

Local Joint Inflammation and Histone Citrullination in a Murine Model of the Transition From Preclinical Autoimmunity to Inflammatory Arthritis

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Objective. Anti-citrullinated protein antibodies (ACPAs) are characteristic of rheumatoid arthritis (RA). However, their presence years before the onset of clinical RA is perplexing. Although multiple putative citrullinated antigens have been identified, no studies have demonstrated the specific capacity of these antigens to initiate inflammatory arthritis. This study was undertaken to recapitulate the transition from preclinical to clinical RA and to demonstrate the capacity of local citrullination to facilitate this transition.

Methods. We performed proteomic analysis of activated human neutrophils to identify citrullinated proteins, including those targeted as part of the RA immune response. Using enzyme-linked immunosorbent assay, we compared RA and osteoarthritis synovial fluid for levels of citrullinated histone H2B and its immune complex. Using macrophage activation assays, we assessed the effect of histone citrullination on immunostimulatory capacity and evaluated the stimulatory capacity of native and citrullin-

ated H2B immune complexes. Finally, we assessed the potential for anti-citrullinated H2B antibodies to mediate arthritis in vivo.

Results. We identified robust targeting of neutrophil-derived citrullinated histones by the ACPA immune response. More than 90% of the RA patients had anti-citrullinated H2B antibodies. Histone citrullination increased innate immunostimulatory capacity, and immune complexes containing citrullinated histones activated macrophage cytokine production and propagated neutrophil activation. Finally, we demonstrated that immunization with H2B was arthritogenic, but only in the setting of underlying articular inflammation.

Conclusion. Our findings indicate that citrullinated histones, specifically citrullinated H2B, are an antigenic target of the ACPA immune response. Furthermore, local generation of citrullinated antigen during low-grade articular inflammation provides a mechanistic model for the conversion from preclinical autoimmunity to inflammatory arthritis.

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Rheumatoid arthritis (RA) is associated with antibodies targeting proteins which have undergone posttranslational modification of arginine to citrulline by a family of enzymes known as peptidylarginine deiminase (PAD) (1–3). Although several lines of evidence implicate these antibodies in RA pathogenesis, the presence of these anti-citrullinated protein antibodies (ACPAs) up to several years before the onset of disease development (4–6) has called into question their direct role in the mediation of synovial inflammation. Similarly, there are multiple citrullinated protein targets of the ACPA immune response with no dominant immunopathogenic antigen identified. Recent studies suggest that proteins generated in multiple pathways of neutrophil activation may serve as antigenic

targets of the ACPA immune response (7–10). However, no studies to date have demonstrated the capacity of these citrullinated products to initiate inflammatory arthritis, nor has a mechanism by which these citrullinated antigens might contribute to the initiation and pathogenesis of RA been elucidated. The presence of neutrophils and their activation products are ubiquitous at sites of inflammation. Thus, we hypothesized that generation of citrullinated products of neutrophil activation could, in the setting of circulating ACPAs, provide the nidus for immune complex generation and the transition from preclinical autoimmunity to clinical RA.

MATERIALS AND METHODS

Sample collection. Serum, plasma, or synovial fluid was obtained from patients with RA, psoriatic arthritis (PsA), or osteoarthritis (OA). All RA patients met the American College of Rheumatology criteria for the disease (11), and all samples were obtained under Institutional Review Board–approved protocols at Stanford University. RA serum was obtained from the VA Palo Alto Health Care system ($n = 62$) or the Auto-immune Biomarkers Collaborative Network cohort of the North American Rheumatoid Arthritis Consortium ($n = 123$) (12). RA, OA, and PsA synovial fluid specimens for quantitation of histone H2B immune complexes were obtained at the VA Palo Alto by the investigators (WHR and JS) or were a generous gift from Dr. David Lee (Brigham and Women's Hospital, Boston, MA), while RA and OA synovial fluid specimens for measurement of H2B levels were obtained as described above, with additional samples purchased from Bioreclamation.

Generation and proteomic interrogation of products of neutrophil activation. Human neutrophils were isolated as described below. Products of neutrophil activation were generated by incubating 3×10^7 neutrophils with $10 \mu\text{M}$ ionomycin, 30 nM phorbol myristate acetate (PMA), or 200 ng/ml tumor necrosis factor (TNF) for 4 hours at 37°C . After removing supernatants, each dish was washed, and the products of neutrophil activation were digested with 10 units/ml of micrococcal nuclease. Samples were centrifuged at $300g$ to remove intact cells, then at $4,000g$ to remove debris. Neutrophil activation and generation of neutrophil extracellular traps was visualized by staining with DAPI, antineutrophil elastase, or anti-citrullinated H3 (Abcam). Alternatively, neutrophil activation was quantitated by measurement of DNA content in the supernatant or by incubation with Sytox green (Invitrogen) followed by measurement of fluorescence at 485 nm (excitation)/ 520 nm (emission).

The products of neutrophil activation induced by ionomycin were separated on parallel sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) gels and stained with Coomassie blue or transferred to PVDF membranes followed by probing with ACPA-positive RA serum IgG, anti-modified citrulline antibody (Millipore), or rabbit anti-H2B polyclonal antibody (Abcam). Coomassie-stained bands corresponding to bands identified by RA IgG and/or anti-modified citrulline were cut from the gel, digested with trypsin, and subjected to analysis by mass spectrometry as previously described (13,14). To confidently identify citrullinated residues, proteomic analysis of products from ionomycin-activated neutrophils was

performed using a filter-aided sample preparation protocol (15) followed by cation exchange chromatography and mass spectrometry as previously described (7).

Immunoprecipitation and immunoblot analysis. Products of neutrophil activation were denatured at 95°C in the presence of 0.1% SDS, 0.5 mM EDTA, and 1 mM dithiothreitol (DTT), incubated with anti-H2B antibody or human RA IgG, then with protein G beads. Beads were eluted by boiling, and proteins were separated by SDS-PAGE and transferred to PVDF membranes, and citrullinated proteins were identified using an anti-modified citrulline antibody (Millipore). Anti-modified citrulline blot was stripped (and re-exposed to confirm stripping), reblocked with 5% milk, and reprobed with rabbit anti-H2B antibody directly conjugated to horseradish peroxidase (HRP) (Abcam).

Detection of antibodies to native H2B and citrullinated H2B. Detection of antibodies to native H2B or citrullinated H2B, as well as a panel of 21 additional citrullinated epitopes (or arginine-containing controls), was performed using a bead-based direct immunoassay as previously described (6).

Measurement of H2B, citrullinated H2B, and H2B immune complexes in RA serum or RA synovial fluid. Levels of citrullinated H2B protein were measured using a novel enzyme-linked immunosorbent assay (ELISA) developed in our laboratory. Plates were coated with $2 \mu\text{g/ml}$ of rabbit anti-H2B capture antibody (Abcam), washed, blocked with 2% bovine serum albumin (BSA), washed again, and incubated with RA or OA synovial fluid diluted 1:20 in phosphate buffered saline (PBS) containing 0.1% BSA and 0.05% Tween 20. Plates were washed and incubated with $2 \mu\text{g/ml}$ of mouse anticitrulline antibody (clone F95; Millipore) followed by incubation with an HRP-conjugated anti-mouse IgM. Levels of H2B immune complex were measured using a C1q capture assay as previously described (13).

H2B immunization studies. All animal studies were performed in adherence to the Guide for the Care and Use of Laboratory Animals and under protocols approved by the VA Palo Alto Institutional Animal Care and Use Committee. DBA/1J mice (The Jackson Laboratory) were immunized with adjuvant alone, $50 \mu\text{g}$ of bovine type II collagen (low dose collagen-induced arthritis [CIA]), $125 \mu\text{g}$ of native or in vitro-citrullinated H2B, or both low-dose collagen and native or citrullinated H2B. Briefly, DBA/1J mice were immunized intradermally in the proximal tail with the above immunogens emulsified in Freund's complete adjuvant. Twenty-one days after immunization, mice were boosted by subcutaneous injection at the base of the tail with the listed immunogens in Freund's incomplete adjuvant. Each limb was graded on a scale of 0–4 (maximum possible score of 16 for each mouse). Hind paw thickness was measured with microcalipers. At the termination of the experiment, pathologic changes were scored by 2 investigators (WHR and JS) who were blinded with regard to immunization condition, as previously described (16). Results presented represent outcomes of experiments performed at least twice.

Serum-transfer experiments. DBA/1J mice were immunized with $125 \mu\text{g}$ of citrullinated H2B or adjuvant alone, followed by booster injection on day 21 as described above. Mice were killed and blood was obtained on experimental day 50, and serum was confirmed to have antibody reactivity with citrullinated H2B. Twenty DBA/1J mice were immunized with $50 \mu\text{g}$ of bovine type II collagen and boosted on day 21 as described above (low-dose CIA). Mice with a score of >4 on day 28 were excluded.

ed, leaving 8 mice per group. To assure randomization, mice with low-dose CIA were ranked by level of disease on day 28 and matched to a mouse with a similar arthritis score before receiving citrullinated H2B or control serum. On days 28 and 30, mice with low-dose CIA received 300 μ l of citrullinated 2B or control serum via intraperitoneal injection. Mice were evaluated and scored for arthritis 3 times weekly until termination (day 46). Mice that failed to manifest any arthritis during the course of the study were excluded, leaving 6 mice analyzed per group.

Cell isolation and culture. Bone marrow-derived macrophages were isolated from wild-type C57BL/6, Toll-like receptor 4 (TLR-4)-deficient B6.B10ScN-*Tlr4^{ps-del}*/JthJ mice, TLR-2-deficient mice (The Jackson Laboratory), or TLR-9-deficient mice (a gift from Dr. Lawrence Steinman, Stanford University) and cultured as previously described (17). Generation of human monocyte-derived macrophages was performed as previously described (18). Neutrophils were isolated from fresh whole blood or buffy coats using the dextran/Ficoll method as previously described (19).

Antibodies and reagents. Lipopolysaccharide (LPS) was from Sigma-Aldrich, and CpG, palmitoyl-3-cysteine-serine-lysine-4 (Pam₃CSK₄), and TLR-4 inhibitor CLI-095 were from InvivoGen. Anti-human CD32 (Fc γ receptor IIa [Fc γ RIIa]) antibody (clone IV.3) was from Stem Cell Technologies. Total histone, as well as histones H1, H2A, H2B, and H3, all purified from calf thymus, were purchased from ImmunoVision. Histones were used in either unmodified native form or citrullinated form. In vitro citrullination was performed as previously described (18) and confirmed by mobility shift on SDS-PAGE and by Western blotting using human ACPA-positive RA sera as well as anti-modified citrulline antibody (Millipore). Native histones underwent sham citrullination in an identical manner to citrullinated proteins but without the addition of the PAD enzyme (Sigma-Aldrich). PAD enzyme incubated with citrullination buffer and DTT but without histones served as a control to assure no contribution or contamination from the small amount of PAD enzyme remaining in histone citrullination reaction mixtures.

Macrophage stimulation. Human macrophages (5×10^4 /well) or murine macrophages (8×10^4 /well) were incubated with native or citrullinated H1, H2A, H2B, or H3 for 16 hours, and TNF levels in supernatants were determined by ELISA (PeproTech). The TLR-4 ligand LPS (1 ng/ml) and TLR-2 ligand Pam₃CSK₄ (10 ng/ml) served as controls for TNF induction by TLR-4 and TLR-2, respectively. Immune complexes were generated in vitro by incubation of native or citrullinated histone (total histones, H2A, H2B, or H3) with a polyclonal rabbit antibody against H2A (Abcam), H2B (eBioscience), H3 (Abcam) or, as a control, with normal polyclonal rabbit IgG (DakoCytomation) at 37°C for 45 minutes. Cross-titration of antibody and antigen yielded an optimal ratio for formation of immune complexes; final concentrations of 20 μ g/ml of histones and 10 μ g/ml of polyclonal antibody were used for immune complex stimulation of monocyte-derived macrophages. Given a molar excess of histone antigen, this likely generates relatively small plate-bound complexes. At final dilutions, all reagents used in the stimulation assays were tested for endotoxin contamination by application of the *Limulus* ameobocyte assay (Associates of Cape Cod), and were shown to possess endotoxin levels below the detectable range (<0.03 endotoxin units/ml). To further exclude endotoxin contamination in select experiments, histones were treated with endotoxin-removal resin

(Detoxi-Gel) or stimulations were performed in the presence of 15 μ g/ml of polymyxin B (Sigma-Aldrich) or after treatment of histones with proteinase K followed by boiling.

For immune complex stimulation of human macrophages, human IgG derived from patients with ACPA-positive RA was used to generate plate-bound human histone immune complexes (total histones, citrullinated H2A, citrullinated H2B, or citrullinated H3). IgG from 3 pooled plasma samples shown by ELISA to contain high levels of anti-citrullinated H2A and citrullinated H2B antibodies was purified on protein G columns (Pierce). Eluted IgG fractions were concentrated and buffer exchanged to PBS (Amicon Ultra) and depleted of endotoxin over an endotoxin-removal column (Detoxi-Gel). IgG concentrations were estimated by optical density at 280 nm; IgG was divided into aliquots and stored at -80°C. For generation of histone immune complexes, flat-bottomed 96-well culture plates were coated with 50 μ l of total histones, citrullinated H2A, citrullinated H2B, or citrullinated H3 (20 μ g/ml), washed, and blocked with 2% low endotoxin BSA, washed, and incubated with 100 μ l of ACPA-positive IgG (1.0 mg/ml) in PBS containing 0.05% Tween 20 or, as a control, IgG isolated from ACPA-negative patients with OA. Wells were again washed and human macrophages (5×10^4 /well) in 200 μ l RPMI containing 10% fetal bovine serum were then added to the wells. To block TLR-4 activation, macrophages were preincubated for 1 hour with the small-molecule TLR-4 inhibitor CLI-095 (InvivoGen). Since prior studies have demonstrated ACPA immune complex-induced macrophage activation to be Fc γ RIIa dependent (20), an anti-human CD32 (Fc γ RIIa) antibody (clone IV.3; Stem Cell Technologies) was used to block Fc γ R activation.

Quantification of products of neutrophil activation.

Freshly isolated neutrophils in RPMI without phenol red were seeded into 96-well plates (5×10^4 cells/well) and incubated with PBS, PMA, ionomycin, LPS, native H2B, citrullinated H2B, native H2B immune complexes, or citrullinated H2B immune complexes for 4–8 hours in the presence of 1 μ M Sytox green (Invitrogen), a non-cell-permeant DNA binding dye, to detect extracellular DNA. The plates were read in a fluorescence microplate reader at 485 nm (excitation)/520 nm (emission).

Statistical analysis. For in vitro cell stimulation assays, an unpaired *t*-test was used to compare cytokine production between groups. Kruskal-Wallis one-way analysis of variance (ANOVA) with Dunn's post hoc test was used to evaluate the association of anti-citrullinated H2B antibodies with circulating TNF levels. Differences in arthritis time points as well as arthritis scores and histology scores were compared by Mann-Whitney 2-tailed U test. These analyses were performed using GraphPad Prism software version 5.

RESULTS

Targeting of the products of neutrophil activation by the autoantibody response in RA. There are multiple pathways that can result in neutrophil activation and histone citrullination, including apoptosis, NETosis, and necroptosis/autophagy, as well as immune-mediated membranolytic pathways, mediated by perforin and the membrane attack complex (7). We tested different stimuli for activating neutrophils, including PMA, ionomycin, LPS, and TNF. We found that ionomycin-activated

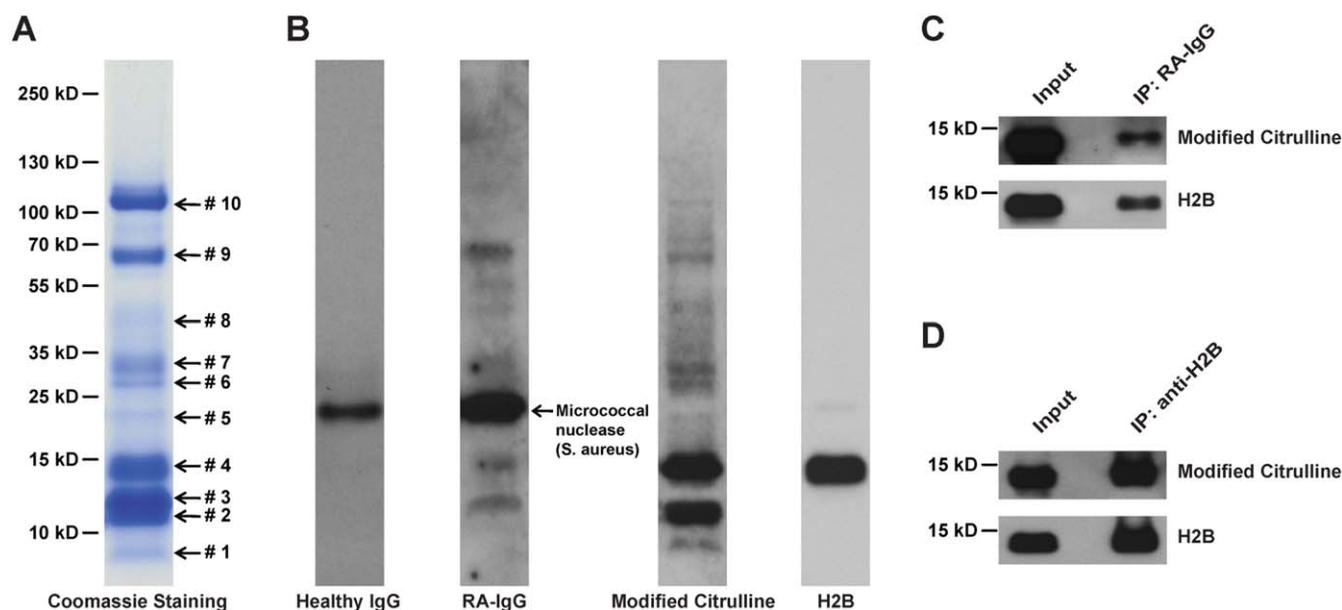


Figure 1. Proteomic analysis of the products of neutrophil activation targeted by the rheumatoid arthritis (RA) immune response. **A** and **B**, Generation of proteins upon neutrophil activation. Neutrophil activation was induced with $10 \mu\text{M}$ ionomycin, and protein products were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and **A**, stained with Coomassie blue or **B**, immunoblotted with healthy IgG, isolated RA IgG, anti-modified citrulline antibody (after citrullinated residues were acid modified), or anti-histone H2B antibody. Mass spectroscopic identification of proteins in **A** is shown in Supplementary Table 1 (available on the *Arthritis & Rheumatology* web site at <http://onlinelibrary.wiley.com/doi/10.1002/art.39283/abstract>). Note that band 5 with prominent staining by RA IgG was confirmed to be micrococcal nuclease (thermonuclease; *Staphylococcus aureus*) used in the digestion of neutrophil activation products. **C**, Immunoprecipitation (IP) of products of neutrophil activation with human RA IgG. **D**, IP of products of neutrophil activation with human anti-H2B antibody and immunoblotting with anti-modified citrulline antibody or anti-H2B antibody.

neutrophils generated a more robust pattern of citrullinated proteins as compared to PMA, while we observed negligible production of citrullinated proteins upon activation by LPS or TNF. Because of its robust and reproducible pattern of citrullination, and to assure the broadest profile of neutrophil activation, ionomycin was used to induce neutrophil activation in subsequent experiments.

We demonstrated the generation of multiple proteins upon neutrophil activation (Figure 1A) (see Supplementary Tables 1 and 4, available on the *Arthritis & Rheumatology* web site at <http://onlinelibrary.wiley.com/doi/10.1002/art.39283/abstract>), including several proteins targeted by ACPA-positive RA IgG (Figure 1B), the majority of which were identified as citrullinated, as assessed by staining with an anti-modified citrulline antibody (Figure 1B). The citrullinated proteins targeted by ACPA-positive RA IgG included the most prominent bands (as defined by densitometry) at 14 kD and 13 kD, subsequently found by mass spectrometry to contain histones, predominantly H2B and H2A, respectively (see Supplementary Table 1, available on the *Arthritis & Rheumatology* web site at <http://onlinelibrary.wiley.com/doi/10.1002/art.39283/abstract>). The identity of H2B was confirmed using a parallel immunoblot probed with a

polyclonal anti-H2B antibody (Figure 1B). Furthermore, immunoprecipitation with either purified human IgG derived from a pool of RA patients or a rabbit polyclonal anti-H2B antibody generated bands at 14 kD which were recognized by both anti-modified citrulline antibody and anti-H2B antibody (Figures 1C and D). Though it is possible that anti-modified citrulline antibodies as well as human RA IgG could recognize other modifications, including carbamylation, use of unbiased mass spectroscopy neutrophil activation products identified citrullination of neutrophil-derived H2B and, to a lesser extent H2A and H3, but not carbamylated histones (Supplementary Table 2, available on the *Arthritis & Rheumatology* web site at <http://onlinelibrary.wiley.com/doi/10.1002/art.39283/abstract>).

Targeting of citrullinated H2B by the ACPA immune response in vitro and in vivo. Guided by proteomic studies of activated human neutrophils, we evaluated plasma and synovial fluid derived from RA patients for the presence of autoantibodies to native or citrullinated H2B. The vast majority of anti-cyclic citrullinated peptide 2 (anti-CCP-2)-positive RA patients possessed antibodies targeting citrullinated H2B, while no such antibodies were found in patients with OA or anti-CCP-2-negative RA ($P < 0.0001$)

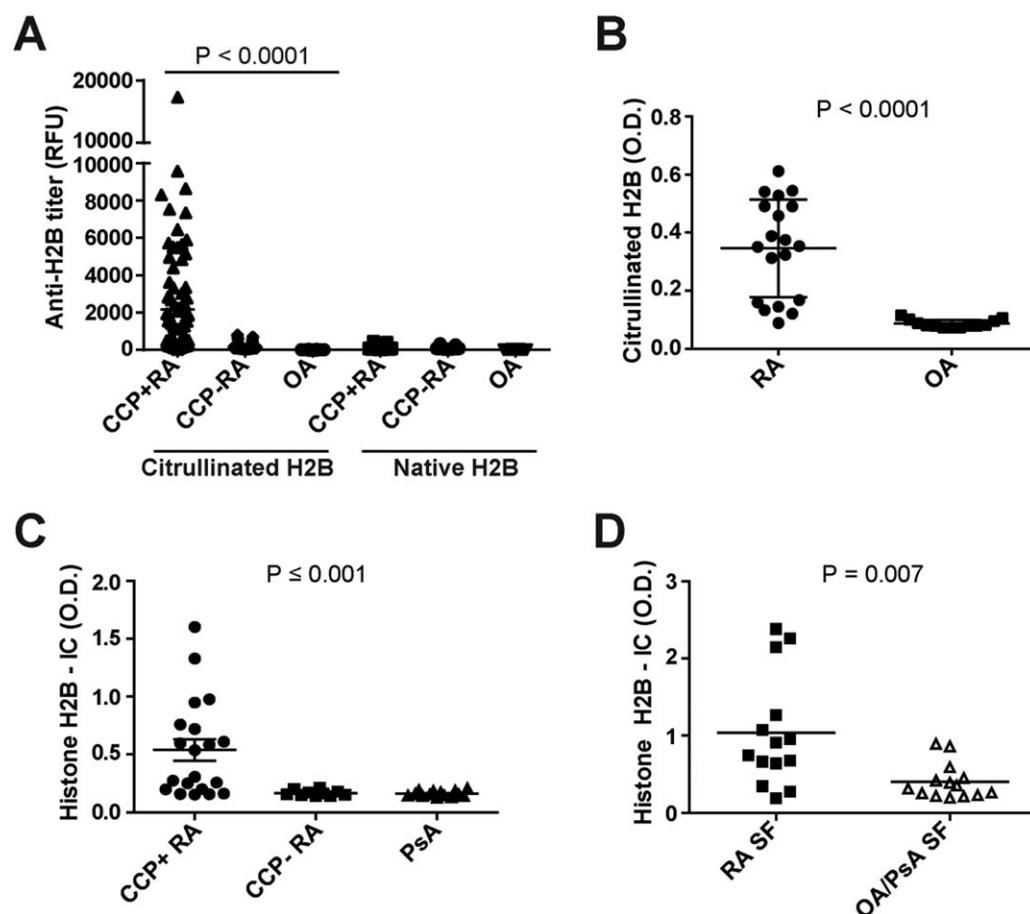


Figure 2. Prominent targeting of citrullinated histone H2B by rheumatoid arthritis (RA)-associated autoantibodies. **A**, Bead-based immunoassay analysis of anti-citrullinated H2B and anti-native H2B autoantibodies in plasma from patients with anti-cyclic citrullinated peptide 2 (anti-CCP-2)-positive RA ($n = 81$), anti-CCP-2-negative RA ($n = 85$), and osteoarthritis (OA; $n = 36$). Symbols represent individual subjects. **B**, Citrullinated H2B enzyme-linked immunosorbent assay analysis of synovial fluid (SF) samples from patients with RA ($n = 19$) or OA ($n = 13$). **C** and **D**, Analysis of H2B immune complexes (ICs) in plasma from patients with anti-CCP-2-positive RA ($n = 20$), anti-CCP-2-negative RA ($n = 10$), or psoriatic arthritis (PsA; $n = 14$) (**C**) and synovial fluid samples from patients with RA ($n = 14$), OA ($n = 10$), or PsA ($n = 4$) (**D**). In **B–D**, symbols represent individual subjects; horizontal and vertical lines show the mean \pm SD. RFU = relative fluorescence units.

(Figure 2A). There was no significant targeting of native H2B in any group (Figure 2A). Consistent with an influx of neutrophils and ongoing neutrophil activation, we observed significantly elevated levels of citrullinated H2B ($P < 0.0001$) (Figure 2B) in RA compared with OA synovial fluid as measured by a citrulline-specific H2B ELISA.

We then measured levels of H2B-containing immune complexes in plasma and synovial fluid from patients with RA, OA, or PsA. Since only citrulline-modified H2B is recognized by RA sera, it was inferred that these immune complexes contained citrullinated H2B (as demonstrated in Figures 1B–D). Such circulating H2B immune complexes were detectable in the plasma of more than half of the anti-CCP-2-positive RA patients tested but not in the plasma of patients with anti-CCP-2-negative RA or PsA (Figure 2C). Similarly, levels of H2B

immune complexes were elevated in the majority of synovial fluid specimens derived from patients with anti-CCP-2-positive RA but not from patients with OA or PsA ($P = 0.007$) (Figure 2D).

Immunization of mice with native or citrullinated H2B is arthritogenic, but only in the setting of low-grade joint inflammation. Having identified citrullinated H2B as a possible target of the RA immune response, we sought to demonstrate that autoimmunity against citrullinated H2B can directly mediate inflammatory arthritis. We immunized DBA/1J mice, with resultant induction of a robust anti-citrullinated H2B antibody response (Figure 3), but without visible induction of arthritis (Figures 4A–C). We hypothesized that a lack of citrullinated H2B expression in the intact joint would not allow generation of citrullinated H2B immune

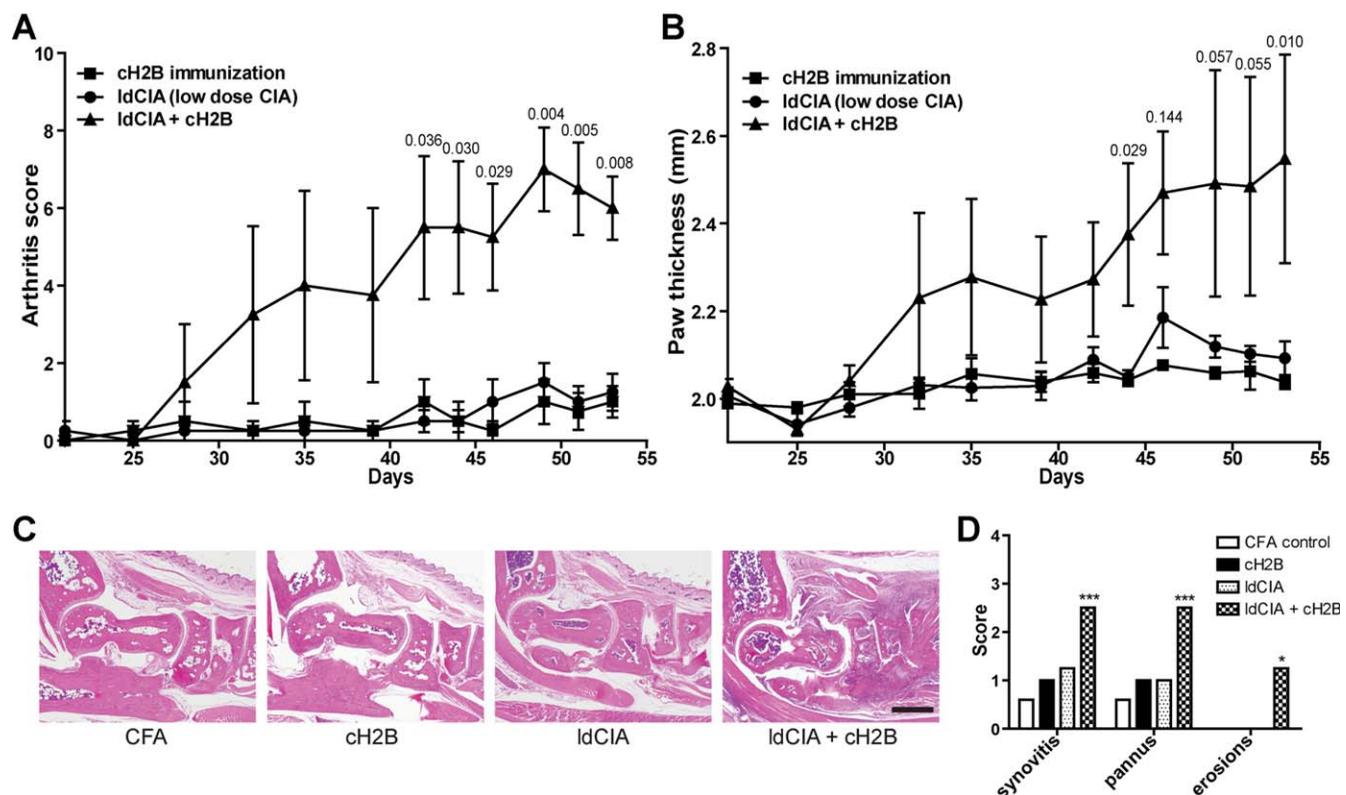


Figure 3. Immunization with citrullinated histone H2B (cH2B) in the presence of low-grade joint inflammation. **A** and **B**, Arthritis score (**A**) and paw swelling (**B**) over time in mice immunized with adjuvant alone (Freund's complete adjuvant [CFA]), citrullinated H2B alone, low-dose type II collagen (low-dose collagen induced arthritis [IdCIA]), or a combination of low-dose collagen and citrullinated H2B ($n = 5$ mice per group). Values are the mean \pm SD. **C**, Representative images of hematoxylin and eosin-stained sections of ankle joints from mice immunized as described in **A** and **B**. Bar = 500 μ m. **D**, Histologic scores for inflammation, pannus formation, and bone or cartilage erosions of ankle joints from mice immunized as described in **A** and **B**. Bars show the mean. * = $P < 0.05$; *** = $P < 0.001$ versus CFA.

complexes and that the conversion from asymptomatic autoimmunity to clinical RA could be the result of a low-grade articular inflammatory response resulting in the generation of intraarticular citrullinated H2B. Supporting this hypothesis, when mice were co-immunized with low-dose type II collagen (low-dose CIA) and citrullinated H2B, we observed development of robust arthritis in the co-immunized mice compared to those immunized with citrullinated H2B alone or low-dose collagen alone ($P < 0.01$) (Figure 4). This effect was observed despite generation of similar levels of anti-citrullinated H2B antibodies and anti-type II collagen antibodies, respectively (Figure 3A).

Using a multiplex ACPA antigen array, we observed a dominant antibody response targeting citrullinated H2B and the closely related citrullinated H2A. We also observed a process of epitope spreading in both citrullinated H2B and low-dose CIA/citrullinated H2B co-immunized mice, with a pattern similar to that observed in studies of preclinical human RA (6) yet,

again, without development of arthritis in mice immunized with citrullinated H2B alone (Figures 3B and C) (see Supplementary Figure 1, available on the *Arthritis & Rheumatology* web site at <http://onlinelibrary.wiley.com/doi/10.1002/art.39283/abstract>). We observed very little cross-targeting of native H2B (Figures 3B and C), thus confirming generation of a predominantly citrulline-specific immune response closely recapitulating the human phenotype.

Notably, immunization of mice with native H2B resulted in generation of robust immunoreactivity with both native and citrullinated H2B and was associated with similar augmentation of low-grade arthritis as was observed with citrullinated H2B immunization (data not shown). This is consistent, given the generation of both native and citrullinated histones in the setting of low-grade inflammation. It is also possible that native H2B immunization generates antibodies that are cross-reactive with both native and citrullinated H2B, though our data demonstrate minimal targeting of native H2B

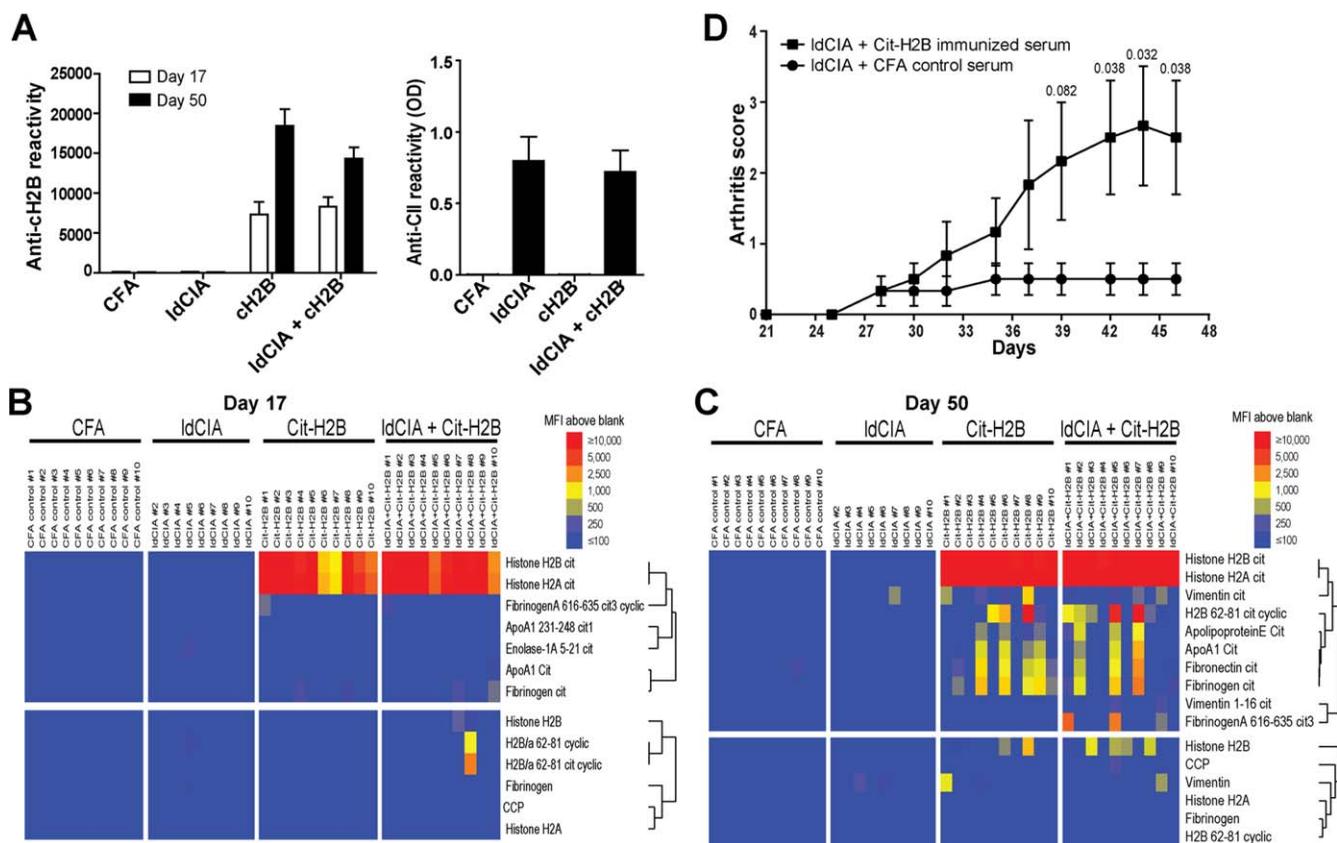


Figure 4. Assessment of immunoreactivity developed after immunization with citrullinated histone H2B (cH2B) and/or low-dose type II collagen (low-dose collagen-induced arthritis [IdCIA]). **A**, Similar levels of anti-citrullinated H2B and anti-type II collagen (CII) antibodies on day 17 and at experimental termination (day 50) in mice receiving a single immunization or co-immunization. Bars show the mean \pm SD. **B** and **C**, Multiplex immunoassay of a panel of citrullinated antigens and their noncitrullinated controls. Significance analysis of microarray comparison between groups identified anti-citrullinated protein antibody (ACPA) subtypes elevated before boost (day 17) (**B**) and at experimental termination (day 50) (**C**) (n = 9–10 per group). **D**, Exacerbation of arthritis in mice with serum transferred from mice immunized with citrullinated H2B compared with mice with serum from mice immunized with adjuvant alone. Values are the mean \pm SD (n = 6 mice per group). CFA = Freund's complete adjuvant; MFI = mean fluorescence intensity; Apo A-1 = apolipoprotein A-1; CCP = cyclic citrullinated peptide.

upon citrullinated H2B immunization (Figure 3), and we did observe relatively more cross-reactivity with citrullinated H2B upon native H2B immunization (see Supplementary Figure 1C, available on the *Arthritis & Rheumatology* web site at <http://onlinelibrary.wiley.com/doi/10.1002/art.39283/abstract>). However, as demonstrated above, human RA is not associated with anti-native histone reactivity. Thus, although native histone immunization of mice does not recapitulate well the human phenotype of anticitrulline autoimmunity, it does further suggest the capacity of low-grade inflammation to generate local antigen immune complexes in the setting of circulating autoantibodies. Finally, we observed that transfer of ACPA-containing serum from mice immunized with citrullinated H2B (Figure 3C) was able to significantly enhance low-grade arthritis compared to control serum (Figure 3D), but again, only in

the setting of an underlying low-grade joint inflammation and generation of intraarticular protein citrullination.

Enhancement of innate immunostimulatory capacity by histone citrullination. Given the plethora of potential citrullinated antigens, we sought to identify mechanisms by which anticitrullinated histone autoreactivity might result in chronic arthritis. Recent studies have identified the ability of extracellular histones to act as mediators of innate immunity (21), including stimulation of cytokine production via TLRs (21–24). Thus, we assessed the stimulatory capacity of extracellular histones and, specifically, the effect of citrullination on stimulatory potency. We observed dose-dependent stimulation of TNF production from human monocyte-derived macrophages stimulated with total histones as well as individually purified mammalian histones with increased stimulation by citrullinated his-

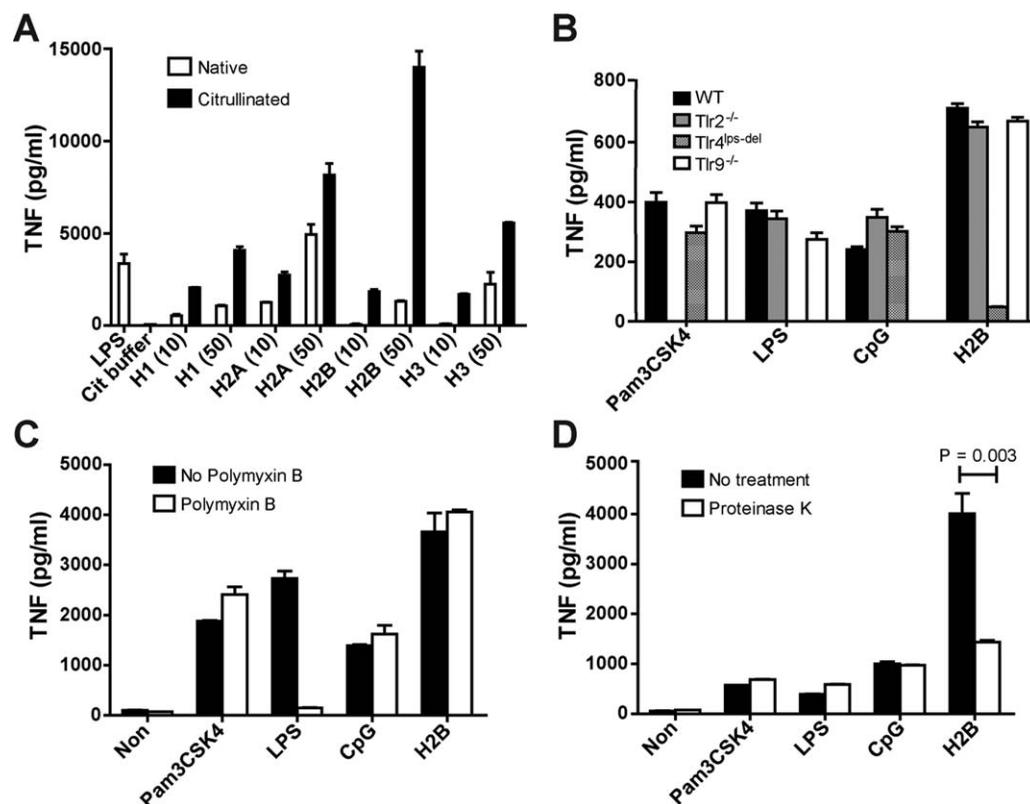


Figure 5. Effect of histone citrullination on innate immunostimulatory capacity. **A** and **B**, Enzyme-linked immunosorbent assay (ELISA) quantification of tumor necrosis factor (TNF) production from human macrophages stimulated with native or citrullinated histones (**A**) or murine macrophages deficient in Toll-like receptor 2 (TLR-2), TLR-4, or TLR-9 (**B**). **C** and **D**, ELISA quantification of TNF production from murine macrophages stimulated with histone H2B in the presence of polymyxin B (**C**) or with H2B after treatment with proteinase K and boiling (**D**). Bars show the mean \pm SD. LPS = lipopolysaccharide; WT = wild-type; Pam₃CSK₄ = palmitoyl-3-cysteine-serine-lysine-4.

tones, most prominently by citrullinated H2B compared to unmodified proteins (Figure 5A). Using macrophages derived from mice deficient in TLR-2, TLR-4, or TLR-9, we observed that H2B-induced TNF production is TLR-4 dependent (Figure 5B), an effect that was not abrogated by the addition of the endotoxin chelator polymyxin B (Figure 5C) but was significantly abrogated after treatment with proteinase K and boiling ($P = 0.003$) (Figure 5D). Interestingly, H2B (Type 1-M) contains 126 amino acids including 8 arginine residues and thus has the potential for citrullination at multiple sites. By mass spectroscopy, only 2 of these arginines were determined to be citrullinated during the process of neutrophil activation, and this pattern of citrullination was closely reproduced by our *in vitro* citrullination (see Supplementary Tables 2 and 3, available on the *Arthritis & Rheumatology* web site at <http://onlinelibrary.wiley.com/doi/10.1002/art.39283/abstract>).

Immune complexes containing citrullinated histones activate macrophages via costimulation of TLR-4 and Fc γ R. To examine whether incorporation of citrullinated histones into immune complexes further enhances

their ability to stimulate cytokine production, we stimulated human macrophages with *in vitro*-generated citrullinated histone or citrullinated H2B immune complexes with or without inhibitors of TLR-4 and/or Fc γ RIIIa. We demonstrated significantly increased induction of macrophage TNF production in response to histone immune complex generated from polyclonal anti-H2B antibody and either native or citrullinated total histones (Figure 6A). Despite similar specificity of this polyclonal antibody for native and citrullinated H2B, we observed significantly increased stimulation by citrulline-containing immune complexes generated in this model. To more closely mimic disease physiology, citrullinated H2B immune complex was generated using human IgG derived from a pool of ACPA-positive RA patients demonstrated to have autoantibody reactivity with citrullinated H2B. Incubation of RA IgG with plate-bound citrullinated H2B isolated only specific ACPAs without the inclusion of nonspecific IgG. Citrullinated H2B immune complex was a powerful inducer of TNF from human monocyte-derived macrophages (Figure 6B). Similar effects were

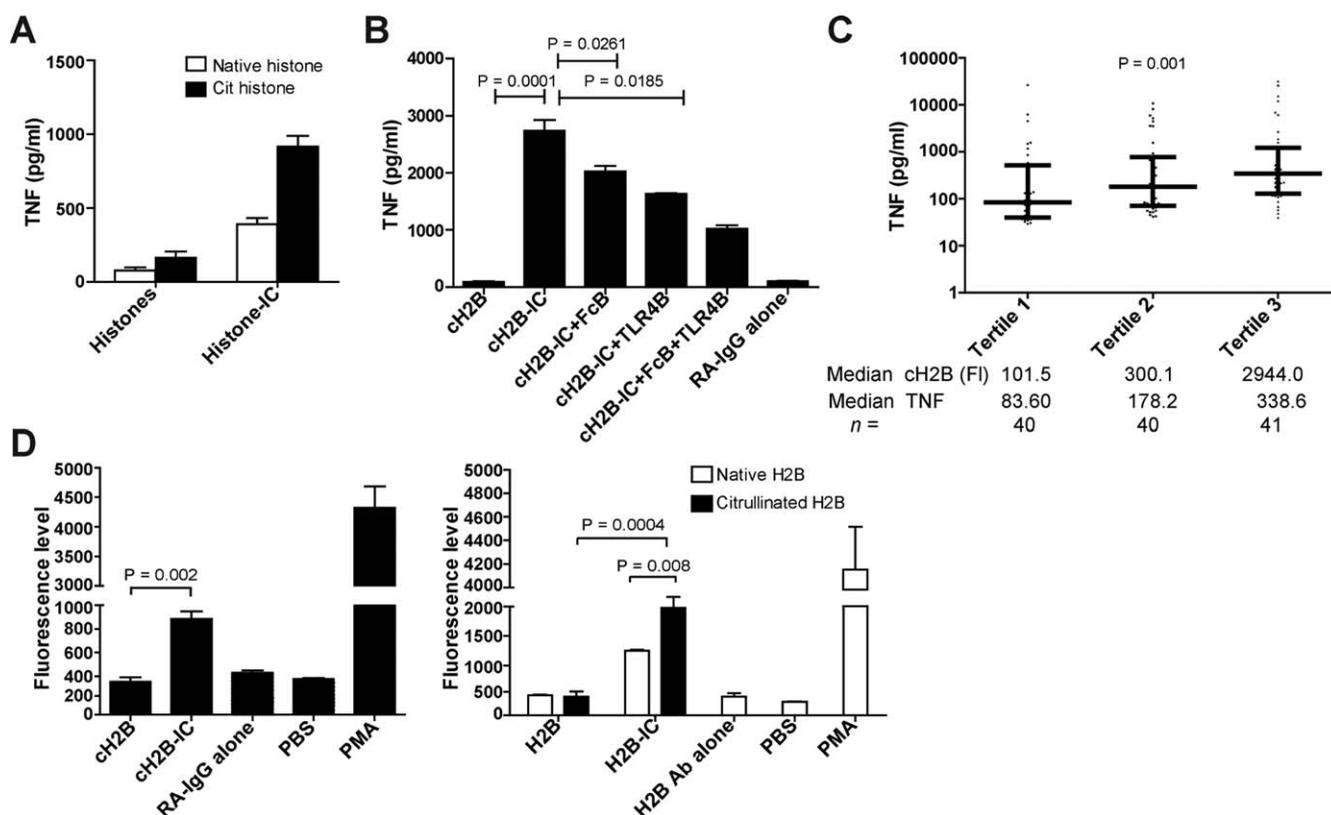


Figure 6. Stimulation of macrophage activation and propagation of neutrophil activation by citrullinated histone-containing immune complexes. **A**, Enzyme-linked immunosorbent assay (ELISA) quantification of human macrophage tumor necrosis factor (TNF) production in response to native or citrullinated histone immune complex generated with a polyclonal anti-histone H2B antibody. **B**, ELISA quantification of human macrophage TNF production in response to citrullinated H2B immune complex (cH2B IC) generated with human rheumatoid arthritis (RA) patient-derived IgG with blockade of Fc γ receptor IIa (FcB), Toll-like receptor 4 (TLR4B), or both. **C**, ELISA quantitation of serum TNF levels from RA patients across tertiles of anti-citrullinated H2B antibody levels. Symbols represent individual patients; horizontal and vertical bars show the mean \pm SD. **D**, Sytox green quantitation of neutrophil activation in response to citrullinated H2B immune complex generated with human RA IgG, or native and citrullinated H2B immune complexes generated with polyclonal anti-H2B antibody (Ab). PBS = phosphate buffered saline; PMA = phorbol myristate acetate. Bars in **A**, **B**, and **D** show the mean \pm SD.

observed for highly homologous citrullinated H2A immune complex but not citrullinated H3 immune complex (data not shown), demonstrating specific reactivity with select citrullinated histones by the RA immune response.

To investigate the requirement of TLR-4 and/or Fc γ R in the stimulation of macrophage TNF production, we evaluated the effect of the small-molecule TLR-4 inhibitor CLI-095 and/or inhibitory antibodies targeting Fc γ RIIa. Inhibition of either TLR-4 or Fc γ RIIa resulted in significant abrogation of the macrophage response to citrullinated H2B immune complex, with further abrogation upon combined inhibition of the TLR-4 and Fc γ R pathways (Figure 6B). These results suggest that immune complexes containing specific citrullinated histones serve as potent inducers of macrophage TNF production by costimulation of TLR-4 and Fc γ R.

To determine whether anti-citrullinated H2B immunoreactivity is associated with increased inflammation in human RA, we evaluated whether levels of anti-citrullinated H2B antibodies were associated with levels of circulating cytokines and/or RA disease activity. Increasing tertiles of anti-citrullinated H2B titers were associated with increasing levels of circulating TNF (Figure 6C), further supporting the immunostimulatory capacity of citrullinated H2B immune complexes. A similar observation was made for interleukin-6 ($P = 0.030$ for the comparison of tertiles of anti-citrullinated H2B antibodies, by ANOVA) as well as disease activity (Disease Activity Score in 28 joints; $P \leq 0.001$ for the comparison of tertiles of anti-citrullinated H2B antibodies, by ANOVA).

Propagation of neutrophil activation by immune complexes containing citrullinated histones. Given the ability of citrullinated H2B immune complex to costimulate

macrophage activation, we hypothesized that they would have a similar capacity to induce neutrophil activation. Citrullinated H2B immune complex generated with human RA IgG stimulated neutrophil activation (Figure 6D). Furthermore, citrullinated H2B immune complex formed with a rabbit polyclonal anti-H2B antibody that equally recognizes native and citrullinated H2B induced greater neutrophil activation from citrullinated H2B immune complex than from native H2B immune complex ($P = 0.008$) (Figure 6D), again supporting the notion of additional stimulatory capacity imparted by histone citrullination. Thus, the generation of citrullinated H2B immune complex may result in additional generation of citrullinated antigens by further induction of neutrophil activation.

DISCUSSION

RA is immunologically characterized by the production of ACPAs targeting a variety of citrullinated proteins, and these antibodies are associated with increased disease activity, disease severity, and disease damage (25). Converging lines of evidence suggest that these antibodies may be directly involved in RA pathogenesis. Emerging literature suggests a common pathogenic mechanism by which induction of local and/or systemic inflammation is produced as a result of autoantibodies with the capacity to bind antigens which themselves possess innate immunostimulatory capacity (18,26–28), including at least some citrullinated antigens such as citrullinated fibrinogen (18,29) and now citrullinated histones. Although others may exist, we have observed that not all citrullinated proteins (including several recognized by ACPAs) possess immunostimulatory capacity after citrullination. The present study provides critical evidence of how ACPAs, and specifically, antibodies targeting citrullinated histones, might participate in the initiation and propagation of synovial inflammation in RA.

Specifically, we identified citrullinated histones, including citrullinated H2A and citrullinated H2B, as novel autoantigens with innate immunostimulatory capacity. We further demonstrated the arthritogenic potential of these antibodies, but only in the setting of low-grade articular inflammation. This observation builds on the findings of a previous study, which showed that administration of an anti-citrullinated fibrinogen antibody alone was unable to induce murine arthritis but was able to significantly enhance submaximal arthritis induced by the administration of anti-type II collagen antibodies (30). Thus, the emerging concept brought to light by our data identifies the requisite role not only for break in tolerance and autoantibody production, but also a critical role for antigen generation to facilitate the onset of clinically apparent autoimmunity and joint inflammation. Our models support the notion of local generation

of citrullinated antigen as the penultimate event leading to generation of ACPA immune complexes and the induction of clinical inflammation.

A limitation of this study is the use of adjuvant to induce a forced immune response against citrullinated antigens, in this case bovine citrullinated H2B. In human RA, the events initiating the immune response against citrullinated antigens are still-unclear environmental factors (including the association of smoking with the generation of citrullinated antigen) (31) as well as genetics (especially the ability of certain HLA haplotypes to preferentially present citrullinated antigen to T cells) (32). As such, this study does not address the origins of anticitrulline autoimmunity in human RA. However, the generation of relatively citrulline-specific autoantibodies supports the utility of this model for studying the effector phase of RA, including the transition from preclinical autoimmunity to clinical arthritis.

Another limitation of this study is the inability of mass spectroscopy to identify all products of neutrophil activation, especially citrullinated proteins, and others investigators have recently reported an overlapping spectrum of protein citrullination during NETosis (7,8). Similarly, it is possible that our analysis may have lacked sensitivity for identifying all citrullinated residues, including those which are not a result of neutrophil activation and the intracellular process of NETosis, but rather due to the potential process of extracellular citrullination. Additionally, the *in vitro* stimulation assays performed do not identify a direct physical interaction between TLR-4 and citrullinated H2B, and although extensive steps were taken to exclude the possibility, there remains a potential for low levels of residual endotoxin. Finally, in our murine models, it was necessary to generate low-grade articular inflammation to observe the arthritogenic effects of anti-citrullinated H2B antibodies. Though potentially advancing our understanding of the events involved in the transition from preclinical autoimmunity, such a window of initiation has not been clearly identified in the development of human RA.

In conclusion, we demonstrated that citrullinated products of neutrophil activation, especially citrullinated H2B, can serve as autoantigens with the capacity to link innate and adaptive immunity, potentially driving the initiation and propagation of RA-associated inflammation. We propose that local histone citrullination generates antigenic fuel, which initiates a feed-forward synergy between innate and adaptive immune activation, resulting in the transition from preclinical ACPA immunity to clinical RA.

AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Sokolove had full access to all of

the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Sohn, Robinson, Sokolove.

Acquisition of data. Sohn, Rhodes, Onuma, Zhao, Sharpe, Gazitt, Shiao, Fert-Bober, Cheng, Lahey, Wong, Van Eyk, Sokolove.

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