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3'4'7-Trihydroxyflavone inhibits RANKL-induced osteoclast formation *via* NFATc1

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3'4'7-Trihydroxyflavone is a flavonoid from ladino clover, alfalfa, and *Albizzia julibrissin*. In the present study, we found that 3'4'7-trihydroxyflavone markedly inhibited the receptor activator of nuclear factor kappa B ligand (RANKL) induced osteoclastic differentiation from mouse bone marrow derived macrophages (BMMs). 3'4'7-trihydroxyflavone also reduced the mRNA expression level of osteoclastic marker genes including calcitonin receptor (CTR), Cathepsin K, v-ATPase V0 subunit d2 (ATP6v0d2), and dendritic cell-specific transmembrane protein (DC-STAMP). In addition, 3'4'7-trihydroxyflavone decreased the bone resorption activity of osteoclasts on dentin slices. We found that 3'4'7-trihydroxyflavone inhibited RANKL-induced expression of nuclear factor of activated T cells c1 (NFATc1), a key transcription factor of osteoclast differentiation. Furthermore, 3'4'7-trihydroxyflavone attenuated RANKL-induced activation of p38 mitogen-activated protein kinase (MAPK) and expression of B lymphocyte-induced maturation protein 1 (Blimp1), a repressor of negative regulators of NFATc1. Taken together, our data suggest that 3'4'7-trihydroxyflavone inhibits osteoclastogenesis *via* NFATc1.

1. Introduction

Bone homeostasis is maintained by bone-resorbing osteoclasts and bone-forming osteoblasts (Tanaka et al. 2005). It is well known that the imbalance in the ratio of osteoclasts and osteoblasts induces bone-related diseases such as osteoporosis, rheumatoid arthritis (RA), and osteosarcoma (Rodan and Martin 2000; Akiyama et al. 2008; Boyle et al. 2003). Osteoclasts are formed by the cell-cell fusion of mononucleated precursors which are derived from the monocyte-macrophage lineage during the differentiation process (Boyle et al. 2003; Väänänen et al. 2000). For osteoclastogenesis, macrophage colony stimulating factor (M-CSF) and RANKL are the two essential key cytokines *in vitro* (Takayanagi et al. 2002). RANK, a receptor for RANKL, is expressed on osteoclast precursor cells (Hsu et al. 1999) and the interaction between RANKL and RANK represents the vital stage in initiating osteoclastogenesis and bone destruction (Boyle et al. 2003; Redlich et al. 2002). The RANKL/RANK binding leads to recruitment of TNF receptor associated factor 6 (TRAF6) to the cytoplasmic domain of RANK (Walsh and Choi 2014; Darnay et al. 1999; Asagiri and Takayanagi 2007). In addition, it leads to the downstream signaling pathways which contain nuclear factor kappa B (NF-κB) and MAPK including p38, extracellular signal-regulated kinase (ERK), and c-jun N-terminal protein kinase (JNK) (Asagiri and Takayanagi 2007). These RANKL-induced signaling pathways cause the induction of NFATc1, which is a master regulator of osteoclast differentiation (Boyle et al. 2003; Takayanagi et al. 2002; Asagiri and Takayanagi 2007; Feng 2005). The final consequence of these signaling pathways is the induction of osteoclastogenic genes, such as tartrate-resistant acid phosphatase (TRAP), CTR, cathepsin K, DC-STAMP, ATP6v0d2, and Blimp1 (Takayanagi 2007; Walsh et al. 2006).

Flavonoids, a group of naturally occurring compounds that are commonly found in a variety of vegetables and herbal medicines, have been extensively reported to possess the ability to treat hepatitis, tumors, diarrhea, and inflammatory diseases without side effects (Xu et al. 2013; Han et al. 2007). They consist of a group of polyphenolic compounds and can be classified based on the oxidation state of the pyran ring which is placed at the center of C_6-C_3-C_6 skeleton (Yao et al. 2004). There are various subgroups of flavonoids and unique compounds (Yao et al. 2004) that include anthocyanins (cyanidin) (Dou et al. 2014), flavones (luteolin (Kim et al. 2011), apigenin (Goto et al. 2015)), flavonols (quercetin (Wong and Rabie 2008; Wattel et al. 2003; Satue et al. 2013), kaempferol (Wattel et al. 2003), flavanones (hesperidin (Chiba et al. 2003), naringenin (Swarnkar et al. 2012)), flavanols (catechins (Nakamura et al. 2010)), flavanonols (taxifolin (Satue et al. 2013)), isoflavones (genistein (Paicherit et al. 2000)). Many reports have demonstrated the suppressive effect of individual compounds of flavonoid subfamilies on osteoclast formation (Garcia et al. 2005; Wattel et al. 2004; Ang et al. 2011; Zhu et al. 2012). 3'4'7-trihydroxyflavone is a member of the class flavones, and has been isolated from ladino clover (Livingston and Bickoff 1964), alfalfa (Bickoff et al. 1965), and *Albizzia julibrissin* (Chamsukasi et al. 1981). Since there are no reports on the effect of 3'4'7-trihydroxyflavone on osteoclasts and bone resorption, we investigated the effect of 3'4'7-trihydroxyflavone on RANKL-induced osteoclastogenesis *in vitro*. Our findings suggest that 3'4'7-trihydroxyflavone could be used as a new therapeutic agent against bone lytic diseases involving increased osteoclastogenesis.

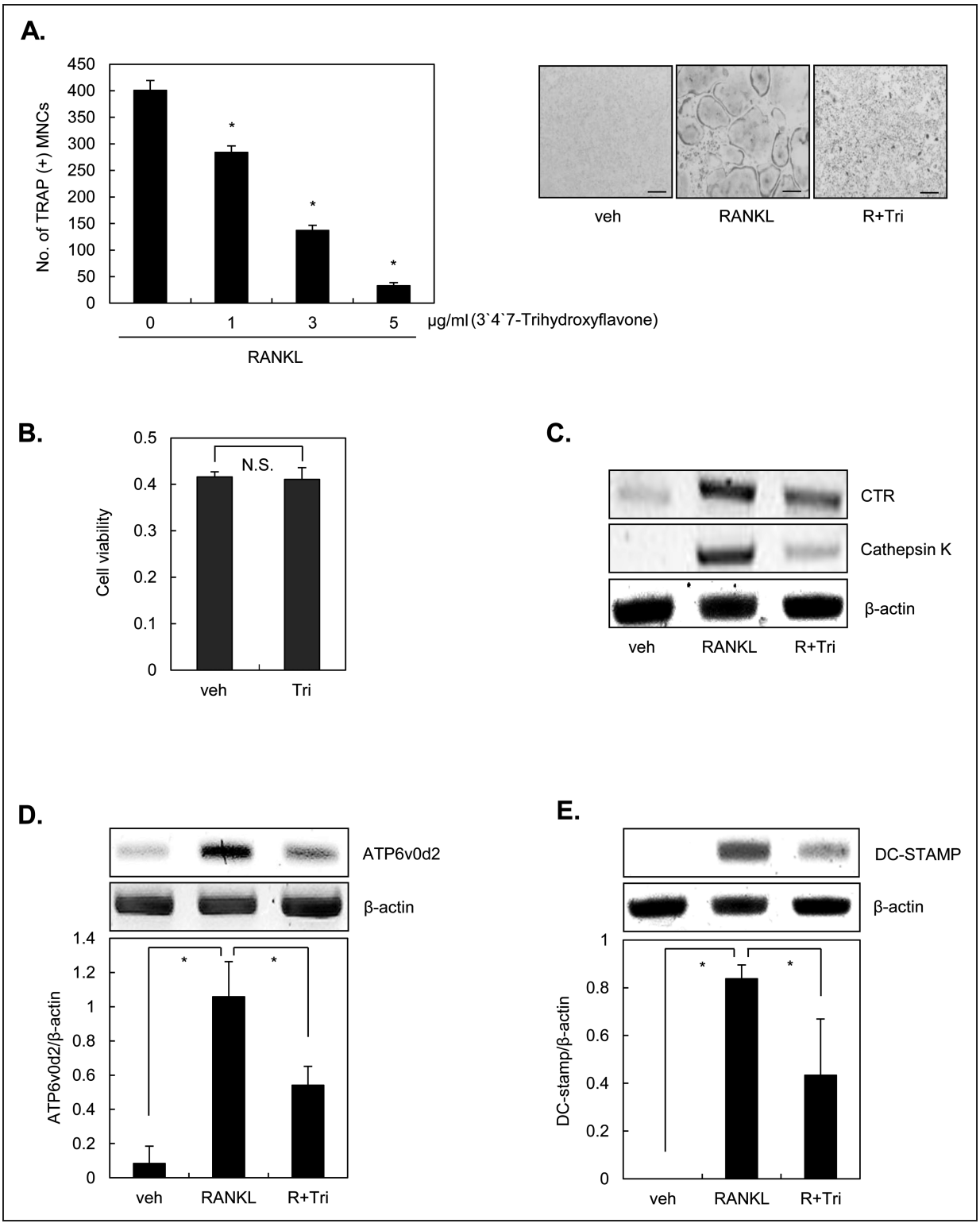

Fig. 1: 3'4'7-trihydroxyflavone inhibits RANKL-induced osteoclast formation. (A) BMMs were cultured with RANKL (100 ng/mL) and M-CSF (30 ng/mL) in the absence or presence of 3'4'7-trihydroxyflavone for 4 days. TRAP$^+$ MNCs containing more than 3 nuclei were counted. (B) BMMs were cultured in the absence or presence of 3'4'7-trihydroxyflavone (5 μg/ml) with M-CSF for 24 h. Cell viability was assessed by MTT assay. (C-E) BMMs were cultured in the absence or presence of 5 μg/ml of 3'4'7-trihydroxyflavone with RANKL (100 ng/mL) and M-CSF (30 ng/mL) for 7 days. The mRNA expression of osteoclast marker genes was detected by RT-PCR using specific primers. Data are expressed as mean ± S.D. of three independent experiments. Veh, vehicle; R, RANKL; Tri, 3'4'7-trihydroxyflavone. (* $p < 0.05$).

2. Investigations and results

2.1. 3'4'7-Trihydroxyflavone inhibits RANKL-induced osteoclast formation and bone resorption

To clarify the effects of 3'4'7-trihydroxyflavone on RANKL-induced osteoclastogenesis, we used a mouse bone marrow-derived macrophages (BMMs) culture system. BMMs can form TRAP$^+$ multinucleated osteoclasts in the presence of M-CSF and RANKL. When BMMs were cultured with M-CSF and RANKL in the presence of various concentrations of 3'4'7-trihydroxyflavone, osteoclast formation was inhibited in a dose-dependent manner (Fig. 1A). To verify that these inhibitory effects were not attributable to cell toxicity induced by 3'4'7-trihydroxyflavone, cell viability was analyzed using the MTT

assay. 3'4'7-trihydroxyflavone showed no cellular toxicity at the highest concentration (Fig. 1B). To further confirm the inhibitory effect of 3'4'7-trihydroxyflavone on osteoclastogenesis, we examined the expression of CTR and cathepsin K, osteoclastogenic marker genes, by RT-PCR. Osteoclasts exhibit the highest CTR-density and binding of calcitonin to CTR causes cessation of osteoclastic activity (Lafont et al. 2011; Dacquin et al. 2004; Davey et al 2008). Cathepsin K is a potent collagenase that is expressed in osteoclasts, and its activity is most evident in osteoclasts that resorb bone tissue (Stroup et al. 2001; Lindeman et al. 2004). In accordance, the mRNA expression levels of CTR and cathepsin K were increased in cultures treated with M-CSF and RANKL for 4 days, which were dramatically suppressed by the presence of 3'4'7-trihydroxyflavone (Fig. 1C). Many other genes are also responsible for osteoclast differentiation. For example, DC-STAMP is highly up-regulated during osteoclastogenesis and has been suggested to be important in osteoclast progenitor cell fusion (Yagi et al. 2005). ATP6v0d2 has been associated with osteoclast fusion, since ATP6v0d2-deficient mice cannot form multinucleated osteoclasts (Lee et al. 2006). Our data show that 3'4'7-trihydroxyflavone significantly suppressed the RANKL-induced expression of DC-STAMP and ATP6v0d2 (Fig. 1D, E), confirming the anti-osteoclastogenic effect of 3'4'7-trihydroxyflavone.

We next examined at which stage 3'4'7-trihydroxyflavone impaired osteoclast differentiation. 3'4'7-trihydroxyflavone was added to osteoclast-generating cultures on different days (D0–4), and TRAP staining was performed on day 4. 3'4'7-trihydroxyflavone effectively inhibited osteoclast formation when it was added on the first 2 days of culture (D0–2) as well as on the last 2 days of culture (D2–4), which suggests that it affects both early and late stages of osteoclastogenesis (Fig. 2A). Osteoclasts have the capacity to resorb bone (Boyle et al. 2003). To examine whether the effect of 3'4'7-trihydroxyflavone on osteoclast formation could be reflected in the osteoclastic activity, we performed an *in vitro* resorption pit assay using dentine slices. Many resorption pits were generated in wells with RANKL-treated cells (Fig. 2B). In contrast, the treatment with 3'4'7-trihydroxyflavone strongly inhibited formation of resorption pits by the RANKL-treated cells (Fig. 2B). Together, these results suggest that 3'4'7-trihydroxyflavone exerts inhibitory effects on osteoclast formation, which leads to reduced bone resorption.

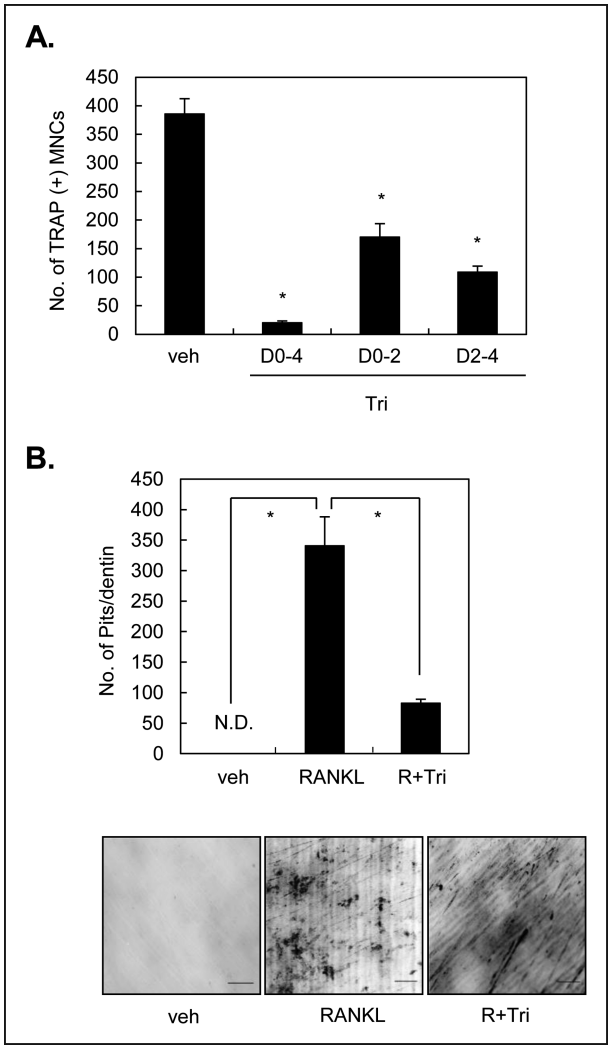

Fig. 2: 3'4'7-trihydroxyflavone suppresses RANKL-induced bone resorption activity. (A) 3'4'7-trihydroxyflavone (5 μg/ml) was added and cultured with RANKL (100 ng/mL) and M-CSF (30 ng/mL) during the indicated culture days. (B) BMMs were seeded on dentin slices and cultured in the absence or presence of 3'4'7-trihydroxyflavone (5 μg/ml) with RANKL and M-CSF for 7 days. Stained pits were counted under a light microscope. Data are expressed as mean ± S.D. of three independent experiments. Veh, vehicle; R, RANKL; Tri, 3'4'7-trihydroxyflavone. (* $p < 0.05$).

2.2. 3'4'7-Trihydroxyflavone suppresses RANKL-induced NFATc1 expression via Blimp-1 and p38 MAPK pathway

The NFATc1 pathway plays a critical and fundamental role in osteoclast development, and the lack of NFATc1 arrests osteoclastogenesis. Furthermore, c-Fos is known to positively regulate the expression of NFATc1 by binding to the NFATc1 promoter (Takayanagi 2007; Matsuo et al. 2004). Therefore, we investigated the effects of 3'4'7-trihydroxyflavone on their expression levels. As reported previously, RANKL stimulation increased the expression of NFATc1 and c-Fos in BMMs (Fig. 3A, B). 3'4'7-trihydroxyflavone abolished RANKL-induced NFATc1 expression, but not c-Fos protein expression (Fig. 3A, B). Several studies indicate that NFATc1 activity is negatively regulated by other transcription factors such as IFN regulatory factor-8 (IRF-8) and v-maf musculoaponeurotic fibrosarcoma oncogene family, protein B (MafB) during osteoclastogenesis (Zhao et al. 2009; Kim et al. 2007). Furthermore, Blimp1 (encoded by *Prdm1*) is known to be crucial for the transcriptional repression of these anti-osteoclastogenic genes. Thus, it acts as a repressor of negative regulators of NFATc1 during osteoclastogenesis (Nishikawa et al. 2010). Since 3'4'7-trihydroxyflavone failed to affect the induction of c-Fos by RANKL, we speculate that Blimp-1 might be involved in the suppression of NFATc1 by 3'4'7-trihydroxyflavone. Indeed, we demonstrated that RANKL increased the mRNA level of Blimp1, which was significantly decreased by treatment with 3'4'7-trihydroxyflavone (Fig. 3C).

To further define the molecular mechanism of the inhibitory effects of 3'4'7-trihydroxyflavone on osteoclastogenesis, we next examined the effect of 3'4'7-trihydroxyflavone on the p38 MAPK pathway induced by RANKL in BMMs. As shown in Fig. 3D, phosphorylation of p38 was observed at 15 mins after RANKL treatment, which was suppressed by pretreatment with 3'4'7-trihydroxyflavone. Taken together, 3'4'7-trihydroxyflavone inhibits RANKL-induced osteoclast formation *via* regulating NFATc1 expression, which is attributable to the modulation of Blimp-1 and the p38 MAPK signaling pathway.

3. Discussion

Flavonoids are polyphenolic compounds that are ubiquitous in nature, and they are found in fruits, flower, and some

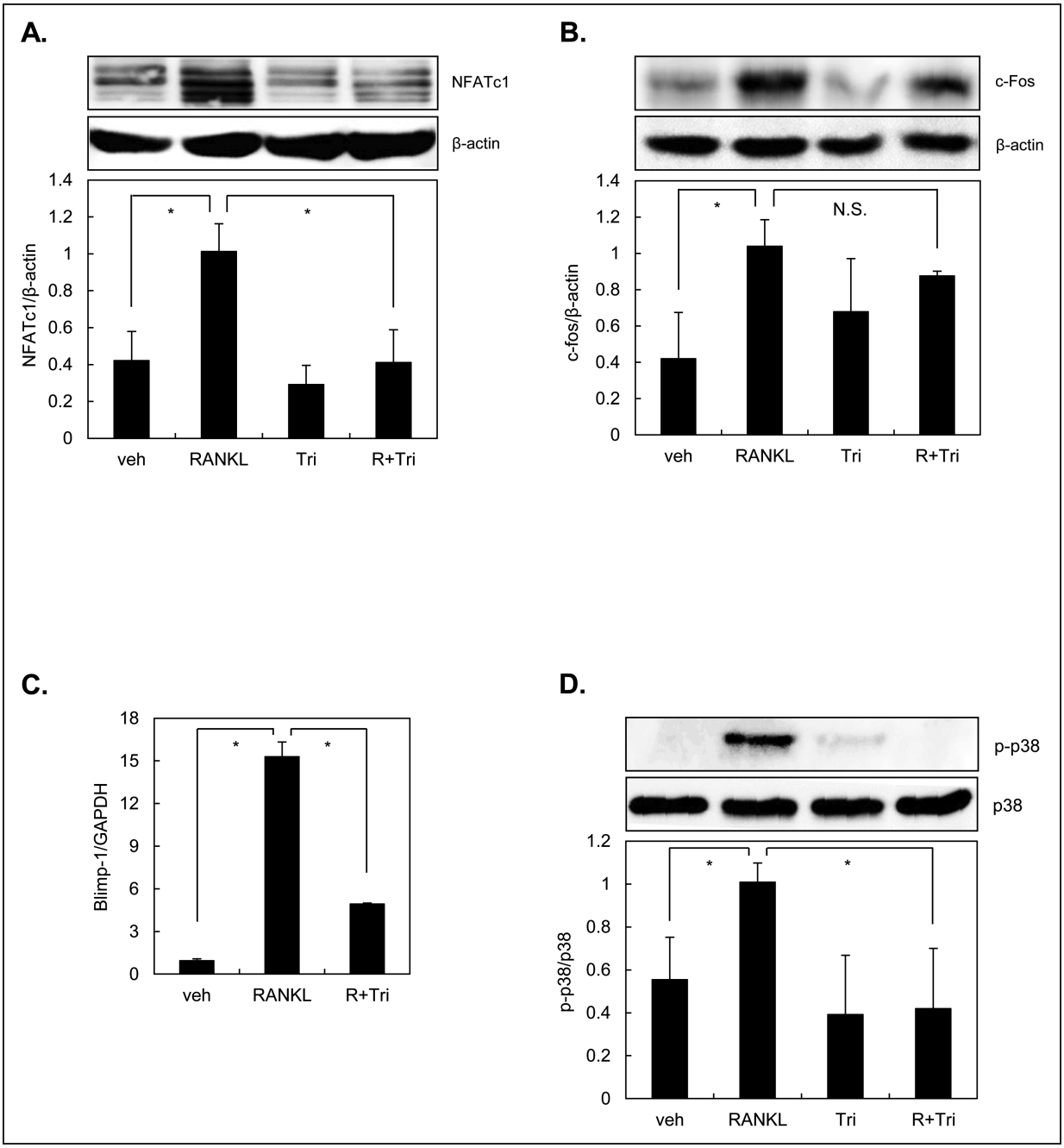

Fig. 3: 3'4'7-trihydroxyflavone decreases RANKL-induced NFATc1, p38 MAPK, and Blimp-1 expression. (A-B, D) BMMs were preincubated with 3'4'7-trihydroxyflavone (5 μg/ml) for 30 min, and then treated with or without 200 ng/mL RANKL for 24 h (NFATc1 and c-Fos) (A-B), or 15 mins (p-p38) (D) in the presence of M-CSF (30 ng/mL). Whole-cell extracts were harvested and subjected to Western blot analysis with specific antibody. (C) BMMs were cultured in the absence or presence of 5 μg/ml of 3'4'7-trihydroxyflavone with RANKL (100 ng/mL) and M-CSF (30 ng/mL) for 7 days. The mRNA expression was determined by real-time PCR using Blimp-1 primers. Data are expressed as mean ± S.D. of three independent experiments. Veh, vehicle; R, RANKL; Tri, 3′4′7-trihydroxyflavone; N.S., not significant. (* $p < 0.05$).

beverages (tea, coffee, beer, wine, and fruit drinks). Several studies have reported their pharmacological effects such as anti-inflammatory, antioxidant, and estrogenic activities (Xu et al. 2013; Gabor 1979; Pietta 2000; Miksicek 1993). In the present study, we demonstrated that 3'4'7-trihydroxyflavone, a member of the flavonoid family, suppressed RANKL-induced osteoclast formation and bone resorption *in vitro*. As an underlying mechanism, we suggested that NFATc1 is a target of the anti-osteoclastogenic effect of 3'4'7-trihydroxyflavone. NFATc1 functions as the key regulator of osteoclast differentiation (Takayanagi et al. 2002; Winslow et al. 2006; Aliprantis et al. 2008) by inducing osteoclastic genes such as Tm7sf4 (encoding DC-STAMP) (Yagi et al. 2007; Kim et al. 2008) and Atp6v0d2 (Lee et al. 2006; Kim et al. 2008) in addition to a number of genes (such as CTR and cathepsin K) expressed in mature osteoclasts that are involved in the regulation of bone-resorbing activity (Takayanagi 2007). In agreement with this finding, 3'4'7-trihydroxyflavone suppressed the RANKL-induced expression of many osteoclastic genes.

During osteoclastogenesis, NFATc1 activity and expression are maintained at a high level (Asagiri et al. 2005). Therefore, the expression of molecules that inhibit NFATc1 activity needs to be repressed. Blimp1, which is induced by the RANKL–NFATc1 axis, represents the repressor of anti-osteoclastogenic genes. It is likely that 3'4'7-trihydroxyflavone suppresses RANKL-induced expression of NFATc1 by acting as an inhibitor of Blimp1, which leads to decreased osteoclast formation.

In our study, 3'4'7-trihydroxyflavone also decreased RANKL-induced p38 MAPK phosphorylation. A pharmacological inhibition experiment using the p38 inhibitor SB203580

revealed direct involvement of p38 in RANKL-induced osteoclast differentiation (Yeon et al. 2012; Li et al. 2002; Matsumoto et al. 2000). Furthermore, a study using both p38 inhibitor SB203580 and over-expression of dominant negative MKK3 and MKK6, which are the upstream kinases of p38, revealed that the p38 signaling pathway could mediate the induction of NFATc1 during RANKL-stimulated osteoclast differentiation (Huang et al. 2006). Thus, the anti-osteoclastogenic action of 3'4'7-trihydroxyflavone could be due to its potential to inhibit both Blimp1 and p38 MAPK signaling pathways that consequently down-regulate the expression of NFATc1.

In osteoclast precursor cells, RANKL signaling increases the intracellular level of ROS, which, in turn, modulates RANKL-induced activation of various signaling pathways (Ha et al. 2004; Kim et al. 2010; Lee et al. 2005). ROS are required for optimal osteoclastogenesis and bone resorption and evidently mediate the bone loss caused by sex steroid deficiency (Lean et al. 2003; Manolagas 2010). 3'4'7-trihydroxyflavone extracted from the stem bark of *Albizzia julibrissin* exerts antioxidant activity by scavenging reactive oxygen species (ROS) (Jung et al. 2003). Thus, it is possible that 3'4'7-trihydroxyflavone inhibits osteoclast formation via the ROS signaling pathway. A further study is needed to elucidate this possibility.

This is the first report on the anti-osteoclastogenic activity of 3'4'7-trihydroxyflavone and its mode of action; 3'4'7-trihydroxyflavone could inhibit RANKL-induced osteoclast differentiation by inhibiting Blimp-1 and p38 signaling pathways, which consequently affect the expression of the osteoclast-specific transcription factor, NFATc1. In a further study, the binding molecules of 3'4'7-trihydroxyflavone may be identified, and the mechanism by which 3'4'7-trihydroxyflavone inhibits the differentiation of osteoclasts *via* the target molecules can be elucidated. Given that down-regulation of RANKL-mediated signals may be a valuable approach to the treatment of pathological bone loss, 3'4'7-trihydroxyflavone could be a drug candidate for treating bone lytic diseases.

4. Experimental

4.1. Reagents

3'4'7-trihydroxyflavone was purchased from EXTRASYNTHESE (France). Antibodies against phospho-p38, p38, c-Fos, NFATc1, and β-actin were purchased from Cell Signaling Technology (Beverly, MA, USA). All other reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA).

4.2. Cells and culture system

Bone marrow cells were obtained from the long bones of 4- to 6-week-old ICR male mice (Samtako, Inc., Osan, Korea). Mice were sacrificed by cervical dislocation, and the bone marrow of tibiae and femora was extracted. Bone marrow cells were cultured overnight in alpha-minimal essential medium (MEM) containing 10% fetal bovine serum (FBS) with M-CSF (10 ng/mL, R&D systems, Inc., Minneapolis, MN, USA). Floating cells were harvested and cultured with 30 ng/mL M-CSF for 3 days. Formed bone marrow–derived macrophages (BMMs) were used to generate osteoclasts in this study. All cells were cultured in alpha-MEM with 10% FBS at 37 °C in 5% CO_2 incubator.

4.3. Osteoclast differentiation

BMMs were treated with M-CSF (30 ng/mL) and RANKL (100 ng/mL, PeproTech, Rocky Hill, NJ, USA) to induce differentiation into osteoclasts. Then, cells were treated with indicated concentrations of 3'4'7-trihydroxyflavone to assess whether it had an effect on RANKL-induced osteoclast formation or not. Cells were fixed with 10% formalin and ethanol-acetone (50:50, v/v), and stained for TRAP, a marker enzyme of osteoclasts. The number of TRAP positive ($TRAP^+$) cells with more than three nuclei was counted under a light microscope.

4.4. MTT assay

BMMs were seeded into 96-well cell culture plate ($2x10^4$ cells/well) and treated with M-CSF (30 ng/mL). After stabilization, cells were treated with 3'4'7-trihydroxyflavone for 24 h. Then, 0.5 mg/ml MTT solution was added and incubated in the dark. After 5 h, solubilization buffer (10% SDS in 0.01 M HCl) was added and cultured overnight. The cell viability was measured based on the O.D. value from ELISA reader at 570 nm.

4.5. Bone resorption assay

BMMs were placed on dentin slices and cultured with M-CSF (30 ng/mL) and RANKL (100 ng/mL) in the absence or presence of 3'4'7-trihydroxyflavone (5 μg/ml) for 7 days. After taking out dentin slices from the plate, they were rubbed and washed to remove the remaining cells. Then, dentin slices were stained with toluidine blue (1 μg/mL, J.T. Baker, UK). Formed pits were counted as a marker of bone resorption activity.

4.6. RT-PCR and real-time PCR (Q-PCR) analyses

Total RNA was extracted from BMMs by Easy-Blue (iNtRON Biotechnology, Inc.). cDNA was synthesized from total RNA by using RevertAid^{TM} first strand cDNA synthesis Kit (Fermentas, EU) and amplified using PCR. Primers for osteoclastogenic genes used in this study were as follows: CTR, 5'- TTTCAAGAACCTTAGCTGCCAGAG-3' (forward), 5'-CAAGGCACGGACAATGTTGAGAAG-3' (reverse); Cathepsin K, 5' -CTTCCAATACGTGCAGCAGA-3' (forward), 5'-ACGCACCAATATCTTGCACC-3' (reverse); β-actin, 5'-TTTGATGTCACGCACGATTTCC-3' (forward), 5'-TGTGATGGTGGGAATGGGTCAG-3' (reverse); ATP6v0d2, 5-TCAGATCTCTTCAAGGCTGTGCTG-3' (forward), 5'-GTGCCAAATGAGTTCAGAGTGATG-3' (reverse); DC-STAMP, 5'-TGGAAGTTCACTTGAAACTACGTG-3' (forward), 5'-ctcggtttcccgtcagcctctctc-3' (reverse). The RT-PCR program was as follows: 30 cycles (ATP6v0d2, DC-STAMP), 28 cycles (CTR), or 22 cycles (cathepsin K, β-actin), after an initial denaturation step at 94 °C for 3 min, then denaturation at 94 °C for 30 s, annealing at 59 °C (ATP6v0d2), 58 °C (CTR, cathepsin K, β-actin, and DC-STAMP) for 45 s, and extension at 72 °C for 60 s, with a final extension at 72 °C for 10 min. After amplification, the PCR reaction mixtures were electrophoresed on 1% agarose gel and visualized by Ethidium Bromide staining and UV irradiation. The relative abundance of mRNA was calculated after normalization to β-actin. The following were performed by quantitative real-time PCR. PCR amplification was performed with specific primers using Applied Biosystems SYBR Green super-mix (Applied Biosystems Inc., Foster City, CA, USA) by an Applied Biosystems 7500/7500 Fast Real time PCR system (Applied Biosystems Ind., Foster City, CA, USA). Specific primer sequences for PCR were as follows: Blimp1, 5'-TGCTTATCCCAGCACCCC-3' (forward), 5'-CTTCAGGTTGGAGAGCTGACC-3' (reverse); GAPDH, 5'-TGCACCACCAACTGCTTAGC-3' (forward), 5'-GGCATGGACTGTGGTCATGAG-3' (reverse). The Q-PCR program was as follows: 40 cycles, after an initial holding stage at 95 °C for 10 min, then cycling stage at 95 °C for 15 s, and annealing at 53-57 °C for 1 min.

4.7. Immunoblot analysis

Whole cell lysates were isolated, loaded into sodium dodecyl sulfate-polyacrylamide gel, and then transferred to Immobilon-P membranes (Millipore, Bedford, MA, USA). The membranes were blocked with 5% non-fat-milk in PBS-T (PBS 0.1% Tween 20), and then incubated with anti-phospho p38 (1:1000), anti-p38 (1:1000), anti-NFATc1 (1:200), anti-c-Fos (1:1000), and anti-β-actin (1:4000), followed by secondary horseradish peroxidase-conjugated antibody (1:5000). The immunoreactive bands were detected with enhanced chemiluminescence (Amersham Bio-Sciences, Buckinghamshire, UK) using an LAS3000 luminescent image analyzer (FUJIFILM Co., Tokyo, Japan).

4.8. Statistical analysis

Data represent the means and the ± S.D. from three independent experiments. Statistical analysis was performed by one-way analysis of variance followed by the Student's *t*-test. A p-value < 0.05 was considered statically significant.

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