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The anti-inflammatory effect of the gut lactic acid bacteria-generated metabolite 10-oxo-*cis*-6,*trans*-11-octadecadienoic acid on monocytes☆

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ABSTRACT

We evaluated the effect of gut bacterial metabolites of polyunsaturated fatty acids on inflammation and found that 10-oxo-*cis*-6,*trans*-11-octadecadienoic acid (γKetoC) strikingly suppressed LPS-induced IL-6 release from bone marrow-derived macrophages (BMMs), which was accompanied by reduced mRNA expression of *Il6*, *TNF*, and *Il1b*. γKetoC decreased the cAMP concentration in BMMs, suggesting that γKetoC stimulated G protein-coupled receptors. A Gq agonist significantly suppressed LPS-induced IL-6 expression in BMMs, whereas a Gi inhibitor partially abrogated γKetoC-mediated IL-6 suppression. Cytosolic Ca^{2+} was markedly increased by γKetoC, which was partly but not fully abrogated by an ion channel inhibitor. Taken together, these data suggest that γKetoC suppresses inflammatory cytokine expression in macrophages primarily through Gq and partially through Gi. γKetoC suppressed osteoclast development and IL-6 expression in synovial fibroblasts from rheumatoid arthritis (RA) patients, suggesting the beneficial effect of γKetoC on the prevention or treatment of RA.

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1. Introduction

Increasing evidence showing the relationship between lipids and inflammation has been reported in studies using various approaches, including epidemiological analysis, molecular biology, and *in vivo* mouse models. Polyunsaturated fatty acids (PUFAs) are the most vigorously studied lipids and are positively associated with inflammatory diseases, especially rheumatoid arthritis (RA), in many epidemiological studies [1]. PUFAs, such as ω3 and ω6, are converted to bioactive metabolites by enzymes in host cells and play important roles in the health of humans and animals. In addition to host metabolism, it was recently found that a gut lactic acid bacterium, *Lactobacillus plantarum*, possesses several enzymes that convert PUFAs to hydroxy fatty acids, oxo fatty acids, conjugated fatty acids, and partially saturated fatty acids [2]. The gut bacterial metabolites of PUFAs exhibit various beneficial effects in host homeostasis, including intestinal epithelial barrier impairment [3], and energy metabolism regulation [4,5]. However, the roles of these metabolites of PUFAs in inflammation are largely unknown. In the current study, we investigated the effects of the bacteria-generated fatty acids on inflammatory responses. Among the various metabolites, we focused on 10-oxo-*cis*-6,*trans*-11-octadecadienoic acid (γKetoC) as the most effective anti-inflammatory fatty acid, which suppressed the expression of inflammatory cytokines by primary macrophages. *In vitro* analyses showed that γKetoC modulates the function of macrophages primarily through Gq and partially through Gi. We discuss the possible use of γKetoC in the prevention and/or treatment of inflammatory

Abbreviations: αKetoA, 10-oxo-*cis*-12,*cis*-15-octadecadienoic acid; αKetoC, 10-oxo-*trans*-11,*cis*-15-octadecadienoic acid; ALA, α-linolenic acid; BMMs, bone marrow-derived macrophages; γKetoA, 10-oxo-*cis*-6,*cis*-12-octadecadienoic acid; γKetoC, 10-oxo-*cis*-6, *trans*-11-octadecadienoic acid; GLA, γ-linolenic acid; GPCRs, G protein-coupled receptors; KetoA, 10-oxo-*cis*-12-octadecenoic acid; KetoC, 10-oxo-*trans*-11-octadecenoic acid; LA, linoleic acid; PUFAs, polyunsaturated fatty acids; RA, rheumatoid arthritis.

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diseases such as RA.

2. Materials and methods

2.1. Mice

Female C57BL/6 mice were purchased from Japan SLC (Hamamatsu, Japan). All animal experiments were performed in accordance with the approved guidelines of the Institutional Review Board of Tokyo University of Science (Tokyo, Japan). The Animal Care and Use Committees of Tokyo University of Science specifically approved this study.

2.2. Cells

Bone marrow-derived macrophages (BMMs) were prepared by a previously described method [6]. RAW264.7 cells obtained from American Type Culture Collection (ATCC, Manassas, VA) were maintained in RPMI 1640-based culture medium. RA patient-derived synovial fibroblasts were purchased from Articular Engineering, LLC (Northbrook, IL) and were maintained in a synovial cell culture medium (Cell Applications Inc., San Diego, CA). Osteoclasts were generated from bone marrow cells as previously described [7]. Murine macrophage colony stimulating factor (mM-CSF) and human sRANKL (hsRANKL) were purchased from PeproTech, (Rocky Hill, NJ).

2.3. Preparation of fatty acids generated by gut lactic acid bacterial enzymes

Hydroxy fatty acids and oxo fatty acids were generated from linoleic acid (LA), α-linolenic acid (ALA), and γ-linolenic acid (GLA) by using the enzymes isolated from *Lactobacillus plantarum* as previously described [2].

2.4. Production of inflammatory cytokines in stimulated BMMs

BMMs incubated in the presence or absence of fatty acids for 24 h were stimulated with 100 ng/mL LPS (*E. coli 011*: B4, Sigma-Aldrich). Culture supernatants were collected 17 h after stimulation, and murine IL-6 concentrations in the collected media were determined with a mouse IL6 HTRF kit (Cisbio, Codolet, France) using an Envision microplate reader (PerkinElmer, Waltham, MA). To determine the mRNA level, BMMs were harvested 3 h after LPS stimulation. Determination of mRNA levels was performed as previously described [8,9] using a Relia Prep RNA cell miniprep system (Promega, Madison, WI), a ReverTra Ace qPCR RT kit (Toyobo, Osaka, Japan), and a StepOne Real-Time PCR system (Applied Biosystems, Waltham, MA). THUNDERBIRD probe qPCR mix (Toyobo) was used with the following TaqMan gene expression assays (Applied Biosystems): #Mm00446190_m1 for mouse *Il6*, #Mm00443258_m1 for mouse *Tnf*, and #Mm99999915_g1 for mouse glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*).

THUNDERBIRD SYBR qPCR mix (Toyobo) was used with the following synthesized oligonucleotides: mouse *Il6* (forward primer: 5′- AATCGTGGAAATGAGAAAAGAGTTG -3′, and reverse primer: 5′- AGTGCATCATCGTTGTTCATACAA -3′), and *Il1b* (forward primer: 5′- AGTTGACGGACCCCAAAAGA -3′, and reverse primer: 5′-GGACAGCCCAGGTCAAAGG -3′).

2.5. Measurement of cellular cAMP concentration

BMMs were suspended in stimulation buffer from a cAMP-Gi kit (Cisbio) and seeded at a density of 2.0×10^4 cells per well in a white v-bottom 384-well plate. After 24 h incubation with fatty acids and/or compounds, the concentration of cellular cAMP was measured with a cAMP-Gi kit according to the manufacturer's instructions using Envision. Pertussis toxin (PTX) and GW-9508 were purchased from Sigma-Aldrich and Cayman Chemical (Ann Arbor, MI), respectively.

2.6. Measurement of cytosolic calcium influx

RAW264.7 cells were seeded at a density of 1×10^4 cells per well in type-1 collagen-coated black 96-well plates and incubated overnight. Fluo-8 NW (AAT Bioquest, Sunnyvale, CA) was added according to the manufacturer's instructions. To inhibit ion channels, 300 μM ruthenium red (Fujifilm Wako Pure Chemical Corporation, Osaka, Japan) was added at the same time as Fluo-8. Fluorescence measurements (emission: 525 nm; excitation: 485 nm) were performed with a FlexStation 3 (Molecular Devices, LLC, San Jose, CA).

2.7. Osteoclast formation

Osteoclasts were stained with a TRAP staining kit (Fujifilm Wako Pure Chemical Corporation) according to the manufacturer's instructions. The number of osteoclasts was counted with Cell^3 iMager duos (SCREEN Holdings Co., Ltd., Kyoto, Japan) and ImageJ (National Institutes of Health, Bethesda, MD).

2.8. IL-6 production by human synovial fibroblasts

Human synovial fibroblasts were incubated in RPMI 1640 supplemented with 0.3% BSA in the presence or absence of fatty acids for 24 h. At 17 h after the addition of 100 ng/mL LPS, supernatants were collected, and the concentration of human IL-6 in harvested supernatants was measured with a human IL6 HTRF kit (Cisbio) using Envision.

2.9. Statistical analysis

A two-tailed Student's t-test was performed with Microsoft Excel. Dunnett's test was performed with Pharmaco Basic (Scientist Press Co. Ltd., Tokyo, Japan). The level of significance was set at $p < 0.05$ and < 0.01.

3. Results and discussion

3.1. Effects of PUFAs on IL-6 secretion by LPS-stimulated BMMs

To evaluate the effect of various PUFAs on the inflammatory response of immune cells, we first determined the amount of the inflammatory cytokine IL-6 released from PUFA-treated BMMs upon LPS stimulation. In this assay, we found that 10-oxo-*trans*-11,*cis*-15-octadecadienoic acid (αKetoC), 10-oxo-*cis*-6,*trans*-11-octadeca dienoic acid (γKetoC), 10-oxo-*cis*-12,*cis*-15-octadecadienoic acid (αKetoA), and 10-oxo-*cis*-6,*cis*-12-octadecadienoic acid (γKetoA), apparently suppressed the LPS-induced IL-6 release from BMMs (data not shown). When the effects of 30 μM αKetoC, γKetoC, and 10-oxo-*trans*-11-octadecenoic acid (KetoC) on IL-6 production were compared, γKetoC and αKetoC reduced IL-6 secretion by approximately one-third and one-half of that of the control, respectively, whereas the 30 μM KetoC did not affect IL-6 production level (Fig. 1A). Based on these results, we selected γKetoC as the most effective metabolite for the suppression of macrophage activation and performed the following detailed analyses primarily using γKetoC.

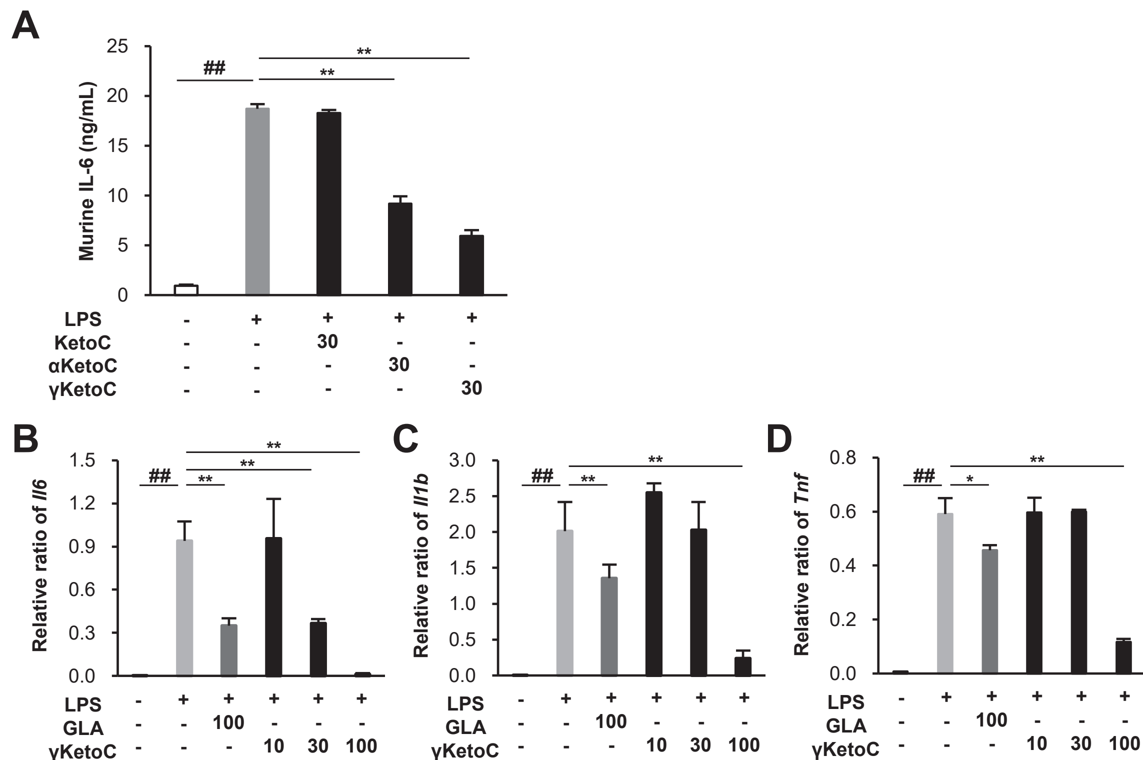

Fig. 1. Effects of gut lactic acid bacteria-generated PUFA metabolites on inflammatory cytokine expression in BMMs.
(A) Effect of KetoC-group compounds on IL-6 production in BMMs. BMMs were incubated in the presence or absence of 30 μM KetoC, αKetoC, and γKetoC for 24 h, and then stimulated with 100 ng/mL LPS for 17 h. n = 3, mean +SD, *t*-test ##p < 0.01 vs. LPS (−), Dunnett's test *p < 0.05,**p < 0.01 vs. LPS (+).
(B–D) Effects of GLA and γKetoC on the mRNA levels of *Il6* (B), *Il1b* (C), and *Tnf* (D) in BMMs. BMMs were incubated in the presence or absence of the indicated concentrations (μM) of GLA or γKetoC for 24 h, and then stimulated with 100 ng/mL LPS for 3 h. n = 3, mean +SD, *t*-test ##p < 0.01 vs. LPS (−), Dunnett's test *p < 0.05, **p < 0.01 vs. LPS (+) vehicle. Similar results were obtained in another independent experiment (A–D).

3.2. Effect of γKetoC on the transcription of inflammatory cytokine genes in BMMs

To investigate whether the γKetoC-mediated reduction in IL-6 occurred at the transcription level, we measured the mRNA expression of *Il6* in BMMs 3 h after LPS stimulation. As shown in Fig. 1B, the LPS-induced upregulation of *Il6* mRNA was significantly decreased in the presence of 30 μM γKetoC and was almost blocked by 100 μM γKetoC. Treatment with 100 μM GLA, the precursor of γKetoC, reduced *Il6* mRNA levels to a similar degree as that of 30 μM γKetoC, suggesting that conversion of GLA to γKetoC increased its anti-inflammatory activity. The suppressive effect of γKetoC was also observed in the LPS-induced transactivation of *Il1b* (Fig. 1C) and *Tnf* (Fig. 1D). These results indicate that γKetoC suppresses LPS-induced transactivation of inflammatory cytokines in macrophages and the subsequent secretion of cytokines and that GLA acquired anti-inflammatory activity through modification by gut lactic acid bacteria metabolism.

3.3. Decrease in cAMP concentration in γKetoC-treated BMMs

Several GPCRs, including GPR40, 41, 43, 84, 109A, and 120, have been identified as free fatty acid receptors [10]. Ligand binding activates GPCR signaling, which up- or down-regulates the intracellular concentration of cAMP by controlling the activity of adenylate cyclase. To clarify the involvement of GPCRs in γKetoC-mediated cellular events, we determined the cAMP concentration in PUFA-treated BMMs and found that the cAMP concentration in BMMs was reduced by incubation in the presence of 100 μM γKetoC (Fig. 2A). These results suggest that γKetoC directly or indirectly decreases the intracellular concentration of cAMP through Gi GPCRs and/or Gq GPCRs.

3.4. Involvement of Gi and Gq GPCRs in γKetoC-mediated suppression of BMMs

The γKetoC-mediated decrease in cAMP concentration indicates the involvement of Gi and/or Gq but not Gs GPCRs in the γKetoC-mediated suppression of BMM activation. To further analyze which GPCR primarily functions in γKetoC-mediated modulation of BMMs, we used an inhibitor and an activator of GPCRs. First, we evaluated the effect of PTX, an inhibitor for Gi GPCR, on γKetoC-mediated suppression. As shown in Fig. 2B, γKetoC-mediated suppression of LPS-induced *Il6* mRNA expression was slightly but significantly inhibited by PTX. Next, we treated BMMs with GW-9508, an activator of Gq GPCRs (especially GPR40), and found that GW-9508 suppressed LPS-induced transactivation of the *Il6* gene similar to that of γKetoC (Fig. 2C). These results indicate that the γKetoC-mediated suppression of BMMs is dependent mainly on Gq GPCRs and partly on Gi GPCRs.

3.5. Effect of γKetoC on Ca^{2+} influx in monocytes

In addition to the decrease in intracellular cAMP, the binding of short-, medium-, and long-chain fatty acids to Gi or Gq GPCRs induces an increase in cytoplasmic Ca^{2+} [10]. It has also been reported that 10-oxo-*cis*-12-octadecenoic acid (KetoA) induces an increase in intracellular Ca^{2+} [4]. These observations prompted us to analyze the effect of γKetoC on Ca^{2+} influx. When the intracellular concentration of Ca^{2+} in RAW264.7 cells (a mouse monocytic

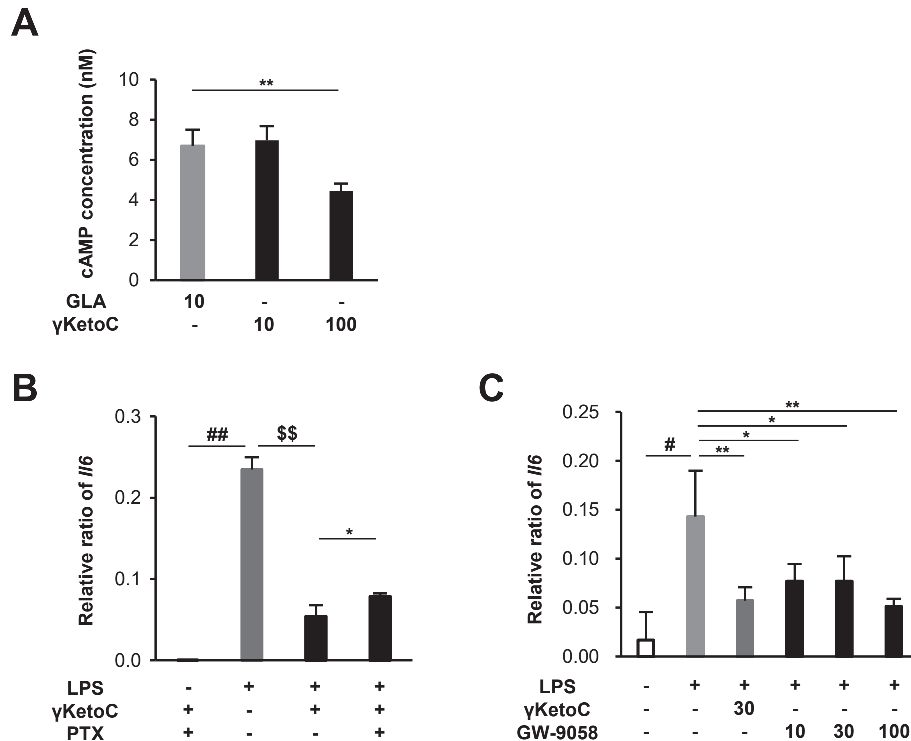

Fig. 2. Effect of γKetoC on intracellular cAMP concentrations in BMMs.
(A) After 24 h incubation of BMMs in the presence or absence of the indicated concentrations (μM) of PUFAs, the intracellular concentration of cAMP was determined by a HTRF kit. n = 3, mean +SD, Dunnett's test *p < 0.05, **p < 0.01 vs vehicle.
(B) Effect of a Gi inhibitor on the γKetoC-mediated reduction in *Il6* mRNA. BMMs preincubated with 100 ng/mL PTX for 1 h were treated with indicated concentrations (μM) of γKetoC for 24 h, and then stimulated with 100 ng/mL LPS for 3 h. n = 3, mean +SD, *t*-test ##, $$, p < 0.01, *p < 0.05.
(C) A Gq agonist reduced *Il6* mRNA levels in BMMs. BMMs were incubated in the presence or absence of the indicated concentrations (μM) of γKetoC or GW-9508 for 24 h and then stimulated with 100 ng/mL LPS for 3 h. n = 3, mean +SD, *t*-test #p < 0.05 vs. LPS (−), Dunnett's test *p < 0.05, **p < 0.01 vs. LPS (+) vehicle. Similar results were obtained in another independent experiment (B and C).

cell line) was monitored by fluorescence, we found that the relative fluorescence increased at approximately 5 s after the addition of γKetoC to the culture medium, and the shape of the curve transiently peaked at 20–30 s (Fig. 3A). The fluorescence increase was caused by γKetoC in a dose-dependent manner, and the magnitude was greater than that of KetoA (Fig. 3A). The mechanism by which KetoA increases intracellular Ca^{2+} is through intake of extracellular Ca^{2+} by the nonselective cation channel TRPV1 [4]. Therefore, to confirm the involvement of ion channels in γKetoC-mediated increases in Ca^{2+}, RAW264.7 cells were stimulated with KetoA or γKetoC in the presence or absence of ruthenium red, an inhibitor of nonselective ion channels. As shown in Fig. 3B, the increase in relative fluorescence in cells treated with 100 μM KetoA was downregulated to the steady state level by the additional treatment with ruthenium red. In this experimental condition, the increase in relative fluorescence in the presence of 100 μM γKetoC was significantly suppressed but apparently higher than that of the steady state level even in the presence of ruthenium red (Fig. 3B). These results suggest that γKetoC induces an increase in cytoplasmic Ca^{2+} probably through the GPCR pathway and partially depends on ion channels.

3.6. *Effect of γKetoC on osteoclast development and the inflammatory response of synovial fibroblasts*

In the current study, the analyses using BMMs and RAW264.7 cells showed that γKetoC suppressed the activation of macrophages/monocytes. To evaluate the effect of γKetoC on different types of cells, we investigated the development of osteoclasts in the presence of γKetoC because we hypothesized that γKetoC would affect osteoclasts derived from monocyte lineages. Bone marrow-derived monocyte precursors were differentiated into TRAP-positive cells, which is a characteristic feature of osteoclasts, following RANKL stimulation (Fig. 4A). As expected, the presence of γKetoC in the culture medium suppressed osteoclast development in a dose-dependent manner, whereas GLA slightly decreased the number of TRAP-positive cells (Fig. 4A).

The continuous overproduction of IL-6, IL-1β, and TNF-α by activated macrophages is the cause of RA, and the blocking antibodies against these cytokines are therapeutic tools for the treatment of RA. Overdevelopment of osteoclasts accelerates RA progression. Based on these findings, γKetoC may prevent and/or improve RA. To confirm the effectiveness of γKetoC in RA treatment, we examined the effect of γKetoC on IL-6 production by human synovial fibroblasts from RA patients upon LPS stimulation. As shown in Fig. 4B, LPS stimulation induced IL-6 release from synovial fibroblasts, and the amount of IL-6 released from activated synovial cells decreased in the presence of γKetoC. These results suggest that γKetoC suppresses the inflammatory responses of macrophages, osteoclasts, and synovial fibroblast in RA patients, and that γKetoC is useful for the prevention and/or treatment of RA. To clarify this issue, we will perform further detailed analyses using an inflammatory response-related *in vivo* mouse model.

It is well known that PUFAs play important roles in a wide

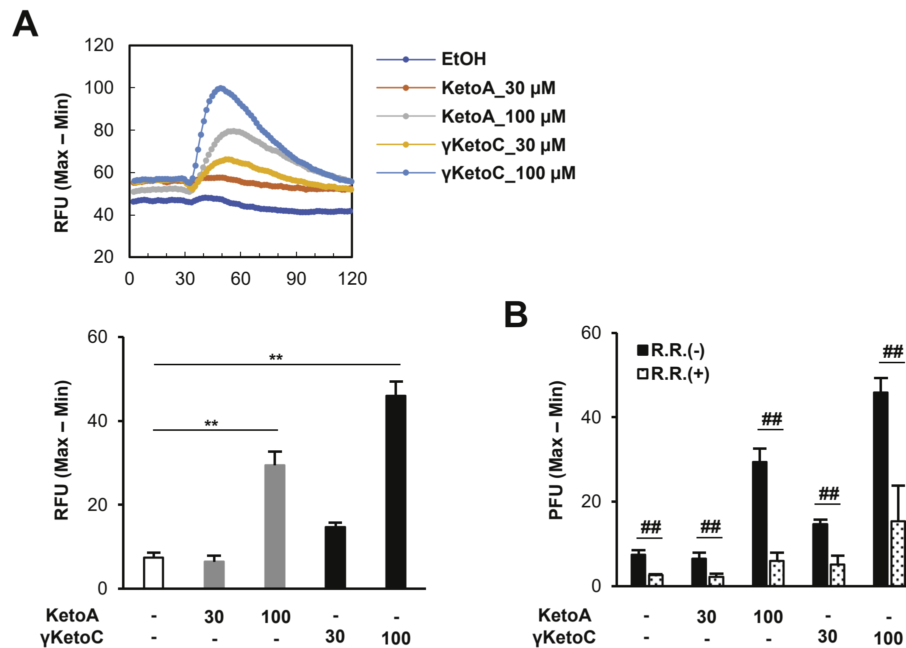

Fig. 3. Effect of γKetoC on cytosolic Ca^{2+} concentrations.
(A) Calcium influx in RAW264.7 cells. Time courses of fluorescence intensity in Fluo-8-treated cells for 120 s upon PUFA stimulation (top) and the relative fluorescence units (RFUs) calculated from the maximum value and minimum value (bottom). n = 3, mean +SD, Dunnett's test **p < 0.01 vs. vehicle.
(B) Effect of an ion channel inhibitor on PUFA-induced Ca^{2+} influx. Fluo-8-treated RAW264.7 cells were preincubated with 300 μM ruthenium red. Measurement of fluorescence and calculation of RFUs were performed in the same manner as (A). n = 3, mean +SD, *t*-test "##p<0.01. Similar results were obtained in another independent experiment (A and B).

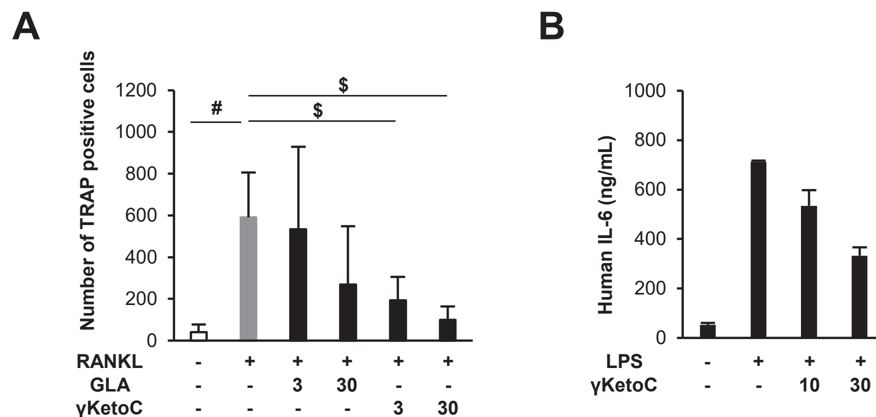

Fig. 4. Effect of γKetoC on osteoclast development and IL-6 release by synovial fibroblasts of RA patients.
(A) Number of TRAP-positive cells. n = 3, mean +SD, *t*-test #p < 0.05, $ p < 0.05.
(B) The concentration of human IL-6 in the culture supernatant of LPS-stimulated synovial fibroblasts treated with the indicated concentrations (μM) of fatty acids. n = 2, mean + range.
Similar results were obtained in another independent experiment (A and B).

range of pro- and anti-inflammatory responses in humans and animals. Among the various PUFAs, n-3 fatty acids, also called ω3, down-regulate inflammation and maintain tissue homeostasis by competing with n-6 fatty acids [11]. γKetoC, which was identified as an anti-inflammatory PUFA in the current study, belongs to the n-7 fatty acid family, indicating that the mechanism by which PUFAs affect inflammation and the immune response cannot be explained by the position of the unsaturated bounds and the n-3/n-6 ratio. Considering that the bacterial metabolites that show anti-inflammatory effects belong to oxo fatty acids (Fig. 1), the oxo structure, especially the enone structure (conjugated oxo structure) in γKectoC, may play a key role in the anti-inflammatory function.

Studies using antagonists and agonists have indicated that Gq-type GPCRs are mainly involved in the γKetoC-mediated suppression of macrophage activation. GPR40 and GPR120, which are Gq GPCRs and have been identified as receptors for medium-long fatty acids [3], may also be receptor(s) for γKetoC because GW-9508 also suppressed the activation of macrophages (Fig. 2C). Alternatively, other GPCRs, which are known to play roles as receptors for eicosanoids released from activated macrophages via COX activity, might suppress the activation of macrophages. In our preliminary experiment using LC-MS/MS, we detected approximately 40 kinds of fatty acids, which were significantly higher in

culture supernatants of γKetoC-treated and LPS-stimulated BMMs compared with that of stimulated BMMs without γKetoC treatment (data not shown). Further detailed analyses of γKetoC may lead to the identification of therapeutic targets for inflammatory diseases.

Declaration of competing interest

The authors declare no conflicts of interest.

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Authorship Contribution

S. H. performed experiments and analyzed the data; K. N. performed experiments and prepared figures, K. K., F. S., and T. Y. performed experiments; S. K. provided experimental tools; J. O. wrote the paper; and C. N. designed the research and wrote the paper.

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