


Affinity Maturation of the Anti-Citrullinated Protein Antibody Paratope Drives Epitope Spreading and Polyreactivity in Rheumatoid Arthritis

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Objective. Anti-citrullinated protein antibodies (ACPAs) are a hallmark of rheumatoid arthritis (RA). While epitope spreading of the serum ACPA response is believed to contribute to RA pathogenesis, little is understood regarding how this phenomenon occurs. This study was undertaken to analyze the antibody repertoires of individuals with RA to gain insight into the mechanisms leading to epitope spreading of the serum ACPA response in RA.

Methods. Plasmablasts from the blood of 6 RA patients were stained with citrullinated peptide tetramers to identify ACPA-producing B cells by flow cytometry. Plasmablasts were single-cell sorted and sequenced to obtain antibody repertoires. Sixty-nine antibodies were recombinantly expressed, and their anticitrulline reactivities were characterized using a cyclic citrullinated peptide enzyme-linked immunosorbent assay and synovial antigen arrays. Thirty-six mutated antibodies designed either to represent ancestral antibodies or to test paratope residues critical for binding, as determined from molecular modeling studies, were also tested for anticitrulline reactivities.

Results. Clonally related monoclonal ACPAs and their shared ancestral antibodies each exhibited differential reactivity against citrullinated antigens. Molecular modeling identified residues within the complementarity-determining region loops and framework regions predicted to be important for citrullinated antigen binding. Affinity maturation resulted in mutations of these key residues, which conferred binding to different citrullinated epitopes and/or increased polyreactivity to citrullinated epitopes.

Conclusion. These results demonstrate that the different somatic hypermutations accumulated by clonally related B cells during affinity maturation alter the antibody paratope to mediate epitope spreading and polyreactivity of the ACPA response in RA, suggesting that these may be key properties that likely contribute to the pathogenicity of ACPAs.

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic autoimmune disorder of unknown etiology (1,2). A hallmark of RA is the presence of anti-citrullinated protein antibodies (ACPAs) that recognize citrullinated antigens (1,2). Citrullination is mediated by peptidylarginine deaminases and plays a role in gene regulation, organization of cell structure, apoptosis, and formation of neutrophil extracellular traps (3–5). In RA, however, citrullination of proteins in the synovial joint and other tissues, coupled with the production of ACPAs, has been hypothesized to contribute to its pathogenesis (1–4).

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Increasing evidence suggests that ACPAs play an important role in the pathogenesis of RA (6). ACPAs have been detected in patients as early as 10 years prior to diagnosis, with increased titers and epitope spreading of the ACPA response preceding the onset of arthritis (7–9). The presence of ACPAs is associated with increased disease severity (10–13) and is a better predictor of erosive disease than rheumatoid factor (14). Finally, in the mouse model of collagen-induced arthritis, administration of a monoclonal ACPA exacerbated the disease, demonstrating that ACPAs can directly promote inflammatory arthritis (15).

Deciphering the mechanisms underlying the development and epitope spreading of the ACPA response could provide new

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insights into the mechanisms underlying RA. Previous studies using RA patient serum demonstrated that epitope spreading of the ACPA response occurs in the years preceding clinical disease (7–9). Of note, during the 2 years immediately preceding the onset of clinical arthritis, the number and breadth of ACPA reactivities sharply increase, correlating with an increase in proinflammatory cytokines in the blood (7). Nevertheless, the mechanisms underlying epitope spreading of ACPAs and their role in the development of clinical RA remain poorly understood.

In the present study, we used barcode-enabled single-cell sequencing to analyze ACPA plasmablast repertoires in RA. We tested the binding capabilities of 69 recombinant monoclonal antibodies (mAb) derived from the plasmablast clonal families. We found that plasmablasts within the same clonal family that possess differential somatic hypermutations encode individual ACPAs that bind different citrullinated epitopes and/or bind distinct sets of citrullinated epitopes, and thus are polyreactive (16). We also found that patient-derived, affinity-matured ACPAs frequently exhibit increased polyreactivity, in which they consistently bind a greater number of specific citrullinated epitopes as compared to predicted ancestral family members. These findings indicate that somatic hypermutations arising through affinity maturation can result in epitope spreading and increase the polyreactivity of individual ACPAs, which in turn increases the ability of the polyclonal serum ACPA repertoire to bind a multitude of citrullinated epitopes in RA. We further used molecular modeling and mutation studies to identify and characterize the key amino acid residues within the complementarity-determining regions (CDRs) and framework regions forming the paratopes within the antigen-binding sites of ACPAs that mediate binding to citrullinated epitopes.

PATIENTS AND METHODS

Collection of human blood specimens. Blood samples were collected from individuals recruited at the VA Palo Alto who met the American College of Rheumatology 1987 classification criteria for RA (17) and who were positive for anti-cyclic citrullinated peptide (anti-CCP) antibodies. The patients' demographic and clinical characteristics are provided in Supplementary Table 1 (available on the *Arthritis & Rheumatology* web site at <http://onlinelibrary.wiley.com/doi/10.1002/art.40760/abstract>). The samples were collected after obtaining informed consent from all patients, and the study was conducted in accordance with human subject protocols approved by the Stanford University Institutional Review Board.

Generation of fluorescent citrullinated peptide tetramers (cit-tets). Cit-tets were generated by incubating phycoerythrin (PE)-conjugated streptavidin with biotinylated citrullinated peptides (a list of the peptides used is provided in Supplementary Table 2, available on the *Arthritis & Rheumatology* web site at <http://onlinelibrary.wiley.com/doi/10.1002/art.40760/abstract>).

These peptides were previously shown to be targeted by ACPAs in RA sera (7,18,19) (for more details, see Supplementary Methods, <http://onlinelibrary.wiley.com/doi/10.1002/art.40760/abstract>).

Single-cell sorting of ACPA-producing plasmablasts.

Cell sorting of CD19+CD20–CD3–CD14–IgD–CD27+CD38^{high} plasmablasts, which were either IgG+ or IgA+, was performed in the same manner as used previously in our prior studies (20,21). A mix of the PE-conjugated cit-tets was included to identify ACPA-producing plasmablasts (18,19) (details in Supplementary Methods, <http://onlinelibrary.wiley.com/doi/10.1002/art.40760/abstract>).

Barcode-enabled antibody repertoire sequencing.

Antibody repertoire sequencing was performed as previously described (20–23).

Bioinformatics pipeline and repertoire analysis.

Paired-chain antibody repertoires were generated from sequencing data using a custom bioinformatics pipeline (22). Clonally related antibodies were defined as sharing heavy-chain and light-chain V–J genes and having a CDR3 nucleotide Levenshtein distance of $\leq 40\%$ of the total CDR3 length for both chains (i.e., CDR3 sequences are at least 60% identical). The bimodal distribution of the nearest distances for the heavy- and light-chain CDR3 sequences was used to set the identity threshold (24). For representing individual clonal family lineages, the heavy- and light-chain sequences of each member were concatenated and analyzed using IgTree (25).

Selection and recombinant expression of mAb.

The antibodies sequenced directly from RA patient blood were produced in-house, as described previously (26). Antibodies sequenced from cit-tet+ clonally expanded plasmablasts were prioritized for expression; however, a handful of cit-tet+ nonclonal and tetramer-negative (tet–) clonal plasmablast antibodies were also expressed. All other antibodies (mutation studies and predicted parent/germline antibody sequences) were commercially produced (at LakePharma).

RA planar array. RA antigen microarrays were printed and probed, and data sets were analyzed as described previously (7,27,28). Positive hits were confirmed by enzyme-linked immunosorbent assay (ELISA).

Molecular modeling. Descriptions of the methods used to model the binding of patient-derived ACPAs to citrullinated epitopes are available in the Supplementary Methods (<http://onlinelibrary.wiley.com/doi/10.1002/art.40760/abstract>).

ELISAs. The CCP3 and peptide ELISAs used are described in the Supplementary Methods (<http://onlinelibrary.wiley.com/doi/10.1002/art.40760/abstract>).

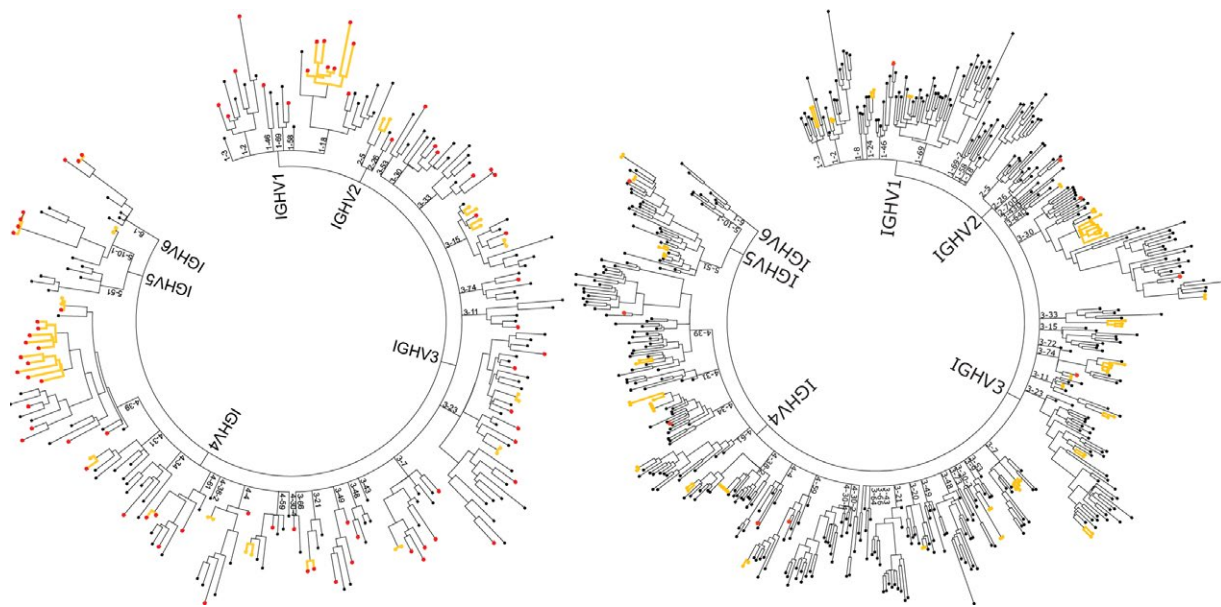


Figure 1. Representative plasmablast antibody repertoires in the blood of cyclic citrullinated peptide–positive (CCP+) rheumatoid arthritis (RA) patients. The plasmablast antibody repertoires of CCP+ RA patients were sequenced using a single-cell, barcode-based antibody repertoire capture method. Bioinformatics analyses of sequencing data sets were performed to obtain consensus sequences, pair cognate heavy-chain (HC) and light-chain (LC) sequences for individual plasmablasts, and generate phylogenetic trees. Two representative trees are presented (RA3 on left, RA11 on right) (see also Supplementary Table 3, <http://onlinelibrary.wiley.com/doi/10.1002/art.40760/abstract>). The tip of each branch denotes the paired HC and LC genes of an antibody expressed by a single plasmablast. Antibodies deriving from clonal families of plasmablasts are highlighted by yellow branches. Antibodies from citrullinated peptide tetramer–positive plasmablasts are colored in red.

Statistical analysis. For 2-group comparisons, the Welch's *t*-test was used. For multiple comparisons, the nonparametric Kruskal-Wallis test was used, followed by Dunn's multiple comparisons test. *P* values less than 0.05 were considered significant.

RESULTS

Sequencing the blood plasmablast antibody repertoire in RA. To study ACPA repertoires from activated, disease-relevant B cells in RA, we sequenced the antibodies expressed by plasmablasts from 6 anti-CCP+ RA patients and generated phylogenetic trees that represent the plasmablast antibody repertoires of each individual RA patient (Figure 1). A total of 2,541 antibodies were sequenced (see Supplementary Table 3, available on the *Arthritis & Rheumatology* web site at <http://onlinelibrary.wiley.com/doi/10.1002/art.40760/abstract>). Raw sequencing data have been deposited in the NCBI SRA database (accession no. PRJNA503739; <https://www.ncbi.nlm.nih.gov/sra>). In total, 182 clonal families, consisting of a total of 510 antibodies, were identified, with the remaining 2,031 antibodies representing nonclonal family “singletons.” These singletons may in fact represent small clonal families that could not be captured at the current sequencing depth; therefore, our estimates of clonality are likely underestimates.

Sequenced antibodies were split nearly evenly between IgA and IgG isotypes (1,200 for IgA and 1,341 for IgG),

although the ratios of IgA to IgG varied between patients. In total, 231 of the sequenced antibodies were derived from tetramer-positive (cit-tet+) plasmablasts. While this represents 9% of the total number of plasmablast antibody sequences, actual percentages of cit-tet+ plasmablasts in individual patients ranged from 1.4% to 34.7%. Of the cit-tet+ plasmablasts sequenced, 28.6% belonged to clonal families; of tet– plasmablasts, 23.8% were clonal. Heavy- and light-chain V-gene usage was comparable between the cit-tet+ plasmablasts and the entire plasmablast repertoires (see Supplementary Figures 1A–D, available on the *Arthritis & Rheumatology* web site at <http://onlinelibrary.wiley.com/doi/10.1002/art.40760/abstract>). Differences in mutation rates between cit-tet+ and tet– plasmablasts were nonsignificant, with the exception of that in subject RA3, whose cit-tet+ plasmablasts were significantly more mutated (see Supplementary Figures 2A–F, <http://onlinelibrary.wiley.com/doi/10.1002/art.40760/abstract>).

Differential binding and unique polyreactive signatures against citrullinated antigens exhibited by recombinant monoclonal ACPAs. Sixty-nine antibodies spanning samples from the 6 RA patients were selected for recombinant expression. Fifty-eight of the recombinant mAb were derived from cit-tet+ plasmablasts, the majority of which were clonally expanded. The remaining 11 were derived from tet– plasmablasts.

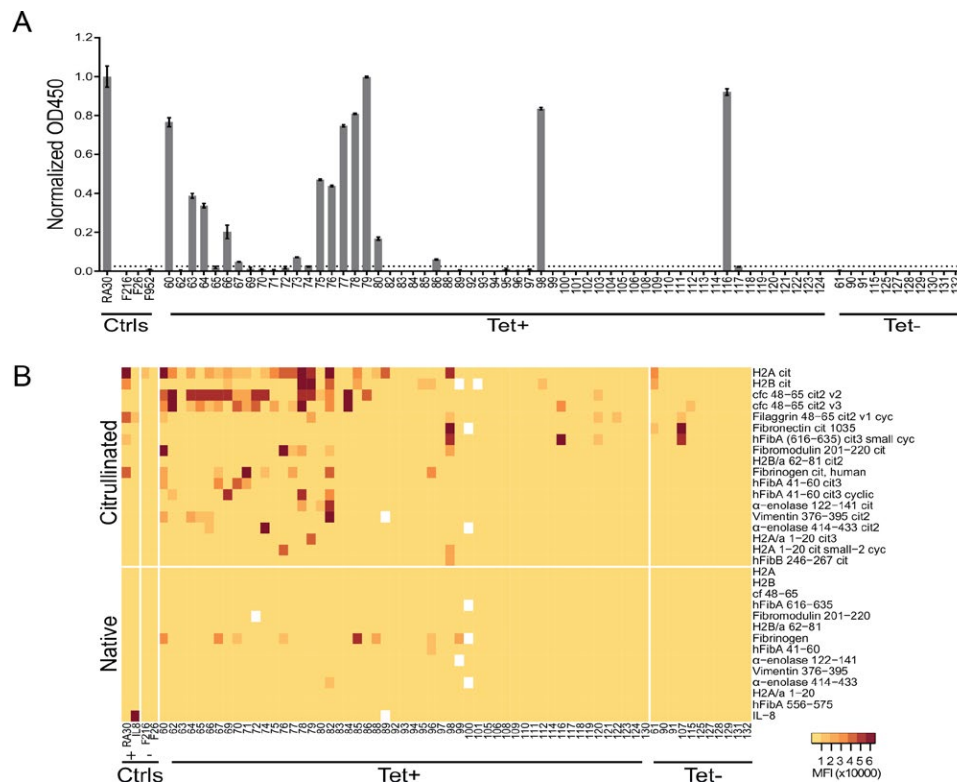


Figure 2. Recombinant monoclonal antibodies sequenced from rheumatoid arthritis (RA) patient plasmablasts exhibit distinct polyreactive binding of citrullinated antigens. **A**, Representative antibodies derived from patient plasmablasts were recombinantly expressed and tested for anti-cyclic citrullinated peptide (anti-CCP) reactivity using a commercial CCP enzyme-linked immunosorbent assay kit. Results are normalized against a monoclonal anti-citrullinated protein antibody (ACPA) control. The cutoff value for positivity was set at 3 SD above the average of the negative controls (antihemagglutinin antibodies). Data are presented as the mean \pm SEM of duplicate assays, and are representative of 3 independent experiments. **B**, Antigen array analysis of recombinant antibody reactivities against RA-associated autoantigens was performed. Arrays were printed with \sim 300 citrullinated and native synovial peptides and proteins, including citrullinated flaggrin cyclic (cfc), a citrullinated flaggrin peptide used in first-generation CCP assays. Arrays were probed with patient-derived recombinant antibodies followed by fluorescent anti-human IgG detection antibody prior to scanning. The median mean fluorescence intensity (MFI) of 4 replicate spots for each antigen is displayed. Ctrls = controls; Tet+ = citrullinated tetramer-positive; Tet- = citrullinated tetramer-negative; H2A = histone 2A; cit = citrullinated; cyc = cyclic; hFibA = human fibrinogen α ; hFibB = human fibrinogen β ; cf = citrullinated flaggrin; IL-8 = interleukin-8.

The recombinant mAb were tested for citrullinated antigen specificity using a CCP assay and synovial antigen planar arrays (27). Of the 69 recombinant mAb expressed, 15 showed positive reactivity on the CCP ELISA (Figure 2A), while 34 recombinant mAb showed reactivity against citrullinated antigens contained on the synovial antigen arrays (Figure 2B). Several of the recombinant mAb exhibited low-level reactivity against native fibrinogen on the planar array.

In comparing the cit-tet+ and cit-tet- recombinant mAb, 33 (56.9%) of 58 cit-tet+ recombinant mAb showed positive reactivity to citrullinated antigens in at least 1 of the 2 assays, as compared to only 3 (27.3%) of 11 tet- recombinant mAb. Intriguingly, of the 33 cit-tet+ recombinant mAb that exhibited ACPA reactivity, 24 (72.7%) bound more than 1 citrullinated antigen, according to the findings on synovial antigen planar array. Taken together, these data indicate that we were able to successfully enrich for, express, and characterize recombinant ACPAs from RA patient plasmablast repertoires.

Differentially mutated antibodies encoded by plasmablast clonal families that exhibit differential binding to citrullinated antigens.

To investigate whether epitope spreading arises from affinity maturation, we characterized, using synovial antigen arrays and with results confirmed by ELISA, the specificities of clonally related, yet differentially mutated, antibodies derived from 3 clonal families of ACPA-producing plasmablasts. Clonally related recombinant mAb exhibited distinct, but overlapping, binding specificities to citrullinated antigens (Figure 3A). For example, 5 of the 7 recombinant mAb derived from clonal family 1 bound the hFibA 41-60 cyclic (cyc) cit3 peptide (Figure 3B). Of these 5, both recombinant mAb 78 and recombinant mAb 80 bound H2A/a 1-20 cit3, but not H2B/a 62-81 cit cyc peptide. In contrast, the reverse was exhibited by recombinant mAb 60 and 76.

For both clonal family 2 and clonal family 3, 3 of the expressed recombinant mAb from each family exhibited shared binding reactivities and recognized the same set of citrullinated

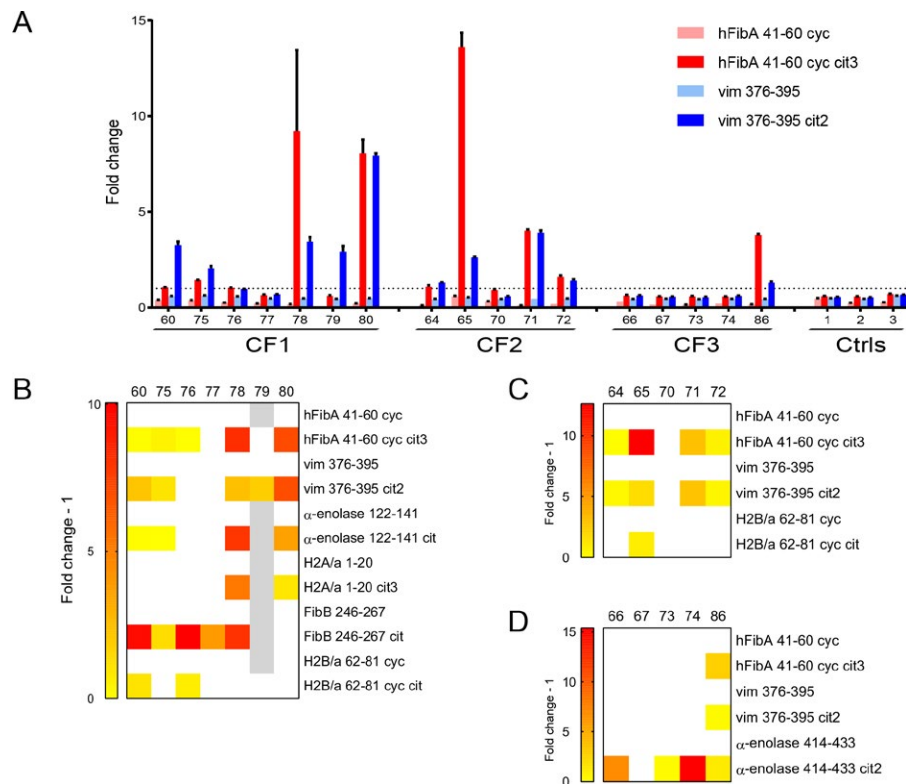


Figure 3. Identification of ACPA-producing plasmablast clonal families (CFs) containing divergent antibodies that exhibit differential polyreactive binding to citrullinated epitopes. **A**, Recombinant monoclonal antibodies (mAb) derived from the 3 ACPA clonal families were expressed and tested by enzyme-linked immunosorbent assay (ELISA) for reactivity against citrullinated peptides and their native counterparts. The cutoff value for positivity for each peptide was set at 3 SD above the average of the negative controls. The horizontal broken line represents the normalized cutoff for positive hits (fold change >1). Data are presented as the mean \pm SEM fold change, and are representative of at least 3 replicated experiments. **B–D**, Heatmaps summarize the ELISA data for the binding of mAb derived from clonal families 1 (**B**), 2 (**C**), and 3 (**D**) to various citrullinated peptides. Data are representative of 3 replicated experiments. The mean reactivity of positive hits (fold change – 1 >0) are displayed in a colored gradient ranging from yellow to red. White indicates no detectable reactivity (fold change – 1 \leq 0). Gray indicates data not available. vim = vimentin (see Figure 2 for other definitions).

peptides (with distinct sets of citrullinated peptides recognized by each family) (Figures 3C and D). However, each family also contained individual ACPAs that exhibited increased polyreactivity by binding additional citrullinated peptides, and 1 antibody that did not bind any peptides. None of the recombinant mAb tested bound the noncitrullinated versions of the peptides (data not shown). These findings provide evidence of overlapping, but distinct, epitope reactivity, including the generation of polyreactive ACPAs of the divergently mutated antibodies encoded within ACPA plasmablast clonal families.

Restricted binding to citrullinated antigens and reduced polyreactivity exhibited by predicted ancestral antibodies. To directly assess the effects of somatic hypermutation on antigen specificity, we reverted clonally related plasmablast ACPAs back toward their germline sequence. We used IgTree (25) to predict the shared parent antibodies for each of the 3 clonal families (Figures 4A, C, and E). All ancestral antibodies, including the inferred germline sequences, were expressed and tested by ELISA to determine their binding specificity. In cases where

the germline sequence was difficult to discern, particularly for the highly variable CDR3 regions, multiple antibodies were produced.

Several of the parent sequences bound citrullinated antigens; however, in general, the ancestral recombinant mAb bound fewer antigens than the affinity-matured recombinant mAb. For example, in clonal family 1, recombinant mAb encoded by parent sequences 60 only bound FibB 246–267 cit and H2A/a 1–20 cit3 peptides, which represent only 2 of the 6 peptides targeted by the corresponding affinity-matured antibodies (Figure 4B). Parent sequences in both clonal family 2 and clonal family 3 bound hFib 41–60 cit3 cyc and vim 376–395 cit peptides, but showed no reactivity against additional peptides bound by the affinity-matured antibodies, namely H2B/a 62–81 cit cyc or α -enolase 414–433 cit2 (Figures 4D and F). Of note, the inferred germline antibodies of 2 of the 3 clonal families did not bind to any citrullinated antigens contained on the synovial antigen arrays (Figures 4B and F). These results demonstrate that the somatic hypermutation and affinity maturation of ACPAs during clonal expansion can lead to epitope spreading of the ACPA response in individuals with RA.

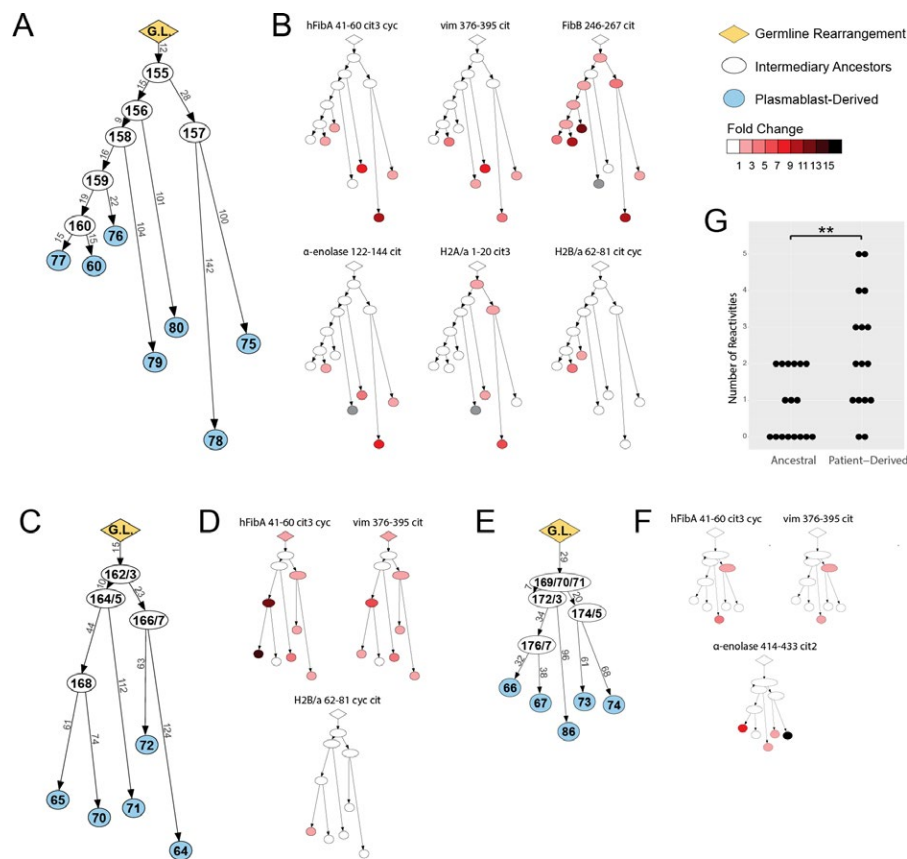


Figure 4. Ancestral antibodies of ACPAs show divergent anticitrulline reactivities and less polyreactivity. **A, C,** and **E,** Lineage tree analysis of the ACPA clonal families. Sequences of clonally related antibodies were analyzed with IgTree to generate lineage trees representing clonal families 1 (**A**), 2 (**C**), and 3 (**E**). The length of each branch has been adjusted to proportionally match the number of mutations present between 2 antibody nodes. **B, D,** and **F,** ACPA reactivity of predicted ancestral B cell antibodies. Lineage trees for clonal families 1 (**B**), 2 (**D**), and 3 (**F**) are shown in various colors based on the antigen specificity as tested by enzyme-linked immunosorbent assay (ELISA). Data are representative of at least 2 independent experiments. The color intensity indicates the mean strength of the ELISA signal. White symbols indicate no binding, and gray indicates data not available. **G,** Polyreactivity graph. The degree of polyreactivity was compared between all ancestral antibodies and all affinity-matured, patient-derived antibodies across the 3 clonal family groups. The degree of polyreactivity between the groups was compared by Welch's *t*-test. ** = $P \leq 0.01$. G. L. = germline (see Figure 2 for other definitions).

There were a few cases in which the predicted ancestral antibodies bound more citrullinated antigens than did the affinity-matured descendants. For instance, ancestral antibodies RA168, RA174, and RA175 bound hFibA 41–60 cit3 cyc and vimentin (vim) 376–395 cit, but several of their descendant antibodies, RA70, RA73, and RA74, did not (Figures 4C–F). This suggests that certain somatic hypermutations lead to loss of specific epitope recognition for some antibodies. Despite this, individual affinity-matured RA plasmablast-encoded ACPAs exhibited significantly increased polyreactivity as compared to ancestral antibodies (Figure 4G). These results demonstrate that the affinity maturation of ACPA B cells can lead to generation of individual B cell clones that encode ACPAs that bind distinct citrullinated epitopes and/or possess increased polyreactivity, which in turn contributes to the increased number of reactivities against citrullinated antigens by the polyclonal ACPA response in the serum of RA patients.

Use of molecular modeling to predict regions within the ACPA paratope involved in epitope specificity.

An antibody's paratope is formed by the specific amino acid residues within the Fv domain that recognize and physically bind to its target antigen. To determine regions within the ACPA paratope responsible for citrullinated antigen binding, we performed molecular modeling using the Rosetta software package to simulate ACPA–citrullinated antigen interactions. We ran docking experiments for 28 antibody–antigen combinations and generated 2 top models for each complex, yielding a total of 56 models. A representative set of 3 docked models is presented in Figures 5A–C. Using PyMOL, we identified for each model the antibody residues predicted to be in contact with the citrullinated antigens; the cumulative results across all antibody–antigen models are shown in Figures 5D–F (29).

As expected, the majority of the contact residues were found in the CDR regions of the antibody sequences (Fig-

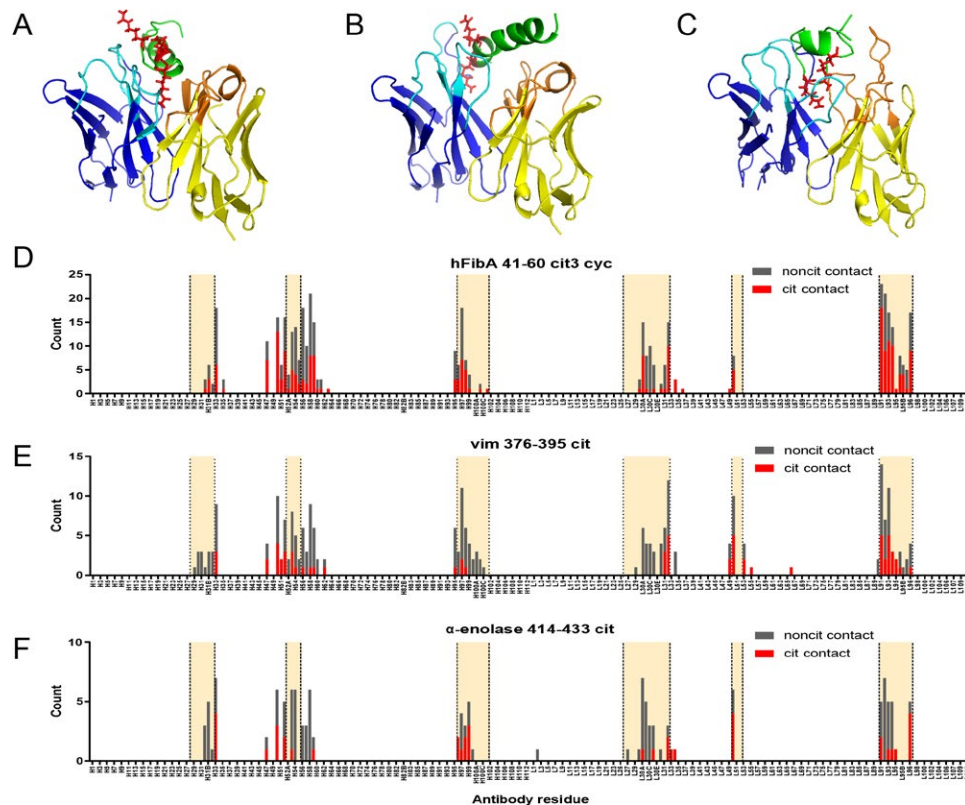


Figure 5. Predicted contact residues of ACPA clonal family antibodies based on molecular modeling. Molecular models of antibodies from clonal families 1, 2, and 3 (see Figure 4) were produced using the Rosetta software package. Three peptides, hFibA 41–60 cit3 cyc (**A** and **D**), vimentin (vim) 376–395 cit (**B** and **E**), and α -enolase 414–433 cit2 (**C** and **F**), were modeled using PEP-FOLD version 2.0 and converted to citrullinated forms using PyMOL. Antibody–antigen docking was performed using Rosetta (see Supplementary Methods, <http://onlinelibrary.wiley.com/doi/10.1002/art.40760/abstract>). Three representative molecular models of patient-derived monoclonal antibodies (mAb) docked with citrullinated peptides are presented: recombinant mAb 80 docked with hFibA 41–60 cit3 cyc peptide (**A**), recombinant mAb 78 with vim 376–395 cit peptide (**B**), and recombinant mAb 66 with α -enolase 414–433 cit2 peptide (**C**). The predicted contact residues for each docked model were counted and the cumulative results of all antibody–antigen combinations are presented (**D–F**). Antibody sequences are presented using the Chothia (post-1997) numbering scheme, with the heavy and light chains concatenated along the x-axis. Complementarity-determining regions are highlighted between the vertical broken lines. See Figure 2 for other definitions.

ures 5D–F). However, although contact residues are predicted in the heavy-chain CDR3 region, which is the predominant region conferring the binding specificity of most antibodies, these data indicate a stronger likelihood of involvement of the light-chain CDR3 (189 counts) than the heavy-chain CDR3 (85 counts), at least with regard to binding to hFib 41–60 cit3 cyc peptide. We also observed that a substantial number of contact residues were predicted to occur in the framework regions flanking the heavy-chain CDR2 (Figures 5D–F). Taken together, these results from our molecular modeling studies identify multiple regions within the ACPA paratopes that may be predicted to be important for citrullinated antigen binding and that confer polyreactivity against specific sets of citrullinated epitopes.

Altered specificity and epitope spreading resulting from mutagenesis of critical ACPA paratope regions. To confirm the relevant ACPA paratope regions identified from molecular modeling, we used the predicted contact residue information

from the docked models to guide mutation studies on 5 pairs of clonally related ACPAs. In each pair, we compared 1 antibody with a particular anti-citrullinated antigen reactivity to a clonally related antibody without that reactivity. Residues predicted to be relevant to binding were transferred from the reactive antibody to the non-reactive antibody (see Supplementary Table 4, available on the *Arthritis & Rheumatology* web site at <http://onlinelibrary.wiley.com/doi/10.1002/art.40760/abstract>). Mutated antibodies along with the subject-derived recombinant plasmablast antibodies were tested using citrullinated peptide ELISAs to determine their antigen specificities (Figures 6A–E).

We were able to generate mutated antibodies that gained novel specificities against the citrullinated antigens and/or epitopes of interest. For instance, from clonal family 2, recombinant mAb 65 bound both hFib 41–60 cit3 cyc and vim 376–395 cit2, whereas recombinant mAb 70 bound neither antigen. Transferring heavy-chain residues 56–59 from recombinant mAb 65 to recombinant mAb 70 induced binding by modified recombinant mAb

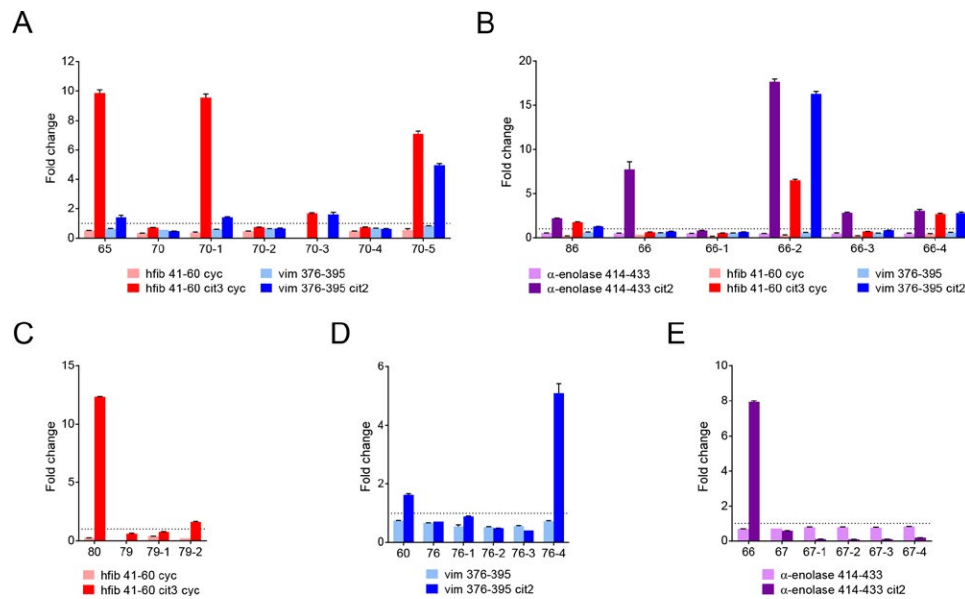


Figure 6. Paratope mutagenesis studies of clonally related ACPAs alter epitope specificity and polyreactivity. For detection of altered citrullinated antigen reactivity of mutated recombinant ACPAs, pairs of antibodies from within the ACPA-producing clonal families were selected based on having divergent reactivity against 1 or more citrullinated peptides. Specifically, recombinant monoclonal antibodies (mAb) 65 and 70 (**A**) were selected from the clonal family presented in Figure 4C. Recombinant mAb pairs 66 and 86 (**B**) and 66 and 67 (**E**) were selected from the clonal family presented in Figure 4E, and recombinant mAb pairs 79 and 80 (**C**) and 60 and 76 (**D**) were selected from the clonal family presented in Figure 4A. Mutated versions of the ACPAs were designed based on the predicted residues identified in the modeling studies that were predicted to be critical for antigen binding (see Figure 5 and Supplementary Table 4, <http://onlinelibrary.wiley.com/doi/10.1002/art.40760/abstract>). The original pair of recombinant antibodies and their corresponding mutated versions were then analyzed by peptide enzyme-linked immunosorbent assays for reactivity against the same antigens used in the modeling studies. Fold change values above the horizontal broken line are considered to be positive hits (fold change >1). Data are presented as the mean \pm SEM, and are representative of at least 2 replicated experiments. vim = vimentin (see Figure 2 for other definitions).

70 against both peptides (recombinant mAb 70-1) (Figure 6A). Importantly, these 4 residues lie in the framework region flanking the heavy-chain CDR2 and were predicted by the modeling studies to be involved in binding. Mutating another region adjacent to the light-chain CDR1 also induced binding to both antigens, although the reactivity against hFibA 41–60 was largely abrogated (recombinant mAb 70-3). Mutating the L31 residue or light-chain CDR3 (recombinant mAb 70-2 and 70-4) did not alter binding. Interestingly, combining all the mutations together (recombinant mAb 70-5) induced not only binding to hFibA 41–60 cit3 cyc but also greater reactivity to vim 376–395 cit2, when compared to either of the original, subject-derived native recombinant mAb.

Similar results were attained for the other pairs of antibodies. Sequences from recombinant mAb 86 were transferred to recombinant mAb 66, resulting in binding activity to hFib 41–60 cit3 cyc and vim 376–395 cit2 (Figure 6B). In particular, transferring residues from the light-chain CDR1 region of recombinant mAb 86 induced strong binding reactivity against the hFib, vim, and α -enolase peptides (recombinant mAb 66-2). The heavy-chain CDR3 from recombinant mAb 80, when grafted into recombinant mAb 79, resulted in a modest amount of binding to hFib 41–60 cit3 cyc (recombinant mAb 79-2) (Figure 6C).

Finally, transferring a combination of the heavy-chain CDR2, heavy-chain CDR3, and light-chain CDR3 from recombinant mAb 60 to recombinant mAb 76 induced strong reactivity against vim 376–395 cit2 peptide (recombinant mAb 76-4) (Figure 6D). Only 1 set of mutated antibodies failed to exhibit new differential reactivities—transferring sequences from recombinant mAb 66 to recombinant mAb 67 resulted in no change in binding against α -enolase 414–433 cit (Figure 6E).

Overall, using the predicted ACPA paratope contact residues identified from the molecular models enabled mutation of clonally related antibody pairs to convey new and/or polyreactive binding to citrullinated epitopes in 4 of the 5 antibodies that had previously shown no reactivity. These mutagenesis studies mimic the somatic hypermutation process, and thus provide further evidence that affinity maturation of ACPAs during clonal expansion can lead to both binding to distinct citrullinated epitopes and/or increased polyreactivity of individual ACPAs in RA.

DISCUSSION

The phenomenon of epitope spreading of the ACPA response against citrullinated antigens is a well-established

feature of RA, but little is understood with regard to how and why it occurs. In the present study, by sequencing the plasmablast antibody repertoire, performing molecular modeling, and characterizing the encoded antibodies, we demonstrate a greater level of ACPA functional diversity at the single-cell level than has been previously reported. We additionally report data suggesting that somatic hypermutations occurring during affinity maturation confer antibody paratopes that mediate both binding to distinct citrullinated epitopes and/or increased polyreactivity, which results in epitope spreading of the serum ACPA response.

To guide our analysis of the ACPA repertoire, we developed cit-peptide tetramers as a sort reagent. This sort reagent enabled identification of ACPA-producing B cells in the blood from individuals with RA, as confirmed by positive reactivities of the encoded recombinant mAb in CCP ELISAs and against citrullinated antigens on synovial antigen arrays. Several of the expressed cit-tet+ plasmablast recombinant mAb did not bind citrullinated antigens either in the ELISA or on planar arrays. There are several potential explanations for this, including potential differences in the conformation of the peptides, which is known to be important for ACPA recognition, as they may differ in solution during flow cytometry as compared to when printed on epoxy slides. Furthermore, some cit-tet-recombinant mAb showed positive reactivity to citrullinated antigens. Since we used a cocktail of 14 citrullinated peptides to generate our tetramers—a relatively small representation of the spectrum of citrullinated antigens known to be present in RA joints—which would be expected to only enrich for a subset of ACPA-producing B cells, we likely missed some ACPA-producing B cells.

Based on the findings from the ELISAs and antigen array analyses, we determined that many of the ACPA recombinant mAb were polyreactive. This is in line with the findings from previous studies, in which ACPAs have been shown to cross-react against multiple citrullinated epitopes (21,30–32). Of note, while most of the studies were limited to analyses of the polyclonal antibodies present in RA patient blood, only a few studies characterized monoclonal ACPAs. Of these, one group identified 36 patient-derived ACPAs, 66.7% of which cross-reacted against multiple epitopes (33). This is similar to our own data, in which 72.2% of our patient-derived ACPAs bound multiple citrullinated epitopes, suggesting that the majority of ACPAs display some degree of polyreactivity. In addition, by testing for reactivity against a greater number of citrullinated antigens via synovial antigen arrays, we demonstrated distinct polyreactivity patterns between individual ACPAs, and showed that affinity maturation can increase the polyreactivity of individual ACPAs.

While the heterogeneity of ACPA reactivity is well established, it is unclear whether the epitope spreading and overall breadth of the ACPA response arises from de novo activation of ACPA B cells

targeting distinct citrullinated epitopes or is the result of diversification of the citrullinated epitope binding specificity within individual clonal families. An important finding of this study is the divergence of reactivities observed among clonally related ACPAs, along with the expanded polyreactivity of individual affinity-matured ACPAs against distinct sets of citrullinated antigens.

By definition, clonal families of antibodies derive from a single, activated parent B cell. They share the same germline genes and junctional rearrangements, differing only in the somatic hypermutations that each antibody has individually accumulated over time. According to this dogma, B cells would be selected for continued clonal expansion if their somatic hypermutations increase the affinity and specificity against the original antigen. However, our data suggest that this is not the case for ACPAs, at least not in terms of their antigen specificity. We demonstrate multiple cases in which clonally related ACPAs exhibited differential binding to citrullinated epitopes. Moreover, we demonstrated differences in the ability of ancestral antibodies to bind citrullinated epitopes, often recognizing fewer targets than the affinity-matured antibodies. Finally, we utilized molecular modeling and mutagenesis to recreate somatic hypermutations, proving that they can change the antigen specificities of ACPAs. Taken together, our results reveal that somatic hypermutation alters the specific citrullinated epitopes bound by clonally related ACPAs. We propose that somatic hypermutation during clonal expansion directly causes epitope spreading of the ACPA response.

In a healthy immune response, the majority of B cells encoding autoreactive antibodies arising from affinity maturation are tolerated (34). In autoimmune diseases, these checkpoints can be disrupted, resulting in the escape of B cells encoding autoreactive antibodies. This phenomenon is not unique to RA; autoantibodies against double-stranded DNA can arise from nonautoreactive B cells undergoing somatic hypermutation in mouse models (35,36) and in patients with systemic lupus erythematosus (37). Similarly, autoantibodies against desmoglein 3 in pemphigus vulgaris can be generated through somatic hypermutation (38). Our study is the first to reveal the role of affinity maturation-mediated somatic hypermutation in the generation of ACPAs. Somatic hypermutations induced novel reactivities and/or polyreactivities against citrullinated antigens, resulting in epitope spreading within clonal families of ACPA B cells. In addition, ACPA paratopes that were artificially reverted to the inferred germline sequences lost their anticitrulline reactivity and polyreactivity, suggesting that ACPA paratopes may arise from nonautoreactive B cells.

It is important to recognize the role of citrullinated epitopes in driving the evolution of ACPAs. Based on our studies, we believe that citrulline acts as a “quasi-hapten,” increasing the antigenicity of an otherwise heterogeneous set of antigens, thereby allowing ACPAs to mutate in ways that increase their polyreactivity, so long as they retain a strong affinity for the citrulline residue itself. In this way, antibodies switching from protective reactivity against foreign citrullinated antigens, such as those arising from *Porphyromonas*

gingivalis infection or smoking, to pathogenic autoreactivity against citrullinated self antigens are easily conceivable (33,39). We hypothesize that citrulline recognition increases the likelihood of an ACPA-producing B cell receiving an activation signal because its B cell receptor is less restricted in terms of which antigens it can bind. Increased B cell activation, coupled with polyreactive ACPAs, leads to the continued expansion of B cell populations targeting multiple citrullinated antigens. Accordingly, our findings suggest that affinity maturation during clonal expansion drives epitope spreading of the ACPA response.

If citrulline is indeed acting as a quasi-hapten, elucidating the mechanisms by which ACPA B cells are generated could give rise to next-generation diagnostics and therapeutics. Conceivably, a common feature responsible for targeting citrullinated epitopes may exist among ACPAs. Identification of a conserved binding paratope could lead to treatments that selectively target ACPA-producing B cells. We characterized the ACPA paratope through molecular modeling and mutagenesis. We were able to predict and verify key contact residues important for binding to citrullinated antigens, including the framework region next to heavy-chain CDR2. This is an interesting finding given that framework regions are believed to primarily maintain loop structures and not direct antigen binding. The role of the framework regions in modulating the specificity of the ACPA paratope should be further investigated. Although our studies identified regions within the ACPA paratope important for citrullinated antigen-specific binding, crystallization studies will be necessary to confirm the critical citrulline-binding residues.

Limitations to our study include the patient population of the VA hospital, which is predominantly male, and thus the subjects utilized do not fully reflect the general RA patient population. Furthermore, at the time of our study, our technology was only optimized to sequence plasmablasts expressing IgG or IgA isotype antibodies, and therefore only B cells expressing these 2 antibody subclasses were analyzed. Additionally, given the limited number of patients analyzed, the repertoire-sequencing depth, and the number of recombinant antibodies expressed, it is difficult to estimate the extent to which affinity maturation within clonal families results in epitope spreading within individual patients and across the entire RA population. In our study, we selected the largest cit-tet+ clonal families with polyreactivity against citrullinated antigens for in depth characterization; however, these clonal families were all derived from a single donor (RA3). Therefore, although we anticipate that multiple clonal families with differentially mutated members will also exhibit epitope spreading in the repertoires of other RA patients, it is also possible that there will be families with differentially mutated members that exhibit a shared specificity without evidence of epitope expansion.

The mechanisms driving the evolution of the antibody repertoire may vary between clonal families within a patient, as well as between patients, based on the antigens targeted or may vary over the course of disease. Nevertheless, our study establishes

that affinity maturation is a mechanism by which epitope spreading of ACPAs can occur. Although it is possible that our observations in the RA3 sample may represent an extreme case, given the ease with which a few mutations to the variable region of an ACPA can alter its epitope specificity, we suspect that epitope spreading via somatic hypermutation is a common occurrence. We anticipate that affinity maturation-driven epitope spreading has the potential to result in diversification of the specificity of the autoantibody response in multiple directions, depending on the specific mutations conferred in the process. Additional studies utilizing deeper sequencing in a larger cohort would help further define the extent to which this mechanism mediates epitope spreading in RA.

Taken together, our findings shed light on a mechanism mediating the pathogenesis of RA, in which somatic hypermutation and affinity maturation result in epitope spreading within lineages of ACPA-encoding B cells, as well as generation of individual ACPAs that have increased polyreactivity and that contribute to the reactivity of the overall ACPA response in RA. Further structural analysis of ACPA-citrullinated antigen interactions may help identify citrulline-specific and epitope-specific paratope regions that could inform the development of next-generation diagnostics and therapeutics. Finally, defining and characterizing the germline versions of ACPAs could reveal the key antigens involved in breaking tolerance to initiate the development of RA, and thereby open avenues for disease prevention.

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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. Robinson 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. Kongpachith, Robinson.

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