SUPPLEMENTARY DATA

SUPPLEMENTARY FIGURE LEGENDS

Figure S1 (A) Purified rPPAD and rPPAD^{C351S} (4 µg per lane) were analyzed by SDS-PAGE, and protein visualized by GelCode Blue Coomassie staining (Pierce) to determine purity. (B) Bacterial proteins in *P gingivalis* are not citrullinated. *P gingivalis* cells were lysed directly in SDS sample buffer, boiled immediately and sonicated. Cell lysate (CL) and culture supernatant (CS) were resolved by SDS-PAGE, transferred onto nitrocellulose and visualized by Ponceau staining (left panel). Anti-PPAD immunoblotting was used to identify PPAD in the same membrane (middle panel). Protein citrullination was detected by AMC immunoblotting (right panel). Purified cit-rPPAD and uncitrullinated rPPAD^{C351S} were included as positive and negative controls for protein citrullination, respectively. Solid and unfilled arrowheads indicate the corresponding molecular weights of cPPAD (CL), sPPAD (CS) and recombinant PPAD (rPPAD and C351S). Open arrowhead denotes protein band likely resulting from PPAD degradation.

Figure S2 Median of differences (MOD) for [anti-cit-rPPAD – anti-rPPAD^{C351S}] antibody levels in RA and controls. Differences in reactivity of individual RA sera (n=83; MOD: 0.0) are shown on the left (expressed as arbitrary units); differences for controls are shown on the right (n=39; MOD: -0.001). The red line represents the MOD.

SUPPLEMENTARY MATERIAL AND METHODS

Mass spectrometry analysis

P gingivalis whole cell lysate, culture supernatant, and purified rPPAD were resolved by 1D SDS-PAGE. PPAD-containing bands (as detected by immunoblotting) were excised from the gel and analyzed by mass spectrometry (Mass Spectrometry and Proteomics Facility, Johns Hopkins University School of Medicine). Potential citrullination sites were identified by mass shift and manually validated by analyzing individual peptide spectra. Deimination sites were confirmed using Scaffold PTM 2.1.2 (Proteome Software) and localization probabilities calculated using the Ascore algorithm. An Ascore of 20 corresponding to 99% certainty in localization (p=0.01) was considered significant.[38]

PPAD activity assay (BAEE)

The enzymatic activity of rPPAD, rPPAD^{C351S} and rPPAD^{Ntx} (0–150 nM) was assayed in parallel using 1 mM Nα-benzoyl-L-arginine ethyl ester (BAEE) substrate in 50 mM CHES pH 9.5, 10 mM DTT as previously described.[S1, S2] Serial dilutions of L-citrulline (0–400 μM) were used as a standard. Plates were incubated for 30 min at 37° Celsius for BAEE deimination to occur. The reaction was quenched by adding freshly prepared detection reagent (one volume of solution A containing 80 mM 2,3-butanedione monoxime, 2 mM thiosemicarbazide and three volumes of solution B containing 3 M phosphoric acid, 6 M sulfuric acid, 2 mM ammonium iron(III) sulfate). Plates were incubated for 15 min at 95° Celsius, and color development measured at 560 nm. The concentration of citrulline was extrapolated from the standard curve.

Immunoprecipitation (IP)

^{35}S-methionine-labeled proteins were generated from pET-28a(+)-rPPAD, pET-28a(+)-rPPAD^{C351S} and pET-28a(+)-rPPAD^{Ntx} by *in vitro* coupled transcription/translation (IVTT) using the TnT® Quick system (Promega). ^{35}S-methionine-labeled products were diluted in IP buffer (20

mM Tris pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% NP-40 Alternative), incubated with 1µL of RA patient serum for 1h at 4° Celsius, and the immune complexes purified using Protein A agarose beads (Pierce). Samples were washed in IP buffer, and immunoprecipitates visualized by SDS-PAGE/ sodium salicylate fluorography.

Competition assay

Polystyrene plates were coated with 50ng/well cit-rPPAD. Plates were blocked with PBS–TM 3%. Patient sera were pre-incubated with 1µg of purified protein ($PPAD^{C351S}$, human alpha-enolase or citrullinated enolase) or buffer alone. Following incubation, sera were added to plates coated with cit-rPPAD at a final dilution of 1:2000. Anti-human IgG was used for detection.

Statistical analysis

Statistical analysis of PPAD ELISA groups was performed using the Wilcoxon matched-pairs signed-rank test (anti-cit-rPPAD vs. anti-$rPPAD^{C351S}$) and Mann-Whitney test for unpaired groups (RA vs. control subjects). Correlation between antibody levels, RA disease activity and disease duration was analyzed using Spearman's correlation. Statistical analyses were performed using GraphPad Prism 6. A p-value <0.05 was considered statistically significant.

REFERENCES

S1. **Boyde TR,** Rahmatullah M. Optimization of conditions for the colorimetric determination of citrulline, using diacetyl monoxime. *Anal Biochem* 1980;**107**:424–31.

S2. **Quirke A-M,** Lugli EB, Wegner N, *et al.* Heightened immune response to autocitrullinated Porphyromonas gingivalis peptidylarginine deiminase: a potential mechanism for breaching immunologic tolerance in rheumatoid arthritis. *Ann Rheum Dis* 2014;**73**:263–9.