***In vitro* characterization of anti-inflammatory activities of 3*RS*, 7*R*, 11*R*-phytanic acid**

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SUPPLEMENTARY FILE

Materials and Methods

Animals and cells

Female C57BL/6J mice obtained from Japan SLC (Shizuoka, Japan) were kept at 25°C on a 12: 12 h light-dark cycle and had free access to a commercial diet (Labo MR stock diet, Nosan Corporation, Yokohama, Japan). Spleens were removed aseptically from mice aged 8 to 13 weeks under the inhalation anesthetic isoflurane. Splenocytes were prepared as single-cell suspensions after treated with ammonium chloride for red blood cell lysis. T-cells and B-cells were purified from splenocytes by negative selection on a magnetic column using the respective isolation kits (Pan T cell isolation kit II and B cell isolation kit, Miltenyi Biotec, Bergisch Gladbach, Germany). The cell purity of >95% was confirmed by flow cytometry. A mouse macrophage-like cell line J774.1 cells was also obtained from the Cell Engineering Division of RIKEN Bioresource Center (Tsukuba, Japan). All cells were suspended in RPMI 1640 medium containing 10% fetal calf serum, 100 U/mL penicillin and 100 μg/mL streptomycin, and incubated at 37°C in a humidified atmosphere of 5% CO2-95% air. Animals were used in accordance with the guidelines for the care and use of laboratory animals at the University of Miyazaki and Law No. 105 of the Japanese government. All experimental protocols were approved by the University of Miyazaki (approval number: 2014-002 and 2020-009).

Cellular toxicity

The 1.5×10^5 mouse splenocytes or 5.0×10^4 J774.1 cells were seeded into flat-bottomed 96-well microtiter plates, and incubated in the presence of 3*RS*, 7*R*, 11*R*-phytanic acid (3*RS*-PHY) or the control palmitic acid at the maximum concentration of 100 μM, where both fatty acids were dissolved in dimethyl sulfoxide (DMSO) and added as a final DMSO concentration of 0.1%. Following 72 h incubation, an Alamar blue assay was carried out as per the manufacturer's instruction (Thermo Fisher Scientific Inc., Waltham, MA, USA). Fluorescence was measured with excitation at 550 nm and emission at 590 nm using a Varioskan Flash 2.4 microplate reader (Thermo Fisher Scientific Inc.).

Cytokine mRNA expression levels in mouse splenocytes and T-cells

The 2.0 × 10^6 mouse splenocytes were stimulated with 10 μg/mL pokeweed mitogen (PWM) or phytohemagglutinin (PHA), and incubated in 24-well plates for 24 h in the presence of 3*RS*-PHY or palmitic acid. Total RNA was then extracted from the cells using TRIzol reagent (Life Technologies, Inc., Grand Island, NY, USA) and applied as a template for cDNA synthesis by a commercially available reverse transcription kit (Toyobo CO., Ltd., Osaka, Japan). Quantitative reverse-transcription polymerase chain reaction (qRT-PCR) was performed in the AriaMx Realtime PCR system (Agilent Technologies, Inc., Santa Clara, CA, USA) where a commercial kit was utilized in accordance with the manufacturer's instructions (Brilliant III Ultra-Fast SYBR Green QPCR Master Mix, Agilent Technologies). The expression levels of interferon (IFN)-γ, interleukin (IL)-2, IL-4, IL-10 IL-17A and GAPDH were assessed using pre-designed primers for each gene (MA025911, IFN-γ; MA155270, IL-2; MA112734, IL-4; MA118529, IL-10; MA157056, IL-17A; MA050371, GAPDH, Takara Bio Inc., Shiga, Japan). The number of cycles required to reach the threshold, which was set in linear part of the amplification curve, was calculated for each gene. Melting curve analysis was also performed to confirm the purity of the PCR products. Data were normalized using GAPDH as a housekeeping gene for cytokine genes. The 1.0 × 10^6 T-cells stimulated with 10 ng/mL phorbol 12-myristate 13-acetate (PMA) and 300 ng/mL ionomycin were also incubated in the presence of test fatty acids. The qRT-PCR assays were similarly done for genes of Th cell differentiation markers (T-bet, MA114313; GATA3, MA146224; RORγt, MA161210, Takara Bio Inc.) in addition to the above cytokine genes.

Cytokine and immunoglobulin secretions of mouse splenocytes and B-cells

The 1.5 × 10^5 mouse splenocytes or B-cells were stimulated with 10 μg/mL PWM and 1 μg/mL lipopolysaccharide (LPS), respectively, and incubated with 3*RS*-PHY or palmitic acid in round-bottomed 96-well plates. After 72 h, culture supernatants were collected and subjected to enzyme-linked immunosorbent assay (ELISA) for determination of cytokine and immunoglobulin concentrations by commercially available ELISA kits (IFN-γ, IL-2, IL-4, IL-10 and IL-17A, Biolegend, Inc., San Diego, CA, USA; IgM and IgG, Bethyl Laboratories Inc., Montgomery, TX, USA). Following the peroxidase reaction with substrate (TMB microwell peroxidase substrate system, SeraCare Life Sciences, Milford, MA, USA) and its stop by 2 M sulfuric acid, absorbance at 450 nm was measured by a Varioskan Flash 2.4 microplate reader. The lower detection limits were as follows: IL-2 and IL-4, 2.0 pg/mL; IL-17A, 15.6 pg/mL; IFN-γ and IL-10, 31.3 pg/mL; IgG, 0.69 ng/mL; and IgM, 1.37 ng/mL.

Nitric oxide (NO) production and cytokine secretion of J774.1

Assays were performed in flat-bottomed microtiter 96-well plates, with each well containing 1×10^4 J77.1 cells. Cells were incubated in medium containing 1 μg/mL LPS and 10 ng/mL IFN-γ (Biolegend, Inc.) along with various concentrations of test fatty acids. After incubation for 48 h, culture supernatants were collected and NO production was detected by a Griess reaction as previously described (Ding et al., 1988) with slight modifications. Briefly, aliquot of the supernatant was mixed with an equal volume of the Griess reagent, 0.2% sulfanilamide, 0.1% N-(1-Naphthyl) ethylendiamine dihydrochloride in 3.0% phosphoric acid. Following incubation for 10 min at room temperature, the NO concentrations were measured as nitrite based on absorbance measurements at 540 nm using a Varioskan Flash 2.4 microplate reader. The known amounts of sodium nitrite were used as standards and linear over this concentration range. The culture supernatant was also utilized for detection of TNF-α and IL-6 by commercially available ELISA kits (Biolegend, Inc.). The lower limit of TNF-α and IL-6 detection was 7.8 pg/ml.

Ding AH, Nathan CF and Stuehr DJ (1988) Release of reactive nitrogen intermediates and reactive oxygen intermediates from mouse peritoneal macrophages. Comparison of activating cytokines and evidence for independent production. *The Journal of Immunology* **141** 2407-2412.

Fig. S1

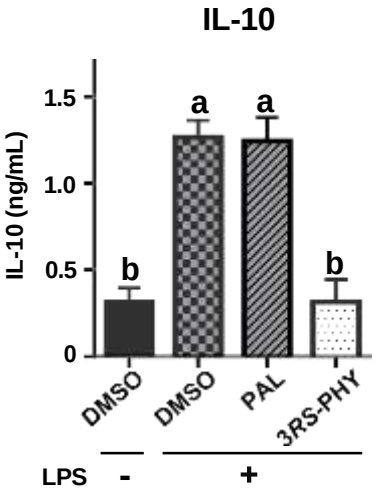

Fig. S1. Effects of 3*RS*, 7*R*, 11*R*-phytanic acid (3*RS*-PHY) on IL-10 secretion by mouse B-cells. After purified B-cells were stimulated with lipopolysaccharide (LPS) and incubated in the presence of 3*RS*-PHY or palmitic acid (PAL), IL-10 concentration in culture supernatant was determined by enzyme-linked immunosorbent assay. DMSO, dimethyl sulfoxide. The data represent means ± SEM. Groups with different letters are significantly different ($P < 0.05$).

Fig. S2

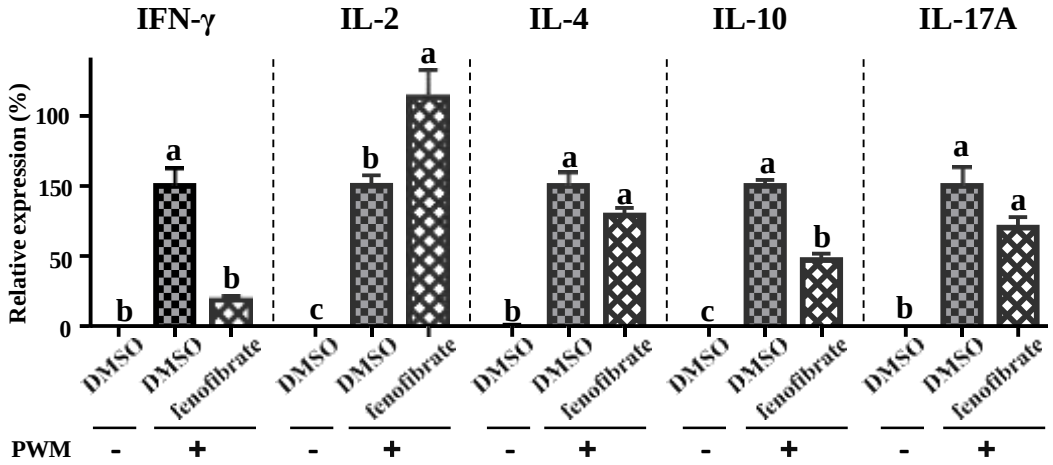

Fig. S2. Effects of fenofibrate on mRNA expression of cytokines in mouse splenocytes. After pokeweed mitogen (PWM)-stimulated mouse splenocytes were incubated in the presence of fenofibrate, mRNA expressions of IFN-γ, IL-2, IL-4, IL-10 and IL-17A were measured by quantitative reverse-transcription polymerase chain reaction. DMSO, dimethyl sulfoxide. The data represent means ± SEM. Groups with different letters are significantly different ($P < 0.05$).