Supporting Information

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SI Materials and Methods

Detection of Isotype ACPAs. For IgE-ACPA detection, IgG was depleted from sera of controls and $ACPA^-$ and $ACPA^+$ RA patients by incubating protein G-coupled beads (Calbiochem) with serum [1:1 (vol/vol)] for 1 h at 4 °C according to the manufacturer's instructions. Microtiter plates were coated with 20 μg/mL native noncitrullinated fibrinogen or citrullinated fibrinogen for 18 h at 4 °C. After blocking with 2% (wt/vol) PBS/BSA for 2 h at room temperature, plates were incubated with 50 μL/well of IgG-depleted serum samples for 18 h at 4 °C. Detection of IgE antibodies was performed by adding monoclonal mouse anti-IgE-HRP (1:800 dilution; Nordic Immunology) for 2 h at 37 °C. In parallel, a CCP2 ELISA detecting IgE-ACPAs was performed using the same detection antibodies. Results were expressed as OD values.

BAT. Aliquots of 100 μL of heparinized whole blood (BD Biosciences) were preincubated at 37 °C for 10 min with 2 ng/mL IL-3 (Peprotech, Inc.) in HBSS with calcium and magnesium (Gibco Invitrogen). Preactivated blood samples were stimulated at 37 °C for 10 min with 10 μg/mL native noncitrullinated fibrinogen (Sigma Aldrich), 10 μg/mL citrullinated fibrinogen, 0.5 U/mL PAD (Sigma Aldrich), 10 μg/mL noncitrullinated MBP, 10 μg/mL citrullinated MBP, 10 μg/mL monomeric IgG (ChromPure Human IgG; Jackson ImmunoResearch Laboratories, Inc.), 10 or 100 μg/mL heat-aggregated IgG (HAIgG), HBSS as a negative control, and 1 μg/mL anti-IgE (Pharmingen, BD Biosciences) as a positive control. After cooling samples on ice for 5 min, cells were stained with monoclonal antibodies to human IgE (Sigma Aldrich) coupled to Alexafluor 488 (Molecular Probes) and monoclonal PE-conjugated anti-human CD63 (Pharmingen) or PE-conjugated irrelevant isotype antibody (Pharmingen) in the dark on ice for 20 min. Red blood cells were lysed, and white blood cells were fixed with FACS Lysing solution (Becton Dickinson) at room temperature for 20 min. Afterward, cells were washed with 1% PBS/BSA and stored in 1% PBS/paraformaldehyde until analysis. Expression of cell surface markers was assessed using flow cytometry (FACSCalibur; Becton Dickinson). Data were analyzed by CellQuest Pro software (Becton Dickinson).

Elution of Igs. Heparinized whole blood was washed twice with 1% HBSS/BSA. Subsequently, basophil-bound Ig was eluted for 30 sec by two serial incubations at room temperature with acid elution buffer (20 mM glycine, 1 mg/mL BSA, 130 mM NaCl, 2 mM $CaCl_2$, pH 2.5), separated by one wash with 1% HBSS/BSA buffer. As a control, noneluted samples were treated with 1% HBSS/BSA instead of elution buffer. Basophils were stained after elution with monoclonal PE-conjugated FcεRI (eBioscience), IgE-Alexafluor 488, and allophycocyanin (APC)-conjugated CD203c antibodies (Becton Dickinson).

RBL Mediator Release. RBL-2H3 and RBL-30/25 cells transfected with human FcεRI (cotransfection of three plasmids encoding the α-, β-, and γ-chains, respectively) were cultured in DMEM (Gibco), supplemented with glutamine (Gibco), 5% (vol/vol) FCS (Gibco), 100 U/mL penicillin G, and 100 μg/mL streptomycin at 37 °C in a humidified atmosphere of 5% CO_2. Briefly, cells were seeded in a 96-well tissue culture plate and incubated for 18 h with human sera or 0.1 μg/mL human IgE (Diatec; Bioporto Diagnostics) as a positive control or DMEM medium alone as a negative control. Subsequently, cells were incubated with 1 μg/mL polyclonal goat anti-IgE (Nordic Immunology), 10 μg/mL noncitrullinated fibrinogen, 10 μg/mL citrullinated fibrinogen, 10 μg/mL noncitrullinated MBP, 10 μg/mL citrullinated MPB or Thyrod's buffer for 1 h at 37 °C in 5% CO2. To determine the enzymatic activity of β-hexoaminidase, supernatants were mixed with p-nitrophenyl-*N*-acetylbeta-d-glucoaminide (Sigma). After 1 h at 37 °C, the reaction was terminated by adding glycine buffer (pH 10.7) and measured at 405 nm. Total β-hexoaminidase content was determined after lysis of the RBLs with Triton-X100 (Sigma Aldrich). Protein (anti-IgE, noncitrullinated fibrinogen, or citrullinated fibrinogen)-specific release is depicted as the percentage of total release after correction for spontaneous release in Thyrod's buffer.

Binding of Noncitrullinated and Citrullinated Fibrinogen to Basophils. Heparinized whole blood was washed twice with 1% HBSS/BSA. Subsequently, blood was stained with $FcεRI^+$-PE and CD203-APC antibodies and incubated with 1% PBS/BSA or 1 or 10 μg/mL citrullinated fibrinogen coupled to FITC (Calbiochem labeling kit; labeling according to the manufacturer's instructions) in the dark on ice for 30 min. Noncitrullinated fibrinogen coupled to FITC and 1% HBSS/BSA was used as a control for staining. Cells were washed with 1% PBS/BSA and stored in 1% PBS/paraformaldehyde until analysis. Expression of cell surface markers was assessed using flow cytometry. Data were analyzed by CellQuest Pro software.

Histamine and IgE Measurements. Quantification of total IgE levels in serum and synovial fluid was performed according to the manufacturer's instructions (ImmunoCap; Pharmacia CAP Systems). Values lower than 2 kU/L were considered negative. Histamine content in synovial fluid was measured with a histamine competitive direct ELISA (Neogen) according to the manufacturer's instructions. The detection limit was 2.5 ng/mL.

Synovium Digestion and Cell Staining. Immediately after harvesting, synovial tissue was collected in sterile PBS. Connective tissue and fat were removed. Synovial tissue was digested in 1 mg/mL collagenase IA (Sigma Aldrich) and 50 μg/mL DNAse (Sigma Aldrich) at 37 °C for 1 h. Afterward, cells were separated from tissue using a 70-μm filter and washed with 1% PBS/BSA. After isolation, 0.5×10^6 cells were stained for 30 minutes in the dark at 4 °C with monoclonal CD117(c-kit)-APC (BD Biosciences), FcεRI-PE, anti-IgE conjugated to Alexafluor 488, or isotype controls with optimal dilutions of each antibody. Expression of cell surface markers was assessed using flow cytometry (LSRII; Becton Dickinson). Data were analyzed by CellQuest Pro software.

Histology. Synovium specimens were fixed by 4% (wt/vol) formaldehyde (Merck) in PBS and stored in 70% (vol/vol) ethanol. Synovial tissue was embedded in paraffin, sectioned at 4 μm, and mounted on glass slides (Polysine slides; Menzel-Gläser). Staining with Naphtol AS-D CAE (Sigma Aldrich) was done at 37 °C in 5% (vol/vol) CO_2 for 30 min. Subsequently, slides were washed with MilliQ H_2O (Millipore bv) and stained with hematoxylin.

Inmmunohistochemistry. Synovium specimens were deparaffinized with xylene (Merck). Endogenous peroxidase activity was blocked with 1% hydrogen peroxide (Merck) in methanol for 10 min. Antigen retrieval was performed for 30 min at 96 °C with a Tris/EDTA solution (pH9; DAKO). Slides were stained with polyclonal rabbit anti-human CD117(c-kit) antibodies (8.6 mg/mL; 1:800 dilution; DAKO) in 1% PBS/BSA for 1 h at room temperature in a humidified chamber. For control sections, matching isotype control (polyclonal rabbit Ig; 15 mg/mL; 1:1,400 dilution; DAKO) was used. Detection was performed using HRP-conjugated goat anti-rabbit Ig (Envision; DAKO). HRP activity was detected using hydrogen peroxide and 3,3′-diaminobenzidine tetrahydrochloride-nickel chloride (Vector

Laboratories) as a substrate. For combined CD117/CAE staining, CAE staining was performed before antigen retrieval. Stained sections were coded and randomly analyzed. The mean number of mast cells was assessed from 10 high-power fields (×400 magnification) and scored blindly by two independent observers (A.J.M.S and A.L.D.). Pearson's correlations between both observers were 0.96 for CAE staining and 0.98 for CD117 staining ($P < 0.0001$ and $P < 0.0001$, respectively). Stained sections were scored semiquantitatively from 0 to 3 (0 indicating lowest expression and 3 indicating highest expression). The percentage of mast cell degranulation was calculated by the difference between the number of $CD117^+$ cells (all mast cells) and the number of CAE^+ cells (nondegranulated mast cells).

Statistical Analysis. Differences of nonparametric data between patient and control groups were analyzed using the Kruskal-Wallis and Mann-Whitney *U* tests. Differences in parametric data between patient and control groups were analyzed using the Student *t* test. Differences for paired samples before and after elution were assessed using the Wilcoxon signed rank test. Correlations were calculated using the Spearman rank correlation for nonparametric data and Pearson's correlation coefficient for parametric data. Statistical analysis was performed using SPSS software, version 16.0.2 (SPSS, Inc.). In all tests, *P* values less than 0.05 were considered significant.

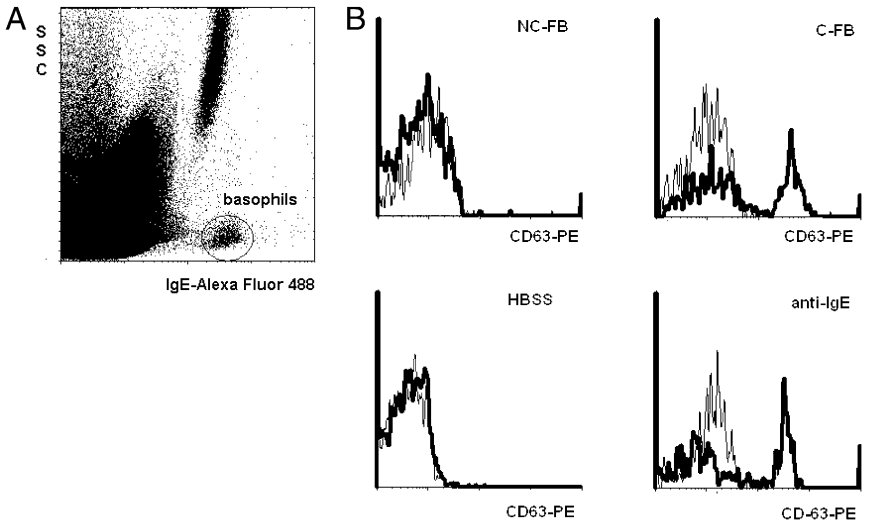

Fig. S1. Flow cytometric analysis of basophil activation. (*A*) Basophils were identified and gated on a dot plot showing side scatter (SSC) and fluorescence intensity of Alexafluor 488-conjugated anti-IgE. (*B*) Histograms show fluorescence intensity of CD63-PE (thick line) and isotype-PE (thin line) after stimulation with HBSS, 1 μg/mL anti-IgE, 10 μg/mL noncitrullinated fibrinogen (NC-FB), and 10 μg/mL citrullinated fibrinogen (C-FB).

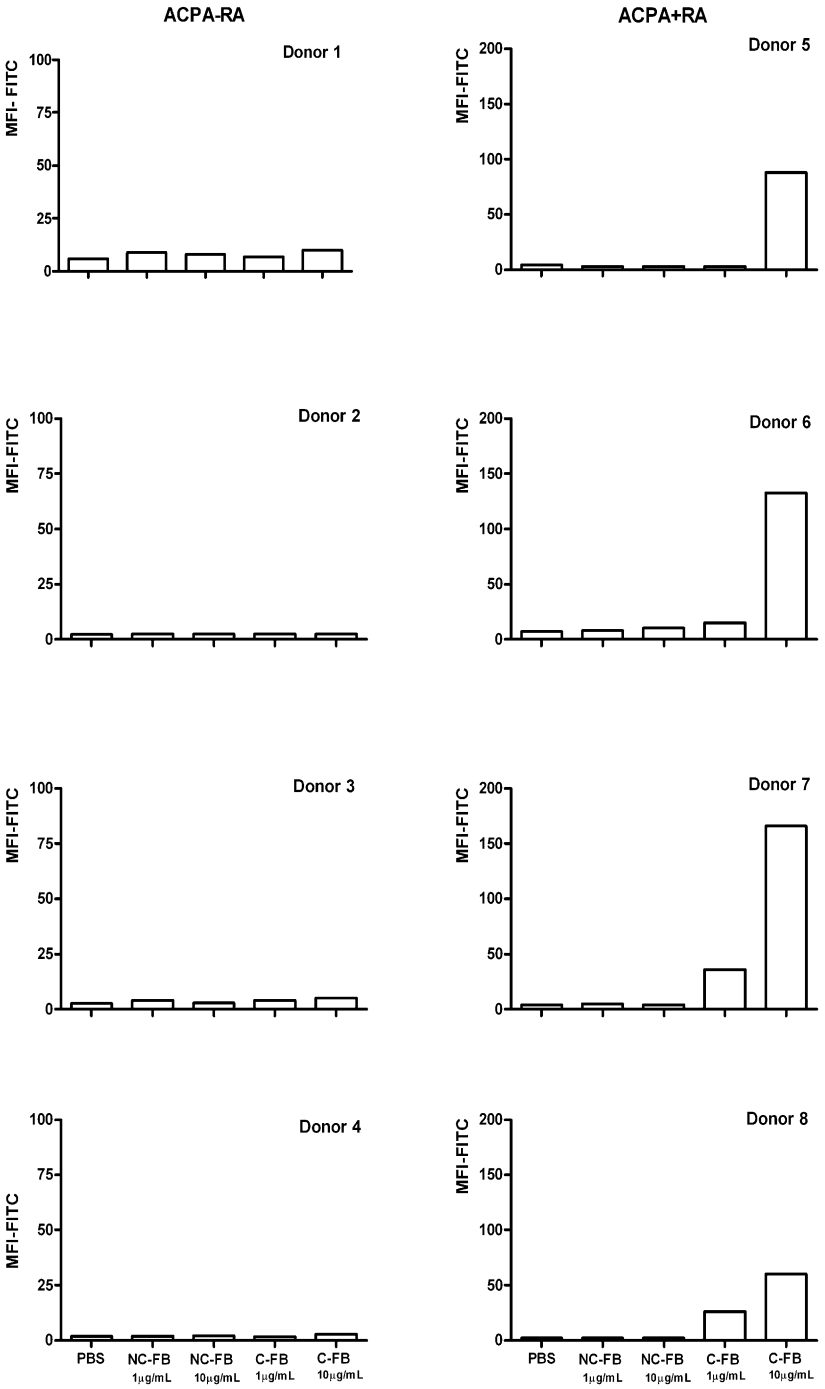

Fig. S2. Binding of citrullinated fibrinogen to basophils. Whole heparinized blood from four ACPA$^-$ RA and 4 ACPA$^+$ RA patients was incubated with 1% PBS/BSA (PBS), 1 or 10 μg/mL noncitrullinated fibrinogen coupled to FITC (NC-FB), and 1 or 10 μg/mL citrullinated fibrinogen coupled to FITC (C-FB). Graphics show individual patient results of eight donors. Basophils were identified as CD203c$^+$ and FcεRI$^+$. Values are the mean fluorescence intensity (MFI) for FITC fluorescence.

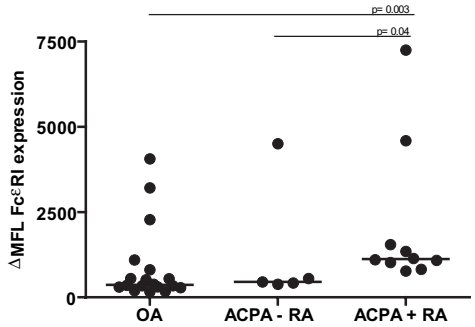

Fig. S3. Surface FcεRI expression on CD117-APC$^+$ synovial mast cells. Synovial mast cells of ACPA$^+$ RA (n = 10), ACPA$^-$ RA (n = 5), and OA (n = 18) patients were stained with PE-conjugated anti-FcεRI. Results are expressed as the difference in mean fluorescence intensity (MFI) between isotype control MFI and anti-FcεRI MFI. C-FB, citrullinated fibrinogen; NC-FB, noncitrullinated fibrinogen.

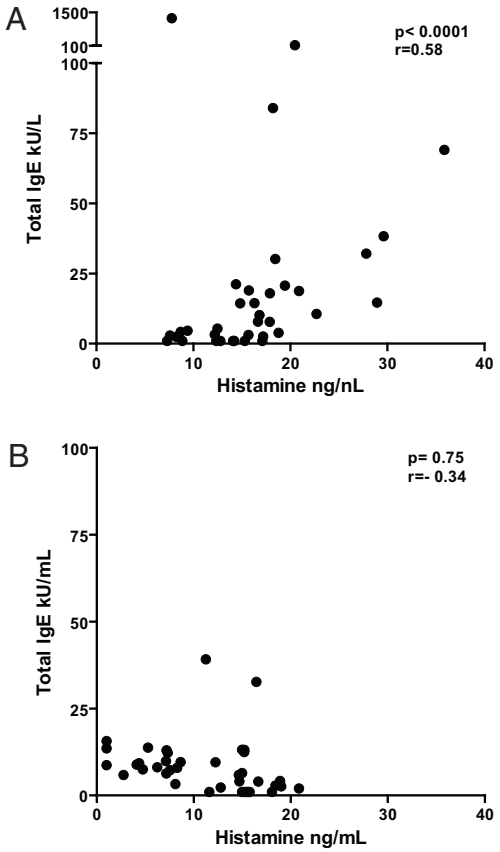

Fig. S4. Correlation of histamine with total IgE. (*A*) Correlation of total IgE levels (kU/L) with histamine level (ng/mL) in synovial fluid of ACPA$^+$ RA patients. $P < 0.0001$, $r = 0.58$. (*B*) Correlation of total IgE levels (kU/L) with histamine level (ng/mL) in synovial fluid of ACPA$^-$ RA patients. $P = 0.75$, $r = -0.34$.