The fungal metabolite (+)-terrein abrogates osteoclast differentiation via suppression of the RANKL signaling pathway through NFATc1

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ABSTRACT

Pathophysiological bone resorption is commonly associated with periodontal disease and involves the excessive resorption of bone matrix by activated osteoclasts. Receptor activator of nuclear factor (NF)-κB ligand (RANKL) signaling pathways have been proposed as targets for inhibiting osteoclast differentiation and bone resorption. The fungal secondary metabolite (+)-terrein is a natural compound derived from *Aspergillus terreus* that has previously shown anti-interleukin-6 properties related to inflammatory bone resorption. However, its effects and molecular mechanism of action on osteoclastogenesis and bone resorption remain unclear. In the present study, we showed that 10 μM synthetic (+)-terrein inhibited RANKL-induced osteoclast formation and bone resorption in a dose-dependent manner and without cytotoxicity. RANKL-induced messenger RNA expression of osteoclast-specific markers including *nuclear factor of activated T-cells cytoplasmic 1 (NFATc1)*, the master regulator of osteoclastogenesis, *cathepsin K*, *tartrate-resistant acid phosphatase (Trap)* was completely inhibited by synthetic (+)-terrein treatment. Furthermore, synthetic (+)-terrein decreased RANKL-induced NFATc1 protein expression. This study revealed that synthetic (+)-terrein attenuated osteoclast formation and bone resorption by mediating RANKL signaling pathways, especially NFATc1, and indicated the potential effect of (+)-terrein on inflammatory bone resorption including periodontal disease.

1. Introduction

Osteoclasts are bone-resorptive, multi-nucleated cells derived from hematopoietic stem cells of the monocyte/macrophage lineage. Together with osteoblasts, osteoclasts are involved in bone homeostasis [1]. Receptor activator of nuclear factor (NF)-κB ligand (RANKL) and macrophage colony-stimulating factor (M-CSF) are two critical factors supplied by osteoblasts that are essential for the differentiation and maturation of osteoclast precursors [2]. During bone resorption, osteoclasts are highly polarized, form tightly sealed compartments on the bone surface, and secrete protons and proteases such as cathepsin K from the ruffled border, resulting in the degradation of bone matrix [1]. The abnormal enhancement of osteoclasts is therefore implicated in a variety of inflammatory bone resorptive diseases, including rheumatoid arthritis, osteoporosis, and periodontal disease [3–5].

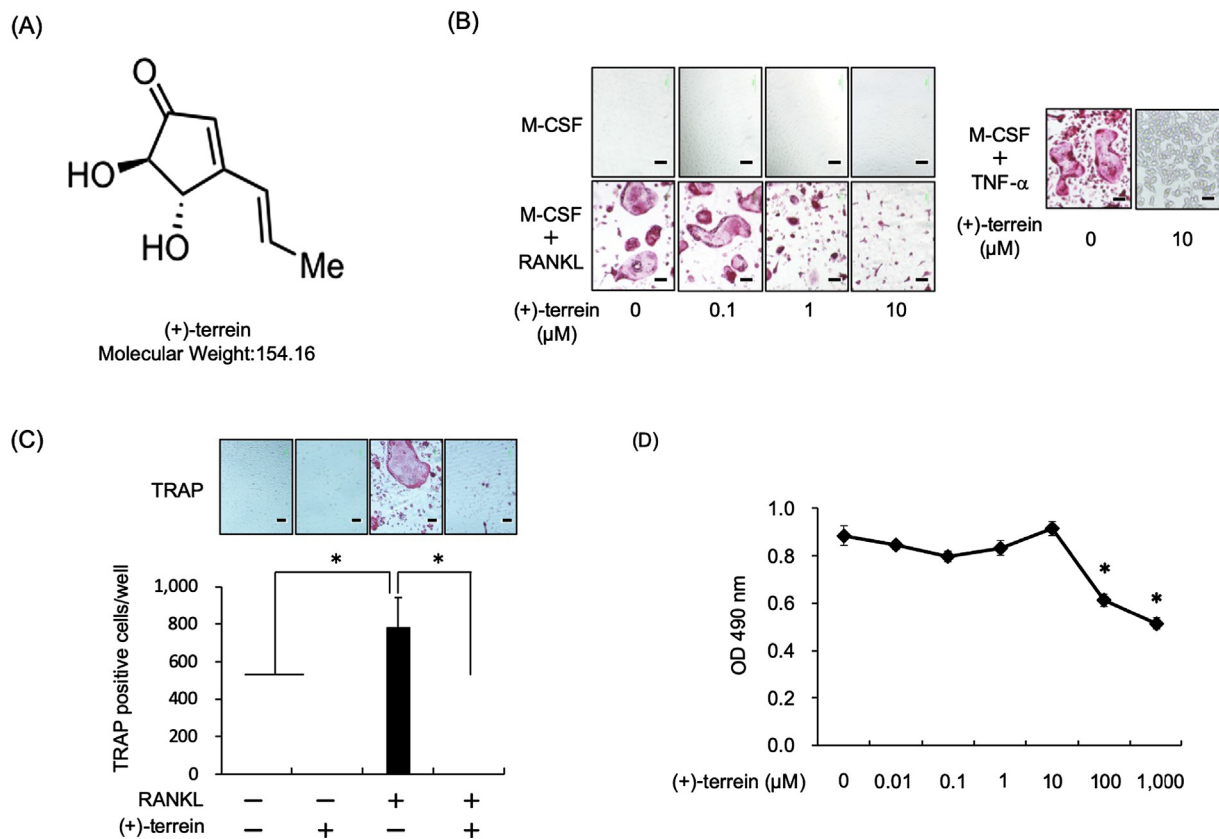Osteoclast-targeting small molecule inhibitors show potential as

Fig. 1. (A) Chemical structure of (+)-terrein; (B) Representative images of a 48-well plate showing the effects of synthetic (+)-terrein (0.01, 1, and 10 μM) on RANKL or TNF-α induced bone marrow macrophage (BMM)-derived osteoclast-like cell formation stained with tartrate-resistant acid phosphate (TRAP). The cells were photographed (original magnification, 100×, scale bar = 100 μm); (C) Analysis of the number of TRAP-positive cells formed in the presence or absence of synthetic (+)-terrein (10 μM). The cells were photographed (original magnification, 100×, scale bar = 100 μm) and TRAP-positive, multinucleated (> 3 nuclei) osteoclasts were counted. Columns represent the mean results of experiments carried out in triplicate and bars represent the standard division (SD) (n = 3, *p < 0.05). and (D) Effect of synthetic (+)-terrein on cell viability as assessed via MTS assay (n = 3, *p < 0.05, control: without (+)-terrein).

therapeutic drugs for bone-related diseases. Bisphosphonates, synthetic analogs of pyrophosphate, are currently the most important and effective anti-resorptive drugs commercially available, and established as the treatments of choice for various diseases of excessive bone resorption, including Paget's disease of bone, the skeletal complications of malignancy, and osteoporosis [6]. However, medicine-related osteonecrosis of the jaw (MRONJ) was reported as one of the serious side effects of bisphosphonate therapy, thus reducing the quality of life of patients receiving such treatment [7]. There is therefore a critical need to develop novel, safe, and effective host modulation treatments to prevent and treat inflammatory bone destructive diseases.

(+)-Terrein is a bioactive fungal secondary metabolite that was first isolated from *Aspergillus terreus* in 1935 (Fig. 1A) [8]. Various biological properties of (+)-terrein have been reported, including antibacterial activities [9], inhibition of angiogenin secretion in prostate cancer cells [10] and head/neck cancer [11], anti-inflammatory immune modulation in pulpal inflammation [12], and inhibition of biofilm formation [13]. Furthermore, we previously reported the effects of synthetic (+)-terrein on anti-interleukin (IL)-6 signaling and inhibition of vascular endothelial growth factor (VEGF) and M-CSF secretion in human gingival fibroblasts (HGFs) [14,15]. IL-6 is an important pro-inflammatory cytokine that induces a wide variety of biological effects including inflammatory bone resorption in rheumatoid arthritis [17] and periodontal disease [18]. We demonstrated that (+)-terrein may be a useful tool in regulating IL-6-associated inflammatory bone resorption, although the precise effects and functional mechanism of (+)-terrein on osteoclastogenesis remain unclear.

In this study, we aimed to investigate the effects of synthetic (+)-terrein on RANKL-induced osteoclastogenesis using mouse bone marrow macrophages (BMMs). Our results suggest that host modulation using synthetic (+)-terrein may be a novel therapeutic modality for the prevention of inflammatory bone destruction in patients with periodontal disease.

2. Material and methods

2.1. Reagents

(+)-Terrein was synthesized from dimethyl L-tartrate. All spectra (^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra, infrared (IR)) and specific rotation of synthetic (+)-terrein were similar to those of natural (+)-terrein and those previously described [14]. The following were used: human soluble RANKL (Wako, Hiroshima, Japan), human M-CSF (Leucoprol, Kyowa Hakko Kogyo, Japan), mouse TNF-α (R&D systems, Minneapolis, MN, USA), rabbit anti-nuclear factor of activated T-cells cytoplasmic 1 (NFATc1) monoclonal antibody (Santa Cruz Biotechnology, Dallas, TX, USA), rabbit anti-phospho ERK1/2, p38-MAPK, and NF-κB p65 monoclonal antibodies (Cell Signaling Technology Danvers, MA, USA), and mouse anti-β-actin monoclonal antibody (Sigma-Aldrich, St. Louis, MO, USA).

2.2. Cell preparation and cell viability assay

Five-week-old male C57BL6/J mice were obtained from Japan CLEA (Japan). Bone marrow cells were collected from the tibiae and femora and were cultured with M-CSF (50 ng/mL) for 3 days in culture dishes containing DMEM (Wako) supplemented with 10% fetal bovine serum (FBS, Invitrogen, Carlsbad, CA, USA). After 3 days, floating cells were

removed by rinsing with phosphate buffered saline (PBS) and attached cells were used as BMMs [18]. Cell viability was evaluated using an MTS assay kit (Promega, Madison, WI, USA). BMMs were seeded at a density of 1×10^4 cells in 100 μL medium in 96-well plates and incubated for 1 day at 37 °C in DMEM containing 10% FBS, M-CSF (100 ng/mL), and RANKL (100 ng/mL). At the same time, (+)-terrein was diluted to concentrations of 0.01–1000 μM. The medium was then removed and 20 μL MTS was added to the wells. Cells were incubated at 37 °C for 4 h to allow the color to develop. Optical density was measured at 490 nm using a microtiter plate-reader (Bio-Rad, Hercules, CA, USA). All animal experiments were performed in accordance with the Guidelines for Proper Conduct of Animal Experiments of the Science Councils of Japan and approved by the Animal Research Control Committee of Okayama University (approval no. OKU-2016277).

2.3. Osteoclast formation

To induce osteoclast differentiation, BMMs were further cultured with RANKL (100 ng/mL) and M-CSF (100 ng/mL) in the presence and absence of (+)-terrein (0.1–10 μM) for 5 days. Cells were then fixed in 4% paraformaldehyde and stained for tartrate-resistant acidic phosphatase (TRAP). TRAP-positive multinucleated cells containing more than three nuclei were considered osteoclasts.

2.4. Real-time polymerase chain reaction (PCR)

BMMs ($1.0 \times 10^5/cm^2$) were harvested to a 12 well-plate, and treated with RANKL (100 ng/mL) and M-CSF (100 ng/mL) in the presence and absence of (+)-terrein (10 μM) for 1 or 2 days. Total ribonucleic acid (RNA) was then isolated using an RNeasy® Mini Kit (Qiagen, Hilden, Germany), followed by deoxyribonucleic acid (DNA) removal using an RNase-free DNase kit (Qiagen). A total of 1 μg high-quality total RNA was then reverse-transcribed using the SuperScript® III First-Strand Synthesis System (Thermo Fisher Scientific, Waltham, MA, USA). Amplification reactions were performed using SYBR® green PCR Master Mix (Thermo Fisher Scientific). Up to 1 ng complimentary (c)DNA was then amplified using specific primers. Reactions were performed using a 7300 Real-Time PCR System (Thermo Fisher Scientific). Ratios of messenger (m)RNA levels to control values were calculated using the ΔCt method ($2^{-\Delta\Delta Ct}$). All data were normalized to the housekeeper control gene, β-actin. PCR conditions used were as follows: maintained for 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C, and 60 s at 60 °C. The primers used were as follows: 5′-CTCGA AAGACAGCACTGGAGCCAT-3′ (forward) and 5′-CGGCTGCCTTCCGTC TCATAG-3′ (reverse) for *Nfatc1*, 5′-TGACCACTGCCTTCCAATACG-3′ (forward) and 5′-TGCATTTAGCTGCCTTTGCC-3′ (reverse) for *cathepsin K*, 5′-ATGCCAGCGACAAGAGGTTC-3′ (forward) and 5′-TGGTTTCCAG CCAGCACATAC-3′ (reverse) for *Trap (Acp5)*, 5′-CCTTGGAAAGCTAGA AGCACAC-3′ (forward) and 5′-AAGAGGAGCAGAACGATGAGAC-3′ (reverse) for *RANK* and 5′-TAGCGGAACCGCTCATTGCC-3′ (forward) and 5′-TTCACCCACACTGTGCCC-3′ (reverse) for *β-actin* [19].

2.5. Western blotting

BMMs ($1.0 \times 10^5/cm^2$) were harvested to a 12 well-plate, and treated with RANKL (100 ng/mL) and M-CSF (100 ng/mL) in the presence and absence of (+)-terrein (10 μM) for 2 days (for detecting NFATc1 expression), 10–30 min (for detecting phospho-ERK1/2, phospho-p38 MAPK, phospho-NF-κB expression). After each time course, cells were lysed rapidly by the addition of ice-cold cell lysis buffer containing 50 mM NaCl, 10 mM Tris-HCl (pH 7.2), 1% Nonidet P-40, 5 mM EDTA-Na, 1 mM sodium ortho vanadate, 1% sodium dodecyl sulfate (SDS), and protease inhibitor cocktail (Sigma-Aldrich) for 10 min, according to a method previously described [20]. Protein concentration was determined via Bradford assay using bovine serum albumin (BSA, Sigma-Aldrich) as the standard. The lysates (30 μg) were

mixed with SDS sample buffer (1% (w/v) SDS, 45 mM Tris-HCl (pH 6.8), 15% (v/v) glycerol, 144 mM 2-mercaptoethanol, and 0.002% bromophenol blue) and samples were boiled for 5 min. The samples were separated via SDS-polyacrylamide gel electrophoresis (PAGE) on 7.5% or 12% polyacrylamide slab gels, and transferred onto polyvinylidene difluoride membranes. Membranes were blocked with 5% skim milk in Tris-buffered saline with Tween 20® (TBST; 20 mM Tris-HCl (pH 7.6) containing 150 mM NaCl and 0.1% (v/v) Tween 20®) for 1 h and incubated with anti-NFATc1 monoclonal antibody (1:1000), anti-phospho-ERK1/2 (1:1000), anti-phospho-p38 MAPK (1:1000), anti-phospho-NF-κB (1:1000) overnight at 4 °C. The membranes were incubated with secondary antibodies (goat anti-rabbit immunoglobulin G (IgG)-horse-radish peroxidase (HRP) conjugate, 1:2000 dilution; GE Healthcare, Chicago, IL, USA) for 1 h. HRP activity was visualized by incubating membranes in an electrochemiluminescence (ECL) detection system (SuperSignal® West Dura Extended Duration Substrate, Thermo Fisher Scientific) followed by autoradiography. At the end of these experiments, the immunodetection system and the bound antibody were removed from the blots by incubating the membranes with re-probing buffer (Restore™ Western Blot Stripping Buffer, Thermo Fisher Scientific). The blots were then stained with anti-β-actin antibody (1:10 000 dilution) to confirm that equal amounts of protein were present in each lane of the gel.

2.6. Resorption assay

BMMs (1×10^4 cells) were cultured for 7 days with M-CSF (100 ng/mL) and RANKL (100 ng/mL) in the presence and absence of (+)-terrein (10 μM) in 96-well, flat-bottomed osteo assay plates (Corning Inc., Corning, NY, USA). To quantitate resorption lacunae, cells were removed using 5% sodium hypochlorite, followed by extensive washing with distilled water and air-drying. The absorbed areas on the well were observed under a microscope and quantified at least 6 wells per condition using Image J software (National Institutes of Health, NIH).

2.7. Statistical analysis

Experimental results are presented as means ± standard deviation (SD). Multiple comparisons were conducted via one-way analysis of variance (ANOVA) and Tukey test. $p < 0.05$ was considered statistically significant.

3. Results

3.1. Synthetic (+)-terrein suppressed osteoclastogenesis

Osteoclastogenesis assays were performed to investigate the effects of synthetic (+)-terrein on the formation of TRAP-positive, multi-nucleated osteoclasts. Treatment with synthetic (+)-terrein resulted in the dose-dependent inhibition of RANKL-induced osteoclast formation, especially in the 10 μM synthetic (+)-terrein-treated group ($p < 0.05$, Fig. 1B and C). In addition, 10 μM synthetic (+)-terrein also inhibited TNF-α induced osteoclast differentiation (Fig. 1B). Cell viability was assessed via MTS cell proliferation assay. No cytotoxic effects were observed in BMMs cultured with ≤100 μM (+)-terrein (Fig. 1D) and the inhibitory effects of (+)-terrein on osteoclast formation were therefore not due to cytotoxicity.

3.2. Synthetic (+)-terrein suppressed RANKL-induced osteoclast function

To investigate the direct effects of synthetic (+)-terrein on osteoclast resorptive function, RANKL-treated BMM-derived osteoclasts were seeded on hydroxyapatite-coated multiwell plates and treated with synthetic (+)-terrein for 7 days. Analysis of hydroxyapatite resorption pits (Fig. 2A) revealed that the resorbed area was significantly decreased in the presence of synthetic (+)-terrein compared to that in the
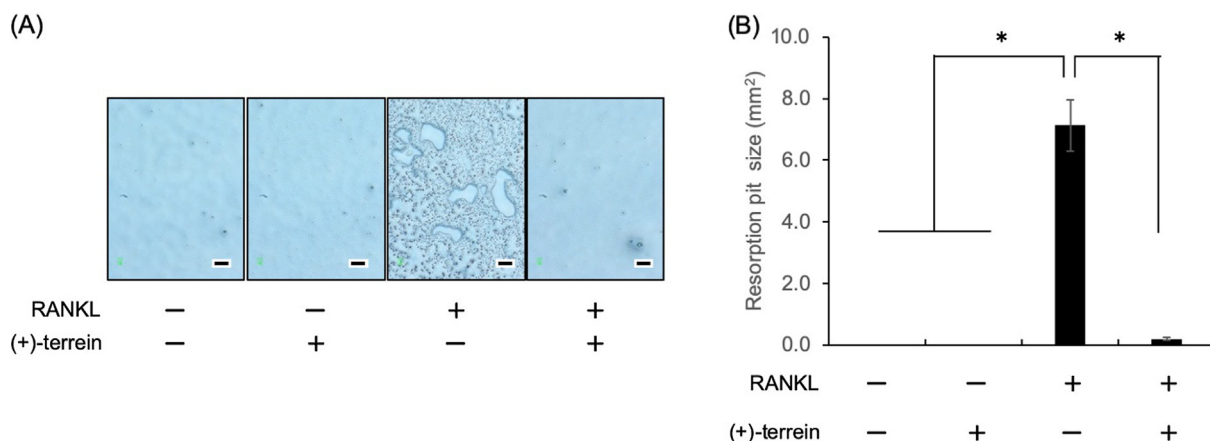
Fig. 2. (A) Synthetic (+)-terrein inhibited RANKL-induced hydroxyapatite resorption. Mouse BMMs (1×10^4 cells/well) were seeded into osteo assay plates and treated with RANKL/M-CSF (100 ng/mL each), in the presence and absence of synthetic (+)-terrein (10 μM), for 7 days. After incubation, images were obtained using a light microscope (original magnification, 200×, scale bar = 10 μm). Representative images of resorption pits are shown. (B) Representative resorption pit areas were measured using Image J software. Columns represent the means of experiments carried out at least 6 wells per condition, and bars represent the SD (n = 6, *p < 0.05).

untreated control group ($p < 0.05$, Fig. 2B).

3.3. Synthetic (+)-terrein inhibited RANKL-induced NFATc1 expression and related osteoclastogenesis related cathepsin K and Trap mRNA expression

In response to the stimulatory effects of RANKL, osteoclast differentiation was associated with up-regulation of *NFATc1*. As shown via quantitative real-time PCR, RANKL dramatically induced NFATc1 mRNA expression in the untreated group ($p < 0.05$, Fig. 3A). However, synthetic (+)-terrein (10 μM) significantly suppressed *NFATc1* expression ($p < 0.05$, Fig. 3A). At the protein level, addition of RANKL increased NFATc1 expression ($p < 0.05$, Fig. 3B), indicating that the NFATc1 signaling cascade was triggered during osteoclastogenesis.

Conversely, synthetic (+)-terrein significantly inhibited RANKL-induced NFATc1 protein expression ($p < 0.05$, Fig. 3B). In addition, RANKL dramatically induced osteoclastogenic factors mRNA expression, cathepsin K and Trap, downstream of NFATc1 (p < 0.05, Fig. 4). However, synthetic (+)-terrein (10 μM) significantly suppressed *cathepsin K* and *Trap* expression (p < 0.05, Fig. 4). While, RANKL and synthetic (+)-terrein did not affect the *RANK* expression (Fig. 4).

3.4. Synthetic (+)-terrein suppressed osteoclast differentiation even after RANKL initiation

To investigate the effects of synthetic (+)-terrein on RANKL-induced osteoclastogenesis at different time-points, synthetic (+)-terrein (10 μM) was added to the culture medium on days 0, 1, or 2 after
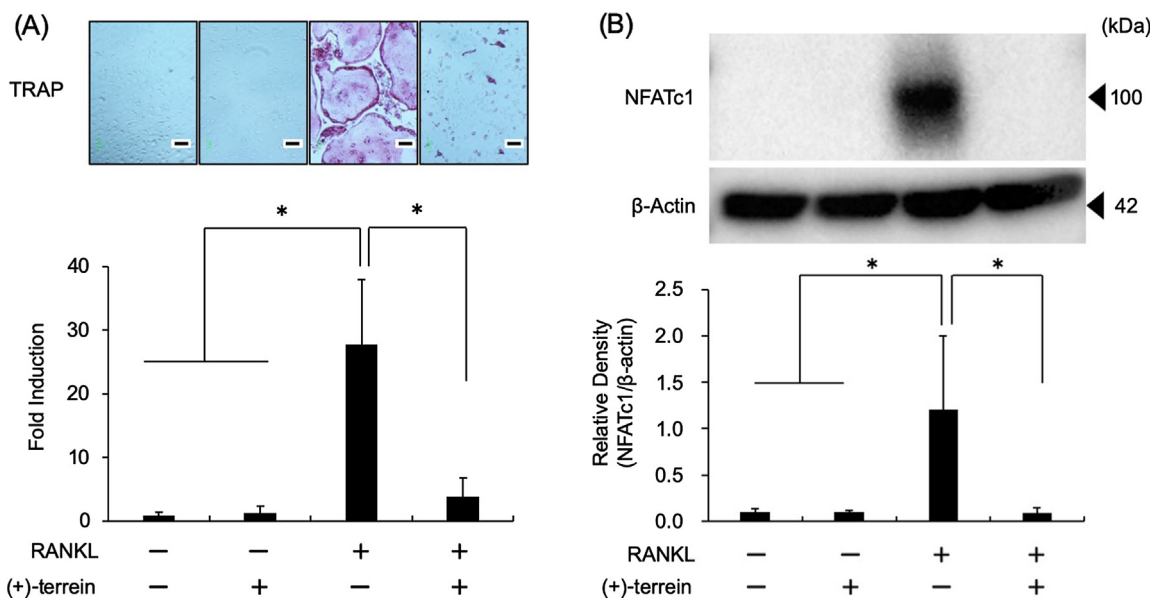

Fig. 3. (A) Synthetic (+)-terrein inhibited RANKL-induced NFATc1 mRNA expression. Mouse BMMs (1×10^5 cells/well) were incubated in serum-free medium containing RANKL/M-CSF (100 ng/mL each) in the presence and absence of synthetic (+)-terrein (10 μM) for 24 h. Cells were lysed and total RNA was subjected to real-time reverse transcription polymerase chain reaction (RT-PCR) for determination of NFATc1 gene expression. Columns represent the mean relative intensity of experiments carried out in triplicate and bars represent the SD (n = 3, *p < 0.05). (B) Synthetic (+)-terrein inhibited RANKL-induced NFATc1 protein expression. Mouse BMMs (1×10^5 cells/well) were treated with RANKL/M-CSF (100 ng/mL each) in the presence and absence of synthetic (+)-terrein (10 μM) for 24 h or were left untreated (controls). Total cell extracts were prepared and subjected to Western blotting using anti-NFATc1 and β-actin. Relative density was measured using Image J software. Columns represent the mean results of experiments carried out in triplicate and bars represent the SD (n = 3, *p < 0.05).
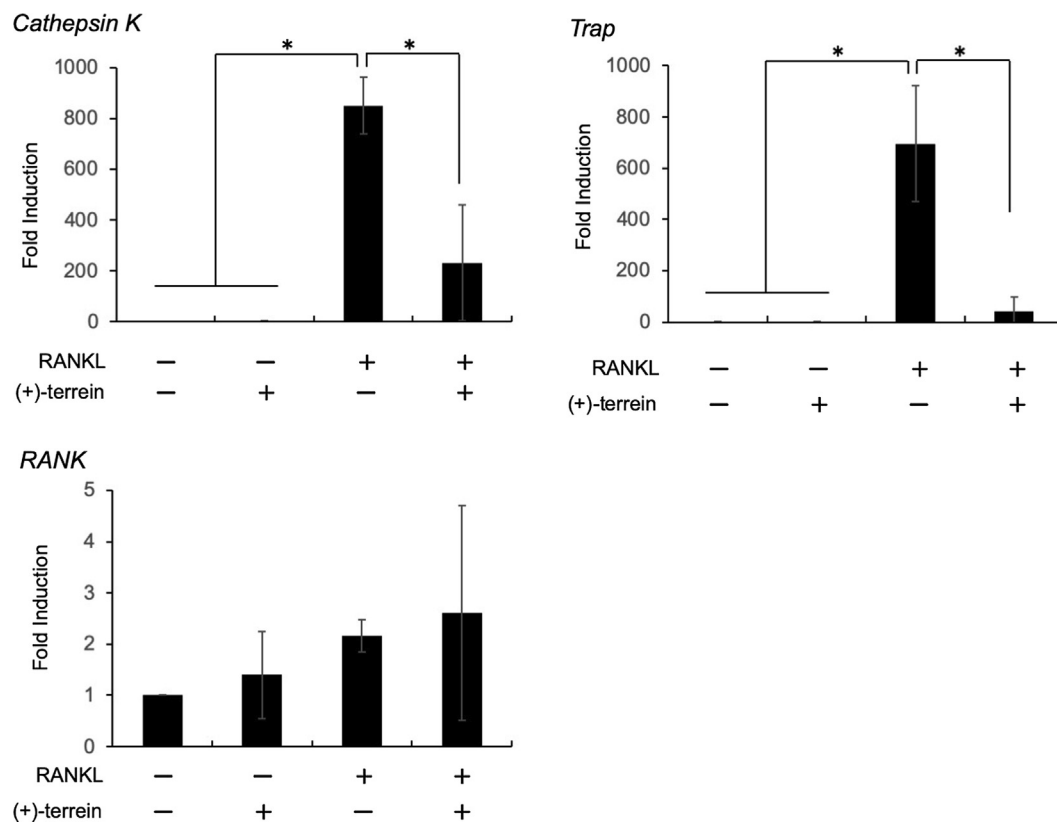
Fig. 4. (A) Synthetic (+)-terrein inhibited RANKL-induced *cathepsin K* and *Trap* mRNA expression. Mouse BMMs (1×10^5 cells/well) were incubated in serum-free medium containing RANKL/M-CSF (100 ng/mL each) in the presence and absence of synthetic (+)-terrein (10 μM) for 48 h. Cells were lysed and total RNA was subjected to real-time reverse transcription polymerase chain reaction (RT-PCR) for determination of *cathepsin K, Trap(Acp5), RANK* gene expression. Columns represent the mean relative intensity of *cathepsin K, Trap, RANK* compared to that of *β-actin* carried out in triplicate and bars represent the SD (n = 3, *p < 0.05).

RANKL stimulation. The maximum inhibitory effects of synthetic (+)-terrein against osteoclastogenesis were observed when synthetic (+)-terrein was added to the culture at the same time as RANKL (Fig. 5A). Exposure of precursor cells to synthetic (+)-terrein at later stages (1 or 2 days after RANKL stimulation) resulted in less effective suppression of osteoclast-induced NFATc1 protein expression (*p* < 0.05, Fig. 5B).

3.5. Synthetic (+)-terrein did not suppress RANKL-induced ERK1/2, p38 MAPK and NF-κB protein phosphorylation

To investigate the effects of synthetic (+)-terrein on RANKL signaling cascade during the osteoclast differentiation, synthetic (+)-terrein (10 μM) was added to the culture medium on 30 min before RANKL stimulation. Addition of RANKL-induced protein phosphorylation of ERK1/2, p38 MAPK and NF-κB p65 (Fig. 6), indicating that the RANKL signaling cascade was triggered during osteoclastogenesis. However, synthetic (+)-terrein did not suppress RANKL-induced protein phosphorylation of ERK1/2, p38 MAPK and NF-κB p65 (Fig. 6).

4. Discussion

In this study, we demonstrated that synthetic (+)-terrein suppressed osteoclastogenesis in mouse BMMs. In addition, synthetic (+)-terrein suppressed RANKL-induced expression of NFATc1, a master transcriptional factor for osteoclastogenesis, and related osteoclastogenic factors, including cathepsin K and Trap. These findings suggest that (+)-terrein may play an important role in suppressing NFATc1 expression, regulating RANKL-induced osteoclastogenesis, and modulating inflammatory bone resorption including periodontal disease.

Excessive bone resorption due to an imbalance between osteoblast and osteoclast activity is characteristic of inflammatory bone resorptive diseases such as periodontal disease. Excessive bone resorption is often caused by increased RANKL levels and subsequent expression of its related downstream genes, and this pathway was therefore confirmed as a useful target for the treatment of osteolytic bone diseases [21,22]. Unlike bisphosphonates, natural compounds exhibit their potential effects by inhibiting osteoclastogenesis and RANKL signaling pathways. Natural compounds present several advantages over current anti-osteolytic treatments such as bisphosphonates and hormone replacement therapies, which may cause severe side effects including MRONJ [23–25]. In this study, we demonstrated that (+)-terrein, a natural secondary bioactive fungal metabolite derived from *A. terreus*, inhibited osteoclast formation and associated resorption by blocking RANKL-induced NFATc1 activities. This indicates that synthetic (+)-terrein may be used as a therapeutic agent to prevent bone resorption in osteolytic bone diseases such as rheumatoid arthritis and periodontal disease.

Various biological properties of (+)-terrein have been reported, including antibacterial [9] and anti-inflammatory, immunomodulatory activities [12,14]. Furthermore, we previously reported the effects of synthetic (+)-terrein on the IL-6 signaling cascade, including inhibition of Janus activated kinase 1 (JAK1) phosphorylation in HGFs [15]. IL-6, as well as TNF-α, is an important pro-inflammatory cytokine that induces a wide variety of biological effects including inflammatory bone resorption in rheumatoid arthritis and periodontal disease [16,17]. Here, we showed that synthetic (+)-terrein is a novel and promising compound for the inhibition of RANKL- and TNF-α-induced osteoclastogenesis (Fig. 1). A recent study revealed that (+)-terrein plays a significant role in suppressing oxidative stress, thus conferring a protective effect on cells and reducing inflammatory molecules in human diploid fibroblasts [26]. Oxidative stress regulates the development of osteoporosis and enhanced osteoclast formation and function [27]. As
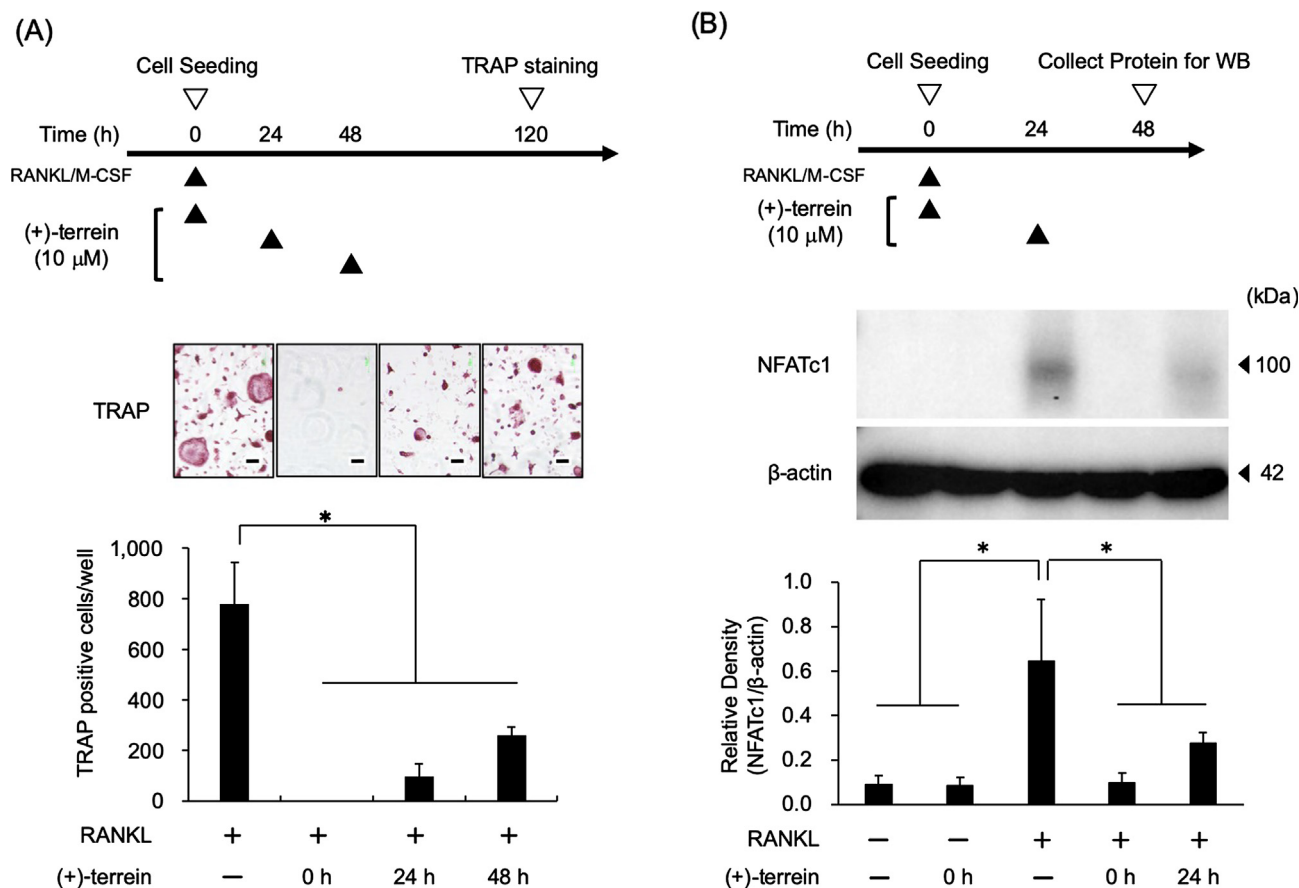
Fig. 5. Delayed addition of synthetic (+)-terrein inhibited RANKL-induced osteoclastogenesis. (A) Mouse BMMs (1×10^5 cells/well) were incubated with RANKL/M-CSF (100 ng/mL each) and synthetic (+)-terrein (10 μM) was subsequently added at 0, 24, or 48 h. After 5 days (120 h), cells were stained to measure TRAP expression. The cells were photographed (original magnification, 100 ×) and TRAP-positive, multinucleated osteoclasts were counted. Columns represent the mean results of experiments carried out in triplicate and bars represent the SD (n = 3, *p < 0.05). (B) RANKL-induced NFATc1 expression was assessed via Western blotting. Mouse BMMs (1×10^5 cells/well) were treated with RANKL/M-CSF (100 ng/mL each) and synthetic (+)-terrein (10 μM) was subsequently added at 0 and 24 h. Total cell extracts were prepared and subjected to Western blotting using anti-NFATc1 and β-actin. Relative density was measured using Image J software. Columns represent the mean results of experiments carried out in triplicate and bars represent the SD (n = 3, *p < 0.05).

RANKL and TNF-α may induce oxidative responses [28,29], it is likely that (+)-terrein has inhibitory effects on RANKL- and TNF-α-induced oxidative stress and osteoclastogenesis.

Osteoclasts formation is triggered by the differentiation and fusion of macrophage precursor cells [22]. RANKL belongs to the tumor necrosis factor family and is the key regulator of bone resorption. RANKL binds with RANK, which activates the downstream signaling pathways regulating osteoclastogenesis [30], including calcium-regulated NFATc1 activity [31]. NFATc1 is a master transcription factor modulating osteoclast differentiation [32] whose activation is dependent on downstream NF-κB activation. NFATc1 nuclear translocation is also mediated by activated calcium signaling and *NFATc1* expression is promoted by NFAT itself via automated feedback [33]. Induced NFATc1 in turn drives the expression of a number of osteoclastogenic genes including dendritic cell-specific transmembrane protein (*Dcstamp*), v-type protein ATPase subunit d2 (*Atp6v0d2*), osteoclast-associated receptor (*Oscar*), integrin β3 (*Itgb3*), osteoclast stimulatory transmembrane protein (*Ocstamp*), tartrate-resistant acid phosphatase (*Acp5*), calcitonin receptor (*Calcr*), and cathepsin K (*Ctsk*) [34]. NFATc1 improved the expression of these genes to promote cellular differentiation and function [34].

In the present study, we showed that RANKL-induced NFATc1 transcriptional activity and NFATc1 protein levels and related osteoclastogenic genes (*cathepsin K* and *Trap*) were dramatically inhibited by synthetic (+)-terrein (Figs. 3, 4). In addition, delayed application of synthetic (+)-terrein also inhibited RANKL-induced osteoclastogenesis

(Fig. 5). However, synthetic (+)-terrein did not suppress RANKL-induced NF-κB and MAPKs phosphorylation (Fig. 6). This may be via direct intervention in the NFATc1 activation pathway or suppression of another RANKL signaling cascade, Ca^{2+}-calmodulin pathway, upstream of NFATc1 activity, required further investigation. These results suggest that (+)-terrein inhibits osteoclast differentiation through NFATc1-mediated pathways. However, reports concerning the role of (+)-terrein on osteoblasts are limited to the fact that (+)-terrein enhanced osteoblasts attachment to a titanium plate [35]. Therefore, the effects of (+)-terrein on osteoblasts require further investigation.

In the present study, we used synthetic (+)-terrein produced from dimethyl L-tartrate. All spectra (^{1}H and ^{13}C NMR, IR) and the specific rotation of synthetic (+)-terrein were similar to those of natural (+)-terrein [14]. Synthesized (+)-terrein presents several significant features: it can be orally administered because of its low molecular weight, has a short half-life, and has a low production cost compared to that of biological products containing natural (+)-terrein extracted from *A. terreus*. The results of the present study provide new insight into the potential of synthetic (+)-terrein to inhibit osteoclastogenesis. However, additional studies should be performed to examine the molecular targets of (+)-terrein in the cytosol.

In summary, the present study demonstrated that synthetic (+)-terrein reduced osteoclast formation and bone resorption by interfering with RANKL-induced NFATc1 activation. The results of this study have provided new insight into the potential use of synthetic (+)-terrein as an anti-resorptive agent for the treatment of osteolytic
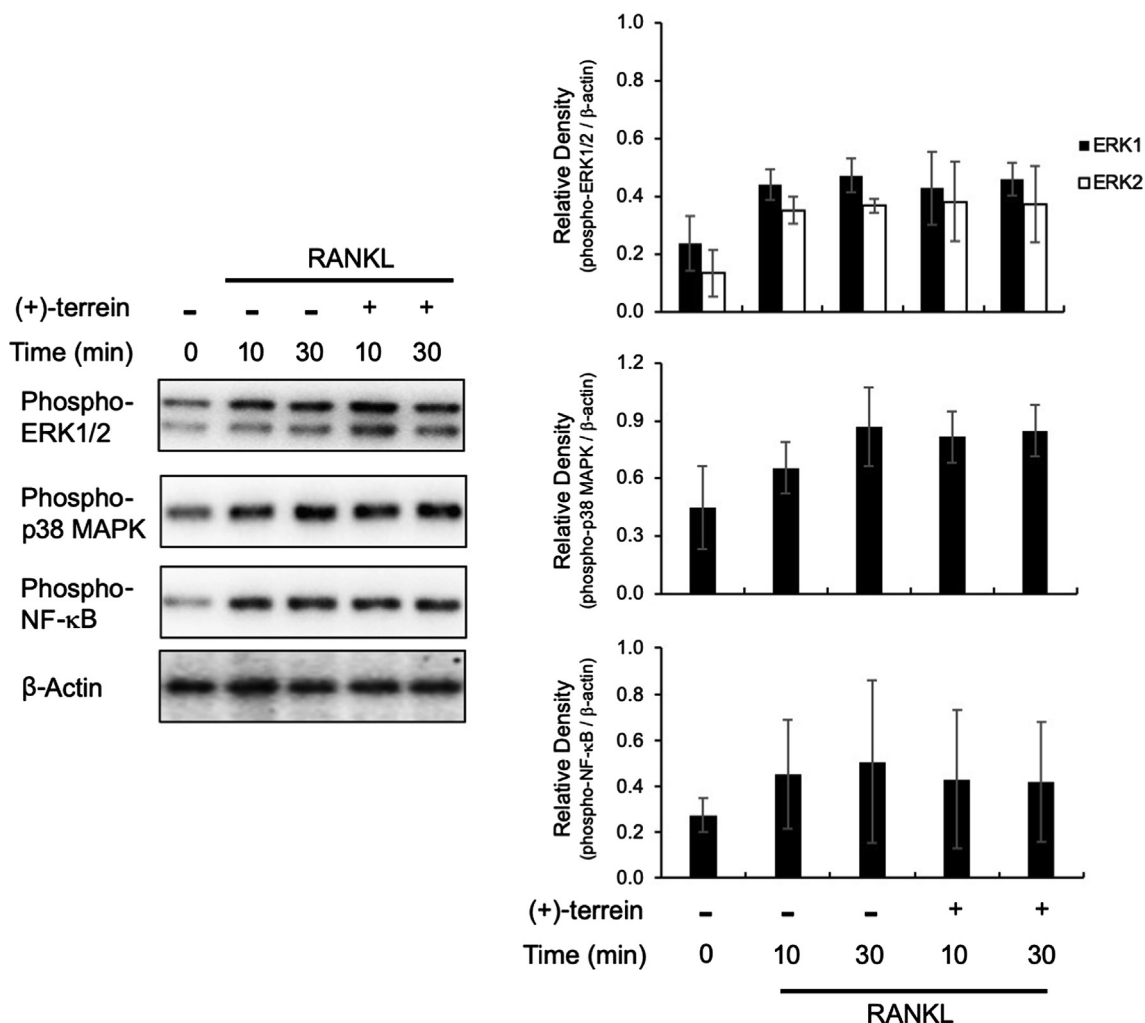
Fig. 6. Synthetic (+)-terrein did not suppress RANKL-induced ERK1/2, p38 MAPK, and NF-κB protein phosphorylation. Mouse BMMs (1 × 10⁵ cells/well) were treated with RANKL/M-CSF (100 ng/mL each) in the presence and absence of synthetic (+)-terrein (10 μM) for 30 min before RANKL stimulation. Total cell extracts were prepared and subjected to Western blotting using anti-phoshpo-ERK1/2, p38 MAPK, NF-κB p65 and β-actin. Relative density was measured using Image J software. Columns represent the mean results of experiments carried out in triplicate and bars represent the SD (n = 3).

bone diseases including periodontal disease.

Author contributions

SN and KO contributed to the conception, design, data acquisition, analysis, and interpretation of the study, and drafted and critically revised the manuscript. MN and HM contributed to the conception, data analysis, and interpretation of the study, and critically revised the manuscript. SY, HK, HS, and KS acquired, analyzed, and interpreted the data. HY and S. Ishii synthesized (+)-terrein. S. Ibaragi, KH, KY, and TY interpreted the data. SS contributed to the conception, data analysis, and interpretation, and critically revised the manuscript. ST contributed to the conception, design, data analysis, and interpretation, and critically revised the manuscript. All authors approved the final manuscript and agreed to be accountable for all aspects of the work.

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Declaration of Competing Interest

None.

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