Effect of *Porphyromonas gingivalis* infection on gut dysbiosis and arthritis exacerbation in mouse model

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Abstract

Background: *Porphyromonas gingivalis* (Pg) infection causes periodontal disease and is involved in the exacerbation of rheumatoid arthritis (RA). Gut microbiota dysbiosis has shown strong associations with systemic diseases, including RA, diabetes mellitus, and inflammatory bowel disease, and inoculation of periodontopathogenic bacteria (i.e. Pg) can alter gut microbiota composition. Therefore, this study investigated dysbiosis-mediated arthritis exacerbation by Pg oral inoculation in an experimental arthritis model mouse.

Methods: Pg inoculation in the oral cavity twice a week for 6 weeks was performed to induce periodontitis in SKG mice. Concomitantly, a single intraperitoneal (i.p.) injection of laminarin (LA) was administered to induce experimental arthritis (Pg-LA mouse). Citrullinated protein (CP) and IL-6 levels in periodontal, intestinal, and joint tissues, and serum were measured by ELISA. Gut microbiota composition was determined by sequencing the bacterial 16S rRNA after DNA purification of mouse feces. Fecal microbiota transplantation (FMT) was performed by transferring Pg-RA-derived feces to normal SKG mice. The effect of the Pg peptidylarginine deaminase (PgPAD) on the level of citrullinated proteins and arthritis progression were determined using PgPAD knockout mutant.

Results: Periodontal alveolar bone loss and IL-6 in gingival tissue were induced by Pg oral infection, as well as severe joint destruction, increased arthritis scores (AS), and IL-6 and CP production in serum, joint, and intestinal tissues. Distribution of Deferribacteres and S24-7 was decreased, while CP was significantly increased in gingival, joint, and intestinal tissues of Pg-inoculated experimental arthritis mice compared to experimental arthritis mice without Pg inoculation. Further, FMT from Pg-inoculated experimental arthritis mice showed the reproduction of donor gut microbiota and resulted in severe joint destruction with increased IL-6 and CP production in joint and intestinal tissues. The avarage AS of FMT from Pg-inoculated experimental arthritis was much higher than that of donor mouse. However, inoculation of the PgPAD knockout mutant inhibited the elevation of arthritis scores, ACPA level in serum and reduced CP amount in gingival, joint, and intestinal tissues compared to Pg wild type inoculation .

Conclusion: Pg oral infection affected gut microbiota dysbiosis and joint destruction via increased CP generation.

Background

Periodontal disease (PD) is a highly prevalent infectious disease caused by periodontopathogenic bacteria, such as *Porphyromonas gingivalis* (Pg), *Tannerella forsythia*, and *Treponema denticola*, called red complex [1]. Due to the host immune response against periodontopathogenic bacterial challenge, severe destruction of dental supportive tissues, including cementum, periodontal ligament, and alveolar bone, occurs [2]. Local and systemic elevation of inflammatory cytokines, interleukin (IL)-6 and TNF-a, affect PD progression through T cell activation [3]. Several studies have shown a strong correlation between PD, especially Pg infection, and systemic disease, including non-alcoholic steatohepatitis

(NASH), rheumatoid arthritis (RA), pre-birth, low weight birth, Alzheimer's disease, and Buerger disease [4-8].

RA is a systemic inflammatory autoimmune disease characterized by chronic inflammation and joint tissue destruction, potentially leading to functional disability [9]. The cause of RA is not fully understood, but a multifactorial pathogenesis, involving both environmental and genetic factors, has been widely accepted [10, 11]. Among the proposed environmental triggers, PD is currently considered as a risk factor for RA and may play a central role in disease initiation [12]. Further, Pg, a Gram-negative anaerobic bacterium, has a central role in the pathogenic hypothesis linking RA and PD, due to the production of peptidylarginine deaminase (PAD) and the strong protease gingipain [13]. PAD derived from Pg (PgPAD) can generate citrullinated proteins (CP), similarly to endogenous PAD (PADI4), which might result in production of the anti-*citrullinated* protein/peptide antibody (ACPA) [14].

We previously showed that systemic or oral Pg administration exacerbated RA-like experimental arthritis in a mouse model [15, 16]. Specifically, with systemic Pg infection, bacterial proteins were detected in swelling joint tissues [15]. However, these proteins were not recovered from the Pg oral administration model, likely because the pathogenic effect of Pg in arthritis progression is not through direct bacterial stimulation. Therefore, C5a was suggested as the indirect pathogenic factor, in which increased C5a in periodontal tissues and systemic sites activated osteoclastogenesis in joint tissues [16]. C5a is the complement cleaved from C5 by C5-convertase. In the previous study, the amount of C5a in serum with anti-Pg antibody positive RA patients showed higher than that with anti-Pg antibody negative RA patients. In vivo study using SKG mouse model, the serum in Pg-infected experimental arthritis mouse also showed the increase of C5a level compared to serum in normal experimental arthritis mouse. Furthermore, C5a in serum from strongly induced osteoclasrgenesis Pg-infected experimental arthritis mouse [16]. Therefore, C5a induced by Pg infection were focused on as tone of the factor for arthritis exacerbation.

Dysbiosis of the gut microbiota was observed in RA patients, and alterations in the microbiome could distinguish RA patients from healthy individuals [17]. The changing of oral microbiota was also reported in RA patients [18]. Furthermore, gut dysbiosis by inoculating Pg has been reported to be involved in the progression of experimental arthritis [17]. Periodontopathogenic bacteria, including Pg, is included in dental plaque and saliva [19]. Therefore, it is plausible that Pg inoculation of the periodontitis patients can be involved in changing gut microbiota. To support this hypothesis, inoculated Pg can also survive in the acid conditions of gastric juice [20]. Previous studies showed the involvement of Pg in the exacerbation of experimental arthritis [15]. The mechanisms of Pg-induced arthritis included the variant immune response in T cells, activation of osteoclastogenesis in joint tissues, systemic and local C5a elevation, and change in gut microbiota [16]. In humans, serum ACPA is used as a marker for RA diagnosis. Immune complex consisting of ACPA and CP is also important molecule for joint destruction. Therefore, monitoring CP generation and ACPA synthesis may be good indicators of RA onset [11, 21]. Of animal models used in RA studies, the SKG mouse model was the only model that reported elevated ACPA during progression of experimental arthritis [22]. Taken together, these immune responses may be

elicited after Pg inoculation and gut dysbiosis. Therefore, this study used the SKG model to investigate the effect of Pg inoculation in the progression of experimental arthritis and analyzed changes in gut microbiota and CP generation in serum and gingival, intestinal, and joint tissues.

Methods

Preparation of bacteria

Bacteria used in this study were purchased from ATCC. Pg W83 and 33277 were cultured on a sheep blood agar plate using the Anaeropack system (Mitsubishi Gas Chemical, Tokyo, Japan) at 37°C. After a 2-day incubation, Pg was inoculated in 40 mL of trypticase soy broth supplemented with 1% yeast extract, hemin (200 mg), and menadion (20 mg). Bacteria were harvested in the exponential growth phase and washed with phosphate buffered saline (PBS) for experiments.

Construction of Pg knockout mutant

The Pg *pad* knockout mutant was constructed by double recombination of the target gene and *ermF* introduction, as previously described [23]. The targeting DNA was constructed as follows. The 0.5 kb-upstream and 0.5 kb-downstream regions of the *pad* gene were amplified with two pairs of primers (pad-Up-F: [GGTCTCACACGAGAGGATACTATGGTCTAT]/pad-Up-R: [CGGGGGATCCTGTTTGATATGTTTTATGAT]; pad-Dw-F: [TAGGGGATCCGGGGCCTTATTTGAGAATAC]/pad-Dw-R: [AGCAGAGGTTACGAGCTTAACCAGAGATGC], where 'Up', 'Dw', 'F', and 'R' indicate upstream, downstream, forward, and reverse, respectively) using the genome of ATCC 33277 as a template. The *ermF* region in the *ermF* DNA cassette was amplified with ermF-F: [ATATCAAACAGGATCCCCCGATAGCTTCCG]/ermF-R: [ATAAGGCCCCGGATCCCCTACGAAGGATGA] using the genome of *gtfF* (PGN_1668)::*ermF* mutant (KDP611) as a template [24]. Using the three purified products, PCR was performed with pad-Up-F/ pad-Dw-R. Finally, the desired PCR product was purified and introduced into Pg ATCC 33277 by electroporation. Transformants were selected on blood agar plates containing 10 μg/mL erythromycin. Proper mutation was confirmed by PCR using pad-Up-R/pad-Dw-R. Correct *ermF* gene insertion of Pg *pad* knockout mutant was also verified by sequencing.

Generation of Pg-infected experimental arthritis mouse model

All animal experimental procedures employed in this study were approved by the Ethical Committee of Hiroshima University (approved No. A16-33) and performed as previously reported [16]. Briefly, SKG mice were maintained under SPF condition until inoculation of bacteria and feces. The food used in this study was MF for mouse with gamma irradiation (Oriental Yeast Co., Ltd, Tokyo, Japan). Laminarin derived from *Laminaria digitate* (LA) was purchased from Sigma-Aldrich (L9634, St. Louis, MO, USA). LA was dissolved in PBS at 100 mg/mL. To induce experimental arthritis, LA (10 mg/100 mL/mouse) was administered to SKG mice by an intraperitoneal (i.p.) injection. Pg was also inoculated (10^8 bacterial cells/50 mL in 2% carboxymethylcellulose (CMC) solution) twice a week for 6 weeks. LA injection and Pg inoculation were started the same day. The same volume of CMC solution was inoculated as a negative

control. All mice were divided into 3–4 groups (5 or 6 mice per group) in each experiments (Ctrl: CMC inoculation, LA: LA i.p injection, Pg: Pg oral inoculation, Pg/LA: LA i.p. injection with Pg oral inoculation, LA-FMT: LA i.p. injection with FMT from the LA group, Pg/LA-FMT: LA i.p. injection with FMT from the Pg/LA group).

Evaluation of alveolar bone level in mouse

The alveolar bone level (ABL) of SKG mice was evaluated by the Kawai's methods described previously [25]. Briefly, after methylene blue staining (Sigma-Aldrich) for 10 min, the upper molar jaw was washed with PBS three times. The length of the blue-stained root surface of all molar teeth from the enamel-cement junction to the top of the alveolar bone was measured. Differences between treated mice and the control group (no treatment) were evaluated.

Clinical assessment of arthritis score (AS) in SKG mice

Joint swelling was monitored by inspection and scored as follows: 0, no joint swelling; 0.1, swelling of one finger joint; 0.5, mild swelling of the wrist or ankle; 1.0, severe swelling of the wrist or ankle. Scores for all digits, wrists, and ankles were totaled for each mouse, as reported previously [26].

IL-6 measurement in mouse tissue

Tissues from gingiva, leg joint, small intestine, large intestine, and serum were collected from each mouse and homogenized by cool-mill (#TK-CM20S, Tokken, Inc., Chiba, Japan) in the RIPA Lysis and Extraction Buffer (#Thermo Fisher Scientific, Tokyo, Japan, 100 mg tissue/100 mL) with 0.1% phenylmethanesulfonyl fluoride (PMSF, Sigma-Aldrich) and 1% proteinase inhibitor cocktail (#87786, Thermo Fisher Scientific). The sera were diluted four times by PBS. Supernatants of the tissue homogenates and sera were used for IL-6 measurement by ELISA (for mouse IL-6, #431304; BioLegend Inc., San Diego, CA, USA), according to the manufacturer's instructions. Briefly, a solid-phase anti-IL-6 monoclonal antibody (diluted in coating buffer to a final concentration of 1 μg/mL) was coated onto a 96-well ELISA plate (BD Falcon, Franklin Lakes, NJ, USA) for target capture. After blocking each well with 1% BSA in PBS supplemented with 0.05% Tween 20 (PBST), the sample or standard (diluted in PBST from 1 ng/mL to zero) was applied to each well. After application of the detection antibody (diluted in PBST to a final concentration of 1 μg/mL), horseradish peroxidase (HRP) conjugated with anti-IgG (2000-fold dilution in PBST) was applied to the wells. Colorimetric reactions were developed with o-phenylenediamine (Sigma-Aldrich) in the presence of 0.02% H_2O_2. Color development was stopped with H_2SO_4 (2N) and measured using an ELISA reader ($OD_{405,}$ Varioskan LUX). The actual concentration of the target was calibrated by referring to a standard curve prepared by serial dilutions. Each sample was examined in triplicate wells of a 96-well ELISA plate. The limits of mouse IL-6 detection for each analyte were 15.6 pg/mL.

Detection of citrullinated protein in mouse tissue

The homogenized tissues from the gingiva, leg joint, small intestine, large intestine, and serum were diluted in sodium bicarbonate buffer (pH 9.4, 10 mg/mL) and coated onto a 96-well ELISA plate. After blocking each well with 1% BSA and sucrose in PBST, anti-citrulline monoclonal IgG (CCP-Ab1 generated from B cells of an RA patient, 10 μg/mL) in PBST at room temperature for 2 h [27]. Subsequently, anti-human IgG conjugated with HRP (2000-fold dilution in PBST) was applied to the wells. Colorimetric reactions were performed as the same as the method of IL-6 measurement.

Measurement of serum ACPA level

Serum from each group was collected to measure ACPA levels. Briefly, cyclic citrullinated peptides (CCP) (Orgentec, Chicago, IL, USA, diluted in PBS pH 7.2 at final concentration of 0.5 μg/mL) was pre-coated onto a 96-well ELISA plate (BD Falcon, Franklin Lakes, NJ, USA) for target capture. After blocking each well with 1% BSA in PBST, the serum (4-fold dilution) or standard (diluted in PBST from 1 ng/mL to zero) was applied to each well. After application of the detection antibody (diluted in PBST to a final concentration of 1 μg/mL), anti-IgG conjugated with HRP (2000-fold dilution in PBST) was applied to the wells. Colorimetric reactions were developed using the same protocol as IL-6 detection. The actual concentration of the target was calibrated by referring to a standard curve prepared by serial dilutions. Each sample was examined in triplicate wells of a 96-well ELISA plate.

Histological observation

Ankle joints were fixed in 4% buffered formalin and embedded in paraffin wax. Subsequently, the tissues were sliced at a thickness of 7 μm and mounted on glass slides. The paraffin-embedded sections were stained with hematoxylin and eosin (HE). The severity of inflammation and cartilage damage were scored using published criteria [28].

Statistical analysis

All experiments were performed at least three times independently. Data are expressed as mean ± standard deviation (SD). Statistical analyses between two groups were performed using Mann-Whitney U test for non-normal distribution. For multiple comparisons, the Tukey-Kramer test or Bonferroni-corrected Mann-Whitney *U* test was used. $P < 0.05$ was considered significant.

Metagenomics of gut microbiota

Bacterial DNA was extracted from feces as described previously [29]. In brief, feces were collected from all the group of mice before Pg inoculation (day 0) and at the end of experiments (day 42). The collected feces were suspended in PBS (10% sodium dodecyl sulfate, 10 mM Tris-HCl, and 1 mM EDTA, pH 8.0), and the bacterial DNA was purified using the DNeasy PowerSoil Kit according to the manufacturer's instructions (the range of total amount of DNA 349.8 ng/ml to 611.8 ng/ml, #12888-100 QIAGEN, Germantown, MD, USA). Following, the V4 variable region (515F–806R) of the bacterial 16S rRNA genes was sequenced on an Illumina Miseq, as the illumine 16s metagenomics sequencing workflow and previously described [30]. Each reaction mixture contained 15 pmol of each primer (16S Amplicon PCR

Forward Primer 5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG, 16S Amplicon PCR Reverse Primer 5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC), KAPA HiFi HotStart ReadyMix (x2) (Hokkaido System Science Co., Ltd., Sapporo, Japan), 50 ng extracted DNA, and sterilized water to reach a final volume of 50 μL. PCR conditions were as follows: 95°C for 3 min; 25 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 min; 72 °C for 5 min. The PCR product was purified by AMPure XP (Beckman Coulter, Inc., Brea, CA, USA) and quantified using a Quant-iT PicoGreen ds DNA Assay Kit (Life Technologies Japan Ltd., Tokyo, Japan). Mixed samples were prepared by pooling approximately equal amounts of PCR amplicons from each sample. The pooled library was analyzed with an Agilent High Sensitivity DNA Kit on an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). Real-time PCR was performed on the pooled library using a KAPA Library Quantification Kit for Illumina, following the manufacturer's protocols, and a sample library with a 20% denatured PhiX spike-in was sequenced by Miseq using a 600 cycles kit to obtain 2 × 250 bp paired-end reads. Taxonomic assignments and estimation of relative abundance of sequencing data were performed using the analysis pipeline of the QIIME software package [31]. An operational taxonomic unit (OTU) was defined at 97% similarity. OTUs indicating relative abundance under 0.05% were filtered to eliminate noise. The OTU was assigned a taxonomy based on a comparison with the Silva database using UCLUST [32].

Fecal microbiota transmission (FMT)

Fresh mouse feces from the LA and Pg/LA group were collected after 6 weeks of Pg inoculation started and mixed from all the same group (n=6), and homogenized in anaerobic resuspension buffer (2% Lab–Lemco powder, 0.1% L-cysteine, 0.045% KH_2PO_4, 0.09% NaCl, 0.045% $(NH_4)_2SO_4$, 0.0045% $CaCl_2$, 0.0045% $MgSO_4$ and 40% glycerol in 1,000 mL) at 10-fold dilution (weight/volume) and kept at −80°C until used. Before FMT, SKG mice were treated with antibiotics (ampicillin (Sigma-Aldrich) 1 g/L,metronidazole (Sigma-Aldrich) 1 g/L, neomycin (Sigma-Aldrich) 1g/L, vancomycin (Sigma-Aldrich) 0.5 g/L) dissolved in water for 1 week. Female SKG mice (6–8 weeks old) were orally inoculated with 250 μL of the fecal suspensions from donor mice twice in a week. Following, these mice were intraperitoneally injected with LA (100 mg) at 1 week after bacterial inoculation. The AS and ankle thickness were monitored every week for 6 weeks after injection. Microbiota composition of the feces from the donor mice (the group of LA and Pg/LA mice after 6 weeks of LA injection with or without Pg inoculation) and the recipient mice at 6 weeks after FMT was analyzed by next generation DNA sequencing of bacterial 16s rRNA. These experiments were performed 3 times independently.

Western blotting

Homoginized tissues from the leg joint were applied to a 12% SDS-polyacrylamide gel by for electrophoresis and the electronically transferred onto nitrocellulose membranes (BioRad Laboratories, CA, USA). The membrane was blocked with 1% non-fat dried milk at room temperature for 1 hour and then reacted with anti-PADI4 rabbit monoclonal IgG (10 μg/ml, EPR20706, abcam, Japan) in PBST at 4°C for 12 hours. The membrane was incubated with HRP-conjugated with sheep anti-rabbit IgG in PBST at

room temperature for 1 hour. Immunodetection was performed according to the manual supplied with ECL Plus Western blotting reagents (GE Healthcare Life Sciences, Japan). As a control, the amount of b-actin was detected by anti-b-actin antibody (10 μg/ml, sc-47778 HRP, Santa Cruz Biotechnology, Inc.) The density of target PADI4 bands were measured by NIH image software.

Results

Evaluation of periodontal tissue in experimental arthritis model mice with Pg infection

Oral infection of Pg induced alveolar bone loss in the Pg and Pg/LA groups (Fig. S1a and S1b). However, the Ctrl and LA groups did not show any alveolar bone loss. Additionally, IL-6 production in mouse gingival tissue was measured, which was increased in the Pg and Pg/LA groups compared to the Ctrl group (Ctrl: 92.2 pg/10 mg tissue, Pg: 318.3 pg/10 mg tissue, Pg/LA: 319.4 pg/10 mg tissue). Although RA affects the condition of periodontitis clinically, especially the only treatment of RA resulted in the improvement of periodontitis [33], IL-6 production in the LA group was similar to the Ctrl group (Fig. 1a).

Assessment of experimental arthritis in mice

To assess experimental arthritis in mice with Pg oral inoculation, serum and joint tissues were examined. AS was determined, which was increased in the LA group after 5 weeks of LA injection. The AS score of Pg/LA group was also significantly elevated from 4 weeks of LA injection and Pg inoculation, same as our previous report [16]. However, AS of Ctrl and Pg groups did not show the induction of joint swelling (Fig. S1c). HE-stained sections of joint tissues in the LA group showed mild infiltration of immune cells and growth of granulation tissue. Furthermore, severe inflammation and pannus formation in the Pg/LA group were observed (Fig. 1b-e). Inflammation and cartilage damage in join tissue were more severe in the Pg/LA group than those in the other groups (Inflammation score: 2.75±0.43, Cartilage damage score: 2.50±0.50) (Fig. 1f and g). The Serum ACPA levels and IL-6 levels in serum and joint tissues were elevated in the LA, Pg, and Pg/LA groups compared to the Ctrl group, with the highest levels in the Pg/LA group (Fig. 1h-j). IL-6 level in serum of the LA group was statistical difference with that of the Ctrl group. This result showed the difference to our previous result [16]. SKG mouse has the mutation of ZAP-70 gene which affect the negative/positive selection of T cell. As the result of the mutation, arthritis occurs spontaneously without LA [34]. In this present study, the experiments were repeated 3 times. However, the baseline of IL-6 was higher than previous study. For this reason, the production of IL-6 in serum of LA group showed high level.

Effect of Pg oral administration on gut microbiota composition

The composition and abundance of gut microbiota in mouse fecal samples were assessed by next-generation sequencing of 16s rRNA. The relative abundance in phylum level in the LA, Pg, and Pg/LA groups was changed compared to the Ctrl group(Fig. 2a). Further, the LA, Pg, and Pg/LA groups showed increased relative abundance at the order level of Bacteroides compared to controls (Fig. 2b). Conversely, the relative abundance of Firmicutes, Deferribacteres, and Clostridiales was decreased in LA, Pg, and

Pg/LA groups compared to Ctrl (Fig. 2c-e). There was a significant decrease in the relative abundance of Deferribacteres in the Pg and Pg/LA groups compared to the LA group (Fig. 2d). At the family level, the relative abundance of S24-7 in the LA, Pg, and Pg/LA groups was significantly increased compared to Ctrl, but Pg and Pg/LA groups were lower than LA (Fig. 2f).

Effect of Pg oral administration on gut inflammation

To assess gut inflammation, IL-6 production in small and large intestinal tissues was measured (Fig. 3a and b). IL-6 production in the small intestine of the Pg/LA group was remarkably elevated compared to other groups (Pg/LA group: 184.8 pg/50 mg tissue). In the Pg group, IL-6 production in the small intestine was slightly increased compared to the Ctrl group (Ctrl group: 36.4 pg/50 mg tissue, Pg group: 67.8 pg/50 mg tissue). Further, IL-6 production in the large intestine of the Pg/LA and Pg groups was remarkably elevated compared to other groups (Pg/LA group: 521.9 pg/50 mg tissue, Pg group: 370.7 pg/50 mg tissue). IL-6 production in the small and large intestine of the group of LA was not elevated in 6 weeks. This is because the induction of experimental arthritis by the single injection of LA takes at least 20 weeks [22]. Therefore, the inflammation in gut would not be appeared.

Effect of Pg oral administration on CP generation

ACPA-activated inflammation depends on CP generation [11]. Therefore, CP generation in serum and small intestinal, large intestinal, and joint tissues was measured by ELISA (Fig. 3c-f). Elevated CP in the Pg/LA group was observed in small intestinal tissue, large intestinal tissue, serum, and joint tissue (1.73-, 1.64-, 3.03-, and 1.65-fold compared to the LA group). CP generation in the small and large intestine of the group of LA was not elevated in 6 weeks. This is same reason as the IL-6 elevation in gut tissue.

Effect of FMT on onset of experimental arthritis

Pg oral inoculation and LA injection in SKG mice induced RA-like experimental arthritis. Further, Pg inoculation resulted in the changes in gut microbiota and inflammation of gut tissue in this model. To determine if the gut microbiota changes as the result of Pg inoculation were important in the exacerbation of experimental arthritis, FMT of feces derived from LA and Pg/LA mice to LA-injected recipient mice was performed. The feces were collected from the donor mice (the group of LA and Pg/LA mice after 6 weeks of LA injection with or without Pg inoculation). The feces from recipient mice were collected after 6 weeks of LA injection with FMT. The composition and abundance of gut microbiota in mouse fecal samples from donor mice and recipient mice were assessed by percentages of order-level and phylum-level bacterial composition (Fig. 4a-d). Composition at the phylum level of LA-FMT and Pg/LA-FMT groups were similar to the LA and Pg/LA groups , respectively. Further, there were no differences in the relative abundance of Bacteroides, Firmicutes (Fig. 4b and 4c). The same tendency in the relative abundance of Deferribacteres was observed between the LA and Pg/LA groups before and after FMT (Fig. 4d). As a control of FMT, the suspension buffer was inoculated into mice (the control group for the FMT experiment). The microbiome between the LA injected group and the control group for the FMT experiment with LA injection did not show the difference (data not shown).

To further assess the effect of FMT on the onset of experimental arthritis, mouse periodontal and joint tissues were analyzed. The route of FMT was beyond the oral cavity. Therefore, FMT does not affect in oral microbiota. To confirm no effect against oral cavity, periodontal tissue was analyzed. FMT did not show any effect in periodontal tissues, as alveolar bone resorption was not observed (Fig. S2 and Fig. 5a). However, severe joint swelling was observed in the LA-FMT and Pg/LA-FMT groups compared to the LA and Pg/LA groups (Fig. 5b-f, Fig. 1c and e). The maximum AS of the LA-FMT and Pg/LA-FMT groups was much higher than that of LA and Pg/LA, respectively. Pannus formation in the Pg/LA-FMT group was found in joint tissues, similar to the Pg/LA group. Further, pannus formation and erosion of joint bone tissues were strongly observed in the LA-FMT group (Fig. 5e and f). Additionally, FMT of feces from the LA and Pg/LA groups increased IL-6 in joint, small intestinal, and large intestinal tissues (Fig. 5g-i). CP levels in joint, small intestinal, and large intestinal tissues were also elevated in the FMT-LA and FMT-Pg/LA groups, with the Pg/LA group showing the highest levels (Fig. 5j-l). FMT of feces from the Pg/LA groups did not show any symptom without LA injection. FMT of feces from Pg group did not show severe arthritis compared with FMT of feces from the LA and Pg/LA groups in the presence of LA injection, either (data not shown).

Effect of PgPAD-deficient mutant on exacerbation of experimental arthritis

To determine the effect of PgPAD on CP generation and exacerbation of experimental arthritis, the PgPAD-deficient Pg strain was inoculated into the oral cavity of SKG mice. Bone resorption around the upper molar teeth of Pg 33277 wild type strain and PgPAD deficient Pg strain was measured (Fig. S3). Oral inoculation of the PgPAD-deficient Pg strain showed slight bone resorption compared to the Ctrl group, which was much lower than that the wild type strain (35.2% lower, Fig. 6a). IL-6 production in gingival tissue of the wild type-inoculated group was elevated. However, this increase was suppressed in the deficient strain (Fig. 6b). Additionally, AS of the PgPAD-deficient Pg strain was significantly lower than the wild type strain (Fig. 6c). CP generation in serum and joint, small intestinal, and large intestinal tissues by PgPAD-deficient Pg strain inoculation was also lower than the wild type strain (55.6%, 38.2%, 35.9%, and 20.2% decrease, respectively, Fig. 6d-g). Serum ACPA levels in the PgPAD-deficient Pg strain was significantly decreased compared to \wild type (45.8% decrease, Fig. 6h). The induced production of IL-6 in gingival tissue, CP generation in gingival tissue, and CP generation in large intestine by the inoculation of Pg wild type strain was suppressed by the inoculation of PgPAD-deficient Pg strain. However, the level of IL-6 and CP was still higher than that in the group of Ctrl. Pg possesses many pathogenic factors including gingipain, outer membrane protein, volatile sulfur compounds [35, 36]. Therefore, the induction by PgPAD-deficient Pg strain may be affected by these pathogenic factors. Because the systemic inflammation in RA upregulate the production of endogenous PADI4, the production of PADI4 in mouse joint tissue were determined. PADI4 level in the group of Pg wild type inoculation increased compared to that in the group of Ctrl. However, the increase of PADI4 level was lower than that in the inoculation PgPAD-deficient Pg strain (Fig. 6i).

Discussion

In this study, Pg oral inoculation induced experimental arthritis in SKG mice due to gut microbiota alterations. Further, FMT from the feces of Pg-inoculated arthritis mice to uninoculated mice caused joint destruction. Concomitantly, CP levels in intestinal and joint tissues were elevated, which was partially suppressed in the absence of PgPAD in the PgPAD-deleted mutant group.

Previous reports showed that periodontitis affected gut microbiota [37]. The inoculation of periodontopathogenic bacteria resulted in the change of gut microbiota and this change induced the destruction of tight junction in the ileum [38]. The bacterial subject in oral cavity and stool bacteria overlapped in nearly 45% in human microbiome project by gemone biology [39]

Clinical studies also demonstrated the effect of periodontopathogenic bacteria on gut microbiota [37], likely due to the daily inoculation of 1–2 L of saliva, including pathogenic bacteria (approximately 10^9 colony-forming units (CFUs) per mL) [19]. While planktonic Pg can be eliminated at low pH (pH 5), the biofilm of inoculated Pg can survive in acidic conditions as low as artificial gastric juice (pH 3) [20].

In this study, Pg inoculation affected gut microbiota composition. Pg inoculation resulted in decreased relative abundance of Deferribacteres and S24-7 in Pg/LA compared to the LA group (Fig. 2d and f). By contrast, the abundance of Bacteroides, Firmicutes, and Clostridiales did not show significant differences between groups, although their relative abundance changed dramatically compared to the control group (Fig. 2). Previous reports showed that Pg administration in the DBA/1J experimental arthritis mouse model resulted in decreased relative abundance of Bacteroidetes (phylum level) and Bacteroides (genus level), but increased Firmicutes (phylum level) [20]. Further, in other systemic disease models (type 2 DM in C57BL/6 mice), Pg inoculation increased the abundance of Bacteroides and decreased Firmicutes [38].

However, the administered Pg DNA fragment and protein were not detected in intestinal feces of the Pg and Pg/LA groups (data not shown). In our previous study, *Escherichia coli* stimulation did not affect the degree of experimental arthritis, as compared to arthritis without *E. coli* stimulation [15]. Further, other periodontopathogenic bacteria *Prevotella intermidia* did not show any differences compared to experimental arthritis without bacterial stimulation [20]. In humans, *Prevotella copri* was isolated from RA patient samples and identified as a key bacteria [40] involved in arthritis progression. However, in our mouse model, *P. copri* or genetically close bacteria was not detected in feces of the arthritis-induced model (data not shown). However, the administered Pg DNA fragment was not detected in intestinal tissues and feces of the Pg and Pg/LA groups after 6 weeks. In detail, the purified DNA from serum, feces, gingival tissue, tongue, esophagus, lung, stomach, small intestine, and large intestine were determined to detect Pg and *P. copri* derived DNA fragment by specific primer to 16s rRNA. Pg derived DNA was only detected from gingival tissue, tongue, esophagus, and lung tissue. This is because the bacteria inoculated in this study was the planktonic condition. Therefore, the way of Pg biofilm inoculation should be performed in the future study.

FMT is used to alter microbiota composition and treat gastrointestinal disorders, such as Crohn's disease and Ulcerative colitis [41]. In FMT-treated patients, an increase in bacterial diversity and abundance of

Bacteroides and Firmicutes were observed, along with improvement of clinical symptoms. In the mouse model, fecal transplantation from RA patients to mice replicated the human gut microbiota of RA and the onset of experimental arthritis via intestinal Th17 cell activation [17]. In our present study, the change of gut microbiota induces gut inflammation and CP generation, then arthritis is exacerbated by these inductions (Fig. S2 and 5). The way of Pg oral inoculation takes 2-3 weeks for changing microbiota. However, FMT can change gut microbiota very quick. This is because the aggressive induction of arthritis from early phase. Despite limitations of comparing differences between human and mouse microbiota, these studies highlight the importance in the progression of systemic disease, including RA, of gut dysbiosis rather than the establishment of specific bacterial species in gut microbial flora. Both periodontitis and RA treatments resulted in changes in the gut microbiota [37]. Therefore, the monitoring of gut microbiota and specific bacterial species during the treatment of both periodontitis and RA would be useful for the marker of the risk factor, prognosis, and therapeutic effect.

ACPAs target proteins/peptides with citrullinated epitopes and serve as informative RA biomarkers, which are useful for RA diagnosis [42]. ACPA are generated within synovium and possibly at extra-articular sites prior to disease onset. Recent investigations have begun to elucidate the different mechanisms by which ACPAs may be directly pathogenic in RA. CP is a specific target of ACPA and involved in ACPA generation. This study found increased CP in gingival, small intestinal, large intestinal, and joint tissues following Pg inoculation in the experimental arthritis model mouse (Fig. 3, S3, and 6). In humans, four citrullinated autoantigens, fibrinogen/fibrin, vimentin, α-enolase, and type II collagen, are now well accepted as ACPA targets [43-46]. In our study, the specific origin of CP is unclear. However, Pg can rapidly generate CP from α-enolase or fibrinogen by proteolytic cleavage at Arg-X peptide bonds using arginine gingipains, followed by citrullination of carboxyterminal arginines by bacterial PAD [13]. Taken together, one of the mechanisms may lead to ACPA generation by PgPAD citrullination. To support this hypothesis, the Pg knockout mutant of PgPAD resulted in less CP generation in gingival tissue, serum, small intestine, large intestine, and joint tissue (Fig. 6d-g). Furthermore, the Pg 33277 wild type-stimulated serum ACPA was diminished by stimulation with the PgPAD knockout mutant (Fig. 6h). However, endogenous PAD, PADI4, is also involved in CP and ACPA generation, for which decrease severity of experimental autoimmune arthritis was found in PADI4 knockout mice [47]. The induction of PADI4 is mediated by TNF-a, IL-6, and neutrophil extracellular traps (NETs, NETosis) [47-49]. Further, increased CP in joint tissue resulted in increased synovial fibroblast migration and spread ability [50]. In lung tissues, CP synthesis and CP-induced inflammation were also reported [51]. Pg inoculation has been shown to induce inflammatory responses in periodontal tissues and in local and systemic sites. In our model, Pg infection activated IL-6 production in the small intestine, large intestine, joint tissue (Fig. 1i, j and 3a, b), resulting in PADI4 induction and subsequent CP and ACPA generation.

Surprisingly, PgPAD knockout showed both decreased CP and ACPA production and suppressed inflammation of periodontal tissues, including alveolar bone resorption (Fig. S3 and 6a), which depends on osteoclast activation via RANKL signaling [52]. A previous report showed that citrullinated vimentin induced endogenous PADI4 and RANKL in fibroblast-like synoviocytes derived from RA patients [53]. However, no previous study has shown a correlation between PgPAD and inflammation. Yet, the

combination of gingipain and PgPAD generated citrullinated a-enolase, and its CP might be an initiator of inflammation. Very interestingly, the inoculation of PgPAD knockout mutant showed the decrease of PADI4 induction in joint tissue (Fig. 6i). The AS of 3 weeks showed no statistical difference between the inoculation of Pg WT and PgPAD knockout mutant. Therefore, one of the mechanism of PgPAD in the exacerbation of RA might be the induction of PADI4 via inducing inflammation like a IL-6 production by increased CP in gingival tissue by PAD possessed Pg and the elimination of Pg as the causative bacteria of CP production might become very important target for RA treatment. In this study, Pg 33277 was used as the knockout mutant. The deletion of PgPAD resulted in the suppression of alveolar bone resorption, arthritis score, IL-6 production, and CP generation. The bacterial phenotype after PgPAD knockout except arthritis symptoms was not analyzed in this study. However, there was a report by using PgPAD knockout mutant in the ACPA generation in mouse model. In that data, the alveolar bone resorption, joint swelling and ACPA production showed the same tendency to our result [54]. The effect of Pg 33277 showed weaker than that of Pg W83 in the production of IL-6 and arthritis score (Fig.1 and 6). For these reasons, there is a limitation to clarify the involvement of PgPAD by using P. gingivalis knockout mutant in the progression of RA. Therefore, the additional use of different pair of PgPAD mutant is needed.

Conclusions

Based on our findings, we propose the following model of arthritis pathogenesis with Pg inoculation (administration) in SKG mice: 1) continued Pg inoculation results in changes in the gut microbiota, 2) dysbiosis of gut microbiota induces inflammation in intestinal tissues, 3) increased CP in the intestine accelerates systemic ACPA production, and 4) severe joint destruction. In addition to inflammation, PgPAD induced CP generation. However, arthritis was also found in the PgPAD knockout mutant, and its AS was higher than the normal arthritis model, indicating Pg as an induction factor for arthritis exacerbation. Therefore, further study is needed to clarify the molecular mechanisms underlying the involvement of Pg infection in the onset of RA.

Abbreviations

ABL: Alveolar bone loss; AS: Arthritis score; CMC: Carboxymethylcellulose; EDTA: Ethylene diamine tetrameric acid; HE: Hematoxylin and eosin; i.p.: Intraperitoneal; LA: Laminarin; Micro-CT: Micro-computed tomography; PD: Periodontal disease; RA: Rheumatoid arthritis; SEM: Standard error of the mean; sRANKL: Soluble recombinant receptor activation of nuclear factor kappa-B ligand

Declarations

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Availability of data and material

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

YH, KO, SM, and MK established the animal experiments in this study. JH, IK and KT analyzed the gut microbiota. MS and KN constructed the knockout mutant of Pg. TO and HK generated the monoclonal antibody against CP. SM, TK, and NM performed the histological experiments. HS and ES evaluated the experimental arthritis symptoms in animal experiments. TF conducted the statistical analysis. HK supervised this study. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Animal experiments were approved by the Ethical Committee of Hiroshima University (approval A16-33). All patients provided written informed consent prior to enrollment.

Competing interests

The authors declare that they have no competing interests.

Consent for publication

The consent of all coauthors was collected before submission.

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Figures

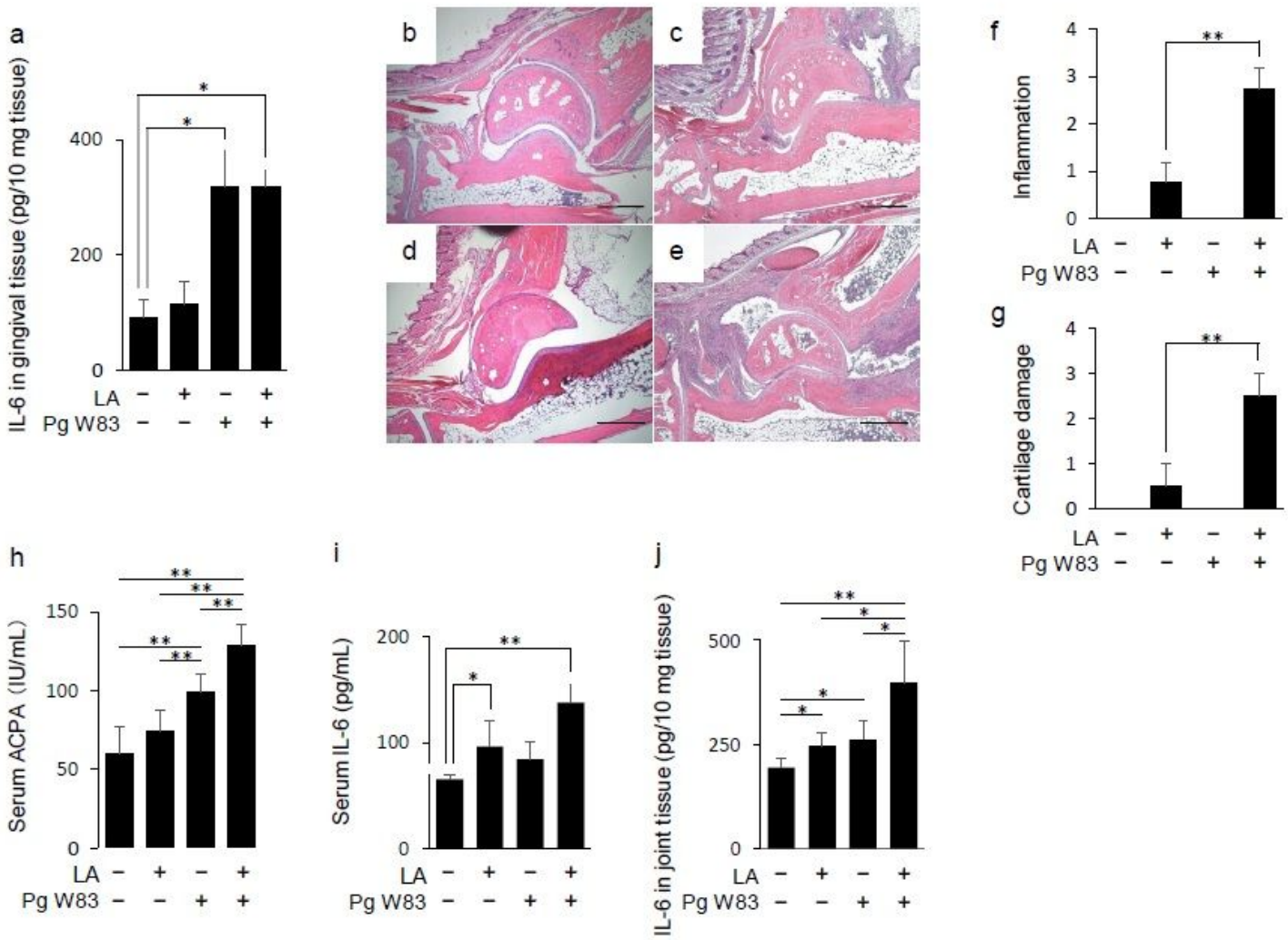
a
IL-6 in gingival tissue (pg/10 mg tissue)
400
200
0
LA
Pg W83
b
c
d
e
f
Inflammation
4
3
2
1
0
g
Cartilage damage
h
Serum ACPA (IU/mL)
150
100
50
i
Serum IL-6 (pg/mL)
200
j
IL-6 in joint tissue (pg/10 mg tissue)
500
250

Figure 1

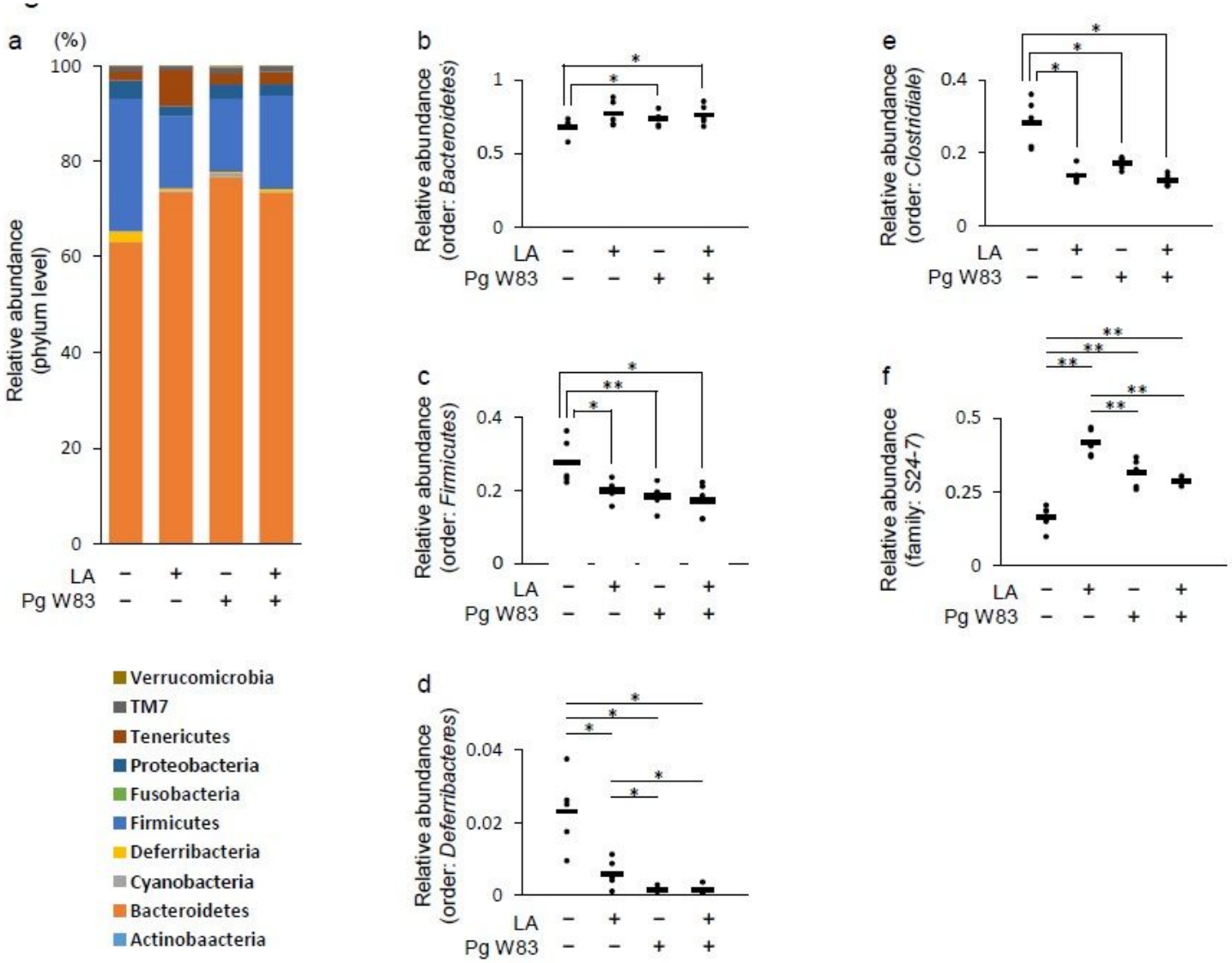
a
(%)
Relative abundance (phylum level)
LA
Pg W83
Verrucomicrobia
TM7
Tenericutes
Proteobacteria
Fusobacteria
Firmicutes
Deferribacteria
Cyanobacteria
Bacteroidetes
Actinobaacteria
b
Relative abundance (order: Bacteroidetes)
c
Relative abundance (order: Firmicutes)
d
Relative abundance (order: Deferribacteres)
e
Relative abundance (order: Clostridiale)
f
Relative abundance (family: S24-7)

Figure 2

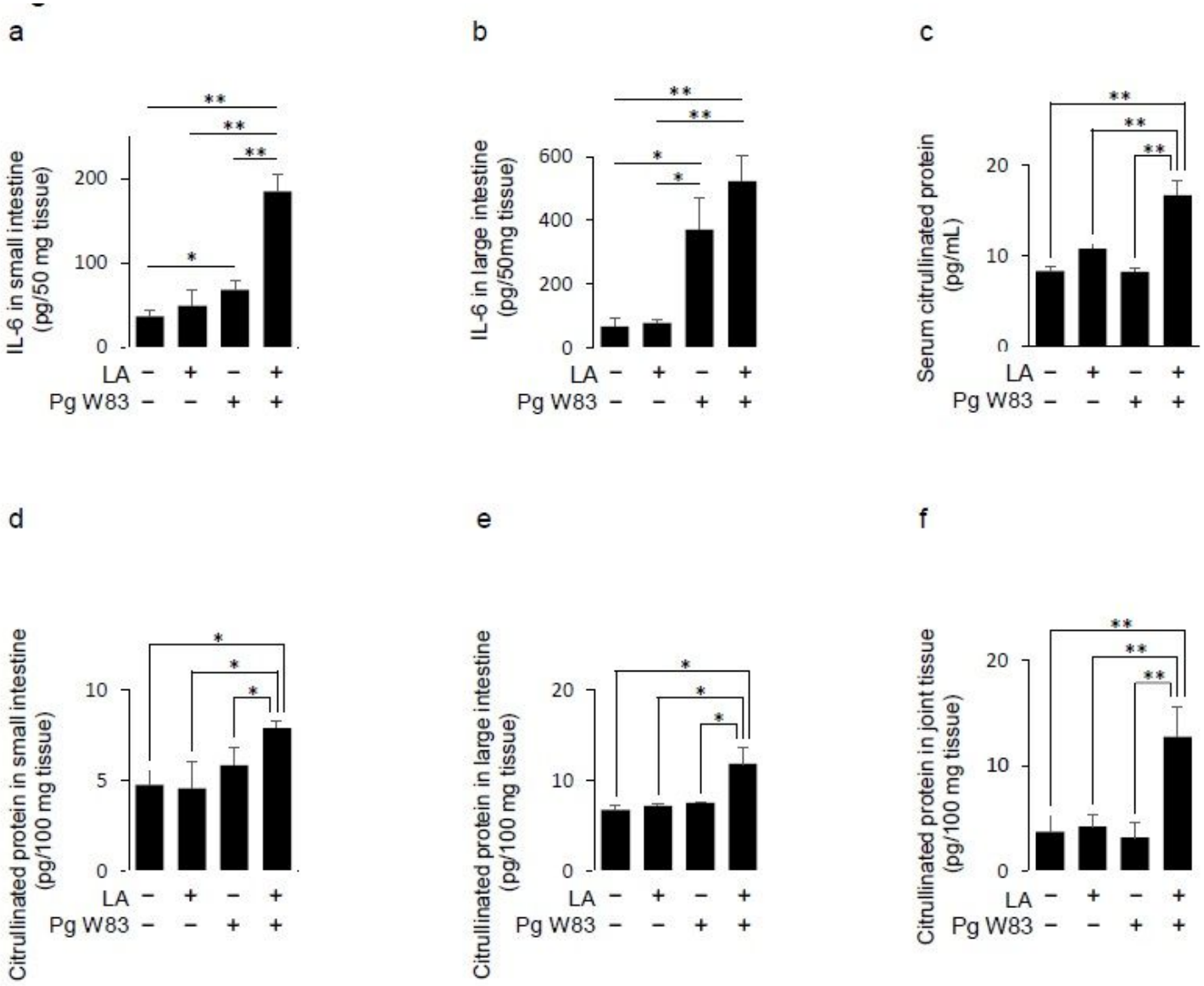
a
IL-6 in small intestine (pg/50 mg tissue)
200
100
0
LA − + − +
Pg W83 − − + +
b
IL-6 in large intestine (pg/50mg tissue)
600
400
200
0
LA − + − +
Pg W83 − − + +
c
Serum citrullinated protein (pg/mL)
20
10
0
LA − + − +
Pg W83 − − + +
d
Citrullinated protein in small intestine (pg/100 mg tissue)
10
5
0
LA − + − +
Pg W83 − − + +
e
Citrullinated protein in large intestine (pg/100 mg tissue)
20
10
0
LA − + − +
Pg W83 − − + +
f
Citrullinated protein in joint tissue (pg/100 mg tissue)
20
10
0
LA − + − +
Pg W83 − − + +

Figure 3

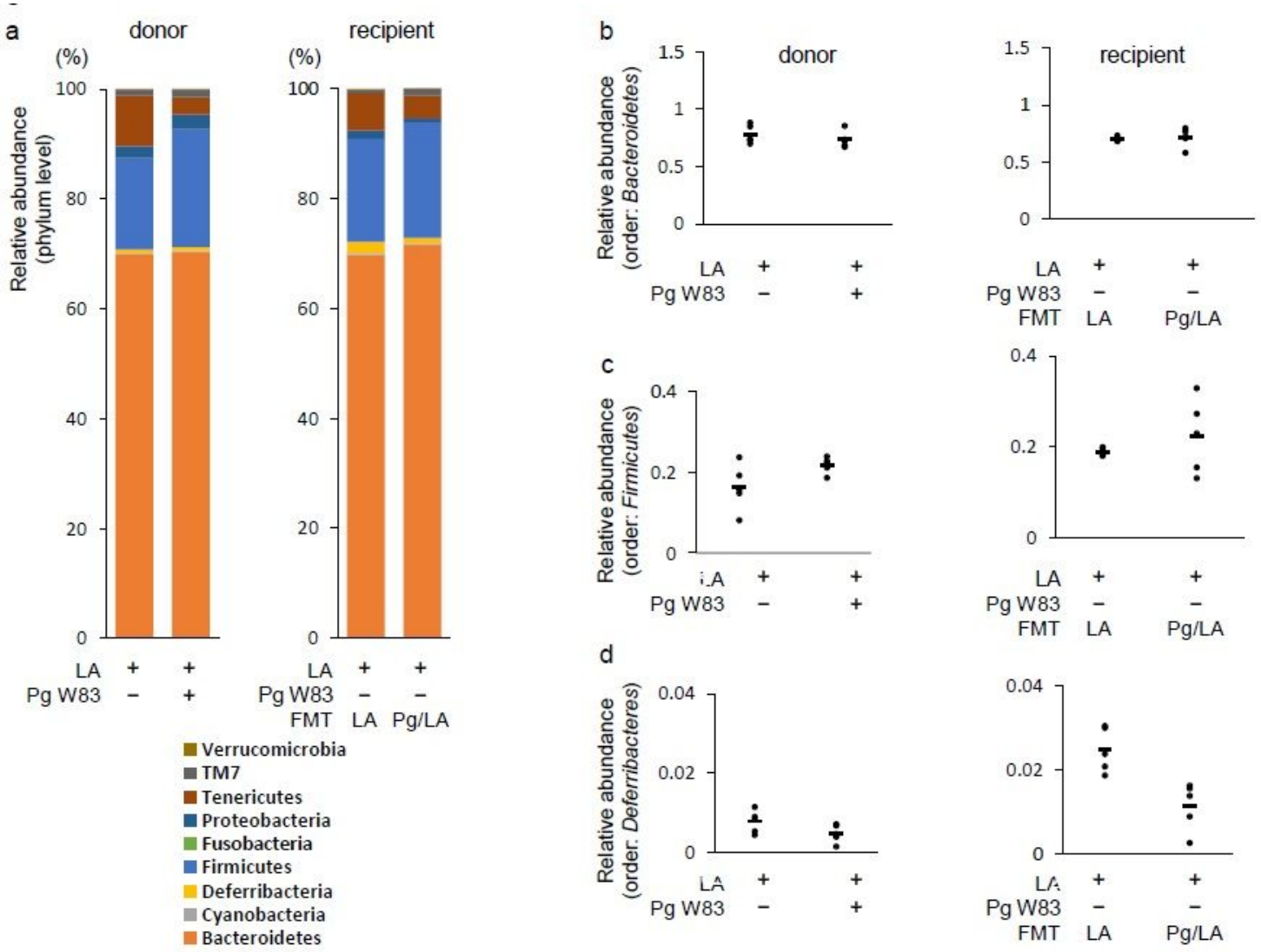
a
donor
recipient
(%)
Relative abundance (phylum level)
LA
Pg W83
FMT
Verrucomicrobia
TM7
Tenericutes
Proteobacteria
Fusobacteria
Firmicutes
Deferribacteria
Cyanobacteria
Bacteroidetes
b
Relative abundance (order: Bacteroidetes)
c
Relative abundance (order: Firmicutes)
d
Relative abundance (order: Deferribacteres)

Figure 4

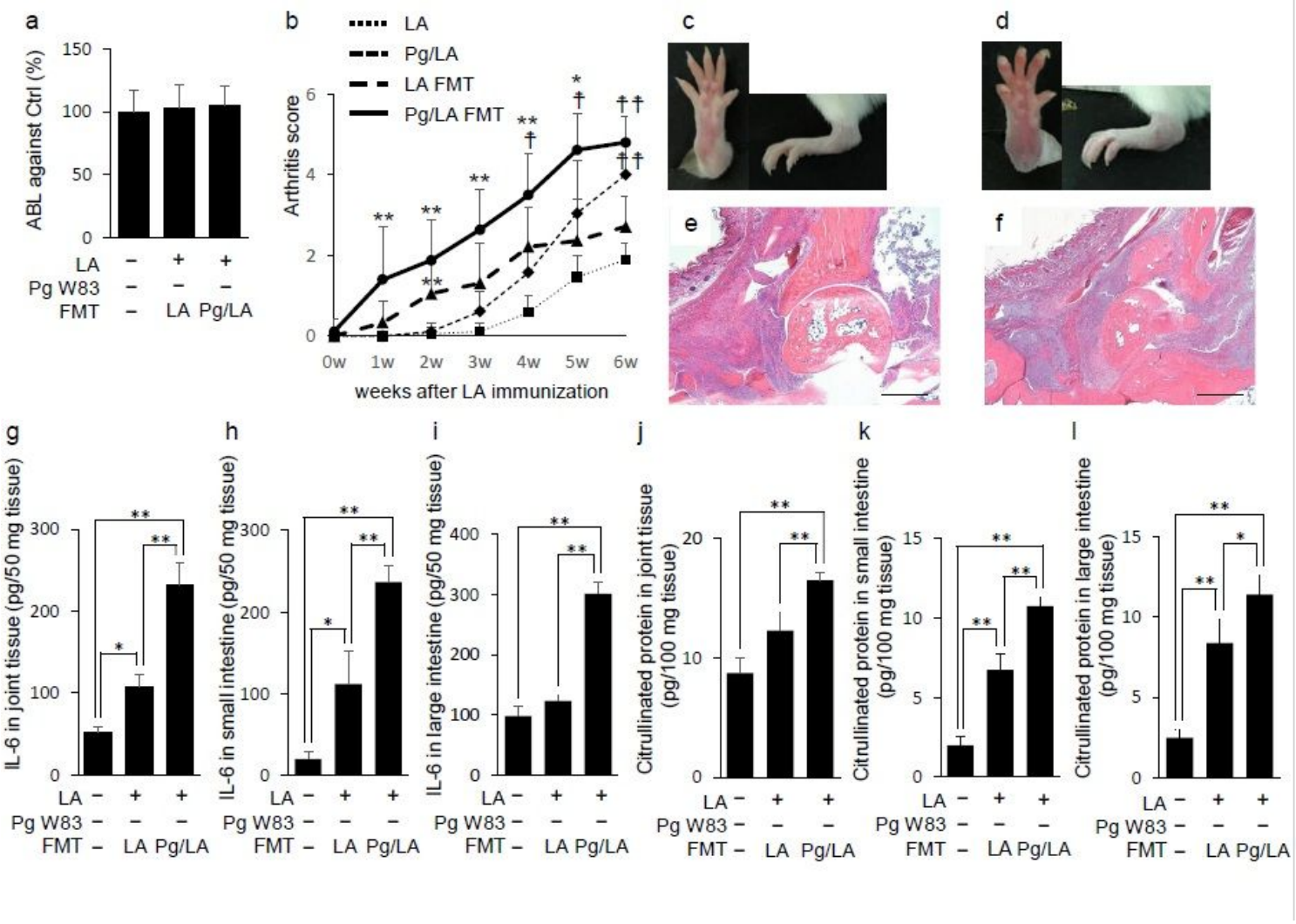
a
ABL against Ctrl (%)
150
100
50
0
LA – + +
Pg W83 – – –
FMT – LA Pg/LA
b
LA
Pg/LA
LA FMT
Pg/LA FMT
Arthritis score
6
4
2
0
0w 1w 2w 3w 4w 5w 6w
weeks after LA immunization
c
d
e
f
g
IL-6 in joint tissue (pg/50 mg tissue)
300
200
100
0
h
IL-6 in small intestine (pg/50 mg tissue)
i
IL-6 in large intestine (pg/50 mg tissue)
400
j
Citrullinated protein in joint tissue (pg/100 mg tissue)
20
10
k
Citrullinated protein in small intestine (pg/100 mg tissue)
15
5
l
Citrullinated protein in large intestine (pg/100 mg tissue)

Figure 5

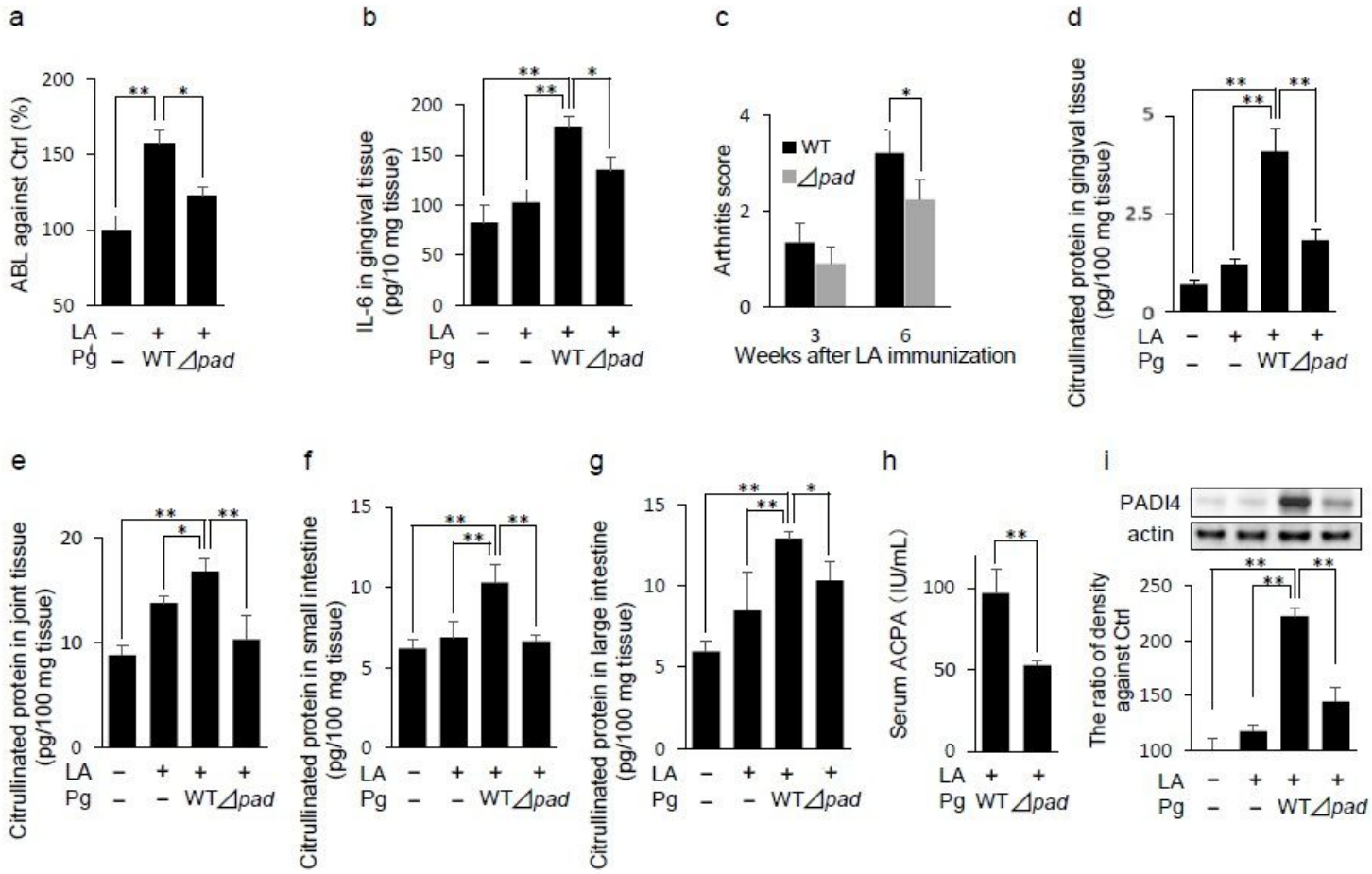

Figure 6

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- FigureS3.jpg
- FigureS2.jpg
- FigureS1.jpg
- HamaART20200105.pdf