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Pathogenic citrulline-multispecific B cell receptor clades in rheumatoid arthritis

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Abstract

OBJECTIVE—Anti-citrullinated protein antibodies (ACPA) have proven highly useful as biomarkers in rheumatoid arthritis (RA). However, the composition and functionality of the associated autoreactive B cell repertoire have not been directly assessed. We aimed to selectively investigate citrullinated autoantigen-specific B cell receptors (BCRs) involved in RA and initiate studies on their pathogenicity.

METHODS—A University of Minnesota ACPA⁺ RA cohort (n = 89 subjects) provided blood samples. Tetramer sets bearing citrullinated filaggrin peptide CFC1 or citrullinated α -enolase

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Author contributions:

PJT designed experiments, conducted the majority of experiments, acquired data, analyzed data, and wrote the manuscript.

GW, NS, NZ, PS, LOB, AB, MH, LI conducted select experiments.

AKS acquired data and analyzed data.

YZ created a software pipeline for data analysis.

TBN, YGP designed experiments and acquired data.

LK analyzed data and edited the manuscript.

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KA acquired data, provided reagents, and edited the manuscript.

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peptide CEP-1 were constructed to specifically capture autoreactive B cells from the unaltered, polyclonal repertoire of RA patients. Citrullinated peptide tetramer-bound B cells were subjected to flow cytometric cell sorting and single-cell *IGH*, *IGK*, and *IGL* gene sequencing for B cell lineage determinations. BCR gene sequences were also expressed as recombinant monoclonal antibodies (mAbs) for direct evaluation of citrullinated autoantigen binding and effector functionality.

RESULTS—Using citrullinated peptide tetramer-enrichment to investigate single autoreactive blood B cells, we identified biased V-region gene usage and conserved junction arrangements in RA subject BCRs. Parsimonious clustering of related immunoglobulin gene nucleotide sequences revealed clonal expansions of rare individual B cell clades in parallel with divergent sequence mutations. Correspondingly, recombinant mAbs generated from such BCR lineages demonstrated citrulline-dependent cross-reactivity extending beyond the citrullinated peptides used for B cell capture. A pair of citrullinated autoantigen-specific mAbs with cross-reactive binding profiles also promoted arthritis in mice.

CONCLUSION—Our findings suggest that broad ACPA specificities in RA arise from a restricted repertoire of evolving citrulline-multispecific B cell clades with pathogenic potential.

Rheumatoid arthritis (RA) is a systemic autoimmune disease marked by chronic inflammation and eventual destruction of the synovial joints. Infiltration by effector T and B lymphocytes and strong genetic association with certain HLA alleles signify the central role of adaptive immune cells in RA disease pathogenesis (1). The successful treatment of RA with anti-CD20 (B cell-depleting) rituximab also suggests critical B cell involvement in the disease (2, 3). Citrulline-modified proteins arising from the post-translational deimination of native arginine residues have been recognized as primary RA autoantigen targets (4–7). Anti-citrullinated protein antibodies (ACPA) can recognize a variety of citrullinated antigens, and serum from individual ACPA⁺ RA patients typically reacts to more than one (8, 9). These targets include citrullinated sequences derived from human filaggrin (CFC1), α -enolase (CEP-1), fibrinogen, vimentin, collagen II, and histone proteins (5, 9–13), as well as several others. How B cell responses toward different citrullinated epitopes evolve at the clonal level and contribute to RA pathogenesis is not yet understood.

Non-specific capture of RA blood or synovial B cells has demonstrated that reactivity to multiple citrullinated targets can be achieved by a single B cell antigen receptor (BCR) (14–18). However, efforts to investigate full autoreactive repertoires have been limited by the difficulty of isolating rare self antigen-specific B cells. We and others have recently shown the utility of antigen tetramer enrichment systems to identify autoreactive B cells of interest by flow cytometry for further analysis (19–21). Based on the success of this approach, we hypothesized that combining citrullinated peptide tetramer capture with single-cell cloning procedures would enable comprehensive analysis of Ig genes from rare blood B cell populations in ACPA⁺ RA. We now show that extended BCR sequence clades arise from permissive heavy/light chain gene rearrangements and constitute the majority of RA blood B cells with citrullinated peptide target reactivity. Individual BCRs within each clade display sequence divergence as a result of progressive and extensive somatic hypermutation (SHM). Finally, recombinant mAbs generated from these sequences demonstrate multi-target citrulline-binding capacity and pathogenicity in a mouse model of inflammatory arthritis.

PATIENTS AND METHODS

University of Minnesota ACPA⁺ RA patient cohort

This investigation reports data obtained from 89 individuals with ACPA⁺ RA, based on the 2010 American College of Rheumatology criteria and at least one positive clinical CCP assay. 9 volunteer blood donors served as healthy controls. This study was approved by the Institutional Review Board at the University of Minnesota (IRB code number 9712M00072) and all individuals gave informed consent to participate.

RA target self antigens

Native arginine-containing and corresponding citrulline-modified peptides, in cyclic form with an N-terminal biotin moiety, were custom ordered from GenScript. Peptides (>95% purity) were reconstituted to 1 mg/ml in sterilized water. Cyclized peptide sequences for CF0 and CFC1 (biotin-HQCHQEST-X-GRSRGRCGRSGS, where X=Arg or Cit respectively) are based on identified targets within human filaggrin (10). Previously described REP-1 and CEP-1 peptide antigen targets from human α -enolase protein (22) were similarly adapted for cyclization (biotin-HQCKIHA-X-EIFDS-X-GNPTVEC, where X=Arg or Cit respectively). All subjects and controls were tested for serological reactivity to each peptide (Supplementary Methods). CFC1, CF0, CEP-1, and REP-1 peptides were also separately loaded onto fluorescently-labeled streptavidin (SA) to generate reagents for antigen-specific B cell detection and capture (Supplementary Methods).

Flow cytometry

PBMC cells were pre-incubated with decoy tetramers (CF0 or REP-1) for 10 minutes, then CFC1 or CEP-1 tetramer were added for an additional 30 minutes. Tetramer-bound B cells were captured on magnetic beads and enumerated by flow cytometry as described (Supplementary Methods). For BCR gene sequencing experiments, single tetramer-bound B cells were isolated by flow cytometry as described (Supplementary Methods). Tetramer-bound and random switched-memory B cells from subjects RA14, RA37, and RA62 were individually sorted on a BD Biosciences FACS Aria into 96-well PCR plates. Additionally, RA37 tetramer-bound and random switched-memory B cells were sorted and captured as individual cells using a small sized C1 mRNA-Seq 96 well chip and the C1 Single Cell Auto Prep System (Fluidigm) (Supplementary Methods).

Ig nucleotide sequence analysis

For PCR plate-sorted samples, individual wells yielding IgH or Ig κ /Ig λ amplicons of expected length were purified (Qiagen or Macherey-Nagel) and read by Sanger sequencing (Eurofins Genomics). Following C1 chip capture, barcoded cDNA libraries were sequenced on an Illumina Hi-Seq 2500 sequencer, and then *IGH*, *IGL*, and *IGK* mRNA sequence reads were analyzed as previously described (23). Trimmed paired-end RNA-Seq reads were filtered based on homology to Ig heavy or light chain loci using Bowtie 2 (24), and then aligned by overlap to generate optimal Ig transcript models using Trinity (25). Plate-sorted and NGS transcripts were evaluated by IMGT/V-Quest (26) to identify productive V-(D)-J arrangements and somatic mutations. Parsimonious alignments of homologous V-(D)-J

nucleotides by the PHYLIP/DNApars algorithm (<http://evolution.gs.washington.edu/phylip.html>) were used to predict the sequence of putative unmutated germline rearrangements within a B cell clade. Finally, select Ig heavy and light chain sequences were expressed as human IgG1 or murine IgG2a recombinant mAbs as previously described (14, 16; Supplementary Methods). Monoclonal antibodies were also examined for binding to citrullinated peptides and proteins (Supplementary Methods).

Animals and injections

BALB/c mice, aged 12–15 weeks, were injected intravenously with either saline or 1 mg IgG2a murinized monoclonal antibody. Eight days later, 1 µg *E. coli* lipopolysaccharide (Sigma) was injected into the ankle joint of the left hind paw, under isoflurane anesthesia. Tactile sensitivity of the ipsilateral hind paw was evaluated, as previously described (27), using von Frey filaments (Marstock OptiHair) and a 50% withdrawal threshold was calculated using the Dixon up-down method (28). Thresholds are displayed as a percent of the baseline withdrawal threshold. Ipsilateral ankle joint arthritis was assigned a score 0–3 based on visible swelling and erythema. Joint histology was also examined as described (Supplementary Methods). Mice were maintained in accordance with Karolinska Institutet and NIH guidelines. Experiments were carried out in accordance with protocols approved by the local ethics committee for animal experiments in Sweden (Stockholm North Animal Ethics Board). Tester was blinded to the treatment of the mice.

Statistical analysis

P-values for parametric sequence mutation rate comparisons and histology inflammation scores were calculated using an unpaired two-tailed Welch's *t*-test. Non-parametric comparisons between two groups for the remaining data were performed using the Wilcoxon Mann-Whitney U test. Statistical comparisons between multiple groups utilized Chi-Square method for proportions and ANOVA for comparisons of means. Correlations were estimated using Spearman's Rank-Order test.

RESULTS

Switched-memory B cells specific for CFC1 or CEP-1 are increased in the blood of ACPA⁺ RA subjects

To identify autoreactive blood B cells that are specific for citrullinated antigens, we designed peptide antigen tetramer probe sets to bind select BCRs with high specificity and avidity. Construction of the citrullinated filaggrin CFC1 and citrullinated α-enolase CEP-1 tetramers relied on the loading of N-terminal biotinylated cyclic peptides onto streptavidin (SA)-phycoerythrin (PE). The cell-binding interaction thereby enables magnetic microbead enrichment of relevant B cells and interrogation by flow cytometry, as we have previously described (19). To exclude irrelevant B cells capable of binding either structural components of the tetramers (e.g., SA, PE, biotin linker) or native (non-citrullinated) polypeptide sequences, B cell samples were always pre-incubated with either native filaggrin CF0– or native α-enolase REP-1–loaded SA-PE*AF647 decoy tetramers. All AF647⁺ B cells were then excluded from the analysis.

Using this antigen tetramer enrichment strategy, we examined peripheral blood from both healthy control subjects as well as from individuals with ACPA⁺ RA. Our ACPA⁺ RA cohort consisted of 89 subjects recruited at the University of Minnesota (see Supplementary Table 1), 49% and 53% carrying serum autoantibodies for CFC1 and CEP-1, respectively (Fig. 1a and Supplementary Fig. 1). More than half of the combined CFC1⁺ and CEP-1⁺ RA subjects were found seropositive for both citrullinated targets (Fig. 1a and Supplementary Fig. 1b, c). In 9 healthy individuals, a small yet defined population of CFC1 tetramer-binding B cells was identified. Only a small minority of these B cells displayed an isotype-switched and differentiated IgD⁻ CD27⁺ (switched-memory) phenotype indicative of previous antigen recognition. A similarly small population of CFC1-reactive B cells was also observed in blood samples from CFC1 seronegative RA subjects, again with only a small fraction of the cells displaying a switched-memory phenotype (Fig. 1c,e,f). In contrast, we detected a ~2.5-fold increase in total CFC1 tetramer-specific B cells in CFC1⁺ RA subjects relative to healthy controls (Fig. 1d–f). The majority of these cells were differentiated to an IgD⁻ CD27⁺ phenotype, corresponding to a nearly 6-fold increase in the number of CFC1-binding switched-memory B cells in CFC1⁺ RA patients as compared to either CFC1⁻ RA subjects or healthy controls. This observed differentiation of CFC1⁺ B cells to a switched-memory phenotype appeared to be citrulline-specific, as no differences were seen between subject groups for either CF0-SA-PE*AF647 decoy tetramer-binding or total blood polyclonal switched-memory B cells (Fig. 1f,g).

The frequency of CFC1-specific switched-memory B cells in CFC1 seropositive RA subjects showed significant direct correlation to the amount of CFC1 antibody detected in their serum (Fig. 1h). Association was also observed between the frequency of CFC1 tetramer-binding switched-memory B cells and the DAS28-ESR ($R = 0.51$, P -value = 0.01) and DAS28-CRP ($R = 0.41$, P -value = 0.01) disease activity indices, as well as the duration of RA ($R = 0.38$, P -value = 0.02), current prednisone use (P -value = 0.01), and subcutaneous nodules (P -value = 0.02) (Supplementary Table 2). Thus, clonal expansion of CFC1 tetramer-binding IgD⁻ CD27⁺ switched-memory B cells in the blood and increased production of CFC1-specific autoantibodies appear associated with more severe disease course among ACPA⁺ RA patients.

To establish the generality of these findings, we also investigated the number and phenotype of peripheral blood CD19⁺ B cells reactive to a second citrullinated antigen implicated in RA: citrullinated α -enolase peptide CEP-1. Using a combination of CEP-1-SA-PE and decoy REP-1-SA-PE*AF647 tetramers, CEP-1 seropositive RA subjects displayed a greater than 3-fold expansion of CEP-1 tetramer-binding B cells as compared to CEP-1⁻ RA subjects (Fig. 1i). Consistent with the CFC1 results, a majority of tetramer-specific B cells in the CEP-1⁺ RA group also expressed a switched-memory IgD⁻ CD27⁺ phenotype, whereas fewer CEP-1-reactive switched-memory B cells were detected in the CEP-1⁻ RA group. Pre-incubation of cell samples with monomeric CEP-1 peptide at high molar excess eliminated the majority (~85%) of CEP-1 tetramer binding with no appreciable effect on REP-1 decoy tetramer binding (Fig. 1j). Similar to the CFC1-specific switched-memory B cell results, ACPA⁺ RA subjects with current prednisone use or a history of subcutaneous nodules were found to have a significantly higher frequency of switched-memory CEP-1 tetramer-binding B cells (P -values = 0.030 and 0.002, respectively, Supplementary Table 2).

Autoreactive B cells can bind to more than one citrullinated peptide

To more critically examine the reactivity of citrullinated peptide tetramer-binding B cells in RA subjects that express both the CFC1 and CEP-1 autoantibodies, we modified our tetramer enrichment strategy to simultaneously detect B cells specific for CFC1 and CEP-1. Here the biotinylated CFC1 peptide was loaded onto allophycocyanin (APC)-conjugated SA and used in parallel with the previously described CEP-1-SA-PE tetramer. B cells binding non-target/non-citrullinated tetramer epitopes were again excluded by pre-incubation of samples with differentially labeled decoy complexes. Gating on decoy-negative B cells in CFC1 and/or CEP-1 seropositive RA patient samples, we identified a small but distinct population of switched-memory B cells that simultaneously bound to both CEP-1 and CFC1 tetramers in many subjects (Fig. 1k). Therefore, the results offered evidence that some autoreactive blood B cells in RA patients can recognize more than one citrullinated peptide antigen.

Citrullinated peptide-binding B cells in ACPA⁺ RA express BCRs with restricted IGH/IGK/IGL V-region gene usage and CDR3 lengths

T cell-dependent B cell activation and differentiation typically commences when productive *IGH*, *IGK*, and *IGL* somatic gene rearrangements encode a BCR with at least low affinity for available antigen. Somatic hypermutation (SHM) and positive selection within germinal centers (GC) then leads to affinity maturation of competitive B cell clones (29). Nevertheless, the citrullinated autoantigen-selected Ig gene rearrangements and subsequent SHM events in ACPA⁺ RA are still unknown. Therefore, BCR *IGH*, *IGK* and *IGL* mRNAs were amplified and sequenced from individual citrullinated peptide tetramer-bound B cells captured from a total of three different RA subjects, as well as from random switch-memory B cells sorted from the same individuals. Single cell Ig heavy (n = 226) and light (n = 228) chain gene sequence reads were obtained for analysis either following nested PCR amplifications or through direct RNA-Seq high-throughput sequencing using a Fluidigm C1 platform.

Shared immunoglobulin V-region gene assignments and predicted complementarity determining region (CDR) 3 lengths served to estimate BCR repertoire diversity in the population of captured B cells. Biased heavy and/or light chain gene rearrangements became apparent in the citrullinated tetramer-selected B cell repertoire of all three RA subjects. For CFC1 tetramer-bound B cells from subject RA14, 71% of heavy chain sequences displayed one of the two highly related *IGHV4-31* or *IGHV4-30-4* genes sharing a CDR3 sequence length of 17 (Fig. 2a). No comparable heavy chain rearrangements were detected in random switched-memory B cells from that same subject, indicating significant bias (P-value <0.001). 71% of light chains from these same tetramer-bound B cells arose from *IGKV4* or *IGKV1* rearrangements with CDR3 lengths of 9 or 10 (Fig. 2b). Here, one light chain clone in the random switched-memory B cells from the same subject also displayed a similar rearrangement (*IGKV4-1*/CDR3=9); nevertheless, light chain recombination still appeared biased in the tetramer pull-down population (P-value = 0.016). Repertoire bias was also observed in the CFC1 tetramer-bound B cells of subject RA62. Here, 77% of heavy chain sequences demonstrated one of two specific gene recombination signatures: *IGHV4-39*/CDR3=11 or *IGHV3-33*/CDR3=18 (Fig. 2a). No comparable heavy chain rearrangements

were detected in the random switched-memory B cells from that same subject (P-value <0.001). Similarly, 75% of the CFC1–selected B cell light chain sequences displayed either a *IGKV4-1/CDR3=9* or *IGKV3-20/CDR3=9* rearrangement, whereas these recombinations were significantly less frequent (P-value <0.001) in the control switched-memory B cells (Fig. 2b). Although heavy chain gene rearrangements in the CEP-1 tetramer–bound B cells of subject RA37 were more diverse, recombination events with particular conserved *IGHV* gene usage/CDR3 length were still significantly more frequent than randomly selected switched-memory B cells (P-value = 0.013). (Fig. 2a). Light chain V-gene usage and CDR3 length were also found to be very significantly biased (P-value <0.001) in CEP-1–specific B cells as compared to random sorted switched-memory B cells (Fig. 2b). Thus, the Ig heavy and light chain gene sequencing data obtained in these three subjects suggest that most citrullinated peptide tetramer-binding RA blood B cells express a restricted set of BCRs consistent with oligoclonal expansion.

Citrullinated peptide–binding B cells undergo extensive immunoglobulin gene hypermutation in ACPA⁺ RA

Further examination of citrullinated peptide tetramer–selected B cell Ig V-region genes in these three RA subjects revealed high rates of SHM consistent with a history of vigorous GC reaction. *IGH*, *IGK*, and *IGL* V-region gene sequences from CFC1 and CEP-1 tetramer–selected B cells on average carried significantly greater total mutations than V-regions from randomly purified switched-memory B cells from the same subjects (Fig. 2c,d). As expected, SHM in all sample groups was concentrated within CDRs, with framework regions (FWRs) more closely resembling the germline (Fig. 2e,f). Furthermore, signs of positive selection pressure were often detected within either the CDR1 or CDR2 V-region sequences of both the heavy and light chains in tetramer–bound B cells, based on average replacement:silent (R:S) mutation ratios that were frequently >2.9 (ref. 30).

Defining individual BCR gene clades within the citrullinated peptide–binding blood B cell repertoire

The biased BCR gene characteristics identified within the citrullinated antigen–binding B cell repertoires could have resulted either from a failure to delete frequent autoreactive precursor cells having similar gene rearrangements (i.e., defects in central and/or peripheral self-tolerance checkpoints) or from the clonal expansion and sequence divergence of rare autoreactive B cells. To determine how SHM directly contributes to citrullinated protein antigen–specific BCR sequence diversity in these RA subjects, we assessed the evolution of expanded heavy and light chain sequence lineages using the DNAPars algorithm of the Phylogeny Inference Package (PHYLIP). In subject RA37, we identified 6 unique B cell clades with 4 cells per group whose members shared recombined gene organization, V- and J-gene usage, and CDR3 sequence length features for both their heavy and light chains (when data were available for both) (Fig. 3a and Supplementary Fig. 2). Based on DNA sequence alignment and maximum parsimony, *IGHV*-D-J nucleotide sequences from a total of 11 unique CEP-1 tetramer–binding BCRs defined one phylogenetic clade with sequential SHM from a putative unmutated *IGHV4-59*01/D3-9*01/J6*02* (CDR3=18) recombination event (Fig. 3a). This clade of CEP-1–binding heavy chains was also associated with a lambda light chain that appeared to arise from an unmutated *IGLV3-21*02/J1*01*

(CDR3=11) precursor via sequential SHM. For both the heavy and light chains, those B cells that appeared more distal in the lineage demonstrated progressively higher numbers of replacement mutations within the CDR3. In addition to this *IGHV4-59*01/D3-9*01/J6*02* and *IGLV3-21*02/J1*01* clade, we identified 5 more B cell clades in RA37 by the same criteria (Supplementary Fig. 2). Taken together, more than 40% of the CEP-1–specific B cells in subject RA37 arose from the iterative SHM and apparent positive selection of descendants from 6 individual unmutated precursor B cells.

We applied the same phylogenetic analysis to the CFC1–selected B cells of subjects RA14 and RA62. In RA14, we identified two extended B cell clades. The dominant clade of 19 B cells demonstrated BCR heavy chains that arose through successive SHM of a putative unmutated *IGHV4-31*03/D3-16*02/J4*01* (CDR3=17) germline rearrangement event (Fig. 3b). This dominant B cell clade in RA14 also expressed light chains that appeared to arise from an unmutated *IGKV4-1*01/J5*01* (CDR3=10) rearrangement. In subject RA62, maximum parsimony analysis suggested that heavy chains in the dominant B cell clade (n = 11) descended from a putative *IGHV4-39*01/D3-10*01/J3*02* (CDR3=11) rearrangement, and light chains in the dominant clade descended from a putative *IGKV4-1*01/J3*01* (CDR3=9) rearrangement (Fig. 3c). In both RA14 and RA62, clonal divergence occurred as a result of SHM with progressive accumulation of CDR3 replacement mutations in both the heavy and light chains. Remarkably, in no case were identical heavy and light chain sequences shared between any two captured B cells within a clade. In summary, the assembled phylogenies suggest that the selection and expansion of a few autoreactive B cell clones with permissive Ig gene recombination events in RA can lead to vast autoreactive BCR diversity through extensive SHM and the accumulation of replacement mutations.

Citrullinated autoantigen binding activity by sequenced BCR clones

Paired heavy and light chain *Ig* gene sequences were available for a total of 105 citrullinated peptide tetramer-captured B cells, with 52 of these B cells (50%) demonstrating membership within one of the extended BCR gene clades. To establish the antigen specificity of these B cells, we initially cloned and expressed 20 BCR gene sequences as human recombinant mAbs. Using ELISA to screen for peptide antigen binding, we determined that 7 of the 20 clones (35%) had specific reactivity to either CFC1 or CEP-1 (data not shown). Importantly, all 7 of these citrullinated peptide-binding mAbs carried sequences derived from one of the expanded BCR gene clades discovered in subjects RA14, RA37, and RA62. In contrast, none of the 11 non-clade-derived mAbs bound to the citrullinated antigens, and this difference from the 9 clade-associated mAbs was highly significant ($\chi^2 = 13.26$, P-value <0.001). Therefore, among all citrullinated peptide tetramer-captured B cells membership in an expanded B cell clade was associated with a greatly increased likelihood for binding to a citrullinated peptide epitope.

The expression of one of these citrullinated peptide-binding clones (14CFCT3F09) could not be maintained *in vitro*; therefore, it was not analyzed further. However, the specificity of the remaining 6 citrullinated peptide-binding mAbs (14CFCT2D09, 14CFCT2H12, 14CFCT3G09, 62CFCT1E04, 37CEPT1G09, 37CEPT2C04) was investigated in greater detail. Based on the initial clade results described above, we also intentionally prepared 7

additional human mAbs for specificity analysis using paired heavy and light chain gene sequences selected from B cells that were associated with expanded BCR gene clades in subjects RA62 (62CFCT1C03) and RA37 (37CEPT1C11, 37CEPF1C22, 37CEPF1C40, 37CEPF1C93, 37CEPF2C05, 37CEPF2C71). Of the 13 clade-derived human mAbs tested, all bound to CFC1 and 8 showed an ability to bind to both CFC1 and CEP-1 (Fig. 4a, b). In contrast, only one of the 21 randomly selected switched-memory B cell mAbs bound to the CEP-1 weakly, and none bound to CFC1.

Taken together with the flow cytometry demonstration of dual CFC1 and CEP-1 tetramer-binding B cells (Fig. 1k) and the genetic evidence of hypermutating and diverging Ig gene sequences within clades (Fig. 3 and Supplementary Fig. 2), we hypothesized that some of these hypermutated BCRs may have gained affinity for citrullinated antigens beyond the citrullinated peptides used for the tetramer selection. To explore this possibility, mAbs were tested for their capacity to specifically bind to an extended panel of citrullinated peptides. As shown in figure 4c, all 13 mAbs bound 2–8 unique citrullinated peptides in excess of the corresponding native non-citrullinated peptide. Thus, RA blood B cells that have undergone clonal expansion and somatic hypermutation as a member of an extended BCR gene clade possess a remarkable degree of citrulline-specific cross-reactivity that we refer to as *citrulline-multispecificity*, similar to what has been previously observed (13, 31, 32).

Arthritogenic potential of RA blood B cell-derived citrullinated protein-specific immunoglobulins in mice

Individuals developing ACPA⁺ RA often experience joint pain (arthralgia) and trabecular bone loss prior to signs of overt synovial inflammation (1, 33, 34). Recently, we demonstrated that the infusion of certain citrullinated peptide-specific immunoglobulins into mice could induce this early pathology, i.e. arthralgia and bone loss (27, 35). This led us to question whether any of the hypermutated and citrulline-multispecific BCR sequences isolated here might also recapitulate symptoms and signs of arthritis in mice. To directly assess the arthritogenicity of tetramer-captured and citrulline-multispecific BCRs, 4 clones (14CFCT3G09, 62CFCT1E04, 37CEPT1G09, and 37CEPT2C04) were expressed as both human IgG1 and murine IgG2a recombinant mAbs. Whereas each of these 4 human mAbs demonstrated some degree of citrulline-multispecificity in the citrullinated peptide ELISA (Fig. 4), only the 37CEPT1G09 and 37CEPT2C04 clones exhibited binding to 4 or more of the tested full-length citrullinated proteins: α -enolase, fibrinogen, vimentin, histone H2A/B, and histone H4 (Fig. 5 and Supplementary Fig. 3).

Each of the 4 murine IgG2a recombinant mAbs was infused into mice. A small amount of LPS (titrated to yield a mild and temporary joint inflammation) was then delivered into one hind ankle joint on day 8 to model an inflammatory stimulus. mAbs 37CEPT1G09 and 37CEPT2C04 induced sustained arthritis with swelling and erythema, as well as new pain-like behavior through day 14 (Fig. 6a, b). The effect in these two groups was localized to the LPS-injected (ipsilateral) mouse hind paw, and led to an increased periarticular immune cell infiltration that could be observed by histological staining (Fig. 6c–e). In contrast, mice injected with either the 14CFCT3G09 or 62CFCT1E04 mAbs, or with a control IgG2a mAb derived from a random switched-memory B cell (62SMT1F05), followed a typical course of

acute arthritis resolution. Thus, select citrulline-multispecific immunoglobulins encoded by hypermutated heavy and light chain BCR genes in citrullinated peptide tetramer-binding blood B cells demonstrated arthritogenic potential.

DISCUSSION

This study of autoreactive BCRs in RA demonstrates the utility of combined tetramer enrichment and single-cell cloning technologies for the detailed analysis of rare clonal B cell lineages in human disease. Our results correlating the number of CFC1 and CEP-1 specific switched-memory B cells to RA disease activity and severity adds to the recent work showing a correlation between circulating CCP2 tetramer-binding B cell frequency and CCP2⁺ RA patient serum Ab titers (20). We now provide further insights into this autoreactive repertoire by evaluating the genetic, biochemical, and functional attributes of encoded immunoglobulin from such B cells. The majority of tetramer-captured B cells were shown to be members of large B cell clades that apparently arose from relatively rare unmutated ancestral clones – ultimately expanding and diverging as a consequence of SHM. Furthermore, we were able to identify for the first time RA patient-derived mAb clones that could promote joint inflammation in mice.

Our work raises several questions concerning the selection and persistence of divergent citrulline-multispecific BCRs in ACPA⁺ RA. Although extensive SHM was frequently seen, B cells carrying BCRs with more modest levels of SHM remained present within the repertoire – implying that strong reactivity to the selecting citrullinated peptide and fitness for positive selection by T cells in germinal centers is rapidly acquired during the evolution of the clade. The co-existence of both mildly and highly mutated sequences in the citrullinated autoantigen-specific B cell repertoire appears consistent with vaccination studies in mice showing that diverse BCR avidities (~40-fold range) from single clades persist in germinal centers even at late time points (36).

What then is the significance of this sequence divergence within BCR clades? The evolution of a broadly neutralizing antibody response to infectious agents may provide important clues. In the case of influenza A, for instance, an Ig lineage has been shown to progress from specific single virus strain-binding to broad cross-neutralization of multiple additional strains based on the accumulation of replacement mutations from sequential SHM (37). Interestingly, SHM within germinal centers following vaccination can also lead to the development of new ‘polyreactivity’ against self antigens by more heavily mutated descendant B cell clones (38). The extensive SHM we observe within clades may therefore work similarly in the context of RA to broaden citrulline-multispecificity of BCRs. Whereas select Ig gene rearrangements and early CDR mutation events may permit initial citrulline reactivity, subsequent CDR3 SHM could provide access to a more diverse array of activating citrullinated self antigen targets and follicular T cell help.

Evidence of gradual ACPA epitope-spreading that occurs over time even before the onset of joint inflammation has suggested that a broader ACPA repertoire may predispose to arthritis progression (32, 34, 39), but causality had not been tested *in vivo*. In support of this notion, we now show that broad citrulline-multispecificity by BCRs can be associated with

functionality to enhance joint inflammation and pain-like behavior in a mouse model of arthritis. Future longitudinal analyses of blood citrullinated peptide tetramer-binding B cell clades and their recombinant mAbs—both before and after the onset of RA—should provide a clearer understanding of how SHM and BCR sequence divergence contribute to the pathogenesis of ACPA⁺ RA. Likewise, the efficient capture of otherwise rare self-reactive B cells clones using a similar autoantigen tetramer-based approach, could prove highly informative in the investigation of other autoimmune diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Identification of RA self-reactive B cells using citrullinated peptide tetramers
(A) CFC1 and CEP-1 serological reactivity within the University of Minnesota ACPA⁺ RA cohort. Shading represents the relative level of citrullinated peptide-specific antibodies. Tetramer-bound B cells from **(B)** healthy control blood donors, **(C)** CFC1⁻ RA subjects, and **(D)** CFC1⁺ RA subjects. **(E)** Frequency of CFC1 (*left*) or CF0 decoy (*right*) tetramer-binding cells per million total blood B cells. **(F)** Frequency of CFC1 (*left*) or CF0 decoy (*right*) IgD⁻ CD27⁺ switched-memory phenotype cells per million total B cells. **(G)** Frequency per million of IgD⁻ CD27⁺ switched-memory B cells without selection or enrichment (flow-through fraction). **(H)** Frequency of tetramer-binding switched-memory B cells from **(F)** plotted as a function of measured CFC1 antibody levels. **(I)** Frequency of total (*left*) or IgD⁻ CD27⁺ switched-memory phenotype (*right*) CEP-1-SA-PE tetramer-binding cells per million total B cells. **(J)** Representative flow cytometric analysis and enumeration of CEP-1 tetramer-specific switch-memory B cells from CEP-1⁺ RA subjects (n = 2 experiments) captured either in the presence or absence of 500 μ M free CEP-1 peptide. **(K)**

Identification of CEP-1 and CFC1 cross-reactive B cells in a single sample using dual tetramer sets.

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Figure 2. Citrullinated peptide-specific BCRs display biased V-gene usage, conserved CDR3 lengths, and extensive SHM

Individual B cells were sorted from blood samples of two CFC1⁺ RA subjects (RA14 and RA62) using the CFC1 tetramer, as well as from one CEP-1⁺ RA subject (RA37) at three separate time points using the CEP-1 tetramer, and then recombined Ig nucleotides were sequenced and analyzed by alignment to human germline heavy and light chain genes using IMGT/V-QUEST. **(A)** Comparison of citrulline tetramer-selected B cells versus random blood switched-memory B cells for VH gene usage and heavy chain CDR3 junction length. **(B)** Comparison of citrulline tetramer-selected B cells versus random switched-memory B

cells for V λ /V κ gene usage and light chain CDR3 junction length. **(C, D)** Enumeration of total point mutations in **(C)** VH and **(D)** V λ /V κ genes. **(E, F)** Average frequency of mutation in FWRs and CDRs for **(E)** VH and **(F)** V λ /V κ genes. The ratio of replacement (R) to silent (S) mutations are also shown for each of these regions. Significant antigen-driven selection pressure is reported to result in R:S ratios exceeding 2.9 within CDR1 and CDR2.

Figure 3. Expanded citrullinated peptide-specific B cell lineages in ACPA⁺ RA patients demonstrate Ig gene sequence divergence

Citrullinated peptide tetramer-selected B cells with equivalent V-gene usage and shared framework and CDR organization were assigned to clades, and their V-(D)-J nucleotide sequences were aligned and clustered based on maximum parsimony using the Phylip/DNApars algorithm. Putative CDR3 V-(D)-J junction organization (*bold type*) was then predicted based on maximum parsimony and the principle of progressive somatic hypermutation. Predicted amino acid sequences are shown to the right of each panel. (A) Phylogenetic clade of CEP-1 tetramer-binding B cells (RA37). (B) Phylogenetic clade of

CFC1 tetramer-binding B cells (RA14). (C) Phylogenetic clade of CFC1 tetramer-binding B cells (RA62). Replacement (*R*) and silent (*S*) V-region nucleotide mutations are indicated. *Only a partial nucleotide sequence could be obtained for some clones. ‡Kappa sequences demonstrating a 15 nt deletion within CDR1. Arrows (<—) illustrate related B cells that demonstrate a progression of CDR3 replacement mutations (*red lettering*). Note that the numbered CDR3 nucleotide and amino acid sequences shown here represent theoretical hypermutation branch points, with sequence “1” predicting the unmutated N-region additions (*underlined nucleotides*) for each clade.

Figure 4. CFC1- and CEP-1-selected clade BCRs display citrulline-multispecific target reactivity as recombinant mAbs

BCR genes from single tetramer-sorted or random CD27⁺IgD⁻ switched-memory B cells were cloned and expressed as human IgG1 recombinant mAbs, and then tested for citrulline specificity by ELISA. (A, B) Native and citrullinated filaggrin (Cf0, CFC1) (A) and alpha enolase (REP-1, CEP-1) (B) ELISA peptide binding activity for mAbs derived from subjects RA14 (n = 3), RA62 (n = 2), and RA37 (n = 8). mAbs cloned from random sorted IgD⁻ CD27⁺ switched-memory B cells (n = 21) derived from these same subjects were also tested as a control. (C) Heat map of ELISA results for all 13 mAbs as a function of target peptide

antigen, RA subject, citrullinated peptide tetramer B cell capture reagent, and expanded BCR gene clade membership.

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Figure 5. CFC1- and CEP-1-selected clade BCRs bind to *in vitro* citrullinated full-length proteins

Full-length protein ELISA Ag-binding results for 4 select human IgG1 recombinant mAbs —14CFCT3G09, 62CFCT1E04, 37CEPT1G09, and 37CEPT2C04. The indicated target proteins (α-enolase, histone H4, histone H2A/B, vimentin, and fibrinogen) were citrullinated *in vitro* with human PAD2 enzyme (or treated with buffer alone as a control). Bars indicate the ELISA relative O.D. 450 nm for each of the native (*open bar*) and citrullinated (*filled bar*) protein substrates.

Figure 6. RA patient-derived citrulline-multispecific mAbs can promote pain-like behavior and joint inflammation in mice

(A, B) Combined data from two experiments (n = 5–9 mice/group) showing the (A) arthritis score (0–3) and (B) mechanical hypersensitivity for the single hind paw receiving intra-articular LPS on day 8 after intravenous injection of saline or 1 mg citrulline-multispecific mAb engineered as a murine IgG2a. (*) represents 37TCEPT1G09 and (#) represents 37TCEPT2C04 compared to saline. (*, #) = $p < 0.05$; (**, ##) = $p < 0.01$; (***, ###) = $p < 0.001$. (C) Representative comparison of hind paw erythema in the same mouse – contralateral (left) or ipsilateral (right) to intra-articular LPS injection – at day 14 after the intravenous administration of 37CEPT1G09 mAb. (D) Representative histology (H&E stain; n = 5 sections/group) comparing the LPS-injected ankle joints of mice that were pretreated with murine mAbs 62CFCT1E04 (left) and 37CEPT2C04 (right). Areas of tissue immune cell infiltration are indicated by the *. (E) Mean tissue inflammation scores (n = 3–4 sections per group).