Background/Aims: The present study aimed to investigate whether tocotrienol regulates interleukin 17 (IL-17)-induced osteoclastogenesis in rheumatoid arthritis (RA).

Methods: We evaluated the effect of tocotrienol on IL-17-induced receptor activator of nuclear factor kappa B ligand (RANKL) production using RA fibroblast-like synoviocyte (FLS), together with real-time polymerase chain reaction and enzyme-linked immunosorbent assay. Osteoclast differentiation was confirmed after culturing IL-17-treated RA FLS and Th17 cells with tocotrienol and monocytes. We analyzed the suppressive effect of tocotrienol on Th17 cell percentage or Th17 cytokine levels among peripheral blood mononuclear cells using flow cytometry.

Results: We found that IL-17 stimulated FLS to produce RANKL and tocotrienol decreased this IL-17-induced RANKL production. Tocotrienol decreased the IL-17-induced activation of mammalian target of rapamycin, extracellular signal-regulated kinase, and inhibitor of kappa B-alpha. When monocytes were incubated with IL-17, RANKL, IL-17-treated FLS or Th17 cells, osteoclasts were differentiated and tocotrienol decreased this osteoclast differentiation. Tocotrienol reduced Th17 cell differentiation and the production of IL-17 and sRANKL; however, tocotrienol did not affect Treg cell differentiation.

Conclusions: Tocotrienol inhibited IL-17-activated RANKL production in RA FLS and IL-17-activated osteoclast formation. In addition, tocotrienol reduced Th17 differentiation. Therefore, tocotrienol could be a new therapeutic choice to treat bone destructive processes in RA.

Keywords: Interleukin-17; Rheumatoid arthritis; Osteoclastogenesis; Gamma-tocotrienol; RANK ligand
genesis indirectly through induction of TNF-α, IL-1, IL-6 and RANKL from macrophages and FLS [5,6]. In our previous study, IL-17 stimulates the production of RANKL from RA FLS and differentiates peripheral blood monocytes into mature osteoclasts [7]. Although IL-17 is essential for development of inflammatory arthritis and joint destruction, the results of human clinical studies for blocking IL-17 are controversial. Ixekizumab, an anti-IL-17A monoclonal antibody, improves sign and symptoms in RA patients, decreasing disease activity score and acute phase reactant at week 12 [8]. Another IL-17 inhibitor, secukinumab, does not achieve the primary efficacy, however, it decreases RA disease activity [9]. After all the analysis, the blockage of IL-17 alone is not sufficient in control inflammation and joint destruction and the ancillary therapeutic modalities are required.

Tocotrienol, a vitamin E family, suppresses inflammatory cytokines. In several experiments, lipopolysaccharides-induced production of inflammatory cytokines and nitric oxide are decreased by tocotrienol [10-12]. In addition, tocotrienol regulates the disease severity of arthritis in the arthritis animal models [13-14]. Moreover, tocotrienol suppresses osteoclast differentiation in mouse bone marrow-derived macrophages by suppression of nuclear factor of activated T cells 1 (NFATc1) and Fos proto-oncogene, AP-1 transcription factor subunit (c-Fos) [15,16] and in tartrate-resistant acid phosphatase (TRAP)+ cells from human CD14+ monocytes [17]. Until now, there is no study showing the effect of tocotrienol in IL-17-induced osteoclastogenesis. Moreover, the effect of tocotrienol has not been studied on human disease cells such as RA FLS.

Thus, the present study aimed to: (1) detect the regulatory effect of tocotrienol on IL-17-induced RANKL expression and osteoclast differentiation; (2) evaluate whether tocotrienol suppresses osteoclast differentiation when osteoclast precursors are incubated with IL-17-treated RA FLSs and Th17 cells; and (3) determine the role of tocotrienol in Th17 cell differentiation.

METHODS

Ethics statement and patients
Informed consent was obtained from all patients, and the experimental protocol was approved by the Institutional Review Board for Human Research, Konkuk University Hospital (KUH1010186), as described previously [18].

Isolation of RA FLS
RA FLS were isolated by enzymatic digestion of synovial tissues obtained from patients with RA undergoing total knee replacement surgery, who fulfilled the American College of Rheumatology (ACR) classification of rheumatoid arthritis of the knee and had advanced rheumatoid arthritis with Kellgren-Lawrens grade 4.

Reagents
Recombinant human RANKL (rhRANKL), rhM-CSF, and IL-17 were purchased from R&D Systems (Minneapolis, MN, USA). Tocotrienol was purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Isolation of peripheral blood mononuclear cells
PBMC were obtained from heparinized blood using Ficoll–Hypaque (GE Healthcare, Philadelphia, PA, USA) density-gradient centrifugation.

Induction of Th17 differentiation
To induce Th17 differentiation, peripheral blood mononuclear cells (PBMCs) were cultured for 48 hours with anti-CD28 and anti-CD3 (BD, Franklin Lakes, NJ, USA), IL-23, IL-6, IL-1β, IL-4-blocking antibodies, and interferon-gamma (IFN-γ)-blocking antibodies. To investigate the suppressive effects of tocotrienol, PBMCs were cultured for 3 hours with tocotrienol, and then incubated as in Th17 differentiation.

Expression of RANKL mRNA by real-time polymerase chain reaction
RA FLS were stimulated with IL-17. For RANKL signal pathway analysis, the FLSs were cultured with or without tocotrienol for 3 hours before the addition of IL-17. After stimulation for 72 hours, mRNA was extracted using RNAzol B (Biotex Laboratories, Houston, TX, USA) according to the manufacturer’s instructions, and then subjected to real-time polymerase chain reaction.

Enzyme-linked immunosorbent assay
Sandwich enzyme-linked immunosorbent assays (ELISAs) were used to quantify sRANKL, IL-1β, TNF-α, IL-6,
IL-8, and IL-17 levels in the culture supernatants from RA FLS or PBMC.

Western blotting analysis
Primary antibodies recognizing to the phosphorylated forms of mammalian target of rapamycin kinase (mTOR), adenosine monophosphate (AMP)-activated protein kinase (AMPK), JUN N-terminal kinase (JNK), extracellular signal-regulated kinase (ERK), or nuclear factor kappa B inhibitor alpha (IκBα; Cell Signaling Technology Inc., Danvers, MA, USA) were diluted 1:1,000 in 0.1% Tween 20/1x Tris-buffered saline (TTBS), and incubated overnight at 4°C. The membranes were washed with TTBS, horseradish peroxidase-conjugated secondary antibody was added, and the membranes were incubated for 1 hour at room temperature. After washing with TTBS, the hybridized bands were detected using an ECL detection kit (Amersham Pharmacia, Piscataway, NJ, USA).

Flow cytometry analysis
Cells were stained with combinations of the following mAbs: anti-CD4–PE/cyanine7 (Cy7) and anti-CD25–allophycocyanin (APC; BD). Cells were washed, fixed, permeabilized, and stained to detect intracellular cytokines with mAbs recognizing IL-17 and forkhead box P3 (FOXP3; eBioscience, San Diego, CA, USA). Cells were analyzed on a FACS Calibur flow cytometry system (BD).

Osteoclast formation
Peripheral blood CD14+ monocytes were cultured with IL-17-treated FLS or Th17-polarizing T cells in media. Monocytes were cocultured in α-minimal essential medium and 10% FBS in the presence of 25 ng/mL of rhM-CSF. The addition of rhRANKL was used as a positive control, which was prepared as described previously [19]. A TRAP Staining Kit was used to stain TRAP in osteoclasts.
Statistical analysis
The data are expressed as mean ± standard error mean (SEM). Statistical difference was assessed using Mann-Whitney U test for analyzing two groups or one-way analysis of variance with Bonferroni’s multiple comparison post hoc test for analyzing more than three groups. A p value < 0.05 was considered statistically significant.

RESULTS

The suppressive effect of tocotrienol on the IL-17-activated RANKL gene and protein in RA FLS
To detect the suppressive effect of tocotrienol on the IL-17-activated RANKL mRNA level, RA FLS were cultured with tocotrienol for 3 hours. The RA FLS were then cultured with 20 ng/mL IL-17 for 72 hours. IL-17 increased RANKL gene expression to its maximum extent at a dose of 20 ng/mL; therefore, we used to IL-17 (20 ng/mL) as an optimal stimulatory dose (data not shown). Tocotrienol suppressed the IL-17-activated expression of RANKL (Fig. 1A). Tocotrienol also reduced the production of TNF-α, but did not affect the IL-17-activated production of IL-6 and IL-8 (Fig. 1C).

Signal pathways involved in the protective effect of tocotrienol in RA FLS
We investigated the intracellular signal pathways of tocotrienol in the IL-17-induced stimulation of RA FLS.
Figure 3. The effect of tocotrienol on osteoclast differentiation from peripheral blood monocytes. CD14⁺ monocytes were isolated from the peripheral blood of healthy donors and then pretreated with tocotrienol for 3 hours, before being cultured with 25 ng/mL of macrophage colony-stimulating factor (M-CSF), (A) 20 ng/mL of interleukin 17 (IL-17), or (B) 30 ng/mL of receptor activator of nuclear factor-κB ligand (RANKL). Osteoclasts were identified using tartrate-resistant acid phosphatase (TRAP) staining. The figures are representative of three independent experiments (original magnification ×100). (C) The expression levels of genes encoding TRAP, cathepsin K, dendritic cell-specific transmembrane protein (DC-STAMP), nuclear factor of activated T cells, cytoplasmic 1 (NF-ATc1), osteoclast stimulatory transmembrane protein (OC-ASTAMP), and V-type proton ATPase subunit D2 (ATP6v0d2) from differentiated osteoclasts were measured using real-time polymerase chain reaction. Data were normalized to the beta-actin mRNA level and reported in relative expression units. The data represent the mean ± SEM of three independent experiments. Toco, tocotrienol. *p < 0.001, b p < 0.05.
The effect of tocotrienol on osteoclastogenesis by interleukin 17 (IL-17)-pretreated rheumatoid arthritis (RA) fibroblast-like synoviocyte (FLS). (A) RA FLS were pretreated with tocotrienol for 3 hours, cultured with 20 ng/mL of IL-17 for 72 hours, and then cocultured with CD14⁺ monocytes from peripheral blood in the presence of macrophage colony-stimulating factor (M-CSF). After 21 days of culture, tartrate-resistant acid phosphatase (TRAP)-positive multinucleated cells were counted. (B) Th17 cells were pretreated with tocotrienol for 3 hours, and then cocultured with CD14⁺ monocytes from peripheral blood in the presence of M-CSF. The figures are representative of three independent experiments (original magnification ×100). (C) The expression levels of genes encoding TRAP, cathepsin K, dendritic cell-specific transmembrane protein (DC-STAMP), nuclear factor of activated T cells, cytoplasmic 1 (NF-ATc1), osteoclast stimulatory transmembrane protein (OC-STAMP), and ATP6v0d2 from differentiated osteoclasts were measured using real-time polymerase chain reaction. Data were normalized to the beta-actin mRNA level and reported in relative expression units. The data represent the mean ± SEM of three independent experiments. *p < 0.05, **p < 0.01, and ***p < 0.001.
IL-17 enhanced the protein levels of AMPK, the phosphorylation of mTOR, ERK, and IκB-α. Tocotrienol significantly reduced the IL-17-activated phosphorylation of mTOR, ERK, and IκB-α (p < 0.05 for each). By contrast, IL-17 did not phosphorylate JNK and tocotrienol significantly increased the IL-17-activated phosphorylation of AMPK (Fig. 2).

The suppressive effect of tocotrienol in IL-17- and RANKL-activated osteoclast formation
The isolated CD14+ monocytes from peripheral blood of three healthy volunteers were incubated with M-CSF and IL-17, which resulted in TRAP+ multinucleated osteoclast differentiation. However, pre-incubation with tocotrienol significantly reduced IL-17-activated osteoclastogenesis (Fig. 3A). To detect the suppressive effects of tocotrienol on RANKL-activated osteoclast formation, CD14+ monocytes were incubated with M-CSF and RANKL. After 21 days of culture, the cells had differentiated into osteoclasts. However, pre-incubation with tocotrienol significantly reduced RANKL-induced osteoclastogenesis (Fig. 3B). The gene expression of osteoclast markers, such as TRAP, cathepsin K, dendritic cell-specific transmembrane protein (DC-STAMP), nuclear factor of activated T cells, cytoplasmic 1 (NF-ATc1), and osteoclast stimulatory transmembrane protein...
(OC-STAMP) increased after IL-17 stimulation; however, their expression was reduced by tocotrienol treatment (Fig. 3C).

The inhibitory effect of tocotrienol in osteoclast formation with coculture of monocytes in addition to RA FLS
RA FLS were incubated with IL-17 and tocotrienol, and then they were cocultured with peripheral blood CD14+ monocytes and M-CSF. When the monocytes were cocultured with IL-17-activated RA FLS without RANKL, TRAP+ multinucleated osteoclasts differentiated and osteoclastogenesis was more pronounced compared with monocytes that were cocultured with untreated RA FLS (Fig. 4A). Tocotrienol suppressed the differentiation of osteoclasts induced by Th17 cells (Fig. 4B). The gene expression of osteoclast markers increased during coculture with IL-17-pretreated RA FLS, while tocotrienol treatment decreased their mRNA expression (Fig. 4C).

The suppressive effects of tocotrienol on Th17 cell differentiation
PBMCs were isolated from normal individuals and incubated under Th17-polarizing conditions in the presence of tocotrienol, and the percent of IL-17 positive and Foxp3 positive cells were counted using flow cytometry. In Th17-polarizing conditions, both Th17 cells and Treg cells were differentiated, and tocotrienol remarkably decreased the proportion of IL-17+CD4+ T cells. However, tocotrienol did not affect the differentiation of the cells into CD25+Foxp3+CD4+ regulatory T cells (Fig. 5A and 5B). Under Th17-polarizing condition, the production of IL-17 and RANKL increased; however, tocotrienol decreased the production of IL-17 and sRANKL in the culture supernatant (Fig. 5C).

DISCUSSION
In this study, we confirmed that tocotrienol decreased IL-17-activated RANKL gene expression and protein production in RA FLS, and inhibited IL-17-induced osteoclast differentiation. IL-17 is an important cytokine-associated with osteoclast formation. Previously, we found that other Th17 cytokines induce RANKL expression in RA FLS [7,20]. Under Th17-polarizing conditions, tocotrienol reduced Th17 cell differentiation and the production of IL-17 and sRANKL, but did not affect Treg cell differentiation. RANKL and M-CSF are essential cytokines for osteoclast differentiation from precursor cells [21]. This result suggested that tocotrienol indirectly interrupts the initial process of the Th17 cytokine-associated bony destructive pathways through inhibition of RANKL production.

In terms of signal transduction pathways, we examined the molecular signaling pathways associated with the regulatory effects of tocotrienol on RA FLS. We focused on the mTOR/AMPK/JNK/ERK/IκB-α pathway, which has an essential role in the activation of RA FLS [18]. In signaling pathways, the suppressive effects of tocotrienol are mediated through the mTOR/JNK/nuclear factor κB (NF-κB) pathway [22,23]. IL-17 activates mTOR, and tocotrienol decreased IL-17-activated mTOR phosphorylation. In addition, tocotrienol increased AMPK phosphorylation. In previous studies, γ-tocotrienol demonstrated an inhibitory effect on the NF-κB activation pathway [24-27]. Moreover, Wu et al. [28] identified that a tocotrienol-rich fraction was capable of inhibiting proinflammatory cytokine production in human monocytes.

We examined the direct inhibitory effect of tocotrienol on the formation of osteoclasts from their precursors. Tocotrienol directly inhibited RANKL and IL-17-activated osteoclast differentiation in a dose-dependent manner. This result suggested that tocotrienol could directly ameliorate cytokine-induced osteoclastogenesis, indicating its possible therapeutic use.

Next, we determined whether tocotrienol modulates Th17 cells under direct stimulation of osteoclast formation. When monocytes were cultured with Th17 cells, osteoclast differentiation increased compared with that in cells cocultured with CD4+ T cells. Without RANKL, Th17 cells directly induced osteoclast differentiation and tocotrienol inhibited this Th17 cell-induced osteoclastogenesis. Tocotrienol controls Th17 cell-induced osteoclastogenesis in different ways. First, tocotrienol inhibited IL-17-induced RANKL expression in RA FLSs. Second, tocotrienol reduced IL-17-induced osteoclast differentiation. Third, tocotrienol inhibited Th17 cell-induced osteoclast differentiation. The ultimate goal of medical care in patients with RA is the prevention of joint destruction. Bone erosion caused by activat-
ed osteoclasts induces joint destruction and patient disability. The results of the present study suggested that tocotrienol could prevent the bony destructive process in RA.

There are some limitations in this study. First, we observed the effect of tocotrienol only in vitro. If preclinical animal study with tocotrienol is performed, more information of therapeutic effect of tocotrienol is produced. Second, we evaluated the effect of tocotrienol using a single target cell, FLS, however, macrophages/monocytes are major cells producing TNF-α, IL-1 and IL-6. The effect of tocotrienol to the macrophages/monocytes needs to be assessed. Third, tocotrienol reduced IL-17-induced production of TNF-α; however, we did not analyze the inhibitory mechanism and we did not focus on TNF-α.

In conclusion, tocotrienol significantly suppressed IL-17-activated RANKL expression and osteoclast differentiation, and attenuated Th17 differentiation. The results suggested that tocotrienol could represent a new therapeutic choice to prevent osteoclast-associated bony destructive diseases, such as RA.

Conflict of interest
No potential conflict of interest relevant to this article was reported.

Acknowledgments
This research was supported by a grant of the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology, Republic of Korea (NRF-2018R1D1A1A02050982) and the Basic Science Research Program through the National Research Foundation of Korea funded by the Ministry of Science, ICT & Future Planning (NRF-2018R1A2B2006820).

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