The *P. gingivalis* Autocitrullinome Is Not a Target for ACPA in Early Rheumatoid Arthritis

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Appendix

Appendix Materials and Methods

Patient and healthy control sera

Serum samples were obtained from 12 DMARD-naïve ACPA+ and 10 ACPA- early RA patients from the 'Pathobiology of Early Arthritis Cohort' (PEAC, http://www.peac-mrc.mds.qmul.ac.uk) and 8 healthy volunteers (controls) after approval from a local ethics committee (REC: 05/Q0703/198) and the Queen Mary Research Ethics Committee (QMREC 2014:61), and informed consent from all donors. RA patient were diagnosed according to the revised American College of Rheumatology (ACR) and European League Against Rheumatism (EULAR) criteria.

Bacterial strains and growth conditions

P. gingivalis W50 and PPAD mutant strain (PG1424) were grown anaerobically (80% N_2, 10% H_2 and 10% CO_2) on blood agar (Oxoid Ltd., Basingstoke, United Kingdom) containing 5% defibrinated horse blood (TCS Biosciences Ltd., Buckingham, United Kingdom) at 37°C for 72h, then in Brain Heart Infusion broth (Oxoid) with hemin (5µg/ml) at 37°C for 48 h. Clindamycin hydrochloride (5µg/mL) was added to blood agar plates for selection of *erm* expression in *P. gingivalis* mutant PG1424.

Generation of *P. gingivalis* mutant strain PG1424

P. gingivalis mutant strain PG1424 was generated using primer pairs designed to separately amplify the 5´- and 3´-ends of the ppad gene region PG1424 and include SstI and XbaI restriction sites, respectively (Appendix Table 1). Following digestion with SstI and XbaI (New England BioLabs Inc., Ipswich, MA, USA), amplicons were ligated to the SstI-XbaI *erm* cassette from pVA2198 by T4-DNA ligase (New England BioLabs Inc.) and introduced into 6h-grown *P. gingivalis* W50 by electroporation, facilitating homologous replacement of *ppad* region PG1424 by the *erm* cassette (Appendix Figure 1). Clindamycin-resistant colonies were selected on blood agar plates, following homologous recombination *via* allelic exchange mutagenesis, and confirmed by PCR.

PPAD activity

N-α-benzoyl-L-citrulline (BC) was prepared by alkaline hydrolysis of the N-α-benzoyl-L-citrulline methyl ester (BCME, Bachem Feinchemikalien AG, Bubendorf, Switzerland) as in (PMID: 18387959).

Colorimetric assay. Bacterial cells from 48h liquid cultures were washed with phosphate buffered saline (PBS) and resuspended in incubation buffer (IB, 1mM EDTA and 1mM flavin mononucleotide in 0.2M Tris-HCl, pH 8.0) at optical density (OD_{600})=1.0. Bacterial cells (1×10^8 colony forming units; CFU) were incubated in the presence of 5mM N-α-benzoyl L-arginine methyl ester (BAME, Chem-Impex Int'l Inc., Wood Dale, IL, USA), N-α-benzoyl L-arginine HCl (BA, Alfa Aesar, Haverhill, MA, USA) or free L-arginine (BDH Biochemical, Lutterworth, Leicestershire, United Kingdom) at 37°C overnight under anaerobic conditions. Detection reagent containing one part reagent A (0.5% 2,3-butanedione monoxime and 0.01% thiosemicarbazide) and two parts reagent B (0.25 mg of $FeCl_3$/mL in 24.5% sulphuric acid and 17% phosphoric acid) was added to the assay products at 105°C for 2min and measured at 515nm using a CLARIOstar spectrophotometer (BMG LABTECH GmbH, Ortenberg, Germany) to measure L-citrulline levels. A standard curve was generated using 0–1,600 µM L-citrulline.

TLC. After incubation of bacterial cells with BAME, BA or free L-arginine HCl, cells were removed by centrifugation, supernatants freeze-dried and extracted into MeOH-$CHCl_3$ (1:2, v/v). Extracts were loaded onto silica gel plates and developed using MeOH-CH_2Cl_2 (8:3, v/v) as a mobile phase for 0.5h. The mobile phase was removed from the plate by drying at 80°C prior to detection of the compounds using a Syngene G-Box gel imager in epi short wave UV mode (Genesnap software V7.04) for incubations with BAME and BA. The products from incubations with L-arginine HCl were visualised by staining with 4 mg/mL ninhydrin in 95% ethanol. TLC plates were heated at 80°C for 10-15 min until coloured spots became visible.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting

Bacterial whole-cell lysates were harvested by centrifugation (13,000rpm, 10min) of 5 OD_{600} of a 48h-liquid culture inoculated with blood agar colonies, resuspended in 135 µL SDS (0.2%) and leupeptin (2mM; (Cai et al., 2018)) added immediately or after 10min of lysis, as stated in the main text. Proteins from supernatant (300 µL) were precipitated with 450µl acetone and then resuspended in 45 µL SDS (0.2%) and leupeptin (670µM). After incubation (10min, RT), proteins from whole-cell lysates and supernatants were diluted in reducing sample buffer at 1/10 and 1/2, separated on 4-12% Bis-Tris Protein Gels (NuPAGE™ Novex™, Carlsbad, CA, USA) transferred onto a polyvinylidene difluoride (PVDF) membrane using an iBlot® Dry Blotting System (Invitrogen, Thermo Fisher Scientific). Membranes were blocked and incubated with 1/250 dilutions of serum from RA patients or healthy controls in 3% BSA-TNT, then with horseradish peroxidase (HRP)-conjugated rabbit anti-human IgG antibody (Dako High Wycombe, Buckinghamshire, United Kingdom). Membranes were developed in 3,3'-diamino benzidine (0.05%) in PBS with hydrogen peroxide (0.02%). Densitometry analysis was performed using the ImageJ software (NIH, Bethesda, MD, USA). After log transformation, student's *t* test was used to determine statistically significant differences between *P. gingivalis*

W50 and PG1424 for each serum group. Moreover, one-way ANOVA was used to determine statistically significant differences between the three serum groups for each strain.

Anti-modified citrulline (AMC) immunoblot

Whole-cell lysates isolated from 48h-bacterial liquid cultures inoculated with BA colonies were separated by SDS-PAGE and transferred to PVDF membranes, as above. The modification of citrulline residues was carried out using an anti-modified citrulline (AMC) kit (Millipore, Merck KGaA, Darmstadt, Germany) according to the manufacturer's protocol and developed using Clarity™ Western ECL Substrate (Bio-Rad Laboratories, Hercules, CA, USA). Native and citrullinated histone H2a were used as controls**.**

Preabsorbed serum ACPA titration ELISA

Immulon 2 HB 96-well flat bottom plates (ImmunoChemistry Technologies, MN, USA) were coated with *P. gingivalis* W50 or PG1424 whole-cell lysates at 100 µg/mL in 0.1M carbonate/bicarbonate buffer, pH9.6, and incubated at 4°C overnight. After washing four times with 0.05% Tween 20 in PBS, wells were blocked (2% BSA in PBS, 1h at RT). After further washes, human serum samples (1:125 in dilution buffer provided with the anti-CCP ELISA kit; Axis Shield, UK) were added to the blocked wells and incubated 2h at RT to facilitate preabsorbtion. Next, the preabsorbed serum samples were transferred to an anti-CCP ELISA plate (Axis Shield, UK) and ACPA content measured as per manufacturer's instructions.

Induction of inflammatory arthritis by K/BxN serum transfer and inoculation with bacteria

11-week-old male specific-pathogen–free C57BL/6 mice were procured from Charles River UK and maintained in individually ventilated cages on a standard chow pellet diet with *ad libitum* access to water, with a 12-hour dark/12-hour light cycle. Animals were allocated to the treatment groups at random and group sizes were minimum size required based on previous experiments using this model (Flak et al., 2019). To induce inflammatory arthritis, mice were injected with arthritogenic K/BxN serum (50µL, i.p.) or vehicle (PBS) on days 0 and 2. In addition, on days –1, 1, and 3, mice were inoculated by oral gavage with 10^9 CFU *P. gingivalis* W50 or PG1424 in 100µL sterile PBS. Vehicle control mice were gavaged on the same days with 100µL sterile PBS.

Disease development was monitored daily by recording clinical scores (sum of inflamed ankles, paws and digits; maximum score/mouse = 26), and swelling index was established by measuring paw edema (width of ankles and paws using calipers). On day 8 post K/BxN serum administration, mesenteric lymph nodes (MLN) were collected for further analyses.

Animal experiments were carried out in strict accordance with the United Kingdom Home Office regulations (Guidance on the Operation of Animals, Scientific Procedures Act) and the

Laboratory Animal Science Association Guidelines (Guiding Principles on Good Practice for Animal Welfare and Ethical Review Bodies) and conform to the ARRIVE guidelines (https://www.nc3rs.org.uk/arrive-guidelines).

16S rRNA gene quantitative PCR (16S qPCR)

DNA was isolated from MLN using physical lysis in phenol/chloroform/isoamylalcohol (25:24:1; pH 8.0; MilliporeSigma) and relative quantitation of bacterial DNA carried out with PowerUp SYBR Green Master Mix and a CFX96 Real-Time System using primers (16S rRNA) specific for a universal bacterial 16S rRNA gene region and genomic sequence of *Gapdh* (gGapdh) as in (Flak et al., 2019). See Appendix Table 2 for primer sequences.

Statistical analysis

All statistical analyses and data derivations were performed using Microsoft Excel and GraphPad Prism 7 and 8. Results shown in figures are expressed as mean±SEM and unpaired Student's *t* test, Mann Whitney test 1-way or 2-way ANOVA, with respective *post hoc* tests as specified in figure legends.

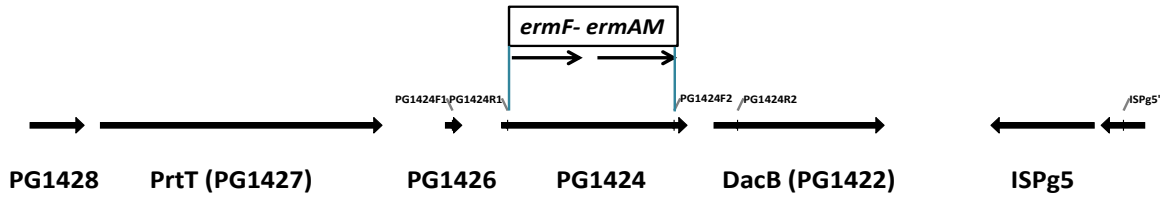
Appendix Table 1. Primers used for the generation of the mutant *P. gingivalis* PG1424.

Primer	Sequence (5´→ 3´)[a]	Size (bp)
PG1424F1	TGTGTCGGATGGCAGGATC	551
PG1424R1 (SstI)	atatat**gagctc**GATTGCGGGCAGTTGGAAG	
PG1424F2 (XbaI)	atatat**tctaga**GTCGCTGCAATGACCAAGG	581
PG1424R2	AAGGCTATCGTCCCGACAG	
ErmFF2	TTCGTTTTACGGGTCAGCAC	2,100[b]
ErmAMR2	ACTTTGGCGTGTTTCATTGC	

[a]Lowercase letters indicate irrelevant sequences used to facilitate restriction digestion and lowercase letters in boldface correspond to sites for restriction enzymes. [b]The amplicon amplified using this pair of primers was previously published (Aduse-Opoku et al., 2006).

Appendix Table 2. Primers used for 16S qPCR

Primer	Sequence (5´→ 3´)	Reference
genomic Gapdh	forward: CATGTTCCAGTATGACTCCA reverse: TGAAGACACCAGTAGACTCC	PMID: 16619041
16S rRNA	forward: ACTCCTACGGGAGGCAGCAGT reverse: ATTACCGCGGCTGCTGGC	PMID: 21998396



Appendix Figure. Map of genetically modified PG1424 locus in mutant *P. gingivalis* PG1424. The region flanking primers PG1424R1 / F2 was replaced with the *erm* cassette, encoding macrolide-lincosamide resistance, by homologous recombination.