

Retraction

MEDICAL SCIENCES

Retraction for “Evidence for a functional role of IgE anti-citrullinated protein antibodies in rheumatoid arthritis,” by A. J. M. Schuerwegh, A. Ioan-Facsinay, A. L. Dorjée, J. Roos, I. M. Bajema, E. I. H. van der Voort, T. W. J. Huizinga, and R. E. M. Toes, which appeared in issue 6, February 9, 2010, of *Proc Natl Acad Sci USA* (107:2586–2591; first published January 25, 2010; 10.1073/pnas.0913054107).

The authors wish to note the following: “Recently, the Department of Rheumatology from the Leiden University Medical Centre discovered that this publication is based upon fraudulent data, as the first author manipulated several assays used in the studies presented in this publication. By adding anti-IgE antibodies into the tubes containing citrullinated fibrinogen, it seemed that the activation of basophils resulted from exposure to citrullinated fibrinogen. Likewise, controls and samples were manipulated through transfer of cells from positive controls to other tubes that now also appeared to contain a positive reaction. This behavior was done in the absence of all other investigators who did not have knowledge of these irregularities. After discovery of these events, several experiments were performed with the goal to reproduce the data indicating the presence of IgE anticitrullinated protein antibody (ACPA). The validity of these data as presented in the manuscript could not be confirmed. For these reasons, the authors wish to retract the paper from the scientific record.

The Leiden University Medical Centre has completed an investigation by an independent investigation committee into the violation of scientific integrity and irregularities in this paper and has concluded that sole responsibility for the irregularities rests with the first author. She has admitted manipulating the data presented in the paper.”

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Evidence for a functional role of IgE anticitrullinated protein antibodies in rheumatoid arthritis

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Rheumatoid arthritis (RA) is a systemic autoimmune disease involving inflammation of the joints. Among the autoantibodies described in RA, anticitrullinated protein antibodies (ACPAs) are highly specific and predictive for RA. In addition, ACPAs have been implicated in the pathogenesis of RA. However, a direct functional response of immune cells from ACPA⁺ RA patients toward citrullinated proteins has not been demonstrated. In this study, we show that exposure to citrullinated antigens leads to activation of basophils from ACPA⁺ RA patients within 20 minutes. This was not observed after exposure of basophils to noncitrullinated control antigens or after stimulation of basophils from ACPA⁻ RA patients and healthy controls. Basophil activation was correlated with the binding of citrullinated proteins to basophils. Furthermore, serum from ACPA⁺ RA patients in contrast to that from ACPA⁻ RA patients could specifically sensitize human FcεRI expressing rat basophil cells (RBL), enabling activation by citrullinated proteins. Mast cell degranulation products such as histamine levels were enhanced in synovial fluid of ACPA⁺ RA patients as compared with ACPA⁻ RA and osteoarthritis patients. In addition, histamine levels in synovial fluid from ACPA⁺ RA patients correlated with IgE levels, suggesting degranulation of mast cells by cross-linking IgE. Immunohistochemistry on synovial biopsies demonstrated an increased number of degranulated CD117⁺ mast cells in ACPA⁺ RA patients; IgE and FcεRI expression in synovial mast cells from ACPA⁺ RA patients was increased. In conclusion, our results show an immunological response of immune cells from ACPA⁺ RA patients in a citrulline-specific manner. Moreover, these data indicate a role for IgE-ACPAs and FcεRI-positive cells in the pathogenesis of RA.

basophil | histamine | mast cell | citrullination | synovium

Rheumatoid arthritis (RA) is a systemic autoimmune disease characterized by chronic inflammation of the joints. Recently, a major development in RA focused on antibodies to proteins modified by citrullination (i.e., an enzyme-mediated posttranslational modification of peptidyl arginine to peptidyl citrulline). Anticitrullinated protein antibodies (ACPAs) have been shown to be highly specific for RA (1), are predictive for the development of RA in patients with undifferentiated arthritis (2), and are associated with the extent of joint destruction (3, 4). In an arthritis mouse model, it has been described that transfer of monoclonal antibodies to citrullinated proteins can enhance arthritis progression, whereas tolerization for citrullinated proteins diminishes arthritis severity (5, 6). Together, these observations indicate a role for ACPAs in RA pathogenesis. In humans, however, in spite of the clinical relevance of ACPAs, the potential role of ACPAs in rheumatoid inflammation has not yet been elucidated.

Previously, it has been shown that in vitro generated immune complexes containing ACPAs are able to activate macrophages. These ACPA-containing and ELISA plate-bound immune complexes could induce TNF-α production in macrophages from healthy donors via the engagement of FcγRIIa (7). However, a direct functional and, more importantly, specific response of immune cells from ACPA⁺ RA patients to citrullinated antigens has not been shown, because it is very difficult to demonstrate in a human setting the direct pathological action of immune

mediators such as ACPAs. Such information would be highly relevant because it would provide evidence for a direct functional role of ACPAs in the immune response underlying RA. Therefore, we reasoned that in case ACPAs can mediate inflammatory reactions in humans, it should be possible to visualize the inflammatory potency of ACPAs by studying the activity of IgE bound to FcεRI-expressing cells. FcεRI is a high-affinity Fc receptor capable of binding monomer IgE molecules. On cross-linking two IgE molecules bound to their Fc receptor, an activatory signal is mediated. Hence, we anticipated that ACPA⁺ RA patients would also harbor FcεRI-bound IgE-ACPAs that can directly trigger FcεRI-expressing cells ex vivo without further manipulation, a phenomenon that would not be present in ACPA⁻ RA. Showing such disease-, phenotype-, and antigen-specific effects would provide compelling evidence for a role of ACPA in the pathogenesis of ACPA⁺ disease and would substantiate the notion that different pathogenic mechanisms are underlying ACPA⁺ and ACPA⁻ RA, as current genetic association studies suggest. Therefore, the aim of the present study is to verify whether IgE-ACPAs can activate FcεRI⁺ immune cells. Using an ex vivo human model, we show that FcεRI⁺ cells from ACPA⁺ RA patients can be directly activated by citrullinated antigens. Moreover, we observe increased levels of histamine within the synovial fluid of ACPA⁺ RA patients as well as increased numbers of mast cells with a degranulated phenotype in the synovia of these patients. Together, these results indicate that ACPAs can directly recruit immune effector mechanisms and provide “translational” evidence for a role of ACPAs and FcεRI⁺ cells in the pathogenesis of RA.

Results

IgE-ACPAs Are Present in RA. IgE antibodies against citrullinated fibrinogen but not against noncitrullinated fibrinogen could be detected in IgG-depleted sera of ACPA⁺ RA patients in contrast to ACPA⁻ RA patients and controls (Fig. 1A). In parallel, a CCP2 ELISA could confirm the presence of IgE-ACPAs in ACPA⁺ RA patients (Fig. 1B). Sixty three percent of IgG-ACPA⁺ RA patients were positive for IgA-ACPAs, and 62% were positive for IgM-ACPAs.

Basophils from ACPA⁺ RA Patients Become Directly Activated After Stimulation with Citrullinated Protein. To determine whether IgE-ACPAs have the potential to activate FcεRI⁺ cells directly, we performed a basophil activation test (BAT) on healthy controls,

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The authors declare no conflict of interest.

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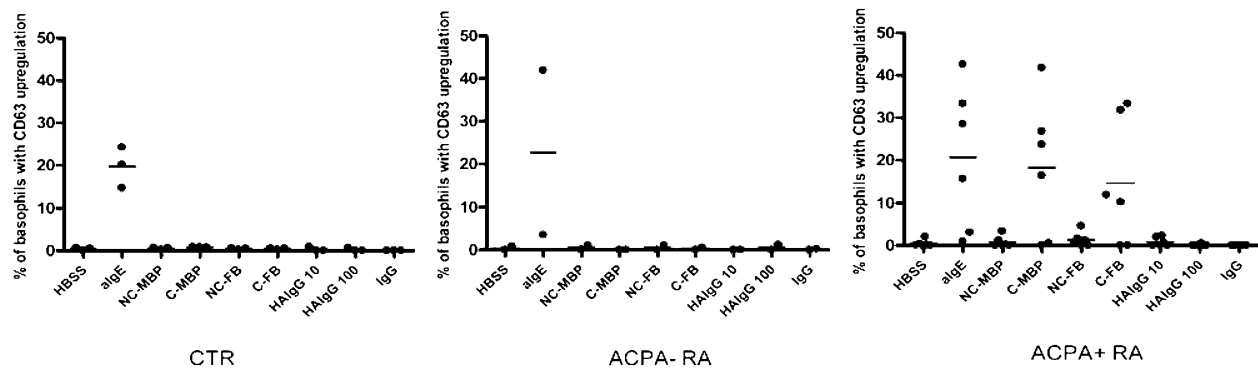


Fig. 3. Basophil activation with citrullinated (C)-MBP, heat-aggregated IgG (HAIG), and IgG. Whole heparinized blood was stimulated with HBSS buffer, 1 μ g/mL anti-IgE, 10 μ g/mL noncitrullinated (NC)-MBP, 10 μ g/mL C-MBP, 10 μ g/mL NC-fibrinogen (FB), 10 μ g/mL C-FB, 10 μ g/mL monomeric IgG, and 10 or 100 μ g/mL HAIG in healthy control (CTR; $n = 3$), ACPA⁻ RA ($n = 2$), and ACPA⁺ RA ($n = 6$) patients. $P = 0.02$ in ACPA⁺ RA vs. CTR and ACPA⁻ RA.

(FITC) was performed (Fig. S2). Basophils from ACPA⁺ RA patients were stained by citrullinated fibrinogen-FITC, whereas basophils of ACPA⁻ RA patients did not bind citrullinated fibrinogen-FITC. Noncitrullinated fibrinogen-FITC did not bind basophils, again demonstrating the potential of basophils from ACPA⁺ RA patients to bind citrullinated antigens.

Enhanced Synovial Mast Cell IgE Expression and Histamine Release in ACPA⁺ RA Patients. The previously discussed results suggest the potential of IgE-ACPAs to activate Fc ϵ RI⁺ immune cells such as mast cells, which are present in synovium of RA patients. Because higher levels of human IgE result in higher expression of both IgE and Fc ϵ RI (10, 11), we next analyzed whether synovial mast cells from ACPA⁺ RA patients display higher levels of both cell-bound IgE and Fc ϵ RI as compared to ACPA⁻ RA patients and osteoarthritis (OA) patients. Significantly higher IgE expression on synovial mast cells of ACPA⁺ RA patients was observed compared with OA patients and ACPA⁻ RA patients (Fig. 6). In parallel, Fc ϵ RI expression was also up-regulated in ACPA⁺ RA patients ($P = 0.04$ vs. ACPA⁻ RA, $P = 0.003$ vs. OA) (Fig. S3).

To verify if activation of mast cells in the synovium had occurred in ACPA⁺ RA patients, histamine levels in synovial fluid from OA, ACPA⁻ RA, and ACPA⁺ RA patients were determined. Histamine levels were significantly increased in ACPA⁺ RA patients compared with controls (i.e., OA and ACPA⁻ RA patients) (Fig. 7). Although total IgE levels in synovial fluid were not different between groups, a significant

correlation between histamine levels and total IgE in synovial fluid was present in ACPA⁺ RA patients only (Fig. S4).

Synovial Mast Cells Are Degranulated in ACPA⁺ RA Patients. To substantiate further the finding that citrullinated proteins might directly activate mast cells and contribute to inflammation, biopsies of inflamed knees of RA patients were microscopically examined. RA synovial biopsies were obtained of 50 patients, of whom 23 were ACPA⁻ and 27 were ACPA⁺; age and disease duration were not significantly different between groups (Table 2). Representative pictures of immunohistochemical staining for CD117 demonstrating c-kit/stem cell receptor on the surface of mast cells, chloroacetate esterase (CAE) staining, and double CD117/CAE staining are shown (Fig. 8). Mast cells were found (although not exclusively) near the synovial vessels. A higher number of mast cells was observed both by semiquantitative scoring and absolute numbers of CD117⁺ mast cells in ACPA⁺ RA patients (Table 2). Intriguingly, when we assessed degranulation by analyzing the difference between CD117 and CAE staining, degranulation was more frequently observed both in absolute as well as relative numbers (i.e., percentage of mast cells) in ACPA⁺ RA synovium compared with ACPA⁻ RA synovium. Overall, these results reveal a higher number of degranulated mast cells in ACPA⁺ RA, suggesting activation of mast cells in ACPA⁺ RA.

Discussion

In this study, we demonstrate that IgE-bearing basophils from ACPA⁺ RA patients are directly activated on exposure to citrullinated antigen. These results most likely reflect the presence of Fc ϵ RI-bound IgE-ACPAs on the surface of basophils, because the high-affinity Fc ϵ RI will bind available free IgE. After cross-linking adjacent IgE molecules by antigen, aggregation of Fc ϵ RI triggers an immediate intracellular signaling process resulting in rapid cellular activation (12). In line with these observations, we also show that IgE-ACPAs are present in sera of ACPA⁺ RA patients, because these sera could readily sensitize an Fc ϵ RI-transfected RBL for recognition of citrullinated antigen. These observations are highly relevant because they show that immune cells from ACPA⁺ patients but not from ACPA⁻ patients or healthy controls can recognize and become activated on binding citrullinated antigens in a highly specific manner.

The presented data do not show that Fc ϵ RI-expressing cells, such as mast cells, are involved in the pathogenesis of ACPA⁺ RA, because the final evidence for this hypothesis can only be obtained by a specific intervention study. However, we believe that our results, obtained from independent lines of evidence, provide a considerable amount of independent data that support the role of IgE-ACPAs and mast cells in the pathogenesis of ACPA⁺ RA. First, only basophils from ACPA⁺ RA patients

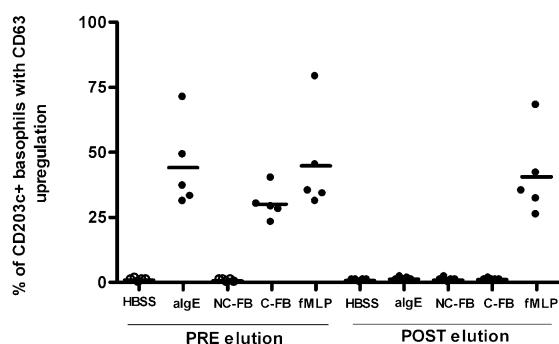


Fig. 4. Elution of IgG. Basophil activation before and after elution of Ig with acid glycine buffer in ACPA⁺ RA patients ($n = 5$). Basophils were stimulated with HBSS buffer, 1 μ g/mL anti-IgE, 10 μ g/mL noncitrullinated fibrinogen (NC-FB), 10 μ g/mL citrullinated fibrinogen (C-FB), and 1 μ g/mL fMLP before and after elution of Ig with acid glycine buffer in ACPA⁺ RA patients ($n = 5$). $P = 0.04$ for C-FB PRE elution vs. C-FB POST elution.

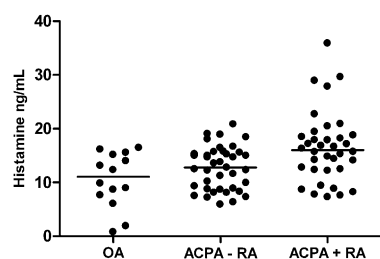


Fig. 7. Histamine levels in RA synovial fluid. Histamine levels (ng/mL) in synovial fluid of OA ($n = 14$), ACPA⁻ RA ($n = 36$), and ACPA⁺ RA ($n = 39$) patients. Bars represent median values. $P = 0.004$ in ACPA⁺ RA vs. OA. $P = 0.008$ in ACPA⁺ RA vs. ACPA⁻ RA.

anti-collagen II-IgE antibodies were observed in several studies (22–24). In contrast to these earlier reports, we now show a direct cellular immune response toward citrullinated antigens.

Because degranulation is the clearest histological hallmark of mast cell activation, the higher number of degranulated mast cells in synovial tissue from ACPA⁺ RA patients as compared with ACPA⁻ RA patients is in keeping with the recent realization that RA represents two main syndromes: ACPA⁺ and ACPA⁻ disease. These two forms of RA are most likely etiologically distinct, because distinct genetic risk factors underlie these distinct disease phenotypes. This notion was initially demonstrated by our finding that different HLA genes predispose to either ACPA⁺ or ACPA⁻ disease (25, 26), and it was subsequently boosted by the results of recent Whole Genome Association (WGA) studies (27). Furthermore, some environmental risk factors underlie only one form of RA but not the other, and the initial evidence is now appearing that ACPA⁺ and ACPA⁻ patients respond differently to therapy (4). Moreover, our observations are in keeping with previous data that RA can be considered a heterogeneous disease with different synovial infiltration patterns (e.g., augmented lymphocytes) (28, 29). Our data further boost this concept by showing that immune cells from ACPA⁺ and ACPA⁻ patients respond differently to citrullinated antigens. Our observation that degranulated mast cell numbers and the level of mast cell products in synovial fluid differ between ACPA⁺ and ACPA⁻ patients provides further support for distinct pathogenic pathways for ACPA⁺ and ACPA⁻ disease.

In conclusion, our data show a direct biological response to citrullinated antigens of immune cells in ACPA⁺ RA patients only. Elucidating this activation pathway mediated by IgE-ACPA in mast cells could lead to previously undescribed treatment strategies in patients with RA.

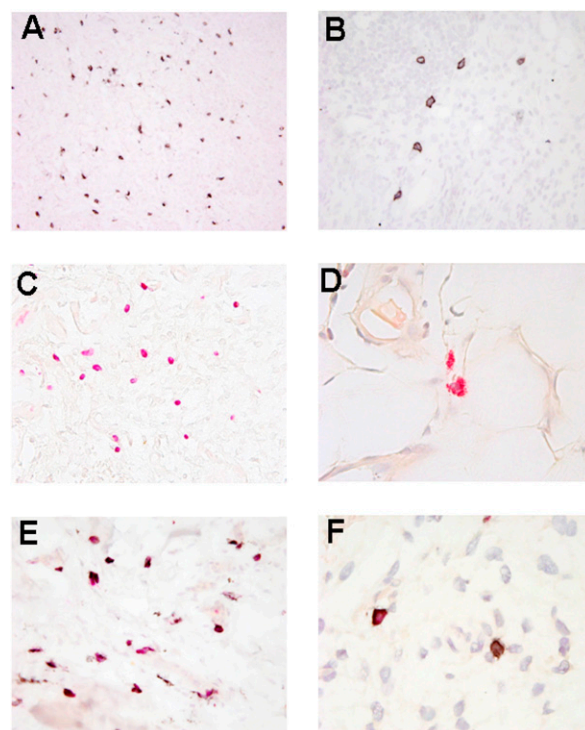


Fig. 8. Microscopic features of the synovial tissue in ACPA⁻ RA and ACPA⁺ RA. (A) CD117 staining of mast cells. (Magnification: $\times 200$.) (B) Staining of surface CD117 on mast cells. (Magnification: $\times 400$.) (C) CAE staining of mast cell. (Magnification: $\times 400$.) (D) CAE staining of granules in mast cells. (Magnification: $\times 1,000$.) (E) Double CAE/CD117 staining (whole granules not visible after antigen retrieval). (Magnification: $\times 400$.) (F) Nondegranulated mast cell positive for CAE and CD117 (Left) and degranulated mast cell positive for CD117 and negative for CAE (Right). (Magnification: $\times 1,000$.)

Materials and Methods

Samples. Fresh peripheral blood was obtained from healthy adult donors (control; $n = 26$) and from patients with established RA ($n = 44$), who were participants in the Leiden Early Arthritis Cohort (EAC) (30, 31). Sera of healthy adult donors ($n = 10$) and RA patients of the EAC ($n = 89$) were used. Synovial fluid was collected from OA patients ($n = 14$) (32) and RA patients ($n = 75$). Synovial tissues of RA patients ($n = 15$) and OA patients ($n = 18$) were obtained from patients undergoing total hip or knee replacement surgery. Synovial biopsies ($n = 50$) were taken from RA patients who underwent therapeutic arthroscopic lavage of an inflamed knee, as described previously (33). Written informed consent was obtained, and the study was approved by the local human ethics committee.

Table 2. Characteristics of synovial biopsies in ACPA⁺ and ACPA⁻ RA patients

	All patients ($n = 50$)	ACPA ⁺ RA ($n = 23$)	ACPA ⁻ RA ($n = 27$)	<i>P</i> value
Age	57 (15)	57 (14)	56 (16)	0.79
Gender, M/F	17/33	8/19	9/14	0.48
Disease duration, years	8 (7)	7 (6)	9 (9)	0.36
Median no. CD117 ⁺ MCs	27 (0–178)	33 (0–178)	11 (0–100)	0.003
MC score CD117 = 0–1 (semiquantitative)	16	4	12	0.004
MC score CD117 = 2–3 (semiquantitative)	34	23	11	
Median no. granulated MCs by CAE	3 (0–86)	4 (0–86)	2 (0–33)	0.63
MC score CAE = 0–1 (semiquantitative)	44	24	20	0.83
MC score CAE = 2–3 (semiquantitative)	6	3	3	
Median no. CAE ⁺ CD117 ⁺ MCs	4 (0–76)	3 (0–75)	6 (0–47)	0.66
Median no. degranulated MCs	21 (0–173)	28 (0–173)	6 (0–67)	0.005
% degranulated MCs	82 (0–100)	89 (0–100)	58 (0–100)	0.03

Results are expressed as mean (SD) or median (minimum–maximum). MC, mast cell, M/F, male/female. Semiquantitative scores are represented as the number of patients with a score of 0 or 1 and the number of patients with a score of 2 or 3.

Detection of Isotype ACPAs. The presence of total IgG-ACPs was tested in all RA patients by an anti-CCP2 ELISA (Eurodiagnostica) according to manufacturer's instructions. IgA-ACPs and IgM-ACPs were measured by a previously described ELISA (34) (see *SI Materials and Methods*).

BAT. As described previously (35), heparinized whole blood was stimulated at 37°C for 20 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 (Jackson ImmunoResearch Laboratories, Inc.), 10 or 100 µg/mL heat-aggregated IgG, HBSS as a negative control, and 1 µg/mL anti-IgE (Pharmingen) as a positive control. Human fibrinogen or MBP was citrullinated as described previously (34).

Elution of Igs. Basophil-bound Ig was eluted with acid elution buffer as previously described (8). As 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 (Molecular Probes), and allophycocyanin (APC)-conjugated CD203c antibodies.

RBL Mediator Release. The sensitization and stimulation of RBLs were performed as described earlier (9) (see *SI Materials and Methods*).

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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 (HA IgG), 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 CaCl₂, 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% CO₂. 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; Bioprote 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-acetyl-beta-D-glucosaminide (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 × 10⁶ 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) CO₂ for 30 min. Subsequently, slides were washed with MilliQ H₂O (Millipore bv) and stained with hematoxylin.

Immunohistochemistry. 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 ($\times 400$ 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.

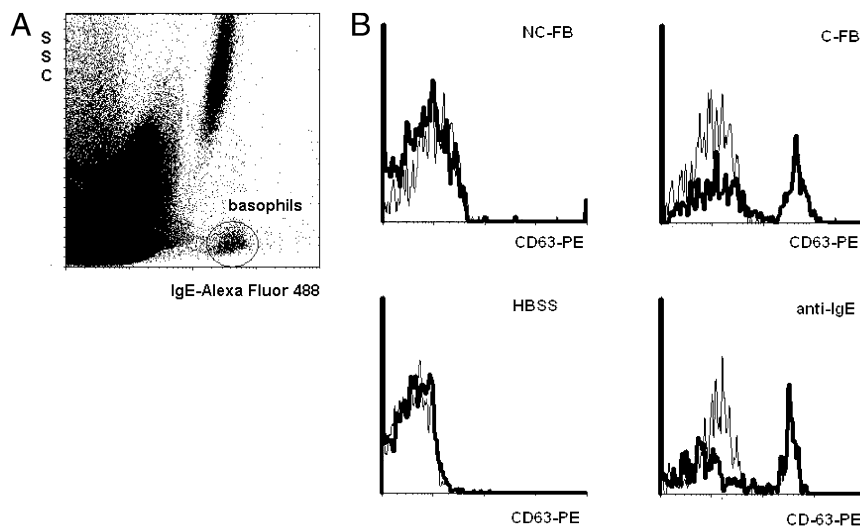


Fig. S1. Flow cytometric analysis of basophil activation. (A) Basophils were identified and gated on a dot plot showing side scatter (SSC) and fluorescence intensity of Alexafluor 488-conjugated anti-IgE. (B) Histograms show fluorescence intensity of CD63-PE (thick line) and isotype-PE (thin line) after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

