

# Monoclonal IgG antibodies generated from joint-derived B cells of RA patients have a strong bias toward citrullinated autoantigen recognition

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**Antibodies targeting citrullinated proteins (ACPAs [anticitrullinated protein antibodies]) are commonly found in patients with rheumatoid arthritis (RA), strongly associate with distinct HLA-DR alleles, and predict a more aggressive disease course as compared with seronegative patients. Still, many features of these antibodies, including their site of production and the extent of MHC class II-driven T cell help, remain unclarified. To address these questions, we have used a single B cell-based cloning technology to isolate and express immunoglobulin (Ig) genes from joint-derived B cells of active RA patients. We found ~25% of synovial IgG-expressing B cells to be specific for citrullinated autoantigens in the investigated ACPA<sup>+</sup> RA patients, whereas such antibodies were not found in ACPA<sup>-</sup> patients. The citrulline-reactive monoclonal antibodies did not react with the unmodified arginine peptides, yet several reacted with more than one citrullinated antigen. A role for active antigen selection of the citrulline-reactive synovial B cells was supported by the strong bias toward amino acid replacement mutations in ACPA<sup>+</sup> antibodies and by their loss of reactivity to citrullinated autoantigens when somatic mutations were reverted to the corresponding germline sequences.**

Rheumatoid arthritis (RA) affects 0.5–1% of the population in most studied communities (Neovius et al., 2011). Today, the detection of prototypic autoantibodies, so-called ACPAs (anticitrullinated protein antibodies; Schellekens et al., 1998), is part of the diagnostic criteria for RA (Aletaha et al., 2010), and approximately two thirds of patients are seropositive (Klareskog et al., 2008). Typically, sera from ACPA<sup>+</sup> RA patients contain antibodies toward several different citrullinated autoantigens (Verpoort et al., 2007; Snir et al., 2010). Anticitrulline antibodies often emerge before onset of disease (Rantapää-Dahlqvist et al., 2003; Nielen et al., 2004; van de Stadt et al., 2011), and we have recently demon-

strated their accumulation in synovial fluid (i.e., active rheumatic joints) as compared with sera, suggesting that they are at least partly produced in the inflamed lesions (Snir et al., 2010). Collectively, the anticitrulline immunity in RA provides an interesting and multifaceted case of potentially pathogenic humoral autoimmunity. To gain a more thorough understanding of the humoral aspect of this autoimmunity, we investigated the cellular and molecular basis of the production of antibodies to various citrullinated autoantigens in RA patients.

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Abbreviations used: ACPA, anticitrullinated protein antibody; CCP, cyclic citrullinated peptide; CDR, complementarity-determining region; FWR, framework region; PEI, polyethylenimine; RA, rheumatoid arthritis; SHM, somatic hypermutation; SPR, surface plasmon resonance.

J. Steen and F. Murray contributed equally to this paper.

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## RESULTS AND DISCUSSION

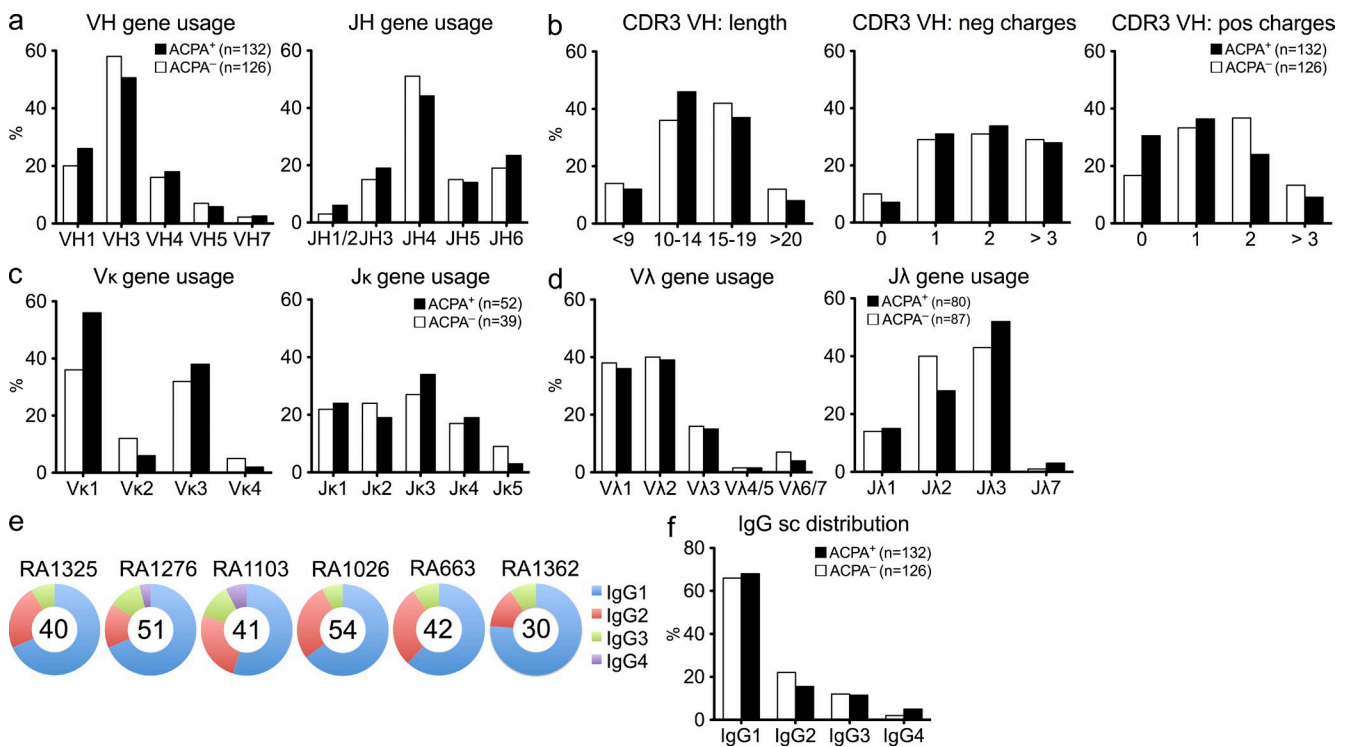
Synovial fluid IgG<sup>+</sup> B cells display extensive clonal diversity

Single cell sorting and subsequent recombinant expression of antibodies from synovial IgG<sup>+</sup>CD19<sup>+</sup> B cells from six RA patients, three ACPA<sup>+</sup> and three ACPA<sup>-</sup>, was performed. Patient demographics are displayed in Table S1. Synovial fluid was shown to contain significantly lower numbers of CD19<sup>+</sup> B cells as compared with peripheral blood, although many B cells were IgG switched and 5–15% of these cells displayed an early plasmablast phenotype (CD19<sup>dim</sup>/CD27<sup>high</sup>; not depicted). Serologically, we have previously demonstrated an enrichment of citrulline-specific IgG antibodies in the joints of ACPA<sup>+</sup> RA patients (Snir et al., 2010) and thus postulated the presence of ACPA-producing B cells/plasma cells in the joints of such patients. After our single B cell approach, we could analyze the synovial B cell repertoire from the analysis of sequences of the variable parts of the Ig genes. Our data demonstrate a wide variation among the patients as well as among individual clones in terms of the gene usage, and overall, the majority of the functional Ig genes were represented among these IgG-expressing B cells (Fig. 1 and Table S3). In total, 258 IgH ( $\gamma$ ) and corresponding IgL gene sequences were generated from ACPA<sup>+</sup> ( $n = 132$ ) and ACPA<sup>-</sup> ( $n = 126$ ) patients. For the Ig heavy chain, *IGHV3* and *IGHJ4* were the

most commonly rearranged genes for both ACPA<sup>+</sup> and ACPA<sup>-</sup> patients (Fig. 1 a and Table S3). The distribution of IgG subclasses of synovial B cells was similar to that of normal human serum, dominated by IgG1 and IgG2 and with low numbers of IgG3 (Fig. 1, e and f). When analyzing the CDR3 (complementarity-determining region 3) features, there were no significant differences between ACPA<sup>+</sup> and ACPA<sup>-</sup> samples and only subtle differences in the CDR3 lengths (Fig. 1 b). In terms of light chain gene usage, *V $\kappa$ 1*, *V $\kappa$ 3*, and *J $\kappa$ 3* were most commonly used among kappa clones and *V $\lambda$ 1*, *V $\lambda$ 2*, and *J $\lambda$ 3* for lambda (Fig. 1, c and d; and Table S3). Collectively, these results indicate that the Ig gene usage in synovial B cells from the inflamed joints of ACPA<sup>+</sup> and ACPA<sup>-</sup> patients display a similarly broad Ig gene diversity.

Citrulline-specific B cells are common in ACPA<sup>+</sup> but not in ACPA<sup>-</sup> RA synovial fluid

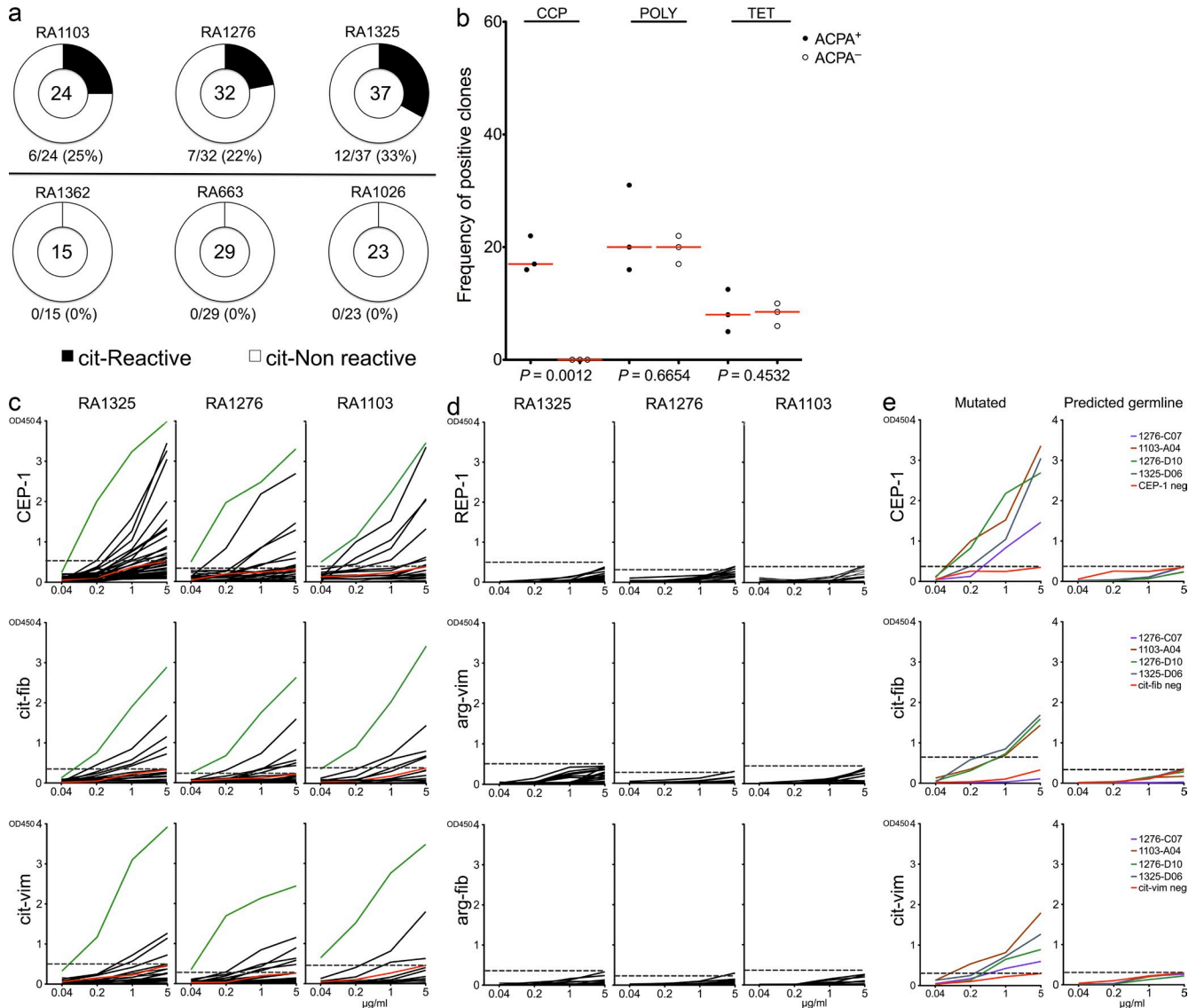
Out of the 258 sequenced antibodies, we expressed 160, 93 from ACPA<sup>+</sup> and 67 from ACPA<sup>-</sup> patient samples, using an Ig $\gamma$ 1 construct as previously published (Wardemann et al., 2003; Tiller et al., 2008). Hereby, monoclonal recombinant human antibodies were generated and could then be assessed for their antigen specificity. We first studied ACPA fine specificity by screening for reactivity toward the citrullinated candidate



**Figure 1. Similar IgG gene characteristic in synovial B cells from ACPA<sup>+</sup> and ACPA<sup>-</sup> RA patients.** (a) Summary of VH and JH family gene usage in seropositive (ACPA<sup>+</sup>) and negative (ACPA<sup>-</sup>) patient samples. (b) Overview of IgH ( $\gamma$ ) CDR3 amino acid characteristics: length (left) and negatively (middle) and positively (right) charged amino acids in ACPA<sup>+</sup> and ACPA<sup>-</sup> patients. (c and d) Light chains data depicting *V $\kappa$* /*J $\kappa$*  (c) and *V $\lambda$* /*J $\lambda$*  (d) gene family usage. (e) Distribution of IgG subclasses displayed per patient with the total number of sequences analyzed represented in the middle of the pie charts. (f) IgG subclass distribution in ACPA<sup>+</sup> and ACPA<sup>-</sup> patient samples. Differences between individual fractions were not statistically significant ( $P > 0.5$ ), as determined by Fisher's exact test.

RA autoantigens  $\alpha$ -enolase (CEP-1; amino acids 5–21), vimentin (amino acids 60–75), and fibrinogen (amino acids 36–52). Overall, CEP-1 was the dominant B cell epitope recognized by these citrulline-specific antibodies, and we identified several

positive clones for one or several of the different citrullinated peptides. Notably, such clones were generated only from ACPA<sup>+</sup> and not from ACPA<sup>-</sup> patient samples (Fig. 2 a). All clones reacting toward citrullinated peptides failed to recognize



**Figure 2. Citrulline-reactive antibodies could be generated from ACPA<sup>+</sup> but not from ACPA<sup>-</sup> RA patients.** (a) Pie charts summarizing the frequency of the citrulline-reactive clones (to any of the tested citrulline peptides) in individual patient samples. The top panel represents the three ACPA<sup>+</sup> patient samples and the three in the bottom panel, the ACPA<sup>-</sup> samples. The absolute number of tested antibodies is indicated in the center of each pie chart. (b) Frequency comparison of antigen reactivity in the clones generated from the different ACPA<sup>+</sup> and ACPA<sup>-</sup> patients (one symbol summarizes all generated antibodies from each individual; range 15–37 clones per patient sample; in total 93 antibodies were tested from ACPA<sup>+</sup> and 67 from ACPA<sup>-</sup> patients). CCP denotes general citrulline reactivity; polyreactivity is based on positivity of two or more of the following: insulin, LPS, and double-stranded DNA; and lastly tetanus toxoid was used as a proxy for recall response. Horizontal lines represent the mean values of reactivity for all RA patients. (c) ELISA graphs of the various ACPA fine specificities: CEP-1 (citrullinated  $\alpha$ -enolase, amino acids 5–21), cit-fib (citrullinated fibrinogen, amino acids 36–52), and cit-vim (citrullinated vimentin, amino acids 60–75). Black lines represent individual IgG<sup>+</sup> memory B cells antibodies. (c) Green lines represent the high positive control (serum pool of ACPA<sup>+</sup> antibodies). (c and e) Red lines represent the negative control antibody (serum pool of ACPA<sup>-</sup> antibodies). Each vertical row represents one ACPA<sup>+</sup> patient sample. (d) ELISA graphs of the corresponding arginine versions of the peptides. (c–e) Dashed horizontal lines show a cutoff of OD450 for positive reactivity. (e) Sequences from four selected antibodies with high reactivity to two or more of the citrullinated peptides that were subsequently reverted into their predicted germline sequence (in both the FWRs and CDRs) and then expressed as recombinant antibodies. ELISA graphs illustrate the citrulline reactivity of the original antibodies (i.e., mutated; left) compared with those encoded by the predicted germline sequences (right). Data are representative of three to six independent experiments.

**Table 1.** Ig gene usage, CDR3 amino acid features, and reactivity of recombinant citrulline-specific IgG antibodies generated from synovial memory B cells of RA patients

| Clone       | Heavy-chain |      |   |      |              |                       |           |            |        |      | Light-chain |             |              |            |               |         |     |                |         |  | Reactivity |  |  |
|-------------|-------------|------|---|------|--------------|-----------------------|-----------|------------|--------|------|-------------|-------------|--------------|------------|---------------|---------|-----|----------------|---------|--|------------|--|--|
|             | V           | D    | J | IgG  | SC           | V-Mut.                | CDR3 (aa) | (+) Length | Family | V    | J           | V-Mut.      | CDR3 (aa)    | (+) Length | CEP-1 cit-fib | cit-vim | CCP | Polyreactivity | Tetanus |  |            |  |  |
| 1276SF-B01  | 3-49        | 3-3  | 3 | IgG1 | 4 (3R/1S)    | EYVDFWSPRARFDI        | 2         | 16         | λ      | 1-44 | 3           | 2 (2R)      | AAWDDSLNGWV  | 0          | 11            | +       | +   | neg            | neg     |  |            |  |  |
| 1276SF-B07  | 3-23        | 4-4  | 4 | IgG1 | 8 (7R/1S)    | DWRHNNYGPHSFDY        | 3         | 15         | λ      | 2-14 | 3           | 4 (3R/1S)   | SSYTSSTWV    | 0          | 10            | -       | +   | neg            | neg     |  |            |  |  |
| 1276SF-C07  | 3-7         | 3-10 | 4 | IgG1 | 6 (4R/2S)    | RKGCFFDC              | 2         | 8          | λ      | 7-43 | 2           | 6 (3R/3S)   | VLYMGSGISV   | 0          | 10            | ++      | -   | pos            | neg     |  |            |  |  |
| 1276SF-D10  | 3-30        | 6-13 | 6 | IgG1 | 6 (4R/2S)    | VRGAAATGYGYGMDV       | 1         | 15         | λ      | 3-1  | 2           | 6 (6R)      | QAWDSSTW     | 0          | 9             | +++     | ++  | pos            | pos     |  |            |  |  |
| 1276SF-F12  | 4-59        | 4-17 | 4 | IgG1 | 3 (2R/1S)    | RLLDGYFDY             | 1         | 10         | λ      | 3-1  | 2           | 4 (4R)      | VRRGTAADV    | 2          | 9             | -       | +   | pos            | pos     |  |            |  |  |
| 1276SF-G08  | 4-39        | 3-10 | 4 | IgG1 | 1 (1R)       | VRGYFDY               | 1         | 7          | κ      | 3-15 | 1           | 3 (2R/1S)   | QHYNNWPPWT   | 1          | 10            | ++      | +   | neg            | neg     |  |            |  |  |
| 1276SF-H10  | 4-39        | 3-22 | 4 | IgG1 | 4 (3R/1S)    | LPNYDSSGARGFDY        | 1         | 17         | κ      | 3-15 | 3           | 1 (1R)      | QQYNNWPRGGFT | 1          | 12            | +       | -   | pos            | neg     |  |            |  |  |
| 1103SF-A03  | 4-39        | 5-18 | 4 | IgG1 | 7 (2R/5S)    | RRGYSYGRARGTIFY       | 5         | 20         | λ      | 1-51 | 3           | 9 (6R/3S)   | GTWDDSLSAGV  | 0          | 11            | +++     | -   | neg            | neg     |  |            |  |  |
| 1103SF-A04  | 1-2         | 3-3  | 3 | IgG1 | 16 (14R/2S)  | DRSPIDYDFWSGSTFYSGMDV | 2         | 24         | λ      | 1-47 | 2           | 6 (4R/2S)   | AAWDDSLSGW   | 0          | 11            | +++     | +   | pos            | pos     |  |            |  |  |
| 1103SF-B02  | 1-69        | 3-9  | 4 | IgG1 | 8 (6R/3S)    | INTSPILGTGYFPGVHDY    | 1         | 18         | κ      | 1-12 | 3           | 6 (4R/2S)   | QQANSFPPT    | 0          | 9             | ++      | +   | pos            | pos     |  |            |  |  |
| 1103SF-B03  | 4-31        | 3-22 | 4 | IgG1 | 3 (2R/1S)    | GAREGLVMDTFDY         | 1         | 14         | κ      | 1-39 | 3           | 4 (4R)      | HQSYSTPQT    | 1          | 9             | +       | -   | neg            | neg     |  |            |  |  |
| 1103SF-B04  | 3-30-3      | 2-2  | 5 | IgG1 | 4 (3R/1S)    | DLGDTSCYT             | 0         | 10         | κ      | 1-39 | 1           | 5 (4R/1S)   | QQSYSTPQT    | 0          | 9             | +++     | -   | neg            | neg     |  |            |  |  |
| 1103SF-H05  | 3-30-3      | 6-6  | 4 | IgG1 | 2 (1R/1S)    | DPYRGKATQDY           | 2         | 11         | κ      | 1-39 | 3           | 7 (6R/2S)   | QQSYSTPPT    | 0          | 9             | +       | +   | pos            | pos     |  |            |  |  |
| 1325SF-A04  | 3-23        | 2-21 | 4 | IgG1 | 34 (27R/7S)  | VKAWELIAS             | 1         | 9          | λ      | 3-21 | 3           | 29 (21R/8S) | QVWDDSSADHPV | 1          | 11            | +++     | +   | pos            | neg     |  |            |  |  |
| 1325SF-B05  | 1-8         | 4-23 | 6 | IgG1 | 25 (18R/7S)  | GGRPYYYYGMDV          | 2         | 17         | λ      | 2-8  | 2           | 26 (19R/7S) | SSYAGGNWV    | 0          | 10            | ++      | -   | pos            | neg     |  |            |  |  |
| 1325SF-B07  | 3-9         | 3-22 | 4 | IgG1 | 2 (1R/1S)    | GARADSSGGSNYFDY       | 1         | 15         | κ      | 1-5  | 5           | 4 (3R/1S)   | QQHNYSPIT    | 2          | 10            | +       | -   | neg            | neg     |  |            |  |  |
| 1325SF-C02  | 1-2         | 3-10 | 4 | IgG1 | 2 (1R/1S)    | SGASITMIRGALEN        | 1         | 14         | λ      | 1-47 | 3           | 7 (6R/2S)   | AAWDDSLRWV   | 1          | 10            | +       | -   | neg            | neg     |  |            |  |  |
| 1325SF-C04  | 3-21        | 4-23 | 4 | IgG1 | 8 (3R/5S)    | GIVTIPGYS             | 0         | 11         | λ      | 3-21 | 2           | 10 (7R/3S)  | QVWDTSSDHHW  | 2          | 12            | ++      | -   | pos            | pos     |  |            |  |  |
| 1325SF-C05  | 1-2         | 4-17 | 5 | IgG1 | 8 (8R)       | VMDPRPYGDAYISH        | 2         | 15         | λ      | 1-47 | 2           | 9 (8R/1S)   | AAWDDSLR     | 1          | 8             | ++      | -   | pos            | neg     |  |            |  |  |
| 1325SF-D06  | 3-74        | 5-24 | 2 | IgG1 | 25 (15R/10S) | DAGNSGHDWYFDL         | 1         | 13         | λ      | 2-23 | 3           | 19 (15R/4S) | CSYAGRGLGV   | 1          | 10            | +++     | +++ | pos            | pos     |  |            |  |  |
| 1325SF-G12  | 4-39        | 5-24 | 4 | IgG3 | 5 (4R/1S)    | HRNPPIAIVFVSPMDY      | 1         | 18         | λ      | 1-51 | 1           | 11 (7R/4S)  | GTWDTSLNVPV  | 0          | 12            | +       | -   | pos            | neg     |  |            |  |  |
| 1325SF-H07  | 3-23        | 3-3  | 4 | IgG1 | 3 (2R/1S)    | GSLYDFWGSYDPSFDY      | 0         | 16         | κ      | 1-5  | 1           | 8 (6R/2S)   | QQYNTYLWT    | 0          | 9             | +       | +   | pos            | pos     |  |            |  |  |
| 1325SF-B109 | 3-49        | 3-3  | 3 | IgG1 | 4 (4R)       | TRDEYDFWVSGPSRAFDI    | 2         | 18         | λ      | 1-44 | 3           | 2 (2R)      | AAWDDSLNGWV  | 0          | 11            | ++      | +   | neg            | neg     |  |            |  |  |
| 1325SF-B118 | 4-59        | 5-12 | 4 | IgG2 | 13 (6R/7S)   | LDVEYSGFDLAYFDS       | 1         | 18         | λ      | 2-23 | 3           | 19 (16R/3S) | CSHARYSLV    | 2          | 10            | +++     | -   | neg            | neg     |  |            |  |  |
| 1325SF-C127 | 3-15        | 2-15 | 6 | IgG1 | 5 (4R/1S)    | TTDPGCSGRCYHFFYGGMDV  | 3         | 22         | λ      | 1-51 | 3           | 5 (2R/3S)   | GTWDDSLSPWV  | 0          | 11            | ++      | ++  | pos            | pos     |  |            |  |  |

For reactivity, increasing signal strength is denoted as + (negative control signal < OD 450 nm < 1.0), ++ (1.0 < OD 450 nm < 2.0), and +++ (OD 450 nm > 2.0).

the native nonmodified arginine version of the peptide (Fig. 2, c and d). Several clones displayed cross-reactivity to one or more citrulline antigens, warranting more detailed affinity analysis in addition to the ELISA. For this purpose, surface plasmon resonance (SPR) measurements were performed to study the kinetics of antibody–peptide interactions, and the analyzed citrulline-specific monoclonal antibodies were shown to display cross-reactivity to various citrullinated peptides with variable affinities. Overall, the  $K_d$  values were in micro- to nanomolar range for all analyzed antibodies: from  $1.36 \times 10^{-6}$  to  $5.94 \times 10^{-10}$  M for CEP-1, from  $3.84 \times 10^{-5}$  to  $3.01 \times 10^{-9}$  M for cit-fib, and from  $1.56 \times 10^{-6}$  to  $1.06 \times 10^{-10}$  M for cit-vim (Table S2). The antibody 1276SF-D10 showed the highest binding affinity for CEP-1 with a  $K_d = 5.94 \times 10^{-10}$  M but exhibited 20-fold and almost 400-fold lower binding affinities to cit-fib ( $K_d = 3.10 \times 10^{-9}$  M) and cit-vim ( $K_d = 1.55 \times 10^{-8}$  M), respectively. The antibody 1103SF-B02 showed the highest binding affinity for cit-vim with a  $K_d = 1.06 \times 10^{-10}$  M but exhibited 3- and 100-fold lower binding affinities to CEP-1 ( $K_d = 3.03 \times 10^{-9}$  M) and cit-fib ( $K_d = 1.01 \times 10^{-8}$  M), respectively. Many antibodies displayed low to medium overall binding affinity and cross-reacted to several citrullinated antigens, whereas the antibodies with high binding affinity for one antigen only displayed low cross-reactivity to other citrullinated antigens. These observations suggest that several different target antigens may initially drive the autoimmune responses before being refined and focused on one particular epitope.

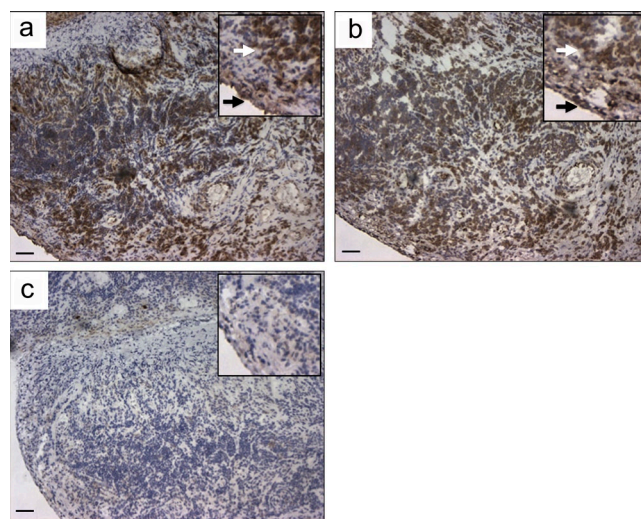
Next, the generated recombinant antibodies were tested for alternative antigen specificities, first with the RA diagnostic anticyclic citrullinated peptide (anti-CCP) assay (Snir et al., 2010) and subsequently also for polyreactivity, using a previously validated panel of ubiquitous antigens toward which RA and systemic lupus erythematosus patient-derived B cells often show reactivity (Wardemann et al., 2003; Tiller et al., 2008), as well as for the recall antigen tetanus toxoid. Reactivity to CCP was only found for antibodies derived from ACPA<sup>+</sup> patients ( $P < 0.001$ ), whereas polyreactivity and tetanus reactivity were found in both groups at similar frequencies (Fig. 2 b and Table S3). However, none of the citrulline-reactive antibodies were cross-reactive to tetanus, whereas 8 out of 25 displayed polyreactivity (Table 1). Two of the citrulline-reactive monoclonal antibodies (1325SF-B109 and 1276SF-D10) were used for the staining of synovial tissue biopsies from three RA patients to analyze the presence of the target antigens in the rheumatic joint. Both antibodies showed distinct staining of inflamed synovial tissues from RA patients, as exemplified in in Fig. 3, whereas staining with the isotype control was negative in all experiments (Fig. 3 c).

#### Citrulline-specific Igs display a different mutational pattern as compared with citrulline-negative Ig derived from the same inflamed joint

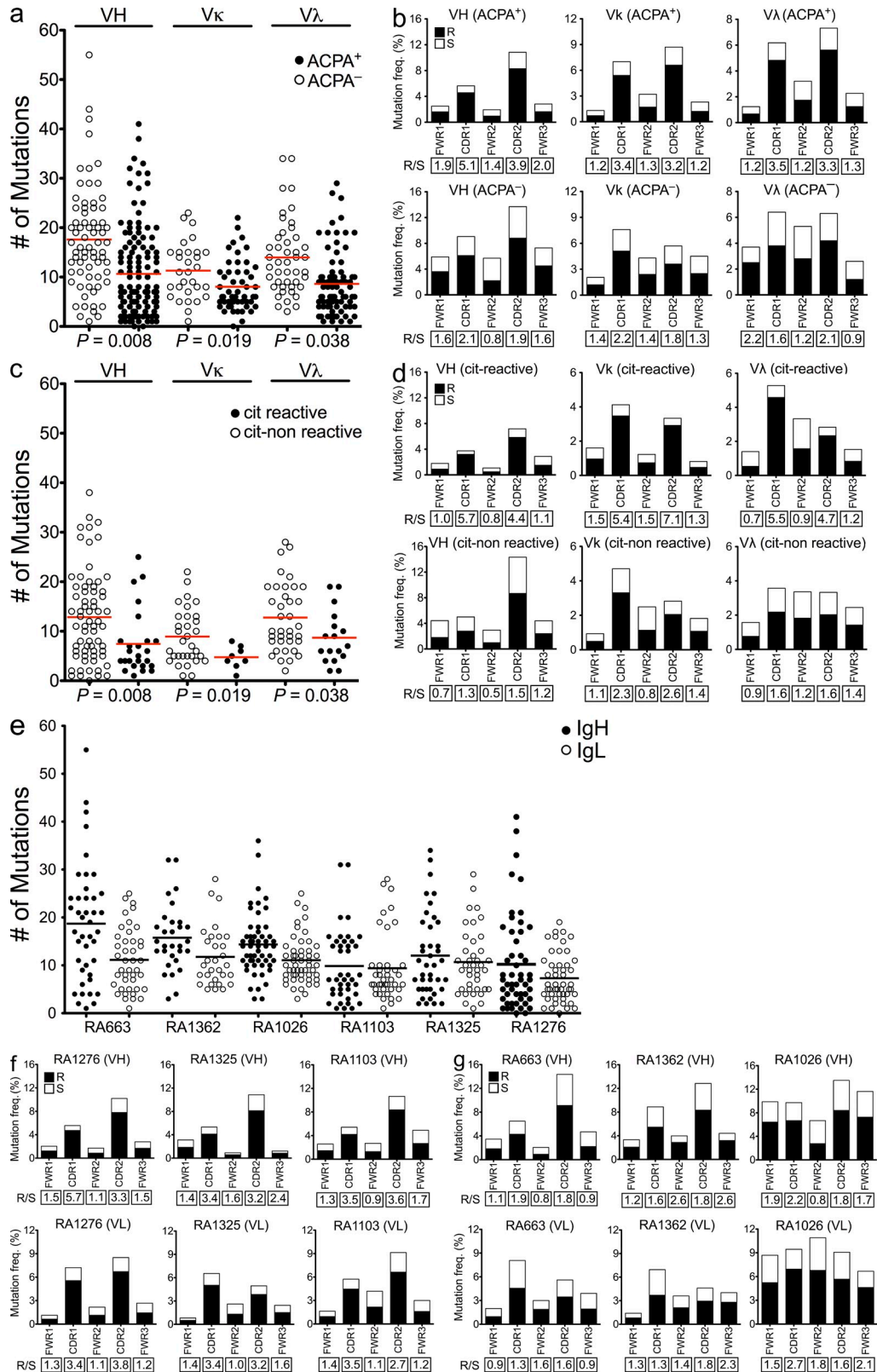
A signature of antigen-driven selection is represented by an increase in the replacement to silent (R/S) mutation ratios in the CDRs as compared with what would be expected for

random mutations (ratio  $> 2.9$ ; Shlomchik et al., 1987). R/S mutation ratios in the framework regions (FWRs) are, however, usually lower than in the CDRs but may have a role in the antibody structure (Berek et al., 1991). In our study, the sequences generated from cells of ACPA<sup>-</sup> patients contained significantly more somatic mutations than those from ACPA<sup>+</sup> patients (Fig. 4, a and e). The overrepresentation of mutations in ACPA<sup>-</sup> patients could be a consequence of recycling of nonreactive or low-affinity B cells within the germinal centers (Allen et al., 2007). Interestingly, sequences generated from ACPA<sup>+</sup> patient samples displayed a bias toward replacement mutations as compared with sequences isolated from ACPA<sup>-</sup> patients, resulting in a striking difference in the R/S mutation ratios, especially in the CDR1 and CDR2 regions, which normally interact with antigens (Fig. 4, b, f, and g). These results suggest that antigen-driven B cell activation and somatic hypermutations (SHMs) are characteristic of antibodies isolated from ACPA<sup>+</sup> patients. Thus, although these data provide supportive evidence for an antigen-driven response, we cannot exclude the possibility of a concomitant component of polyclonal B cell stimulation in these synovial samples (Shlomchik et al., 1987; Randen et al., 1992; Bridges et al., 1993).

Next we performed reanalyses of the mutation patterns of the monoclonal antibodies generated from ACPA<sup>+</sup> patient samples based on citrulline reactivity. As seen in Fig. 4 c, the citrulline-specific antibodies displayed fewer overall mutations but higher R/S ratios in their CDRs as compared



**Figure 3. Immunohistochemical localization of citrullinated proteins in synovial tissues of RA patients.** (a and b) Immunohistochemistry using two of the recombinant citrulline-specific antibodies, 1276SF-D10 (a) and 1325SF-B109 (b), brown staining of both the lining (black arrows) and sublining (white arrows) layers in an inflamed synovial biopsy, obtained at the time of joint arthroplasty from a RA patient. (c) Staining of a matched irrelevant IgG2a-negative control used at similar concentration. Insets show the same samples at higher magnification. Similar results were observed in two other RA synovial tissues (not depicted). Data presented in this figure are representative of three independent experiments from four patients. Bars, 68  $\mu$ m.



**Figure 4. Citrulline-reactive antibodies display a bias toward nonsynonymous mutations in the CDRs.** (a) Comparison of the absolute numbers of somatic mutations in individual VH, V<sub>k</sub>, and V<sub>λ</sub> genes of the antibodies generated from ACPA<sup>+</sup> and ACPA<sup>-</sup> patients. (b) Frequencies of replacement (R) and silent (S) mutations in the CDRs and FWRs of VH, V<sub>k</sub>, and V<sub>λ</sub> genes of synovial IgG<sup>+</sup> memory B cells from ACPA<sup>+</sup> and ACPA<sup>-</sup> RA patients. (c) Comparison of the absolute numbers of somatic mutations between citrulline-positive versus -negative antibodies generated

with the citrulline-negative clones (Fig. 4 d). Traditionally, selection of high-affinity B cells during affinity maturation is primarily dependent on the presentation of antigens to T cells and takes place in germinal centers (Liu et al., 1997). However, it is known that such affinity maturation may also take place at extrafollicular sites (William et al., 2002). To address whether the citrulline-specific antibodies had undergone affinity-driven maturation, we reverted four antibodies with high citrulline reactivity to their predicted germline sequences. Reversion of mutations in these four citrulline-specific monoclonal antibodies led to a loss of reactivity to all the autoantigens (Fig. 2 e). The above observations suggest that the generation of citrulline-specific antibodies may result from T cell-dependent B cell immune responses (Shlomchik et al., 1987; Weiss and Rajewsky, 1990; Berek et al., 1991). Moreover, SHM was likely required for development of high-affinity citrulline-binding antibodies because reversion of these four representative citrulline-specific antibodies to the corresponding germline led to complete loss of binding to the citrullinated peptides.

We and others have previously postulated that autoimmunity against citrullinated autoantigens may initially be triggered at sites distant from the joints, e.g., in lungs during smoking (Klareskog et al., 2008) or in gums during infection with *Porphyromonas gingivalis* or other bacteria (Lundberg et al., 2008; Berthelot and Le Goff, 2010), whereas the factors behind the transition to arthritis are still unclear. Our data provide the first evidence that specificity of IgGs of B cells from joints of ACPA<sup>+</sup> RA patients can be drastically biased toward reactivity with the candidate citrullinated autoantigens. More extensive investigations of B cell/plasma cell specificities in the blood or other lymphoid organs would be required to determine the degree to which this bias is caused by local immune reactions driven by T cells present in inflamed joints or whether immune activation against citrullinated autoantigens also happens outside the joint. The analysis of SHM patterns suggests that at least antibodies generated from synovial fluid B cells are driven by T cell immunity. In this context, it is of interest that T cell responses to the same sets of RA-associated citrullinated autoantigens have been described and associate to the same set of HLA-DR alleles (Sebbag et al., 2006; James et al., 2010; Snir et al., 2011; Pieper et al., 2012).

RA patients are known to display a B cell repertoire generally biased toward autoimmunity (Samuels et al., 2005; Menard et al., 2011), and some underlying factors have been identified such as increased frequency of the *C1858T* *PTPN22* risk allele in ACPA<sup>+</sup> RA patients (Menard et al., 2011). Our patient samples were selected and characterized concerning the presence of the RA-associated HLA-DRB1 alleles and

carriage of the *PTPN22* risk allele (two out of three ACPA<sup>+</sup> and one out of three ACPA<sup>-</sup>). ACPA<sup>+</sup> patients were also selected for having high titers in the CCP test (>300, cut off for positivity 25). Still, our data demonstrate a considerable heterogeneity in the fine specificity of the citrulline-reactive B cells. None of the antibodies generated appeared to be clonally related based on the dissimilarity of the VH-DH-JH joints, a finding which could be explained by the many different candidate autoantigens that have been found to be targeted by ACPA in RA patients (Lundberg et al., 2012). Clearly, understanding the role of ACPAs in the pathogenesis of RA will require careful dissection of several different epitopes, a notion well-compatible with observations in the mouse, where different anticitrulline antibodies are able to trigger or enhance development of arthritis (Kuhn et al., 2006; Hill et al., 2008; Uysal et al., 2009) or specific features of arthritis such as bone erosions (Harre et al., 2012). Extended studies on synovial tissue and possible differences to synovial fluid would also be needed to fully dissect the local contribution of B cells within the rheumatic joint.

Our study gives rise to several downstream questions relating to the mechanisms that determine accumulation of ACPA-producing B cells and plasma cells in joints and how and where these cells differentiate and undergo SHM. Key factors for driving plasma cell differentiation such as IL-6, IL-21, APRIL, and CXCL13 are present (and elevated) in the rheumatic joint (McInnes and Schett, 2007; Humby et al., 2009), and plasma cell differentiation (without assessment of antigen specificity) has been demonstrated to occur in the rheumatic joint by extensive V gene analysis (Scheel et al., 2011). Indeed, a subset of the IgG<sup>+</sup> B cells we analyzed displayed an early plasmablast phenotype, which is indicative that at least some of the antibodies we have generated are also secreted *in vivo*.

Besides the role of antibodies as effector molecules in RA pathology, it is also tempting to speculate that the synovial IgG-expressing B cells specific for citrullinated autoantigens may function as local antigen-presenting cells important for T cell reactivation. Here the B and T cell epitopes may be expected to overlap, as has been shown to be the case for citrullinated vimentin (Verpoort et al., 2007; Snir et al., 2011). A similar scenario has been discussed in the context of celiac disease, another condition under which high numbers of autoantigen-specific B cells are found in the target organ (Di Niro et al., 2012).

In conclusion, the present findings have demonstrated the utility of a single cell approach for generation of monoclonal antibodies from the target organ of a classical autoimmune disease, with resulting new insights on the specificity as well as mutational features of the resulting IgGs. Notably, the

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from ACPA<sup>+</sup> patients. (d) R/S mutation ratios in the CDRs and FWRs of the VH, VK, and VL genes in the citrulline-positive and -negative clones generated from ACPA<sup>+</sup> patients. (e) Absolute numbers of somatic mutations in individual VH and IgL genes of the antibodies generated from the individual patient samples. (a, c, and e) Horizontal bars indicate mean. (f and g) Frequencies of R and S mutations in the CDRs and FWRs of VH and VL of synovial IgG<sup>+</sup> memory B cell from the individual ACPA<sup>+</sup> (f) and ACPA<sup>-</sup> (g) RA patients. The R/S ratios are indicated below the respective graphs.

generated antibodies will also provide the immunological/rheumatological community the opportunity to investigate the role of specific immunity to citrullinated autoantigens in arthritis and to identify ways to counteract the emergence, as well as the effects, of such immunity.

## MATERIALS AND METHODS

### Patients

RA patients attending the Rheumatology Clinic at Karolinska University Hospital and fulfilling the American College of Rheumatology criteria for the diagnosis of RA (Arnett et al., 1988) were included in the study. Informed consent was obtained from all patients in accordance with a protocol approved by the Ethical Review Committee North (KI forskningsetikkommitte Nord) of Karolinska University Hospital.

Synovial fluid samples from six HLA-DR shared epitope-positive RA patients (five females and one male) with a median age of 39 yr (range 35–47 yr) and median disease duration of 10 yr (range 3–28 yr) were included. The joint fluid was taken at a time point of local disease activity that required arthrocentesis and subsequent injection of local corticosteroids.

The demographic and clinical features of all six patients are summarized in Table S1. In brief, three of the patients were ACPA<sup>+</sup> based on a CCP test, whereas three were negative. The ACPA<sup>+</sup> patients showed variable positivity for citrullinated  $\alpha$ -enolase (CEP-1; Kinloch et al., 2005; Lundberg et al., 2008), vimentin (amino acids 60–75), and fibrinogen (Table S1; Verpoort et al., 2007).

### Flow cytometric analysis

Mononuclear cells were prepared from synovial fluid samples by Ficoll-Paque Plus preparation (GE Healthcare). Immunofluorescence labeling for flow cytometry was performed by incubating cell suspensions with anti-human CD19 conjugated to PE-Cy7 (BioLegend), anti-human IgG conjugated to APC (Miltenyi Biotec), anti-human CD3 (BioLegend) and anti-human CD14 (BioLegend) both conjugated to PB, anti-human CD27 conjugated to APC-Cy7 (BioLegend), anti-human IgD conjugated to FITC (BD), and anti-human IgM conjugated to PE (BioLegend). Cell incubation with antibodies was performed at 4°C for 30 min in PBS/1% human serum. Flow cytometric analysis was performed using a Cyan flow cytometer (Dako) and FlowJo software version 8.8.7 (Tree Star).

### Single B cell sorting

Cryopreserved synovial mononuclear cells were thawed, and the cell suspensions were stained with anti-human CD19 conjugated to PE (Miltenyi Biotec), anti-human IgG conjugated to APC (Miltenyi Biotec), and anti-human CD3 (BD) and anti-human CD14 (BD) both conjugated to FITC. Single synovial B cells expressing CD19 and IgG, while lacking CD3 and CD14, were sorted by flow cytometry using a Cytomation MoFlo (Dako) into 96-well PCR plates containing 5  $\mu$ l/well of 0.5 $\times$  PBS containing 10 mM DTT and 8 U RNasin inhibitor (Promega) as previously described (Wardemann et al., 2003; Tiller et al., 2008).

### cDNA synthesis and PCR amplification

Single cell cDNA was synthesized in a total volume of 15  $\mu$ l in the original 96-well PCR plate using the SuperScript III RT (Gibco Invitrogen). Individual IgH ( $\gamma$ ) and IgL chain ( $\kappa$  or  $\lambda$ ) gene rearrangements were amplified independently, using the cDNA as template, by two successive rounds of PCR (50 cycles each) using primers as previously described (Tiller et al., 2008).

### Ig gene sequence analysis

From each 96-well PCR plate, wells from which matching IgH ( $\gamma$ ) and Ig $\kappa$ /Ig $\lambda$  amplicons were obtained were sequenced (Eurofins MWG Operon) and analyzed for Ig gene usage, CDR3 features, and number of V gene SHMs by IgBLAST comparison (Table S3). The Ig CDR3 length was determined as indicated previously (Kabat et al., 1983; Kabat and Wu, 1991). Replacement (R) and silent (S) mutation frequencies in FWRs and CDRs were calculated

for each region based on the absolute number of nucleotides in all analyzed sequences as defined by IgBLAST. IgG isotype subclasses were determined using the international ImMunoGeneTics database (Table S3).

### Cloning and sequencing

Restriction sites for expression vector cloning were introduced by using gene-specific primers and first PCR products as template as previously described (Wardemann et al., 2003; Tiller et al., 2008). The digested PCR products from each single cell were cloned into expression vectors containing human Ig $\gamma$ 1, Ig $\kappa$ , or Ig $\lambda$  constant regions as previously described (Wardemann et al., 2003; Tiller et al., 2008). Ligation reactions were performed using the quick ligase kit (New England Biolabs, Inc.) according to the manufacturer's instructions. Expression vectors containing IgH and the corresponding IgL genes were transformed into DH5 $\alpha$  bacteria (Gibco Invitrogen) and sequentially isolated using NucleoSpin plasmid DNA purification kits (Macherey-Nagel) according to the manufacturer's instructions. To confirm identity with the original PCR products, the isolated IgH and IgL plasmids from each clone were sequenced.

### Antibody production and purification

Antibodies were produced by transient cotransfection of exponentially growing 293 human embryonic kidney fibroblasts (Gibco Invitrogen) using the polyethylenimine (PEI)-precipitation method as described previously with some modifications (Boussif et al., 1995; Mouquet et al., 2011). Adherent (293A) as well as suspension (Freestyle 293F) cells were used to generate recombinant proteins (Gibco Invitrogen). 293A cells growing in DMEM + GlutaMAX (Gibco Invitrogen) supplemented with 10% ultra-low IgG FBS (Gibco Invitrogen) and antibiotics/antimycotics (Gibco Invitrogen) were washed at 70% cell confluency using serum-free DMEM for 5 min. The medium was then replaced with serum-free DMEM + GlutaMAX supplemented with 1% Nutridoma-SP (Roche) and antibiotics/antimycotics (Gibco Invitrogen) before PEI-mediated transfection with equal amounts (10  $\mu$ g) of IgH and corresponding IgL chain vector DNA. Antibodies were alternatively produced by transient transfection of suspension cultured 293 Freestyle cells (Gibco Invitrogen) with PEI-Max (Polysciences) as transfection agent and a total of 20  $\mu$ g of vector DNA, as previously described (Corti et al., 2011). Supernatants were collected after 6 d of culture, and antibodies were purified by binding to protein G-Sepharose (Sigma-Aldrich) and eluted with 0.1 M glycine buffer, pH 3, into storage buffer (1 M Tris-HCl, pH 8). Antibody concentrations were determined by anti-human IgG1 ELISA using human monoclonal IgG1 as standard (Sigma-Aldrich) as previously described (Wardemann et al., 2003; Tiller et al., 2008). Expression of antibodies consisting of both heavy and light chain, as well as the protein purity, was verified by PAGE.

### Reversion of hypermutated sequences to germline

Antibodies for reversion experiments were chosen according to their level of reactivity to the citrullinated autoantigens (Table 1). Germline sequences were determined by reverting mutations to the germline sequence while retaining the original CDR3 junctions and terminal deoxynucleotidyl transferase (TdT) N nucleotides. Germlined VH and VL nucleotide sequences were codon optimized and synthesized by Genscript and their accuracies were confirmed by sequencing. Recombinant mutated and germline-reverted antibodies were tested for citrulline reactivity by ELISA as described above in comparison.

### Antibody specificities

**Assessment of IgG antibodies reactivity against  $\alpha$ -enolase.** Anti- $\alpha$ -enolase reactivity was determined by ELISA as described previously with some modifications (Snir et al., 2010). In brief, 96-well Nunc plates were coated with 2.5  $\mu$ g/ml of the  $\alpha$ -enolase peptide 1 in its native (REP-1) or citrullinated (CEP-1) form (Kinloch et al., 2005; Lundberg et al., 2008). Purified antibodies, diluted in blocking buffer, were used at concentrations of 5, 1, 0.2, and 0.04  $\mu$ g/ml to generate dilution curves. Positive and negative controls included sera from patients and healthy individuals, respectively. In all ELISAs, HRP-conjugated goat anti-human IgG (Jackson ImmunoResearch



Laboratories, Inc.) was used as the detecting antibody and visualized using the chromogenic substrate 3,3',5,5'-tetramethylbenzidine (Bio-Rad Laboratories). Reactivity was detected at 450 nm with a reference of 650 nm, and the minimum OD<sub>450</sub> at which antibodies were considered reactive is indicated in each graph by the red line (Fig. 2, c and e). To be considered reactive, the results for any given antibody had to be confirmed in at least two independent experiments.

**Assessment of IgG antibody reactivity against fibrinogen and vimentin.** Anti-vimentin and -fibrinogen reactivity was determined by ELISA as described previously with some modifications (Snir et al., 2010). In brief, streptavidin-coated high binding-capacity 96-well plates (Thermo Fisher Scientific) were coated with 1 µg/ml of biotinylated vimentin (amino acids 60–75) or fibrinogen (amino acids 36–52) peptides in their native and citrullinated forms (Verpoort et al., 2007). All other stages of the vimentin and fibrinogen ELISAs were performed exactly as for the α-enolase ELISA described above.

**Anti-CCP assay.** Anti-CCP reactivity was determined using a commercial anti-CCPplus ELISA kit according to the manufacturer's instructions (Euro-Diagnostica). Purified antibodies were used at concentrations of 5, 1, 0.2, and 0.04 µg/ml in blocking buffer. Positive and negative controls included sera from patients and healthy individuals, respectively. Reactivity was detected at 450 nm with a reference of 650 nm, and the minimum OD<sub>450</sub> at which antibodies were considered reactive is indicated in each graph. To be considered reactive, the results for any given antibody had to be confirmed in at least two independent experiments.

**Assessment of IgG antibodies polyreactivity.** IgG antibodies were screened for reactivity against specific antigens as previously described (Wardemann et al., 2003). Three different control antibodies were used in all ELISAs: mGO53 (nonreactive), JB40 (weak reactive), and ED38 (high reactive; Wardemann et al., 2003; Meffre et al., 2004). Antibodies were considered polyreactive when they recognized at least two antigens out of the three analyzed antigens that include double-stranded DNA, insulin, and LPS. In all ELISAs, HRP-conjugated goat anti-human IgG (Jackson ImmunoResearch Laboratories, Inc.) was used as detecting antibody and revealed using the chromogenic substrate 3,3',5,5'-tetramethylbenzidine (Bio-Rad Laboratories). Reactivity was detected at 450 nm with a reference of 650 nm and the minimum OD<sub>450</sub>. To be considered reactive, the results for any given antibody had to be confirmed in at least two independent experiments.

**Assessment of IgG antibody reactivity against human tetanus.** Anti-tetanus antibodies were detected using a commercial anti-human tetanus ELISA kit according to the manufacturer's instructions (MyBioSource). Purified antibodies were used at a concentration of 0.5 µg/ml. Samples were added to the appropriate microtiter plate wells and incubated for 20 min at 37°C. ELISAs were developed with HRP-conjugated tetanus antigen and revealed using the chromogenic substrate 3,3',5,5'-tetramethylbenzidine. Reactivity was detected at 450 nm with a reference of 650 nm and the minimum OD<sub>450</sub>. To be considered reactive, the results for any given antibody had to be confirmed in at least two independent experiments.

### Immunohistochemical analysis

Immunohistochemical staining was performed on synovial tissue sections to investigate the presence of citrullinated proteins. Biopsy specimens were obtained from three RA patients at the time of joint replacement. Serial cryosections (7 µm) were fixed for 20 min with 2% (vol/vol) formaldehyde (Sigma-Aldrich) and stored at -70°C until use. For the immunostaining, synovial tissue sections were blocked with 1% H<sub>2</sub>O<sub>2</sub> and 20% AB human serum (Akademiska pharmacy) for 20 min and incubated overnight in a moist chamber at 4°C with the purified recombinant "murinized" antibodies (range 3–10 µg/ml). The murinization of the human monoclonal antibodies was performed by replacing the full human IgG1 Fc by the murine IgG2a

Fc. Parallel sections were stained with irrelevant origin-, mouse monoclonal IgG2a isotype-, and concentration-matched antibody as negative control (Sigma-Aldrich). The next day, sections were first blocked with 1% normal goat serum and then incubated for 30 min with biotin-conjugated goat anti-mouse secondary antibody (Invitrogen). Staining was performed using the VECTASTAIN Elite ABC kit (Vector Laboratories) and visualized with 3,3'-diaminobenzidine (DAB). Sections were counterstained with Mayer's hematoxylin, permanently mounted, and viewed by a light microscope (Reichert Polyvar 2 type 302001; Leica).

### SPR analysis of antibody affinities

To analyze the interactions between the citrullinated autoantigens and the citrulline-specific monoclonal antibodies, we performed an SPR analysis on a Biacore T200 (GE Healthcare) using a streptavidin capture (CAP) sensor chip according to the manufacturer's instructions. Initially, Biotin CAPture reagent, which is a modified form of streptavidin, was immobilized on the CAP sensor chip for 5 min at a flow rate of 2 µl/min. Next, to immobilize the biotinylated citrullinated peptides on the streptavidin surface of the CAP-chip, the CEP-1 cit-fib and cit-vim (50 nM concentrations in 0.3 M sodium phosphate buffer, pH 7.4) were injected for 3 min at a flow rate of 10 µl/min. Once the citrullinated peptide surface on the CAP-chip was prepared, five different concentrations of each of the citrulline-specific monoclonal antibodies (ranging from 5 nM to 1.5 µM) were injected into the flow cells at a flow rate of 30 µl/min. For each concentration used, cycles of injection for 3 min and dissociation period were performed. All SPR analyses were performed at 25°C. Binding datasets from five different concentrations of monoclonal antibodies were collected using a single-cycle kinetics mode (Karlsson et al., 2006). The binding data were analyzed using the Biacore T200 Evaluation software version 1.0 (GE Healthcare) and were fitted with a 1:1 binding model.

### Statistics

Ig gene repertoire analyses, analysis of positive and negative charges in IgH CDR3, and antibody reactivity were calculated by Fisher's exact test or  $\chi^2$  test. Differences in CDR3 length and V gene mutations were calculated by paired two-tailed Student's *t* test (with Wilcoxon's signed rank test). Ratios of V gene SHM were calculated by one-way nonparametric Kruskal-Wallis test followed by Dunn's multiple comparison tests. Differences were considered to be statistically significant at values of  $P \leq 0.05$ . All statistical analyses were performed by Prism software version 5.0c (GraphPad Software).

### Online supplemental material

Table S1 shows patient characteristics. Table S2 shows kinetic rates and affinity of synovial IgG antibodies to CEP-1, cit-fib, and cit-vim measured by SPR. Table S3, included as a separate Excel file, shows sequence data and reactivity of IgG antibodies from synovial memory B cells of RA patients. Online supplemental material is available at <http://www.jem.org/cgi/content/full/jem.20121486/DC1>.

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The authors have no conflicting financial interests. However, a patent application covering the citrulline-specific monoclonal antibodies has been submitted by K. Amara, L. Klareskog, and V. Malmström.

Author contributions: K. Amara conceived the study, performed experiments, analyzed data, prepared figures, and contributed to the writing of the manuscript. J. Steen, F. Murray, and H. Morbach performed experiments and revised the manuscript. B.M. Fernandez-Rodriguez and L. Israelsson performed experiments and revised the manuscript. V. Joshua and M. Engström performed immunohistochemistry experiments. O. Snir recruited study subjects and performed experiments. A.I. Catrina provided the associated clinical data from the subjects. D. Corti performed the germline sequence reversion, revised the manuscript, and provided valuable suggestions. H. Wardemann and E. Meffre revised the manuscript and provided valuable suggestions. L. Klareskog conceived and supervised the study and revised the manuscript. V. Malmström conceived and supervised the study, analyzed data, and prepared the manuscript. All authors discussed the results, commented on the manuscript at all stages, and approved the final manuscript.

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