DATA SUPPLEMENT

METHODS

Recombinant PPAD expression

Following expression of tagged PPAD in *E.coli* BL21 (DES) cells, cleavage of both tags proved inefficient compared to simple elution of the tagged protein from the resin. Expression and purification of vectors without PPAD inserts were carried out as controls. The protein yield from GST-His tagged PPAD was higher than the Trx-His tagged PPAD yield, therefore the GST-His PPAD was used in most experiments.

Immunoblotting

After SDS-PAGE proteins were transferred electrophoretically to a nitrocellulose membrane (Invitrogen) and blocked in 5% (w/v) non-fat dried milk in Tris-Buffered Saline 0.05% Tween (TBS-T) for 2 hours at RT or overnight at 4^{o}C. The sera were diluted (1:60) in TBS-T and incubated with the membrane-bound proteins for 1 hour at RT. The membranes were thoroughly washed with TBS-T. The secondary HRP-conjugated antibodies, peroxidase-conjugated mouse anti-human IgG (Hybridoma Reagent Laboratories, Baltimore, MD) (1:5,000) and Goat-anti-Rabbit HRP conjugated IgG antibody (1:5,000), were diluted in TBS-T and incubated for 1 hour at RT. The membranes were washed and bound secondary antibody detected using ECL Plus (Amersham) according to the manufacturer's instructions.

Citrullinated proteins were detected on nitrocellulose membranes using the anti-modified citrulline (AMC) detection kit (Upstate, Charlottesville, VA, USA) according to the manufacturer's protocol.

PPAD enzyme activity

The reaction was carried out using 1mM substrate (BAEE, Fib-A: ESSSHHPGIAEFPSR or Fib-B: PAPPPISGGGYR) in PPAD activity buffer (50 mM CHES pH9.5 containing 10 mM DTT) and 0.5-10 µM PPAD. A standard curve was created using free L-citrulline (0, 10, 50, 100, 200, 400 µM) in PPAD activity buffer. The plate was sealed with adhesive film and incubated at 37˚C for 15 min. Freshly prepared "citrulline detection reagent" containing 1 volume of Solution A (80 mM diacetyl monoxime and 2 mM thiosemicarbazide) and 3 volumes Solution B (3 M phosphoric acid, 6 M sulfuric acid, 2 mM ammonium iron (III) disulfate) was used to quench the reaction. The plate was sealed with adhesive film, incubated at 95^{o}C for 15 min then allowed to cool on ice (5-10 min) and the absorbance read at 540 nm. The slope from the standard curve was used to determine the concentration of citrulline in the unknown sample wells, after adjusting for background from substrate alone (control). A sample containing enzyme but no substrate was run to control for possible background from the enzyme itself.

Mass spectrometry

Recombinant GST-tagged PPAD was digested both in gel and in solution. Bands were excised from 1D-SDS-PAGE stained, reduced, alkylated and digested by trypsin using standard protocols [S1]. In solution digestion was carried out using 3 proteases (GluC, pepsin B and trypsin). For each digestion 10 µg fusion protein was digested at protease:protein ratios of 20:1. The proteins were digested by GluC and

trypsin and reduced by dithiothreitol (DTT) and alkylated by iodoacetamide, followed by digestion in 50 mM ammonium bicarbonate at 37ºC overnight. The pepsin digest were performed in 1% trifluoroacetic acid, at 37ºC overnight and reduced by DTT. All samples were ziptipped (C18 ZipTip®, Millipore Corporation, Billerica, MA, USA) before analysis by mass spectrometry (MS).

Peptides corresponding to 1 pmol protein were injected onto in-house packed 10 cm long fused silica tip columns (SilicaTips™ New Objective Inc.) packed with 3 µm C18-AQ ReproSil-Pur® (Dr. Maisch GmbH, Germany) using an Easy-nLC system (Thermo Scientific). The peptides were separated using an acetonitrile gradient. The C18 columns were coupled on-line to a hybrid LTQ Orbitrap XL mass spectrometer (Thermo Scientific, Bremen, Germany). The instrument was operated in a data dependent mode where survey spectra were acquired in a range of m/z 300 to m/z 2000 with a resolution of 60,000 at m/z 400, followed by MS/MS spectra of the 5 most intense peptide ions using collision induced dissociation (CID) as fragmentation method.

Mass lists were extracted by Raw2MGF, an in-house developed program, and searched against the SwissProt database (UniProtKB/Swiss-Prot protein knowledgebase release 2011_08) using the Mascot search engine (v. 2.3.0). Several parameters for the database searches were adopted for maximising identification of citrullination of arginine. Only peptides with a Mascot score of at least 20 were considered. All peptides that were identified as citrullinated were manually validated by 1) verifying correct assignment of precursor mass, 2) comparing retention time of unmodified, deaminated and citrullinated versions of the same peptide, which should not coincide, 3) comparing MS/MS spectra from unmodified, citrullinated and, where

available, deamidated peptides, to find fragment ions able to distinguish the different modification states.

Generation of anti-PPAD antibody

Briefly, 2 rabbits were immunised subcutaneously every 2 weeks, for 10 weeks, with 1.5 mg Thioredoxin-His PPAD in Freund's incomplete adjuvant per boost. One week after each injection, blood was collected and sera were analysed for the presence of anti-PPAD antibodies. Final serum was affinity purified on a thiopropyl-Sepharose column coupled with GST-His PPAD. Bound antibody (0.19 mg/ml) was eluted with TAE. The eluate was evaluated for sensitivity and specificity to PPAD by immunoblotting, where 1:1,000 dilution resulted in strongest reactivity towards cleaved PPAD.

Immunohistochemistry.

An immortalised oral keratinocyte cell line (OKF6-TERT2 cells, kind gift of the Rheinwald laboratory, Brigham and Woman's Hospital, Boston) were cultured to 80% confluence in a 4 well chamber slide with defined-keratinocyte serum-free medium (KSFM) (Invitrogen, Paisley, UK). *P.gingivalis* ATCC 33227 was grown at 37^{o}C in Schaedler anaerobe broth (Oxoid, Cambridge, UK) in an anaerobic chamber (85% N_2, 10% CO_2 and 5% H_2, [Don Whitley Scientific Limited, Shipley, UK]) for 24 hours, then washed, resuspended in KSFM and added to OKF6 cells at a ratio of approximately 100 bacteria to 1 epithelial cell. The epithelial-bacteria co-culture was cultured in 5% CO_2 for 12 hours, gently washed with PBS then fixed with 4% paraformaldehyde at 4^{o}C and incubated with 2.5% human serum for 30 mins at room

temperature. Cells were allowed to react with 2 µg/ml anti-PPAD antibody or polyclonal rabbit IgG. Detection was performed using ImmPRESS anti-rabbit polymer detection kit followed by ImmPACT DAB peroxidase substrate (both Vector laboratories, Peterborough, UK) according to the manufacturer's instructions. Between steps, cells were washed with TBS-T for 5 minutes. Slides were counter stained with haematoxylin.

Cell fractionation of *P.gingivalis* W83

The method of cell fractionation by Nguyen KA[S2] was modified by replacing sarkosyl with 1% Triton X-100, 20 mM $MgCl_2$ (4℃ for 30 min) to solubilize the inner membrane. Samples were adjusted to contain the same amount of protein (20 ug) and were electrophoresed using Novex® 10-20% Tricine Gel (Invitrogen) at 125 V for 90 min. The proteins were electrotransferred onto a nitrocellulose membrane (Bio-rad) at 100 volts for 60 min. Non-specific binding sites were blocked with 5% skimmed milk in PBS supplemented with 0.1% Tween 20 (PBST) at 4℃ overnight. Subsequently, a membrane was incubated with anti-PPAD antibody at room temperature for 3 hours, washed 3-times with PBST, then probed with alkaline phosphatase-conjugated anti-rabbit IgG (Sigma,A3687, Lot#16H8840, 2,000-fold dilution in PBST) for 3 hours at room temperature. A membrane was developed by using APConjugated Substrate Kit (Bio-Rad, #1706432).

Serum samples from patients and control subjects.

In addition to the serum cohort used in the ELISA analysis, a small independent cohort was used to validate the antigen specificity of the assay consisting of ten serum samples from RA patients and ten serum samples from healthy volunteers (control) in the UK. Seven PD serum samples were obtained from Jagiellonian University, Krakow, Poland, with a clinical diagnosis of PD. All serum samples were obtained with informed consent under approval from the local ethics committee [S3]. .

ELISA data analysis

For each serum the average 'background' (coating buffer with no antigen) was taken from the average sera reactivity. Absorbance values were converted into arbitrary units per millilitre using either pooled anti-RgpB positive sera standards on anti-RgpB antibody plates or anti-PPAD antibody-positive standard on anti-PPAD plates. To establish a cut-off to define a positive sample we tested sera for reactivity towards C351A as well as PPAD. To determine the citrulline specific portion of reactivity, we subtracted the inactive C351A reactivity from the reactivity against active PPAD for each serum. We subsequently calculated the cut-off as the ninety fifth percentile of the control group and all sera with values above this were considered 'positive'. To examine the correlation of antibody detection by ELISA relative to immunoblotting, 10 control, 7 PD and 10 RA sera were examined by both immunoblotting and ELISA.

PPAD peptides

Thirteen cyclic 19-26-mer peptides were synthesized at either Cambridge Research Biochemicals (Billingham, Cleveland, UK) or Genscript (120 Centennial Avenue,

Piscataway, NJ 08854, USA) and purified by HPLC to a purity > 85% with the exception of CPP7 which was 62% pure. The peptides encompassed all the arginine bases in PPAD, with arginine substituted by citrulline (Table 1). Peptides were resuspended in 2.5 mg/ml sterile distilled water or 50% DMF and stored at -20^{o}C.

PPAD peptide ELISA procedure

ELISA was carried out as with recombinant PPAD and RgpB with the following modifications: Peptides were diluted at 10 µg/ml in coating buffer. Serum was diluted 1:100 in RIA buffer. On each plate duplicate wells were coated with RIA buffer without sera (background). For each serum the average 'background' was taken from the average sera reactivity on each plate. Results were expressed as average OD450.

References

S1 Shevchenko A, Wilm M, Vorm O, et al. Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. Anal Chem. 1996;**68**:850-8.

S2 Nguyen KA, Travis J, Potempa J. Does the importance of the C-terminal residues in the maturation of RgpB from Porphyromonas gingivalis reveal a novel mechanism for protein export in a subgroup of Gram-Negative bacteria? J Bacteriol. 2007;**189**:833-43.

S3 Lundberg K, Kinloch A, Fisher BA, et al. Antibodies to citrullinated alpha-enolase peptide 1 are specific for rheumatoid arthritis and cross-react with bacterial enolase. Arthritis Rheum. 2008;**58**:3009-19.