2-related factor 2, NAD(P)H quinone oxidoreductase 1 and heme oxygenase 1 in the BMMs. Furthermore, the administration of britanin significantly reduced osteolysis in a Ti particle-induced calvarial osteolysis mouse model. Based on these findings, it is suggested that britanin may be a potential therapeutic agent for wear particle-induced osteolysis and osteoclast-associated disease.

## Introduction

Osteolysis is a pathological bone disorder primarily caused by the abnormal activation of osteoclasts and is widely reported in patients with dental implants and orthopedic prostheses. The long-term use of titanium (Ti)-based implants may produce wear debris, which subsequently causes osteolysis and aseptic loosening associated with orthopedic implant failure and dental peri-implantitis (1).

Wear particles are phagocytosed by monocytes/macrophages around the prosthesis, which, in turn, release pro-inflammatory cytokines such as tumor necrosis factor (TNF)- $\alpha$ , interleukin (IL)-1 $\beta$  and IL-6, resulting in the proliferation and differentiation of bone marrow-derived macrophages (BMMs) (2,3). In addition, wear particles can disrupt the balance of antioxidant systems via the generation of large amounts of reactive oxygen species (ROS) and the promotion of osteoclast formation. Endogenous ROS generated following receptor activator of nuclear factor  $\kappa$ B (NF- $\kappa$ B) ligand (RANKL)-induced stimulation can activate the NF- $\kappa$ B/mitogen-activated protein kinase (MAPK)/phosphoinositide 3-kinase pathway. Conversely, ROS also activate the nuclear factor erythroid-2-related factor 2 (Nrf2)-Kelch-like ECH-associated protein 1 pathway to relieve oxidative

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stress (4,5) and suppress osteoclast formation and activity (6). In summary, these findings demonstrate that wear particles promote osteoclast differentiation via the secretion of inflammatory cytokines and the disruption of the antioxidant system balance.

Osteoclasts are multinucleated cells derived from the bone marrow macrophage/monocyte lineage (7). Two essential cytokines regulate osteoclastogenesis: Macrophage colony-stimulating factor (M-CSF) and RANKL. M-CSF plays a critical role in the survival and proliferation of osteoclast progenitor cells. The binding of RANKL to RANK in BMMs activates downstream targets, including MAPK, NF- $\kappa$ B and c-Fos (8). Activation of NF- $\kappa$ B and c-Fos induces the expression of nuclear factor of activated T-cells cytoplasmic 1 (Nfatc1); subsequently, Nfatc1 functions in conjunction with other transcription factors such as PU.1 activator protein 1, microphthalmia-associated transcription factor and cAMP-responsive element binding protein (9). Nfatc1 is a master transcriptional regulator of osteoclast differentiation, which upregulates osteoclast-specific genes such as cathepsin K (*Ctsk*), tartrate-resistant acid phosphatase 5 (*Acp5*), matrix metalloproteinase 9 (*Mmp9*) and dendritic cell-specific transmembrane protein (*Dc-stamp*). Moreover, Nfatc1 regulates the expression of B lymphocyte-induced maturation protein-1 (Blimp1), which is a transcriptional repressor of negative regulators of osteoclastogenesis, including interferon regulatory factor-8 (Irf8), v-maf musculoaponeurotic fibrosarcoma oncogene family, protein B (MafB) and B-cell lymphoma 6 (Bcl6), during osteoclast formation (10,11). Therefore, precise control of bone turnover is pivotal for bone health and is an efficient strategy for the inhibition of abnormal osteoclast formation and activity.

Britanin is a natural product that has a pseudoguaianolide sesquiterpene structure with an exomethylene group, and has been shown to exert preventive effects against inflammation, asthma and allergy (12-14). Britanin also inhibits lipopolysaccharide-induced nitric oxide production, prostaglandin E2 and pro-inflammatory cytokines via the inactivation of NF- $\kappa$ B and MAPK in macrophages (15), and exerts anti-tumor effects by inhibiting the proliferation and migration of breast, gastric and pancreatic cancer cell lines (16,17). In addition, britanin demonstrated the ability to inhibit tumor growth in a pancreatic cancer xenograft mouse model (17). Furthermore, britanin has been reported to protect organs against myocardial ischemia/reperfusion injury and cerebral ischemic reperfusion injury via the activation of Nrf2 signaling (18,19). These reports suggest that britanin exhibits multiple biological activities, including anti-inflammatory and anti-oxidative effects.

In the present study, the effects of britanin on *in vitro* osteoclastogenesis and *in vivo* Ti particle-mediated osteolysis were investigated in a mouse model. The mechanism underlying the effect of britanin on osteoclast formation was also investigated.

## Materials and methods

**Chemicals and reagents.**  $\alpha$ -modified Eagle's medium ( $\alpha$ -MEM) was obtained from Gibco; Thermo Fisher

Scientific, Inc. Recombinant murine M-CSF and RANKL were purchased from R&D Systems, Inc. The Leukocyte Acid Phosphatase kit [tartrate-resistant acid phosphatase (TRAP)] kit, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), dimethyl sulfoxide (DMSO) and all other unspecified reagents were purchased from Sigma-Aldrich; Merck KGaA. Specific antibodies against phospho-extracellular signal-regulated kinase (ERK; cat. no. 9101S), EK1/2 (cat. no. 9102S) phospho-c-Jun N-terminal kinase (JNK; cat. no. 9251S), JNK (cat. no. 9252S), phospho-p38 (cat. no. 9211S), p38 (cat. no. 9212S), phospho-AKT (cat. no. 4058S), AKT (cat. no. 9272S) and phospho-inhibitor of nuclear factor  $\kappa$ B ( $\text{I}\kappa\text{B}\alpha$ ; cat. no. 2859S) were purchased from Cell Signaling Technology, Inc. Antibodies against  $\text{I}\kappa\text{B}\alpha$  (cat. no. sc-847), Irf8 (cat. no. sc-6058) and Nfatc1 (cat. no. sc-7294) were purchased from Santa Cruz Biotechnology, Inc. Antibodies against cathepsin K (cat. no. MAB3324) and  $\beta$ -actin (cat. no. A5441) were purchased from MilliporeSigma. Britanin, which is also known by the International Union of Pure and Applied Chemistry name of 9-acetyloxy-8-hydroxy-5,8a-dimethyl-1-methylidene-2-oxo-4,5,5a,6,7,8,9,9a-octahydro-3aH-azuleno[6,5-b]furan-6-yl) acetate, was isolated from *Inula japonica* (Inulae Flos), as described previously (20) and its purity was confirmed by one-dimensional nuclear magnetic resonance data comparison. Britanin was dissolved in DMSO for further experiments. Ti particles (purity, 99.9%; diameter, 30-50 nm; US Research Nanomaterials, Inc.) for *in vivo* experiments were prepared as previously described (21).

**Cell culture and osteoclast differentiation.** BMMs were isolated from mouse bone marrow as described previously (22). Briefly, cells were extracted from the tibiae and femurs of 4- to 6-week-old ICR male mice. The mice were purchased from Dae Han Bio Link Co., Ltd., and maintained under conventional housing conditions at 22-24°C and 50-60% humidity, with a 12-h light/12-h dark cycle and free access to water and food pellets. The mice were euthanized by cervical dislocation following CO<sub>2</sub> treatment. The flow rate of CO<sub>2</sub> was maintained at 40-60% chamber volume/min. Subsequently, the isolated cells were cultured in  $\alpha$ -MEM (cat. no. 11900) containing 10% fetal bovine serum (cat. no. 16000; Gibco; Thermo Fisher Scientific, Inc.) for 24 h on a Petri dish. Non-adherent cells were centrifuged on a Histopaque density gradient medium (Sigma-Aldrich; Merck KGaA) and cultured for 3 days in the presence of M-CSF (30 ng/ml) to obtain BMMs. The BMMs were cultured with RANKL (20 ng/ml) and M-CSF (10 ng/ml) in the presence of different concentrations of britanin (1 or 5  $\mu$ M) in 96-well plates. After 4 days, the cells were fixed in 4% paraformaldehyde for 15 min at room temperature and then stained using a TRAP staining kit according to the manufacturer's instructions. TRAP-positive multinucleated cells with  $\geq 3$  nuclei were considered osteoclasts. To examine signaling pathway in response to britanin, BMMs were stimulated with DMSO or 5  $\mu$ M of britanin for 0, 5, 15, and 30 min, then the reaction was terminated using ice-cold PBS.

**Cell viability assay.** The cytotoxic effects of britanin were evaluated using the MTT assay. BMMs were cultured with M-CSF (10 ng/ml) with or without britanin (1 or 5  $\mu$ M) for

3 days at 37°C. MTT reagent was then added to each well, and the cells were incubated at 37°C for 2 h. The formazan was subsequently solubilized with DMSO. The absorbance at 570 nm was measured using an Epoch 96-well microplate reader (BioTek Instruments, Inc.).

**Western blotting.** BMMs were lysed using RIPA lysis buffer (Pro-Prep™; Intron Biotechnology, Inc.) containing protease and phosphatase inhibitors. Protein concentrations were determined using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Inc.). Proteins (30 µg/lane) were separated on 10% gels using sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to 0.2-µm nitrocellulose membranes (Whatman plc; Cytiva). Before incubation with the primary antibody, the membranes were blocked with a blocking buffer [3% non-fat milk in Tris-buffered saline containing 0.1% Tween 20 (TBS-T)] for 1 h at room temperature. The membranes were incubated with specific primary antibodies (1:1,000 by volume) overnight at 4°C in blocking buffer. The membranes were then washed with TBS-T and incubated with a secondary antibody (HRP-conjugated; cat. no. PA1-74362; 1:5,000 by volume; Thermo Fisher Scientific, Inc.) in blocking buffer for 1 h at room temperature. Immunoreactive bands were analyzed using a WesternBright ECL kit (Advansta, Inc.) and recorded using a chemiluminescent imager (cSeries Capture Software, version 1.9.5.0606; Azure Biosystems, Inc.).

**Immunofluorescence staining.** BMMs were plated on glass coverslips and cultured with RANKL (20 ng/ml) and M-CSF (10 ng/ml) in the presence or absence of britanin (5 µM). After 4 days of culture, the cells were fixed with 4% paraformaldehyde for 15 min at room temperature and then treated with 0.25% Triton X-100 in phosphate-buffered saline (PBS). The cells were blocked with 5% normal goat serum (S-1000; Vector Laboratories, Inc.) in PBS for 1 h at room temperature, after which the cells were incubated with Nfatc1 primary antibody (1:500) overnight at 4°C, followed by Alexa Fluor 488-conjugated secondary antibody (1:100; cat. no. A-11059; Thermo Fisher Scientific, Inc.) for 1 h at room temperature. Rhodamine-conjugated phalloidin (1:1,000; Cytoskeleton, Inc.) and 4',6-diamidino-2-phenylindole dihydrochloride (1:10,000; Santa Cruz Biotechnology, Inc.) were used to stain actin and nuclei, respectively. Fluorescence images were obtained using a Leica DM2500 microscope (Leica Microsystems GmbH). The images were aligned using ImageJ software (version 1.52a; National Institutes of Health).

**Bone resorption pit assay.** Briefly, 2.5x10<sup>4</sup> BMMs were placed on each bone slice (IDS Nordic) and incubated with RANKL (20 ng/ml) and M-CSF (10 ng/ml) for 3 days. After osteoclast formation, the cells were treated with or without 5 µM britanin for 2 days at 37°C. The bone slices were washed and soaked in hematoxylin solution for 30 sec at room temperature to visualize the resorption pits. The pit area was quantified using ImageJ software (version 1.52a).

**Reverse transcription-quantitative PCR (RT-qPCR).** Total RNA was extracted from britanin or DMSO-treated cells for 1 day using TRI-Solution™ (Bio Science Technology), and

1 µg RNA was converted into cDNA using SuperScript II reverse transcriptase according to the manufacturer's protocol (Invitrogen; Thermo Fisher Scientific, Inc.). The cDNA and each primer set were mixed with SYBR Premix Ex Taq (Takara Bio, Inc.). qPCR was performed using a LightCycler 1.5 Real-Time PCR System (Roche Diagnostics). The amplification conditions consisted of an initial denaturation step at 95°C, followed by 40 cycles of denaturation at 95°C, annealing at 60°C and extension at 72°C. The primers used for the qPCR are listed in Table I and included *Gapdh* as the reference gene. The relative gene expression data were calculated using the 2<sup>-ΔΔC<sub>q</sub></sup> method (23).

**Ti particle-induced calvarial osteolysis model.** Animal experiments were approved by the Committee on the Care and Use of Animals in Research at Kyungpook National University (Daegu, Korea; approval no. 2021-0071). As previously described, a mouse calvarial osteolysis model was established to determine the protective effect of britanin on osteolysis *in vivo* (24). Briefly, 6-week-old C57BL/6 male mice (n=20) were divided into four groups: Sham (DMSO control; n=4), Ti + vehicle (DMSO) (15 mg Ti particles; n=6), Ti + 2 mg britanin (15 mg Ti particles plus 2 mg/kg/day britanin; n=4) and Ti + 25 mg britanin (15 mg Ti particles plus 25 mg/kg/day britanin; n=6). The mice were purchased from Dae Han Bio Link Co., Ltd., and maintained under conventional housing conditions at 22-24°C, with 50-60% humidity, a 12-h light/12-h dark cycle, and free access to water and food pellets. For mouse anesthesia, 240 mg/kg avertin was injected intraperitoneally. The mouse heads were shaved and disinfected using a 10% povidone-iodine solution under anesthesia. A periosteal pocket was created using a 28-gauge needle, followed by an injection of pure Ti particles (15 mg) dissolved in 30 µl PBS and embedded in the center of the calvarium under the periosteum. Britanin (2 or 25 mg/kg) was administered intraperitoneally daily for 9 days, while DMSO was administered daily to the mice in the sham and vehicle groups. No adverse events were detected during the animal experiments, and the mice were sacrificed at the end of the experimental period (10 days). To euthanize the mice, 480 mg/kg avertin was injected intraperitoneally, and cervical dislocation was performed under anesthesia. The calvariae were isolated and fixed for 1 day in 4% paraformaldehyde at 4°C for micro-computed tomography (CT) and histological analyses.

**Micro-CT scanning.** Harvested mouse calvariae were fixed and then analyzed using a high-resolution micro-CT scanner (Skyscan 1272; Bruker Corporation). The scan was set to a resolution of 10 µm with a voltage source of 70 kV and current of 142 µA. The three-dimensional image was reconstructed using CTvox software (version 3.0.0 r1114; Bruker), and a rectangular region of interest (3.2x2.1x0.1 mm) around the midline suture was assessed for further quantitative analysis as previously described (25). Bone volume as a percentage of tissue volume and the number of pores in each sample were measured as previously reported (26).

**Histological and histomorphometric analysis.** Calvarial samples were decalcified in 10% EDTA for 10 days and then

Table I. PCR primer sequences.

| Gene                           | Primer sequence (5' to 3')                                 | Amplicon size (bp) | RefSeq NCBI no. | Amplification factor (E) |
|--------------------------------|--|--------------------|-----------------|--------------------------|
| <i>Nfatc1</i>                  | F: ACCACCTTTCGCAACCA<br>R: TTCCGTTTCCCGTTGCA               | 72                 | NM_001164112.1  | 2.03                     |
| <i>Ctsk</i>                    | F: GGCTGTGGAGGCGGCTAT<br>R: AGAGTCAATGCCTCCGTTCTG          | 66                 | NM_007802.4     | 1.94                     |
| <i>Mmp9</i>                    | F: AAAGACCTGAAAACCTCCAACCT<br>R: GCCCGGGTGTAAACCATAGC      | 77                 | NM_013599.5     | 1.97                     |
| <i>Acp5</i>                    | F: TCCCAATGCCCATTC<br>R: CGGTTCTGGCGATCTCTTTG              | 63                 | NM_001102405.1  | 2.10                     |
| <i>Dc-stamp</i>                | F: CTTCCGTGGGCCAGAAGTT<br>R: AGGCCAGTGCTGACTAGGATGA        | 64                 | NM_029422.4     | 2.13                     |
| <i>TNF-<math>\alpha</math></i> | F: GGTGCCTATGTCTCAGCCTCTT<br>R: GCCATAGAAGTATGAGAGGGAG     | 139                | NM_013693.3     | 2.03                     |
| <i>Nrf2</i>                    | F: CAGCATAGAGCAGGACATGGAG<br>R: GAACAGCGGTAGTATCAGCCAG     | 107                | NM_010902.5     | 1.89                     |
| <i>Nqo1</i>                    | F: GCCGAACACAAGAAGCTGGAAG<br>R: GGCAAATCCTGCTACGAGCACT     | 120                | NM_008706.5     | 2.08                     |
| <i>HO-1</i>                    | F: CACTCTGGAGATGACACCTGAG<br>R: GTGTTCCCTCTGTCAGCATCACC    | 115                | NM_010442.2     | 2.14                     |
| <i>TRAF6</i>                   | F: AACTGTGCTGTGTCCATGGC<br>R: CAGTCTCATGTGCAACTGGG         | 246                | NM_001303273.1  | 1.89                     |
| <i>Blimp1</i>                  | F: TTCTTGTTGGTATTGTCGGGACT<br>R: TTGGGGACACTCTTTGGGTAGAGTT | 148                | NM_001405929.1  | 1.90                     |
| <i>Irf8</i>                    | F: GATCGAACAGATCGACAGCA<br>R: AGCACAGCGTAACCTCGTCT         | 214                | NM_001301811.1  | 1.91                     |
| <i>Bcl6</i>                    | F: ATGAGATTGCCCTGCATTTTC<br>R: TTCTTCCAGTTGCAGGCTTT        | 202                | NM_009744.5     | 1.89                     |
| <i>Gapdh</i>                   | F: ATGACATCAAGAAGGTGGTG<br>R: CATACCAGGAAATGAGCTTG         | 177                | NM_001411843.1  | 1.90                     |

*Nfatc1*, nuclear factor of activated T-cells, cytoplasmic 1; *Ctsk*, cathepsin K; *Mmp9*, matrix metalloproteinase 9; *Acp5*, tartrate-resistant acid phosphatase 5; *Dc-stamp*, dendritic cell-specific transmembrane protein; *TNF- $\alpha$* , tumor necrosis factor- $\alpha$ ; *Nrf2*, nuclear factor erythroid-2-related factor 2; *Nqo1*, NAD(P)H quinone oxidoreductase 1; *HO-1*, heme oxygenase 1; *TRAF6*, TNF receptor-associated factor 6; *Blimp1*, B lymphocyte-induced maturation protein-1; *Irf8*, interferon regulatory factor-8; *Bcl6*, B-cell lymphoma 6; NCBI, National Center for Biotechnology Information.

embedded in paraffin. The paraffinized samples were cut in the coronal direction to a section thickness of 6  $\mu\text{m}$ . Histological sections were stained with hematoxylin and eosin (H&E; hematoxylin for 5 min and eosin for 1 min at room temperature) or a TRAP kit (cat. no. 387A; MilliporeSigma) according to the manufacturer's instructions. The stained area was examined using a bright-field light microscope (Leica Microsystems GmbH), and the number of TRAP-positive osteoclasts in each sample was quantified using ImageJ software.

**Statistical analysis.** Experiments were performed in triplicate, and all data are presented as the mean  $\pm$  standard deviation. Statistical analyses were performed using a two-tailed unpaired Student's t-test or one-way analysis of variance with Tukey's multiple comparison post hoc test.  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**Britanin inhibits RANKL-induced osteoclastogenesis.** Britanin was originally identified through the screening of natural product-derived compounds in an osteoclast differentiation model, with testing conducted at a concentration of 5  $\mu\text{M}$  (22,27,28). Once its inhibitory effect on osteoclast differentiation was confirmed, its cytotoxicity was assessed across a range of concentrations from 1 to 10  $\mu\text{M}$ . No cytotoxic effects were observed on BMMs at these concentrations, as determined by MTT assay. In addition, a 5- $\mu\text{M}$  concentration of britanin significantly inhibited osteoclast differentiation in the presence of M-CSF (30 ng/ml) when applied for 3 days *in vitro*. As a result, concentrations of 1 and 5  $\mu\text{M}$  were selected for further investigation to demonstrate the inhibitory effect on osteoclasts *in vitro* in the present study. Moreover,



5  $\mu\text{M}$  britanin treatment significantly increased cell growth by 19% at 24 h (data not shown) and 11.5% at 72 h in BMMs when compared with that in the control group (Fig. S1).

The effect of britanin on osteoclast differentiation in BMMs was evaluated using TRAP staining. The BMMs were incubated with RANKL (20 ng/ml) and M-CSF (10 ng/ml) in the presence or absence of britanin. Multinucleated osteoclasts were formed in the control group, whereas britanin significantly inhibited osteoclast formation in a concentration-dependent manner, even when cell growth was enhanced. Compared with the control group, treatment with 1 and 5  $\mu\text{M}$  britanin significantly reduced the number of TRAP-positive multinucleated cells by 25 and 98.7%, respectively (Fig. 1). These results suggest that britanin directly suppressed osteoclast formation without affecting cell viability.

*Britanin suppresses the expression of osteoclast-specific genes but induces the expression of antioxidant marker genes.* To further confirm that britanin inhibits osteoclast differentiation, the mRNA expression levels of osteoclast-associated genes were examined by RT-qPCR. The results revealed that the expression of *Nfatc1* was increased in the RANKL-treated group, and britanin significantly suppressed this expression (Fig. 2A). Likewise, britanin significantly suppressed the RANKL-induced expression of osteoclast marker genes, namely *Acp5*, *Dc-stamp*, *Mmp9* and *Ctsk* (Fig. 2B-E). Following 3 days of treatment, britanin markedly decreased the protein levels of *Nfatc1* and *Ctsk* when compared with those in the osteoclastogenesis control (RANKL-treated) group (Fig. 2K). Inflammation and oxidative stress are major events that mediate osteolysis. Therefore, the effect of britanin on the expression of genes associated with inflammation and oxidative stress, namely TNF receptor-associated factor 6 (*TRAF6*) and *TNF- $\alpha$* , was examined. The results demonstrated a significant reduction in the mRNA expression levels of *TRAF6* and *TNF- $\alpha$*  in the RANKL-induced cells upon treatment with britanin (Fig. 2F and G).

*Nrf2* is a transcription factor that regulates cellular defense against oxidative stress, and its deficiency has been shown to promote RANKL-induced osteoclast differentiation (29,30). In BMMs undergoing osteoclastogenesis, britanin increased the mRNA expression of *Nrf2*, NAD(P)H quinone oxidoreductase 1 (*Nqo1*) and heme oxygenase 1 (*HO-1*) on day 4 (Fig. 2H-J). Taken together, these findings suggest that britanin may inhibit osteoclast differentiation via downregulation of the expression levels of genes associated with inflammatory cytokines and oxidative stress *in vitro*.

*Britanin suppresses F-actin ring formation.* RANKL-induced mature osteoclasts attach to the bone matrix, reorganize the cytoskeleton and form circular actin ring structures that provide a sealing zone for osteoclast bone resorption (31,32). Frequently, large osteoclasts indicate more bone resorption, but in certain cases, small osteoclasts can actively resorb bone (33). Therefore, the effect of britanin on F-actin ring formation was investigated using immunofluorescence staining. While the control group exhibited large F-actin rings, the formation of F-actin rings was impaired in the britanin-treated group (Figs. 3A and S2). Compared with

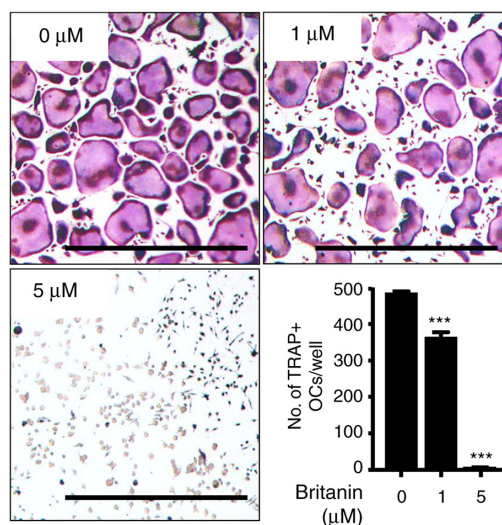


Figure 1. Britanin inhibits RANKL-induced osteoclast formation. Bone marrow-derived macrophages were cultured with macrophage colony-stimulating factor (10 ng/ml) and RANKL (20 ng/ml) in the presence of 0, 1 and 5  $\mu\text{M}$  concentrations of britanin for 4 days. The cells were stained for TRAP, and TRAP-positive multinucleated cells ( $\geq 3$  nuclei) were counted as OCs. Scale bar, 100  $\mu\text{m}$ . \*\*\* $P < 0.001$  vs. the vehicle-treated control as analyzed by one-way analysis of variance with Tukey's multiple comparison post hoc test. RANKL, receptor activator of nuclear factor  $\kappa\text{B}$  ligand; TRAP, tartrate-resistant acid phosphatase; OC, osteoclast.

the RANKL control group, britanin significantly reduced the number of F-actin rings and nuclear *Nfatc1*-positive cells by 85.1 and 87.1%, respectively (Fig. 3B and C). In addition, bone resorption was monitored to determine whether it was influenced by the effect of britanin on F-actin ring formation. Britanin treatment was performed after initial osteoclast formation, and under these conditions, britanin markedly reduced the resorption ability of bone slices compared with that in the RANKL control group (Fig. 3D). Collectively, these findings suggest that britanin may attenuate osteoclast function.

*Britanin inhibits osteoclast differentiation via the repression of transcriptional negative regulators.* To elucidate the molecular mechanism underlying the inhibitory effect of britanin on RANKL-induced osteoclastogenesis, RANKL downstream signaling pathways, including ERK, JNK, p38, AKT and NF- $\kappa\text{B}$ , were analyzed using western blotting. The phosphorylation of ERK was notably decreased by britanin at 5 and 15 min; however, the phosphorylation levels of p38, JNK, AKT and  $\text{I}\kappa\text{B}\alpha$  were not markedly altered (Fig. 4A and B). These data suggest that britanin suppresses the ERK signaling pathway, which may underlie its ability to attenuate osteoclast differentiation and function.

The expression of *Nfatc1* can also be suppressed by anti-osteoclastogenic genes such as *Irf8*, *Bcl6* and *MafB*, and the expression levels of these anti-osteoclastic genes can be downregulated by *Blimp1* (11-13). Britanin treatment for 4 days was confirmed to induce *Irf8* protein expression associated with M-CSF and RANKL (Fig. 4C). In the case of mRNA expression, compared with the control group, britanin significantly decreased the expression of *Blimp1* and significantly increased the expression of *Bcl6* and *Irf8* (Fig. 4D).

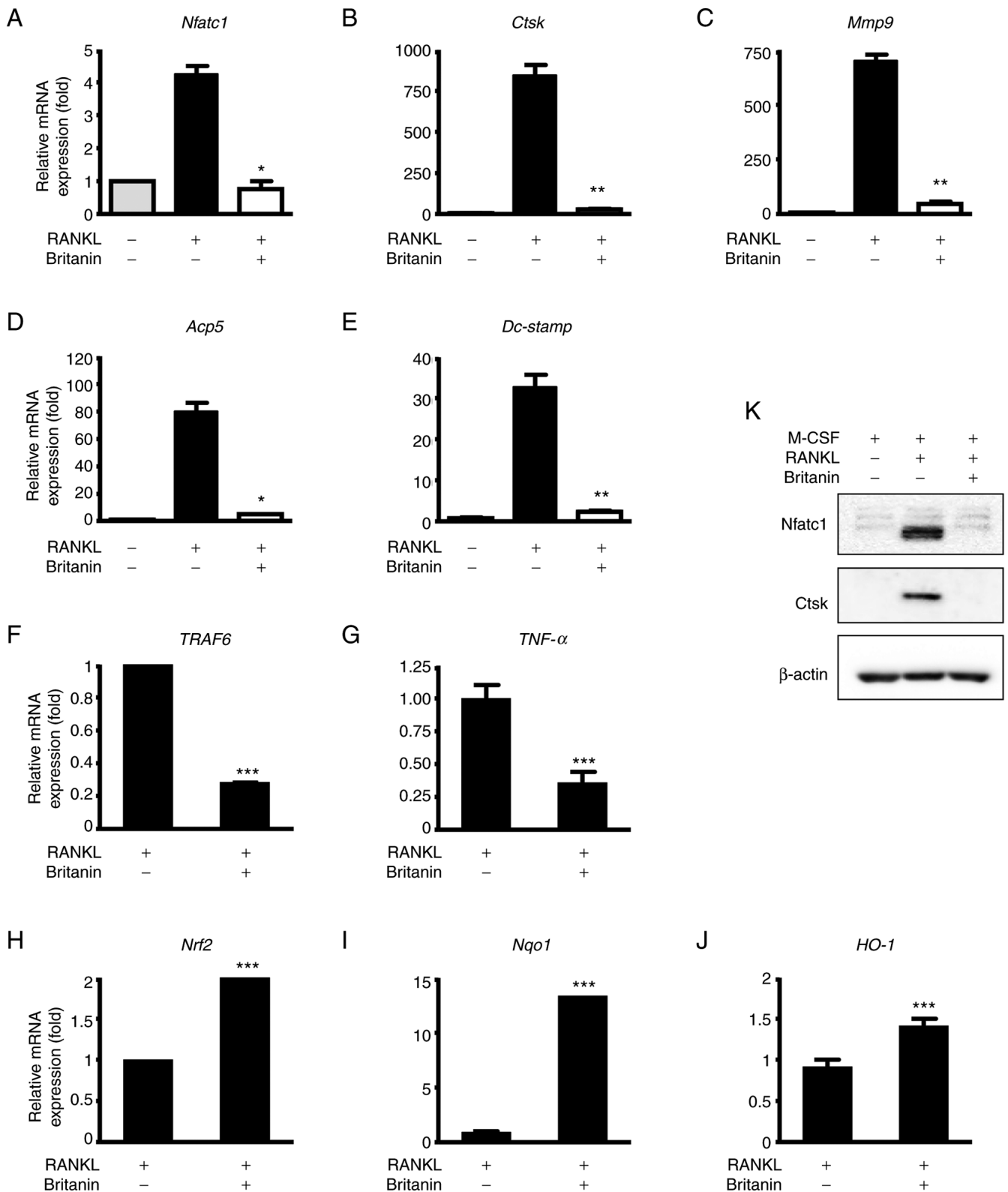


Figure 2. Britanin suppresses the expression of osteoclast and Nrf2-associated markers during osteoclastogenesis. (A-E) BMMs were cultured with M-CSF (10 ng/ml) and RANKL (20 ng/ml) in the absence or presence of britanin for 4 days and RT-qPCR was performed to analyze the expression of (A) *Nfatc1*, (B) *Ctsk*, (C) *Mmp9*, (D) *Acp5* and (E) *Dc-stamp*. (K) BMMs were incubated in an osteoclastogenic medium with vehicle or britanin for 3 days and the protein expression levels of *Nfatc1* and *Ctsk* were determined using western blotting. BMMs were cultured with M-CSF (10 ng/ml) and RANKL (20 ng/ml) in the absence or presence of britanin for (F and G) 1 day and (H and I) 4 days, and RT-qPCR was performed to analyze the expression of (F) *TRAF6*, (G) *TNF- $\alpha$* , (H) *Nrf2*, (I) *Nqo1* and (J) *HO-1*. \* $P < 0.05$ , \*\* $P < 0.01$  and \*\*\* $P < 0.001$  vs. RANKL-treated control as analyzed by (A-E) ANOVA with Tukey's multiple comparison post hoc test and (F-J) two-tailed unpaired Student's t-test. Nrf2, nuclear factor erythroid-2-related factor 2; BMMs, bone marrow-derived macrophages; M-CSF, macrophage colony-stimulating factor; RANKL, receptor activator of nuclear factor  $\kappa$ B ligand; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; *Nfatc1*, nuclear factor of activated T-cells, cytoplasmic 1; *Ctsk*, cathepsin K; *Mmp9*, matrix metalloproteinase 9; *Acp5*, tartrate-resistant acid phosphatase 5; *Dc-stamp*, dendritic cell-specific transmembrane protein; *TRAF6*, tumor necrosis factor receptor-associated factor 6; *TNF- $\alpha$* , tumor necrosis factor- $\alpha$ ; *Nqo1*, NAD(P)H quinone oxidoreductase 1; *HO-1*, heme oxygenase 1.

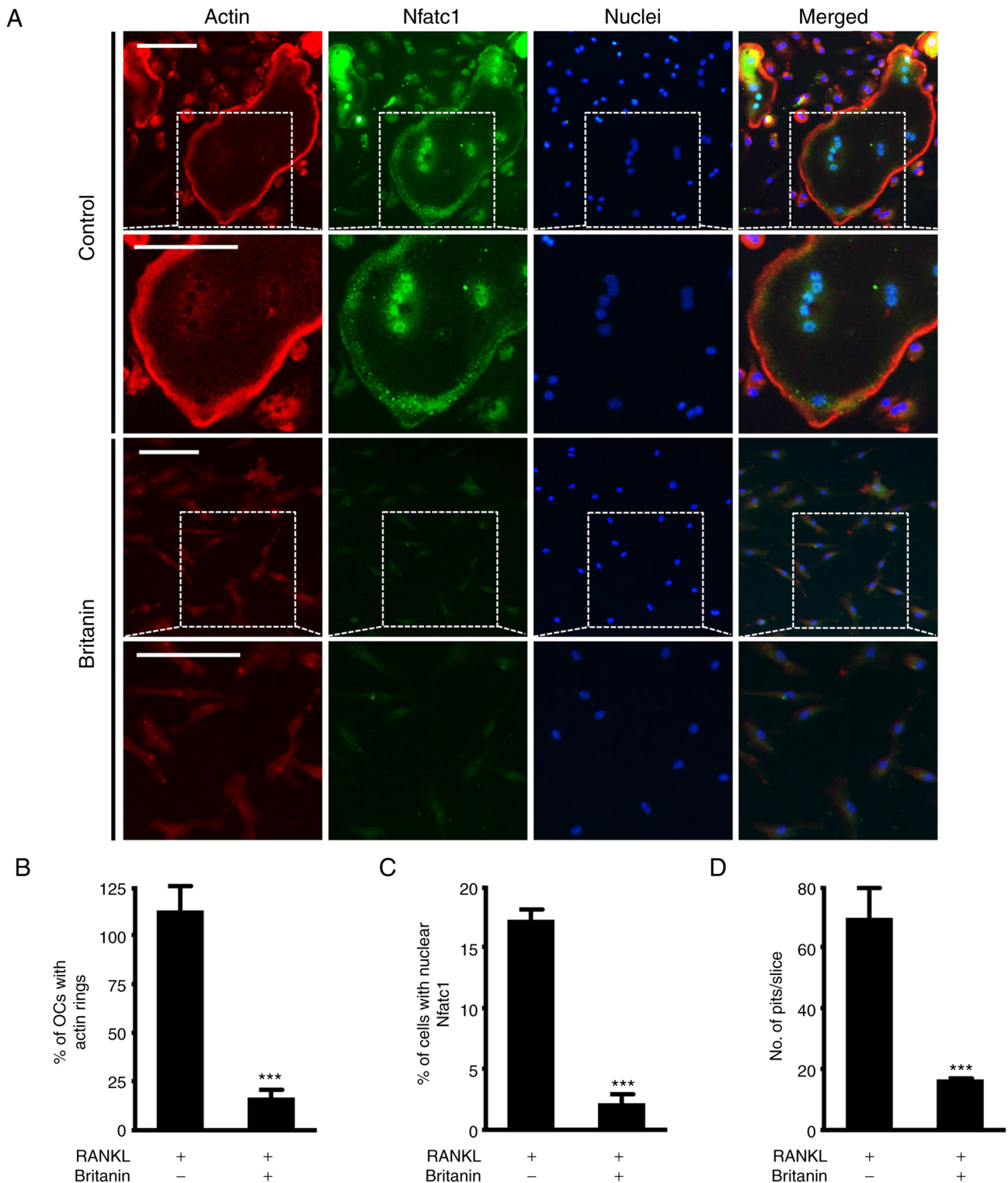


Figure 3. Britanin attenuates the formation of actin rings and resorption pits. (A) BMMs were incubated on glass coverslips with M-CSF (10 ng/ml) and RANKL (20 ng/ml) for 4 days in the absence or presence of britanin. Actin, nuclei and Nfatc1 were stained with rhodamine-phalloidin (red), 4',6-diamidino-2-phenylindole dihydrochloride (blue) and anti-Nfatc1 antibody (green), respectively. The white dashed box indicates the magnified region. Scale bar, 50  $\mu$ m. The percentage of (B) OCs displaying actin rings and (C) nuclear Nfatc1-positive cells was assessed. (D) BMMs were seeded on bone slices and cultured with an OC-inducing medium for 3 days. The cells were then treated with or without britanin for 2 days. Bone slices were stained with hematoxylin to observe the resorption pits. The number of resorption pits per bone slice was counted using ImageJ software. \*\*\* $P < 0.001$  vs. RANKL-treated control as analyzed using two-tailed unpaired Student's t-test. BMMs, bone marrow-derived macrophages; M-CSF, macrophage colony-stimulating factor; RANKL, receptor activator of nuclear factor  $\kappa$ B ligand; Nfatc1, nuclear factor of activated T-cells, cytoplasmic 1; OC, osteoclast.

These findings suggest that britanin may suppress osteoclast differentiation via the upregulation of negative regulators, including Irf8 and Bcl6.

*Britanin exhibits inhibitory effects on a Ti particle-induced mouse calvarial osteolysis model.* After confirming that britanin reduced osteoclast formation and function *in vitro*, the

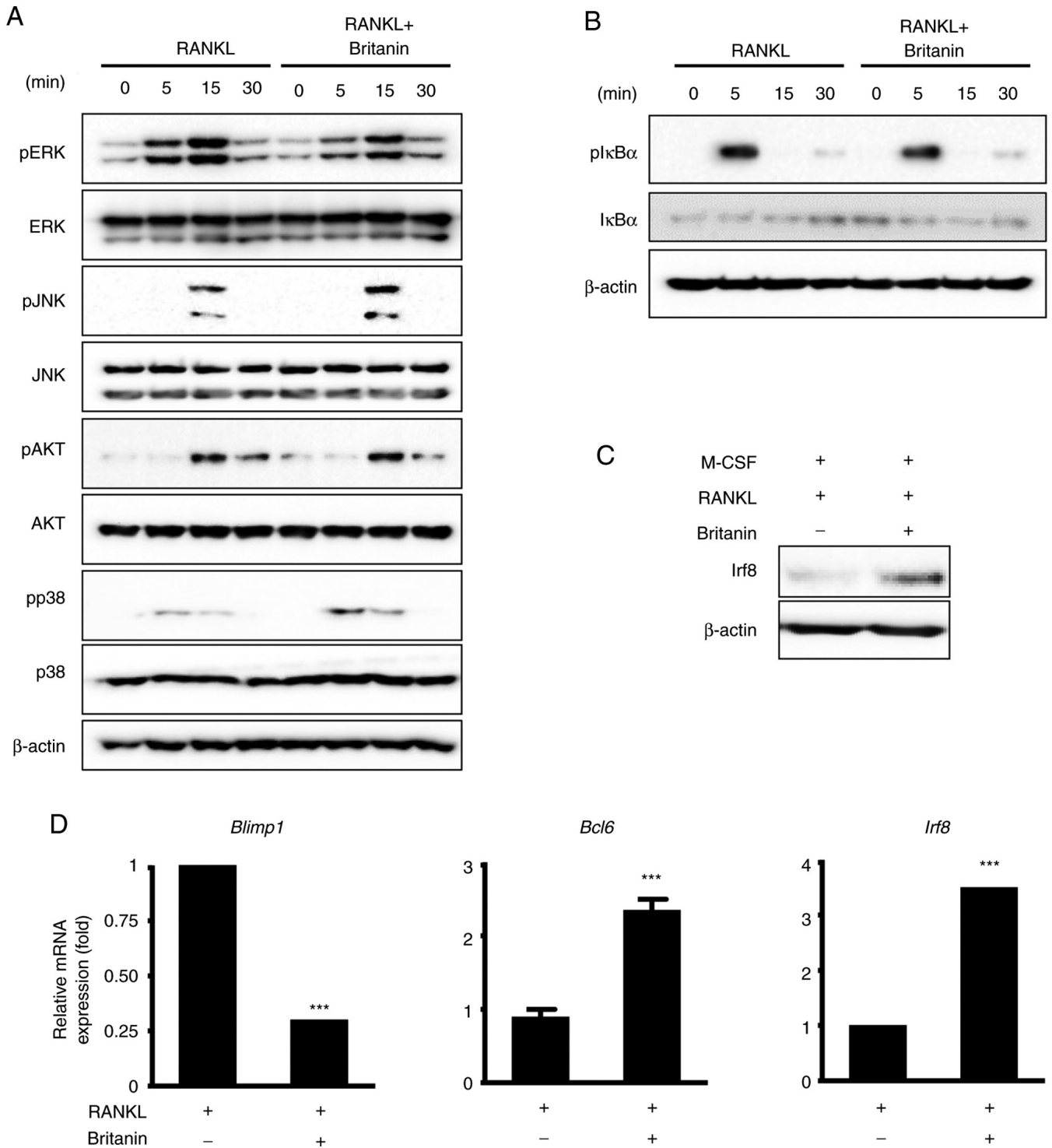


Figure 4. Britanin inhibits the suppression of negative mediators of RANKL-induced osteoclast differentiation. (A and B) BMMs incubated in serum-free medium were pretreated with britanin or vehicle for 1 h. Next, several time points were examined following treatment with RANKL (50 ng/ml). Phosphorylation levels of ERK, JNK, Akt, p38 and IκBα were determined using western blotting. (C) BMMs were incubated in an osteoclastogenic medium with vehicle or britanin for 3 days. The protein expression of Irf8 was examined using western blotting. (D) BMMs were cultured with M-CSF (10 ng/ml) and RANKL (20 ng/ml) in the absence or presence of britanin for 4 days. Reverse transcription-quantitative PCR was performed to analyze the expression of *Blimp1*, *Bcl6* and *Irf8*. \*\*\* $P < 0.001$  vs. RANKL-treated control as analyzed using two-tailed unpaired Student's t-test. RANKL, receptor activator of nuclear factor κB ligand; BMMs, bone marrow-derived macrophages; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; IκBα, inhibitor of nuclear factor κB; Irf8, interferon regulatory factor-8; M-CSF, macrophage colony-stimulating factor; *Blimp1*, B lymphocyte-induced maturation protein-1; *Bcl6*, B-cell lymphoma 6; p-, phosphorylated.

effect on osteolysis was investigated in a Ti particle-induced osteolysis mouse model (Fig. 5). In previous experiments (21), compounds that affected cell responses at a concentration

of  $\sim 5 \mu\text{M}$  *in vitro* also demonstrated effective results *in vivo* within the range of 20-30 mg/kg. Consequently, an initial dose of 25 mg/kg was selected for *in vivo* testing, whereas

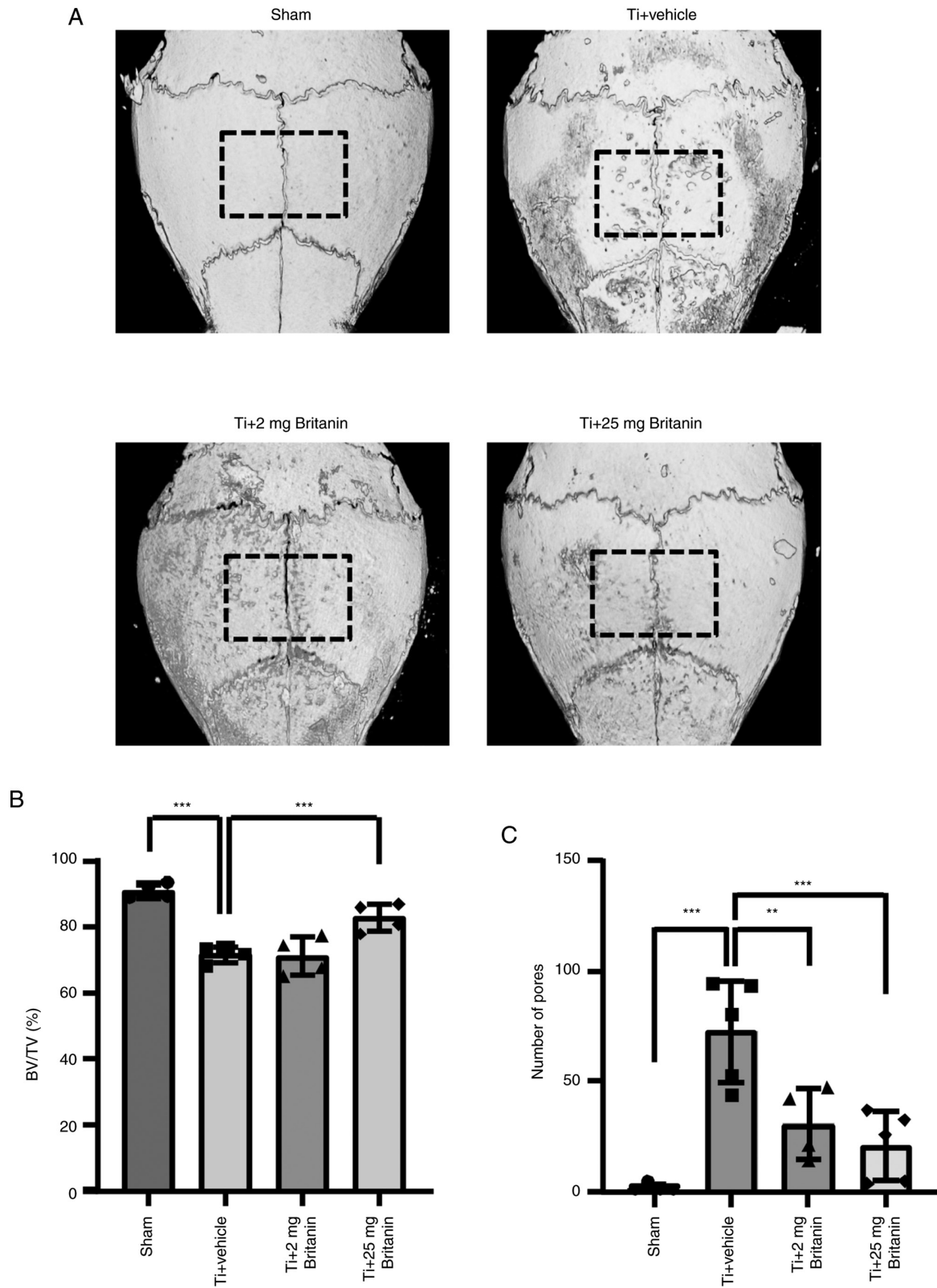


Figure 5. Britanin prevents Ti particle-induced mouse calvarial osteolysis. (A) Representative micro-computed tomography three-dimensional reconstructed images from each group. (B) BV/TV percentage and (C) the number of pores were measured for each sample within the area in the black dashed boxes. \*\* $P < 0.01$  and \*\*\* $P < 0.001$  as analyzed by one-way ANOVA with Tukey's multiple comparison post hoc tests. Ti, titanium; BV, bone volume; TV, tissue volume.

an additional dose of 5 mg/kg was selected to assess the dose-dependent efficacy of britanin. To induce osteolysis, Ti particles were implanted into mouse calvaria. This model allowed the measurement of bone resorption *in vivo* owing to

Ti particle-induced activated osteoclasts, which are typically generated in the Ti-implanted sites of patients (34). Despite excluding the load-bearing effect that can induce additional osteoclast-mediated bone resorption, Ti-particle-induced

inflammation and osteoclast differentiation were detected. Micro-CT analysis was performed in Ti particle-loaded sites, revealing the presence of extensive bone resorption in the Ti particle-implanted group (vehicle). However, britanin appeared to reduce bone resorption in a dose-dependent manner (Fig. 5A). Based on quantitative analysis, the administration of high-dose britanin (Ti + britanin; 25 mg/kg/day) significantly preserved bone volume compared with that in the Ti-loading group (Fig. 5B). The number of bone-resorbing pits was significantly elevated in the Ti group, and the effect of Ti was reduced in the britanin-treated groups in a dose-dependent manner (Fig. 5C).

Histochemical analysis demonstrated that britanin protected bone from Ti particle-induced resorption in a dose-dependent manner (Fig. 6A). In the H&E stained tissue, the periosteum layers of the calvarial bone were clean and flat in the sham group. By contrast, a deep and wide area of periosteum side bone was resorbed in the Ti + vehicle group. Treatment with britanin substantially and dose-dependently reduced the Ti particle-induced resorbed area. Moreover, TRAP staining showed that the number of TRAP-positive osteoclasts was reduced by 29 and 56% in the Ti + britanin 2 mg (2 mg/kg/day) and Ti + britanin 25 mg (25 mg/kg/day) groups, respectively, compared with that in the Ti-vehicle group (Fig. 6B). These results suggest that britanin inhibits *in vivo* osteoclast formation and indicate that britanin may protect the bone against Ti particle-induced osteolysis via the suppression of osteoclast formation in mice.

## Discussion

Inflammation and oxidative stress are known to mediate periprosthetic osteolysis (24). Ti is widely used in medical devices, including dental and joint implants, which are used to recover the function of damaged tissues. However, weight or load bearing can gradually generate wear particles of various sizes from the implants over time. These nano- to micro-sized wear particles induce inflammation, oxidative stress and periprosthetic osteolysis, and may subsequently result in implant failure (34-36). To the best of our knowledge, the present study is the first to demonstrate that britanin exerts antiosteoclastic activity, indicating its therapeutic potential against wear particle-induced osteolysis.

Oxidative stress is typically involved in osteoclast differentiation (37). Wear particles induce ROS-mediated immune responses (24) and are crucial for RANKL-induced osteoclast differentiation (38). Elevated ROS levels decrease *Nrf2* expression, thereby contributing to increased *Nfatc1* expression during osteoclast formation (39,40). Accordingly, ROS signaling is indicated to be a suitable target for the pharmacological treatment of bone loss (30). The pathogenesis of periprosthetic osteolysis involves several inflammatory events. Specifically, Ti particles induce inflammatory cytokines such as TNF- $\alpha$  and ILs (1). TNF- $\alpha$  promotes osteoclast differentiation in the presence of ROS, and the expression of TNF- $\alpha$  is increased by ROS in the presence of large quantities of iron-like wear particles. In the present study, britanin not only upregulated the expression of ROS defense molecules, including *Nrf2*, *Nqo1* and *HO-1*, in the absence of excess iron

*in vitro*, but also significantly inhibited the RANKL-induced expression of *TNF- $\alpha$* .

Considering the anti-inflammatory and antioxidative properties of britanin, it was speculated that britanin would be able to suppress abnormal osteoclast differentiation effectively, *in vitro* and *in vivo*. The inhibition of inflammation and ROS can suppress osteoclastogenesis; however, the regulation of osteoclast function can be challenging. Therefore, osteoclastogenesis is an important therapeutic target in periprosthetic osteolysis. The present study examined the effects of britanin on the differentiation and function of osteoclasts to explore its potential mode of action. The binding of RANK to RANKL leads to osteoclast differentiation and activation. The RANK-RANKL interaction activates the NF- $\kappa$ B and MAPK downstream signaling pathways by recruiting the signaling molecule *TRAF6* and subsequently inducing the expression of the master osteoclast regulator *Nfatc1* (41-44). In the present study, britanin significantly reduced the expression of *Nfatc1* and its target genes, as well as ERK phosphorylation, without impacting NF- $\kappa$ B activity. In previous studies, britanin was found to exhibit antitumor effects via the inhibition of NF- $\kappa$ B activity in pancreatic, gastric and prostate cancer cell lines (16,17,45). NF- $\kappa$ B inhibition has also been shown to mediate mast cell-mediated inflammatory responses (12,13). However, in the present study, the results indicated that britanin inhibited osteoclast differentiation via suppression of the ERK pathway. Accordingly, these results suggest that the effects of britanin on NF- $\kappa$ B and MAPK may differ according to the cell type or environment.

*Blimp1* is a transcriptional repressor that suppresses the expression of anti-osteoclastogenic genes, including *Irf8*, *MafB* and *Bcl6*. *Blimp1* deletion has been shown to increase *Bcl6* expression, resulting in osteopetrosis due to failure of osteoclast function (10,46). Conversely, *Irf8* binds to TRAF6 through physical interactions and induces TRAF6 ubiquitination (47). *Irf8* deficiency activates the expression of *Nfatc1*, resulting in osteoporosis *in vivo* owing to excessive osteoclast formation (48). In the present study, britanin inhibited *Blimp1* gene expression and increased the expression of *Bcl6* and *Irf8*, suggesting that its inhibitory effect on osteoclast differentiation may be partially associated with an attenuating effect on the *Blimp1*-mediated repression of anti-osteoclastogenic genes.

In addition, the present study evaluated the therapeutic potential of britanin in periprosthetic osteolysis in a mouse calvarial model. Ti particles significantly induced osteolysis in the examined mouse model, whereas britanin reduced bone resorption. Histological analysis revealed that britanin prevented Ti particle-induced osteolysis. In addition, TRAP staining indicated that britanin suppressed osteoclast formation *in vivo*. These results strongly suggest that britanin not only inhibits osteoclast differentiation but also suppresses inflammatory signals, such as those mediated by TNF- $\alpha$  and ROS. These britanin-mediated activities effectively inhibited osteolysis *in vivo*. Although the inhibitory effect of britanin on osteolysis *in vivo* was examined using a mouse calvaria osteolysis model, this may not be the most suitable model for fully mimicking human osteolysis. In this model, the absence of load-bearing prevents the gradual generation of titanium particles. Moreover, it does not replicate physiological



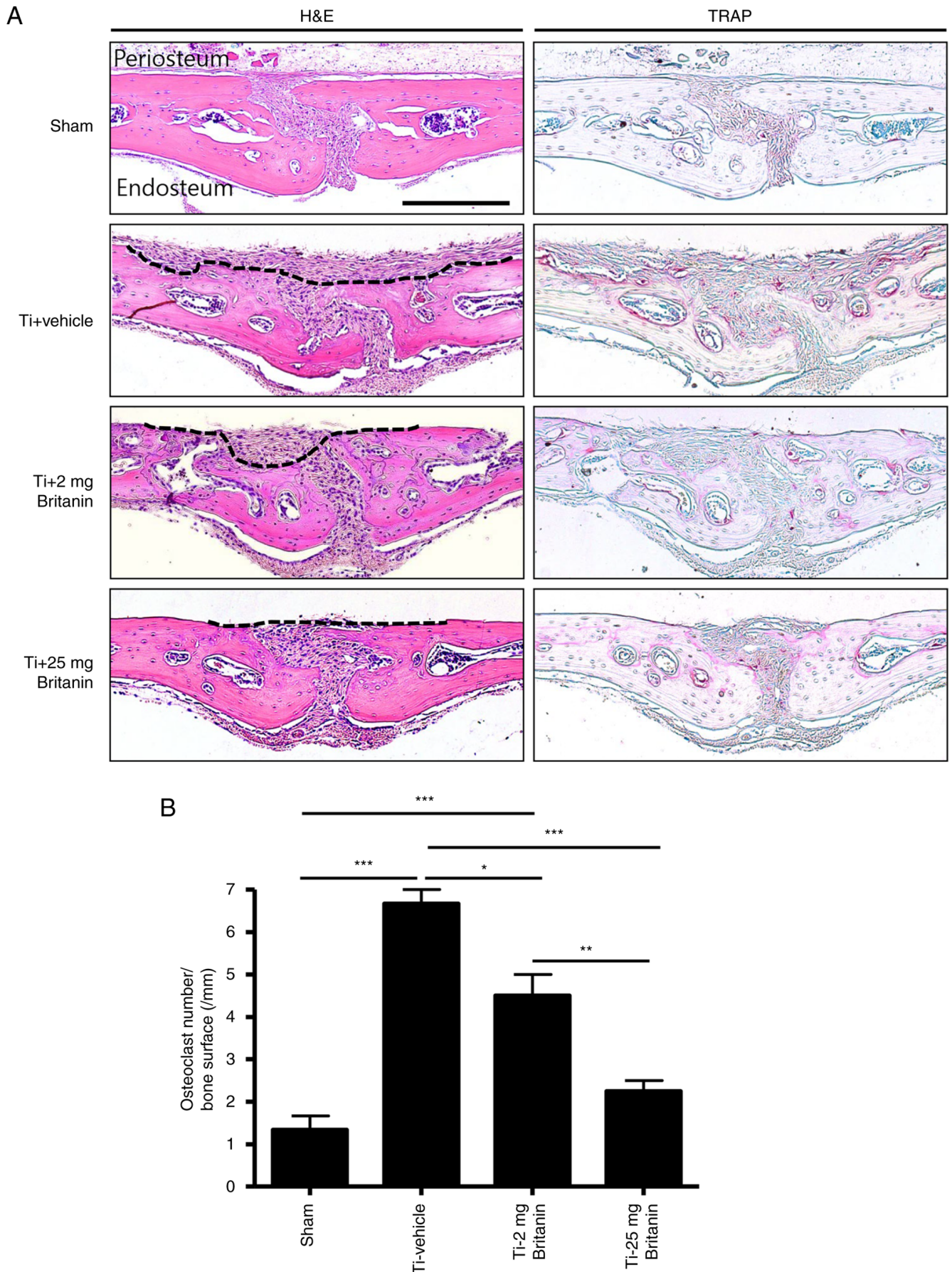


Figure 6. Britanin attenuates Ti particle-induced mouse calvarial osteolysis, assessed using histological and histomorphometric analysis. (A) H&E and TRAP staining were performed on the calvarial bone sections. The resorbed bone area is marked with a black dashed line. Scale bar, 250  $\mu$ m. (B) Histomorphometric analysis of the number of TRAP-positive cells. \* $P$ <0.05 \*\* $P$ <0.01 and \*\*\* $P$ <0.001 as analyzed by one-way ANOVA with Tukey's multiple comparison post hoc tests. Ti, titanium; H&E, hematoxylin and eosin; TRAP, tartrate-resistant acid phosphatase.

dynamic environments such as the movement of joints in activities such as running and jumping.

In summary, the present study indicated that britanin regulates inflammation and oxidative stress signaling and inhibits osteoclast differentiation by the downregulation of *Blimp1-Nfatc1* *in vitro*, thereby suppressing osteoclast differentiation and the expression of osteoclast-specific marker genes. In addition, britanin was demonstrated to protect bone from Ti particle-induced calvarial osteolysis *in vivo*. These results strongly suggest that britanin is a potential candidate for the treatment of osteoclast-associated diseases and wear particle-induced osteolysis.

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### Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

### Authors' contributions

JAK, SL and EKP were responsible for conceptualization. JAK, SL and HJI designed the study methodology. Validation was performed by JAK, SL and HJI. Formal analysis was performed by JAK, SL and EKP. JEK, KY and HC carried out the investigation. JM and HC contributed resources, and extracted and analyzed the britanin. The original draft of the manuscript was prepared and written by JAK and SL, and was reviewed and edited by HC and EKP. HC and EKP supervised the study. HC and EKP confirm the authenticity of all the raw data. All authors have read and approved the final version of the manuscript.

### Ethics approval and consent to participate

Animal experiments were approved by the Committee on the Care and Use of Animals in Research at Kyungpook National University (Daegu, Korea; approval no. 2021-0071).

### Patient consent for publication

Not applicable.

### Competing interests

The authors declare that they have no competing interests.

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