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Elevated IgA Plasmablast Levels in Subjects at Risk of Developing Rheumatoid Arthritis

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Objective. **The disease process in rheumatoid arthritis (RA) starts years before the clinical diagnosis is made, and elevated levels of disease-specific autoantibodies can be detected during this period. Early responses to known or novel autoantigens likely drive the eventual production of pathogenic autoimmunity. Importantly, the presence of disease-specific autoantibodies can identify individuals who are at high risk of developing RA but who do not currently have arthritis. The goal of the current study was to characterize plasmablasts from individuals at risk of developing RA.**

Methods. **We investigated antibody-secreting plasmablasts derived from a well-characterized cohort of individuals who were at risk of developing RA, based on RA-related serum autoantibody positivity, as compared to patients with early (<1 year) seropositive RA as well as healthy control subjects. The plasmablast antibody repertoires of at-risk subjects were analyzed using DNA barcode–based methods with paired heavy- and light-chain gene sequencing. Cells were single-cell sorted, the cell- and plate-specific DNA barcodes were sequentially added, and next-generation sequencing was performed.**

Results. **Total plasmablast levels were similar in the antibody-positive (1%) and control (0.4–1.6%) groups. However, increased frequencies of IgA+ versus IgG+ plasmablasts were observed in the antibody-positive group (39% IgA+ and 37% IgG+) as compared to other groups (1–9% IgA+ and 71-87% IgG+). Paired antibody sequences from antibody-positive subjects revealed cross-isotype clonal families and similar sequence characteristics in the IgA and IgG plasmablast repertoires. Antibody-positive individuals also demonstrated elevated serum levels of IgA isotype anti–cyclic citrullinated peptide 3 antibodies.**

Conclusion. **The IgA plasmablast dominance in these antibody-positive individuals suggests that a subset of RA-related autoantibodies may arise from mucosal immune responses and may be involved in early disease pathogenesis in individuals who are at risk of developing RA.**

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Dr. Robinson has received consulting fees from Atreca (more than $10,000), owns equity in the company, and serves on the board of directors of the company.

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Rheumatoid arthritis (RA) is an autoimmune disease that affects ~1% of the population (1) and is associated with disease-specific autoantibodies, including anti–citrullinated protein antibodies (ACPAs) and rheumatoid factors (RFs). The disease process begins many years before the clinical diagnosis of RA, with RA-specific autoantibodies present for as long as 5–10 years (2–8). This phase is followed by the development of increased systemic levels of proinflammatory cytokines and chemokines as well as epitope spreading of individual ACPAs, culminating in the onset of clinically apparent arthritis and classifiable disease (7). RA-related autoantibodies play a key role in the pathogenesis of RA. For example, murine models of RA show that anti–citrullinated protein/peptide responses can lead to and exacerbate arthritis (9–13). T cell

responses to citrullinated proteins can also enhance disease (14).

First-degree relatives of RA patients and other subjects identified during screening programs may be classified as being at high risk of developing RA at some point in the future when they exhibit 1 or more of these RA-related autoantibodies, despite having no current signs or symptoms of arthritis (15,16). Importantly, these high-risk subjects have features consistent with a preclinical disease state, including an expanded ACPA repertoire (17) and elevated cytokine/chemokine levels (18). They also demonstrate pulmonary inflammation (19) and expression of RA-related autoantibodies in the lung (20), suggesting the presence of an ongoing mucosal immune response. Individuals who are within 1 year after their RA diagnosis (early RA) are also of interest because of their recent transition from preclinical to clinically apparent RA, which is a poorly understood process (21).

Several groups of investigators have sequenced single B cells from the synovium (22) and circulating plasmablasts of patients with established RA (23). However, in terms of our efforts to understand the origins of RA, it is important to determine the earliest citrullinated and other potentially novel epitopes to which B cells respond.

During an immune response, peripheral blood plasmablasts arise from both naive and memory B cells activated by inciting antigens (23–28). In order to study an ongoing antibody response, it is highly informative to examine the antibody repertoires of circulating plasmablasts. In the present study, we identified and characterized plasmablasts derived from 3 groups: RA-related antibody-positive subjects with no history or current evidence of inflammatory arthritis (antibody-positive at-risk group), patients with early RA, and normal control subjects. Using a recently developed method of DNA barcode–enabled single-cell antibody sequencing (29), plasmablasts sorted from antibody-positive at-risk individuals were sequenced and characterized.

PATIENTS AND METHODS

Study subjects and samples. Subjects were selected under the aegis of the Studies of the Etiology of RA (SERA) project (30), a natural history study of individuals at elevated risk of developing RA because of having a first-degree relative with RA, a genetic risk factor for RA (shared epitope), or RA-related serum autoantibody positivity identified through community screening efforts. Subjects were selected for the 3 study groups from the Colorado site of the SERA cohort, as follows. Antibody-positive at-risk subjects were defined as being positive for anti–cyclic citrullinated peptide (anti-CCP) on 1 or more commercial tests (anti–CCP-2 or anti–CCP-3.1) and/or having 2 or more RF isotypes in the absence of a history or the presence of inflammatory arthritis on examination at the time of the study. These autoantibody criteria are sensitive (~56%) and highly specific (>96%) for future RA and therefore serve as appropriate criteria for identifying subjects for evaluation of early mechanisms of disease initiation and evolution (7). Patients with early RA were seropositive for CCP and/or RF, met the American College of Rheumatology 1987 classification criteria for RA (31), and were within 1 year of the onset of symptoms attributed to clinically apparent RA. Normal control subjects were recruited from the Denver community through advertising efforts and had no personal history of autoimmune rheumatic disease, no evidence of inflammatory arthritis upon examination at the time of the study, and were negative for ACPA and RF.

Study approval. Written informed consent was obtained from the participants prior to their inclusion in the study. Study samples were collected according to the human subject protocols approved by the Investigational Review Board at the University of Colorado Denver Anschutz Medical Campus.

Autoantibody testing. Anti–CCP-3.1 (IgG/IgA Inova Diagnostics) and anti–CCP-2 (IgG Diastat; Axis-Shield) enzyme-linked immunosorbent assays (ELISAs) were performed and the results analyzed according to the manufacturers' instructions. RF was measured using nephelometry (Siemens), with a cutoff for positivity of ≥15 IU/ml. RF isotypes (IgG, IgA, and IgM) were measured by ELISA (Quanta Lite) following the manufacturer's recommendations (Inova Diagnostics). Cutoffs for positivity for RF nephelometry and isotypes were defined using a threshold that was higher than that observed in 95% of 490 randomly selected blood donor controls from the Denver area. All subjects were tested for ACPA IgG, IgA, and IgM using ELISAs coated with the CCP-3.1 antigen (research use only; donated by Inova Diagnostics); a technician (MCP) who was blinded with regard to the sample status performed these tests. A standard curve was established by performing serial dilutions on a pooled serum sample from patients with RA with known high serum levels of anti-CCP.

Single-cell sorting of plasmablasts. Cell sorting was performed as previously described (29). Peripheral blood mononuclear cells (PBMCs) were stained with fluorochrome-conjugated antibodies against CD19 (HIB19; BioLegend), CD3 (UCHT1; eBioscience), CD33 (WM53; BioLegend), CD14 (M5E2; Fisher Scientific), CD20 (L27; Fisher Scientific), CD27 (M-T271; Fisher Scientific), CD38 (HB7; Fisher Scientific), IgA (IS11-8E10; Miltenyi Biotec), and IgM (G20-127; Fisher Scientific). Analyses were performed on a doublet cell–excluded, live, and lymphoid cell gate. IgG+ and IgA+ plasmablasts were single-cell sorted into 96-well plates using a MoFlo XDP100 cell sorter (Beckman Coulter), with plasmablasts defined as CD19+CD3–CD33–CD14–CD20–CD27+CD38^{high}. As IgG-producing plasmablasts have low surface expression of B cell receptors, IgG+ cells were identified by the absence of IgA and IgM staining. The isotype of sorted cells was subsequently confirmed by gene-specific polymerase chain reaction (PCR) and by the identification of isotype-specific sequences in the final data (Supplementary Table 1, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.39771/abstract).

Barcode-enabled antibody sequencing. Antibody repertoire sequencing was performed as previously described (23,29). Briefly, plasmablasts were sorted into 96-well plates containing lysis buffer (10 m*M* Tris with 1 unit/μl of RiboLock RNase inhibitor) and stored at −80°C. One of 96 unique

Table 1. Characteristics of the study subjects*

	Normal subjects (n = 22)†	Antibody-positive first-degree relatives (n = 33)‡	Early RA patients (n = 13)§
Age at visit, mean ± SD years	38.9 ± 13.4¶	52.9 ± 11.2	52.6 ± 9.6
% female	86.36	76.47	69.23
% non-Hispanic white	59.09	85.29	69.23
% ever smokers	22.73	35.29	84.6#
% current smokers	4.55	5.88	38.5#
No. of pack-years, mean ± SD	1.0 ± 0.6¶	8.5 ± 7.3	12.0 ± 11.1
CCP-2			
% positive	0	14.71	76.9#
Mean ± SD units	NA	163.7 ± 171.8	250.1 ± 242.6
CCP-3.1			
% positive	0	61.8**	92.3#
Mean ± SD units	NA	241.5 ± 557.2	700.0 ± 978.1
Rheumatoid factor			
% positive	0	32.4	53.8#
Mean ± SD IU/ml	NA	128.0 ± 193.0	272.7 ± 319.3

* Samples were compared and significance was determined by chi-square test for comparisons of prevalence and by analysis of variance for continuous variables.
† Normal subjects (recruited through community screening efforts) were individuals who had no personal history of autoimmune rheumatic disease, had no evidence of inflammatory arthritis at the time of the study, and were anti–citrullinated protein antibody and rheumatoid factor negative (<15 IU/ml).
‡ Antibody-positive subjects (recruited from the Studies of the Etiologies of Rheumatoid Arthritis project) were first-degree relatives of probands with rheumatoid arthritis (RA) or were seropositive for RA-related autoantibodies as identified through community screening efforts. They were designated seropositive based on results of serum testing for RA-related autoantibodies at the time of original sample collection and had positive results on ≥1 commercial anti–cyclic citrullinated peptide (anti-CCP) antibody test (CCP-2 or CCP-3.1) and/or had ≥2 rheumatoid factor isotypes in the absence of a history or presence of inflammatory arthritis at the time of the study.
§ Early RA was defined as <12 months since the onset of symptoms attributed to clinically apparent RA.
¶ $P \leq 0.05$ versus antibody-positive first-degree relatives and versus early RA patients, by nonparametric test.
# $P \leq 0.05$ versus normal subjects and versus antibody-positive first-degree relatives, by nonparametric test.
** $P \leq 0.05$ versus normal subjects and versus early RA patients, by nonparametric test.

well-specific DNA barcodes was added to the complementary DNA (cDNA) of each cell by template switching during reverse transcription with Maxima Reverse Transcriptase (ThermoFisher Scientific). Well ID–tagged cDNA was pooled from each plate, and plate-specific indices were added during PCR with primers specific for the heavy-chain and light-chain constant regions. The γ, κ, and λ primers were as described previously (23), and the nested α gene–specific primer sequences were ATT-CGT-GTA-GTG-CTT-CAC-GTG (forward) and CTA-TGC-GCC-TTG-CCA-GCC-CGC-GGG-AAG-ACC-TTG-GGG-CTG-GT (reverse). Barcoded amplicons from multiple plates were pooled prior to the addition of sequencing adaptors and final purification with AMPure XP beads (Beckman Coulter). Samples sequenced prior to fall 2014 underwent Roche 454 sequencing using Lib-L adaptors and Titanium chemistry. Following the development of 600-bp read lengths on the Illumina MiSeq platform, samples underwent 2× 300 MiSeq analysis.

Compound barcode assignment and assembly of sequences. Compound barcode assignment and assembly of amplicon sequences was performed as previously described (23). Sequence data were demultiplexed using a custom software pipeline to separate reads from each single cell according to its unique compound (plate plus well) barcode ID. Sff output files from 454 sequencing were read into Python by using the Biopython package, and sequences were grouped and parsed into separate sff files based on their compound IDs. To correct for sequencing errors, the Newbler 2.6 program was used to assemble reads from each sff file into consensus sequences by using the "-cdna," "-ud," and "-urt" options. For MiSeq, poor-quality reads and bases were trimmed (Trimmomatic-0.32), and the remaining paired reads were stitched together (Flash-1.2.10). The stitched sequences were then read into Python, grouped by compound ID, and parsed into separate fastq files. Consensus sequences were identified by clustering reads within each fastq file into operational taxonomic units (32–34).

V(D)J assignment and production of phylogenetic trees. Heavy-chain V(D)J and light-chain VJ sequences were analyzed with version 1.3.1 of the ImMunoGeneTics (IMGT) HighV-Quest database (35). IMGT HighV-Quest outputs including gene segment usage and the numbers of silent and nonsilent mutations from germline were further analyzed and plotted using GraphPad Prism software and the ggplot2 package in R. Sequences were binned according to their heavy-chain V gene usage, and heavy and light chains were then concatenated and aligned with the use of Muscle to produce phylogenetic trees, as previously described (23,36). Tree images were drawn using ETE (37).

Analysis of clonality. IMGT HighV-Quest data were read into R, and B cells with shared heavy-chain VJ and light-chain VJ gene segments were grouped together. Within these groups, complementarity-determining region 3 (CDR3) amino acid sequences were compared using the stringdist package to

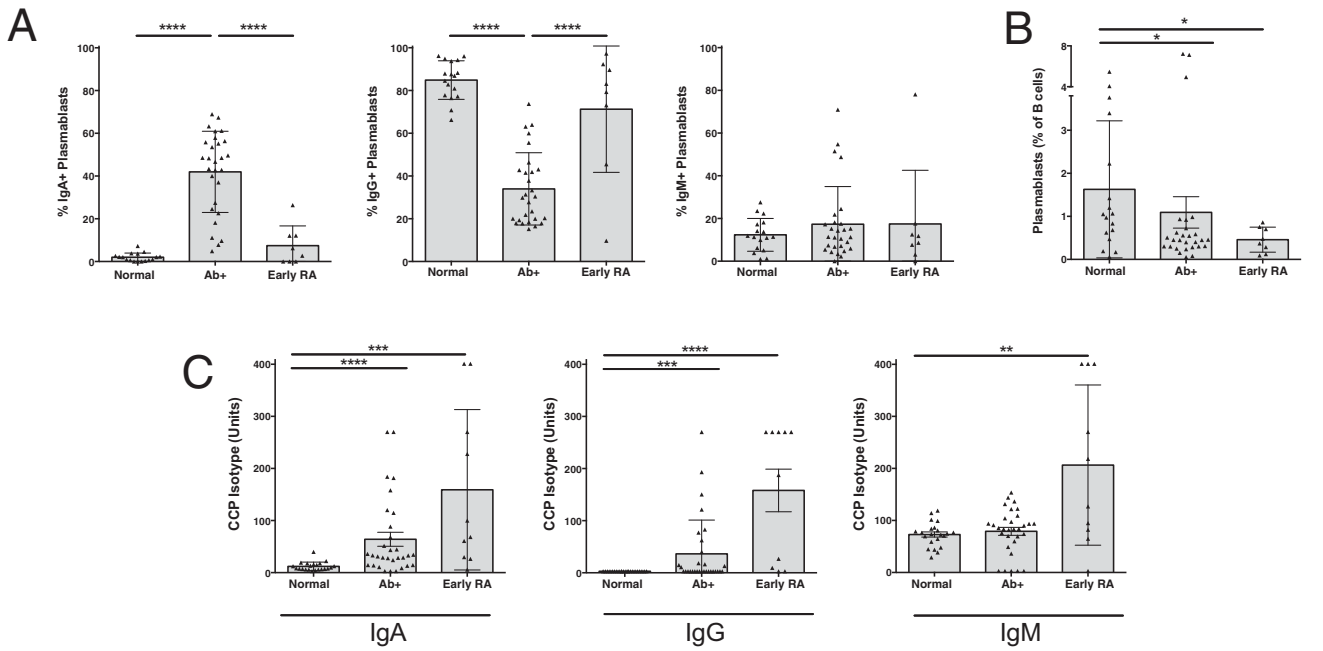

Figure 1. Elevated levels of IgA+ peripheral blood plasmablasts and serum anti–citrullinated protein antibody reactivity in normal control subjects (n = 17), antibody-positive (Ab+) at-risk subjects (n = 28), and early rheumatoid arthritis (RA) patients (n = 8) (see Patients and Methods for details of study groups). **A,** Antibody isotype distribution of plasmablasts. Individual isotypes were determined by their expression of IgA or IgM. IgG plasmablasts were defined as IgA–IgM– plasmablasts. Individuals for whom insufficient data were available to accurately define plasmablast isotype frequencies were excluded. **B,** Total peripheral blood plasmablasts (CD19+CD3−CD33−CD14−CD20–CD27+CD38^{high} cells) as a percentage of total B cells. **C,** Serum levels of cyclic citrullinated peptide 3.1 (CCP-3)–reactive autoantibodies, as measured using isotype-specific detection antibodies. Each symbol represents a single subject; bars with lines show the mean ± SD. $* = P < 0.05$; $** = P < 0.01$; $*** = P < 0.001$; $**** = P < 0.0001$, by analysis of variance using the Geisser-Greenhouse correction, followed by Tukey's (**A**) or Dunn's (**B** and **C**) multiple comparisons test.

calculate the Levenshtein distance. Clonal families were defined as sharing heavy-chain and light-chain VJ genes and having a CDR3 AA Levenshtein distance of ≤2 for both heavy and light chains. Clonal families were numbered and counted in R prior to statistical analysis with GraphPad Prism.

Confirmation of antibody isotypes. In addition to fluorescence-activated cell sorter analysis of plasmablast antibody isotypes, the production of IgG or IgA by cells from a given patient was confirmed in two ways: by gene-specific PCR during library preparation for sequencing, in which sorted IgA plates were not amplified with IgG primers and vice-versa and by the presence of isotype-specific sequences (ASTKG for IgG versus ASPTS for IgA).

Multiplex autoantigen arrays. Levels of IgG and IgA antibodies targeting 51 putative RA-associated autoantigens were measured using a custom Bio-Plex assay as previously described (38) and were analyzed on a Luminex 200 instrument running Bio-Plex Manager software version 6.1. Three subjects (2 normal controls and 1 early RA patient) were excluded from analysis because of broad, non–citrulline-specific IgG reactivity (≥12 native antigens elevated ≥3-fold over the cohort average).

Statistical analysis. Data are presented as the mean ± SD. Student's unpaired 2-tailed *t*-test was used to assess differences between 2 groups, substituting the nonparametric Mann-Whitney U test when the data did not follow a normal Gaussian distribution or Welch's correction when the SDs were not equal. For multiple comparisons, 1-way analysis of variance (ANOVA) was used, substituting the nonparametric Kruskal-Wallis test when the data did not follow Gaussian distribution, followed by the Geisser-Greenhouse method to correct for violations of the sphericity assumption and either Dunn's or Tukey's multiple comparisons test. All tests were performed using GraphPad Prism software. *P* values less than 0.05 were considered significant. For comparisons of prevalence, Fisher's exact test was used to determine the significance, and ANOVA was used for comparisons of continuous variables in tables. Significance Analysis of Microarrays (SAM) version 4.0 was used to run a multiclass comparison between subject groups in the multiplex autoantigen arrays. SAM hits with a q value of less than 0.1% were chosen for display.

RESULTS

Characteristics of the study subjects and results of autoantibody analyses. The characteristics of the study subjects are given in Table 1. The antibody-positive and early RA groups were of similar age, and the normal control group was significantly younger. There were no significant differences in sex or race. Patients with early

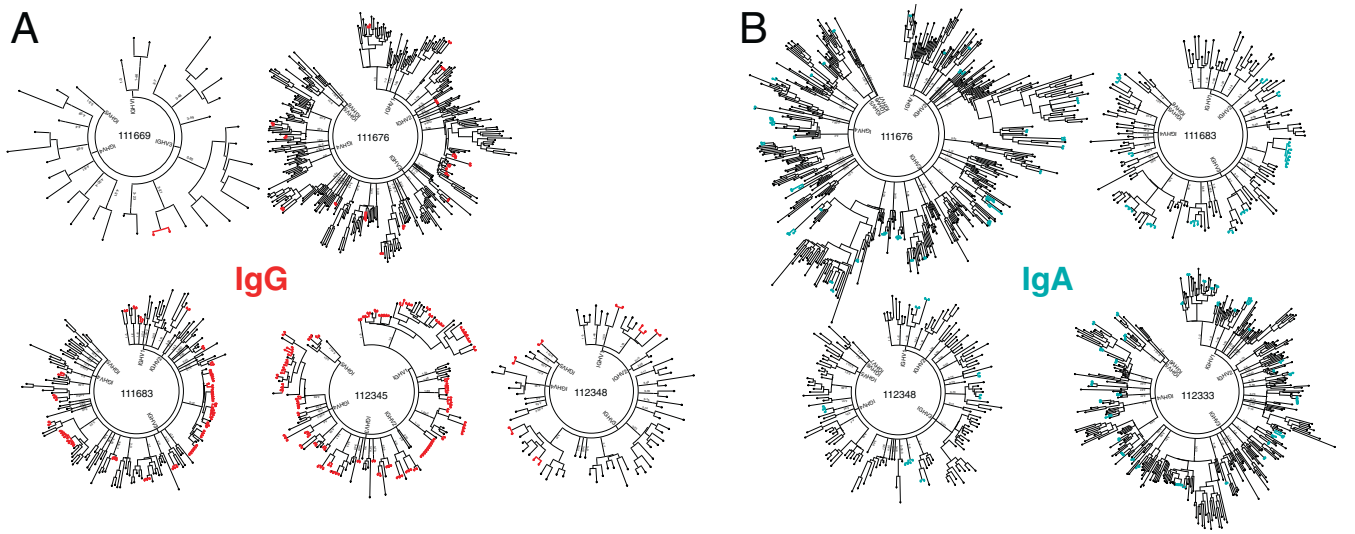

Figure 2. Sequencing of plasmablast antibody repertoires in antibody-positive at-risk subjects. **A,** Phylogenetic trees of paired IgG antibody sequences from peripheral blood plasmablasts derived from 5 antibody-positive at-risk subjects. **B,** Phylogenetic trees of paired IgA antibody sequences from peripheral blood plasmablasts derived from 4 antibody-positive at-risk subjects. Clonally related sequences (defined as sharing heavy- and light-chain V and J genes and heavy- and light-chain complementarity-determining region 3 amino acid sequences with a Levenshtein distance of ≤2) are shown in red for IgG and in blue for IgA. Phylogenetic trees were generated by concatenating and clustering (using the Muscle alignment tool) the heavy- and light-chain sequences and arranging them by heavy-chain V gene family. Each peripheral node represents a single paired antibody.

RA reported significantly higher rates of ever smoking and current smoking as compared to the other 2 groups.

Patients with early RA exhibited the highest prevalence of seropositivity for CCP-2, CCP-3.1, and RF of any isotype. Normal controls were negative for all antibodies tested. The results of CCP and RF testing for all groups are shown in Supplementary Figure 1 (available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.39771/abstract).

Elevation of IgA+ peripheral blood plasmablasts in the antibody-positive at-risk group. Plasmablasts, defined as CD19+CD3–CD33–CD14–CD20–CD27+ $CD38^{high}$ (Supplementary Figure 2, available at http://onlinelibrary.wiley.com/doi/10.1002/art.39771/abstract), were stained and single-cell sorted from PBMCs into 96-well plates, as previously described (29). Plasmablasts were sorted into separate plates based on isotype (IgA, IgM, or IgG). A difference in the relative frequency of total plasmablasts between the antibody-positive at-risk group and the normal control group, as well as between the normal control group and the early RA group, was noted (Figure 1B).

Although the total plasmablast numbers were not elevated in the antibody-positive at-risk group compared to other groups, the antibody-positive group demonstrated a significant increase in IgA+ plasmablasts compared to the other 2 groups (Figure 1A). Conversely, those with early RA had significantly lower relative levels of IgA+ plasmablasts and instead demonstrated much higher relative levels of IgG+ plasmablasts (Figure 1A). No differences in the relative levels of IgM+ plasmablasts between any of the groups were observed. The pattern of plasmablasts found in individual subjects within the different groups is displayed in Supplementary Figure 3 (available at http://onlinelibrary.wiley.com/doi/10.1002/art.39771/abstract).

Follow-up data were available on 17 subjects in the antibody-positive at-risk group; 2 of the 17 subjects (~12%) developed classifiable RA at 4 and 13 months, respectively, after the plasmablast assessment visit. Overall follow-up of this group was for a median of 24 months (range 4–35 months), and follow-up is ongoing.

Serum samples tested for isotype-specific reactivity with CCP-3.1 showed levels of both IgA and IgG anti–CCP-3.1 to be elevated above normal control levels in the antibody-positive at-risk group as well as the early RA group (Figure 1C). These results are consistent with our findings for plasmablast isotype and total ACPA levels and may suggest that circulating plasmablasts contribute to IgA ACPA production in antibody-positive at-risk individuals.

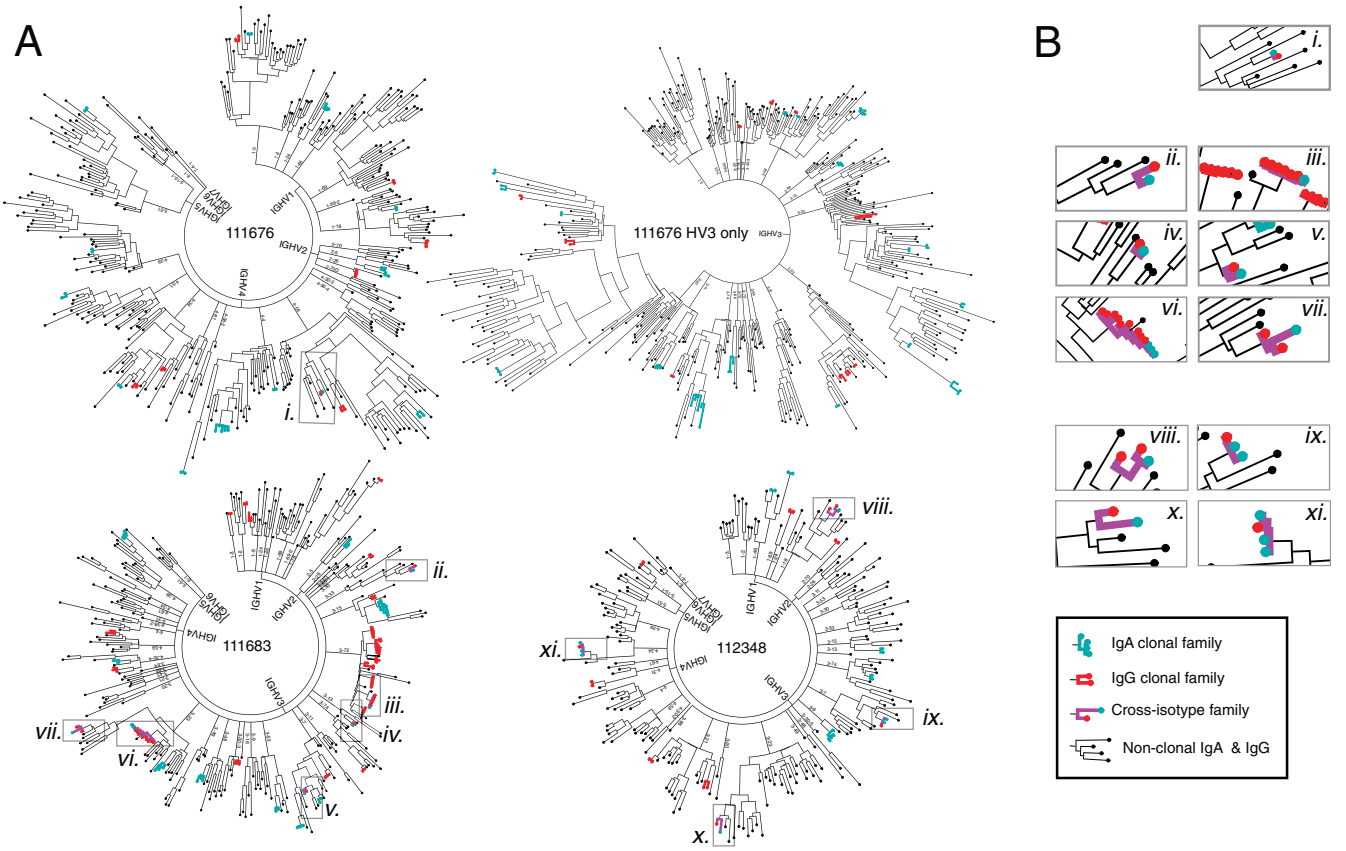

Figure 3. Shared clonality among IgG and IgA repertoires of antibody-positive at-risk subjects. **A,** Combined trees of IgA and IgG sequences from 3 antibody-positive at-risk individuals. The tree for subject 111676 was divided into 2 trees (IgHV3 family and IgHV1,2,4-7) for better viewing (top row). **B,** Higher-magnification views of cross-isotype clonal families showing IgG/IgA shared clonality (the boxed and numbered areas of 3 of the images in **A**).

Sharing of clonal families between IgA and IgG isotypes in paired heavy- and light-chain antibody gene sequences from antibody-positive at-risk subjects. Plasmablasts from 5 antibody-positive at-risk subjects were used for paired heavy- and light-chain repertoire sequencing (Figure 2). Phylogenetic trees of paired IgG (Figure 2A) and IgA (Figure 2B) sequences were generated as previously described (29). In addition to isotype-specific flow cytometric staining, the isotype of IgA+ and IgG+ plasmablasts was confirmed by gene-specific PCR performed during the sequencing process and by identification of isotype-specific constant-region sequences in the final data. These methods confirmed the high fidelity of the method for sequencing the desired isotype (see Supplementary Table 1). From these data, we were able to identify clonal families of antibodies that use the same heavy-chain V and J gene segments, the same light-chain V and J gene segments, and have a Levenshtein distance of ≤ 2 in the CDR3 amino acid sequences. Combined IgG plus IgA phylogenetic trees were generated to demonstrate the relationship between sequences across these isotypes (Figure 3A). IgG/IgA cross-isotype clonal families of varying sizes were observed in all 3 antibody-positive at-risk subjects for whom both isotypes had been sequenced (Figure 3B).

Similar sequence characteristics in IgG and IgA repertoires. Plasmablast antibodies produced by antibody-positive at-risk subjects contained mutations from the germline gene segments consistent with affinity-matured plasmablasts (Figure 4A). Mutation rates did not differ between clonal and nonclonal sequences, nor did they differ between IgG and IgA repertoires. However, within cross-isotype clonal families, the IgA family members were more mutated from the germline sequence

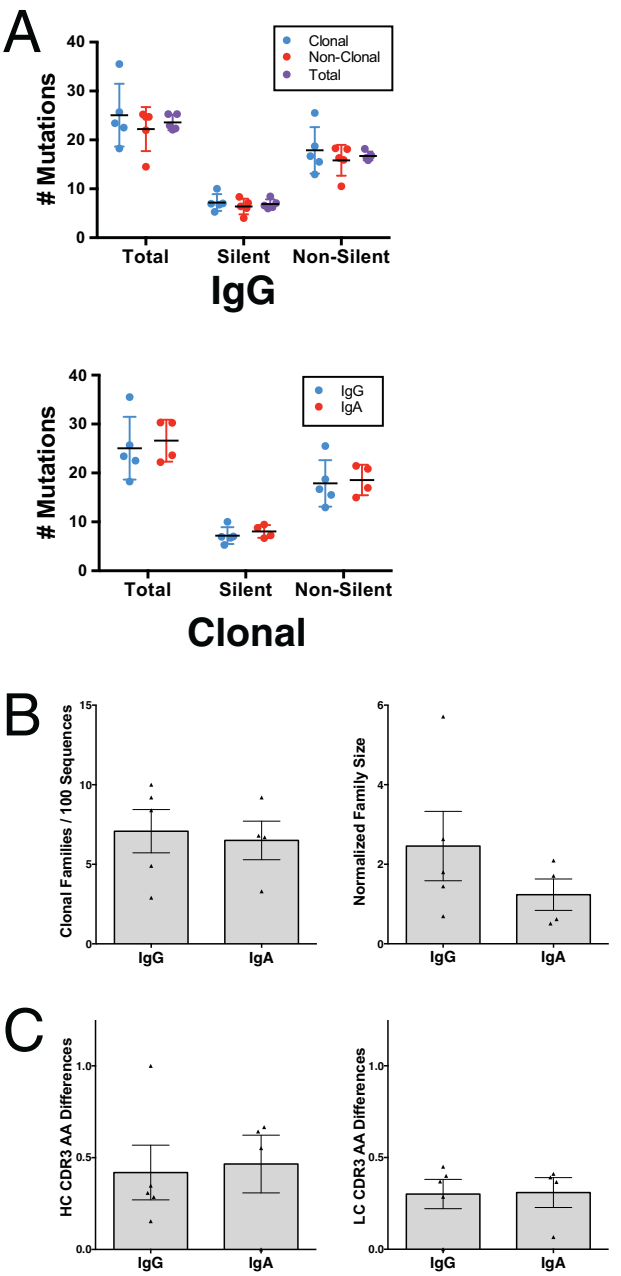

Figure 4. Characteristics of the antibody repertoire. **A,** Numbers of mutations from germline for IgA versus IgG and for clonal versus non-clonal antibody sequences. Each symbol represents the mean number of mutations in a single subject (5 subjects for IgG; 4 subjects for IgA); horizontal lines with bars show the mean ± SD. **B,** Number, size, and sequence percentage of clonal families in the antibody repertoire of each antibody-positive at-risk subject. Each symbol represents a single subject (5 for IgG; 4 for IgA); bars with lines show the mean ± SD. **C,** Amount of variation (Levenshtein distance) within the complementarity-determining region 3 (CDR3) amino acid (AA) sequences of clonal families from each antibody-positive at-risk subject. Each symbol represents the average level of difference for all clonal families within a single subject (5 for IgG; 4 for IgA); bars with lines show the mean ± SD. HC = heavy chain; LC = light chain.

than their closest IgG relatives (Figure 4A). This is consistent with the fact that IgG-producing cells can class switch to IgA production, but once switched, the IgA-producing cells cannot switch back to IgG. Neither the number of clonal families per tree sequence (Figure 4B) nor the degree of similarity within the CDR3 sequences of each family (Figure 4C) differed between IgG and IgA repertoires.

Reactivity of serum IgA and IgG with known and putative RA-related antigens. To identify possible IgA autoantigens associated with antibody-positive at-risk status, we used a multiplex antigen array to compare IgA and IgG ACPA specificities present in the serum. Figure 5 demonstrates that levels of IgG antibodies to 21 of the 51 known or putative RA-related autoantigens were differentially observed in normal controls, antibody-positive at-risk, and early RA subjects. Levels of IgA antibodies to 13 of the 51 antigens were also differentially observed in these groups. Antibodies detected in the antibody-positive at-risk individuals targeted many of the same antigens that were targeted by antibodies in the early RA patients.

DISCUSSION

We report herein the sequence characterization of paired heavy- and light-chain immunoglobulin genes expressed by single B cells in subjects at high risk of developing clinically apparent RA. Identification of clonally related antibodies enables analysis of the evolution of responses to potential known and unknown RA-related autoantigens that could characterize the initial phases of clinical autoimmunity and eventually lead to clinical disease.

RA-related autoantibodies can be elevated in the serum years prior to the onset of clinical disease during a period of autoimmunity that has come to be classified as preclinical RA (4,5). Previous studies of preclinical RA have demonstrated that the progression of autoimmunity is characterized by initial reactivity with a limited number of known citrullinated antigens, with epitope spreading over time to the point where clinically apparent articular RA appears (4,5,39). Still not known, however, are the mechanisms by which autoimmunity develops, a process that may be brought into better understanding by identifying the potential inciting antigens and ultimately targeting these for disease prevention.

Examining plasmablasts may prove useful in elucidating the initiation and propagation of autoimmunity during preclinical RA. Prior studies in patients with established RA have revealed multiple clonal families of antibodies, many of which bind to known RA-related autoantigens. Thus, this method is capable of isolating

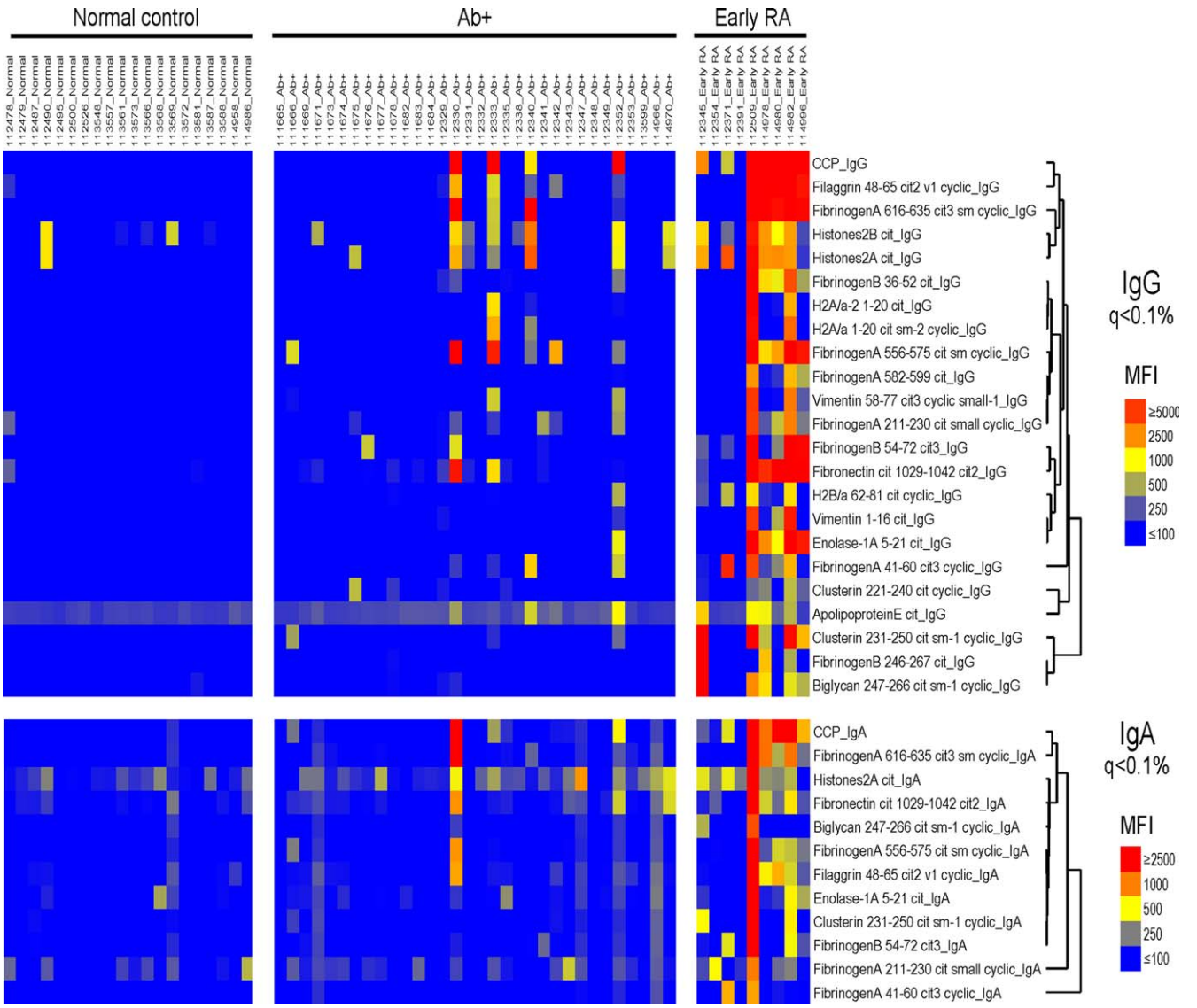

Figure 5. Reactivity of serum IgG and IgA with anti–citrullinated protein antibody (ACPA). Heatmap shows ACPA levels in the serum of normal control subjects (n = 20), antibody-positive (Ab+) at-risk subjects (n = 32), and early rheumatoid arthritis (RA) patients (n = 9) (see Patients and Methods for details of study groups). Levels of autoantibodies against 51 putative targets of the RA immune response were compared between groups by use of a bead-based multiplex antigen microarray. Significance Analysis of Microarrays (SAM) software was used to sort output based on false discovery rates in order to identify antigens with the greatest differences in autoantibody reactivity between groups. SAM hits with a q value of <0.1% were chosen for display. Colors indicate mean fluorescence intensity (MFI) relative to the average values in the cohort evaluated. CCP = cyclic citrullinated peptide.

monoclonal autoantibodies and identifying their cognate antigens (23,40–43).

Similar approaches can be used in preclinical RA to identify plasmablast abnormalities with relevance to the pathogenesis of RA. While we did not identify specific antigenic targets of these plasmablasts, it is particularly intriguing that the IgA+ plasmablasts are increased in antibody-positive at-risk individuals without inflammatory arthritis. The predominant antibody of mucosal immunity is IgA (44), and IgA ACPAs have been shown to be highly specific for RA when present both in patients with preclinical RA and in those recently diagnosed as having RA (45,46). Furthermore, our findings correlate with previous data, in that we observed increased levels of IgA ACPAs using a CCP-3.1 assay in samples from our at-risk population (46).

This observation suggests several possibilities as to the origin of autoantibodies that are seen years prior

to the onset of joint disease. In particular, the associations between smoking and RA as well as emerging data regarding the potential role of oral, lung, and gut inflammation in the development of RA all point to a mucosal site of origination of disease (19,20,47–50). In particular, the occurrence of severe periodontitis is increased in patients with RA, and these patients also exhibit higher joint disease activity scores (51). Moreover, several oral pathogens are expanded in patients with untreated early RA, and antibodies to these pathogens are increased in these patients (48,52,53). Evidence that the lung may play a role in the mucosal origin of ACPA is also quite strong. Specifically, exposure to cigarette smoke is highly associated with the future development of RA (54,55). Individuals with RA also often present with pulmonary abnormalities (56), and individuals positive for RA-related autoantibody but without RA demonstrate airways abnormalities on imaging studies (19). Analyses of sputum from at-risk individuals have also demonstrated lung production of RA-related antibodies, which again supports a mucosal origin (20). Finally, bacteria that reside in the gut help drive autoimmune arthritis in mice (57), and changes in the microbiota of the gut have been associated with early untreated RA in humans (47,58).

Steady-state levels of IgA+ plasmablasts exist in normal individuals, and these levels are not affected by systemic vaccination against nonmucosal pathogens, which may indicate that systemic and mucosal humoral immune responses are regulated independently of each other (40). Our results demonstrating relatively low levels of IgA+ plasmablasts in normal individuals are not consistent with all previous studies (40); however, we did use 3 analytical methods (flow cytometry, isotype-specific primers, and DNA sequence analysis) to assure the correct isotype identification in our cohort.

Plasmablast repertoires in antibody-positive at-risk subjects show equivalent mutation rates to those that have been reported for established RA (23), suggesting that these cells are part of an ongoing chronic immune response and are not an unrelated gastrointestinal infection. Similarly, the size and frequency of clonal families in antibody-positive at-risk subjects were lower than what we have observed for acute infection and vaccination responses (29,59) and were more consistent with chronic autoimmune activation (ref. 23 and Kinslow JD, et al: unpublished observations). Whether plasmablasts in at-risk subjects are induced by a sudden trigger (such as viral or bacterial infection) or progress steadily toward autoreactivity over time due to low-level stimulation of the gingival and pulmonary mucosa is not yet clear. However, sequence characteristics consistent with an acute triggering infection were not observed in this study.

An unexpected finding was that individuals with early RA in our study did not have elevated frequencies of IgA+ plasmablasts. It may be that once clinically apparent RA is present, the disease has already progressed past the early mucosal inflammatory stage. In addition, we speculate that the disease-modifying antirheumatic drugs used to treat RA could affect total plasmablast levels as well as the different isotypes. It is also not clear why IgA anti–CCP-3.1 levels were elevated in the at-risk subjects while IgA-RF levels were not. One possible explanation is that these 2 autoantibody pathways are generated at different sites and/or at times in the evolution of RA. These issues will need to be addressed in future studies.

We determined that there was no correlation between shared epitope positivity and IgA/IgG predominance of the subject's plasmablasts. Additionally, there was not a specific pattern of elevated ACPA or RF levels in individuals with elevated IgA+ plasmablasts, nor were there differences in total serum IgG, IgA, or IgM (data not shown). At-risk individuals with high levels of IgA+ plasmablasts were not more likely to have ever smoked (data not shown). Our conclusions may be limited by the small sample size; however, these findings lead us to speculate that there is a causal relationship between elevated IgA+ plasmablast levels and an as-yet-unknown factor. Our results demonstrate elevated serum IgA anti-CCP antibody levels and show peripheral blood IgA+ plasmablasts of as-yet-unknown specificity, so we cannot directly link the source of IgA anti-CCP to the IgA+ plasmablasts. In addition, whether IgA+ plasmablasts are in fact producing the RF antibodies that are detected serologically will be answered in future studies by expressing and testing monoclonal antibodies derived from IgA+ plasmablast sequences.

Although IgA+ cells were increased and IgG+ cells decreased in the antibody-positive at-risk group relative to other groups, no significant differences were observed in the antibody sequence characteristics of IgA+ versus IgG+ plasmablasts. Given that IgG-expressing cells represent an earlier class-switching state than IgA+ cells, the equivalent sequence characteristics of these isotypes implies that nascent IgG responses are forming at the same time that other IgG+ cells are class switching to IgA. This is consistent with the transition toward IgG predominance in plasmablasts from patients with early RA. The presence of cross-isotype clonal families supports the idea that we have captured a snapshot of the incipient immune response as it transitions from IgG to IgA. Although the average mutation rates did not differ between the IgG and IgA repertoires in the

antibody-positive at-risk group overall, IgA members of cross-isotype families were more mutated than their IgG counterparts. This is to be expected, given that class-switch recombination can only move in one direction, in this case from IgG to IgA.

We previously observed large clonal expansions following acute infection or vaccination and smaller clonally expanded families in patients with chronic infection or autoimmunity (23,29,59). Cells of the small clonal families retained during a chronic response may be better able than their nonsurviving clonal relatives to bind their cognate antigens and compete for resources. The clonality of both IgG+ and IgA+ plasmablast repertoires from antibody-positive at-risk individuals is similar to what has previously been reported in established RA (23). Although we have measured an actively developing immune response in the process of switching from IgG to IgA, we have not captured the absolute earliest time point (within weeks of initiation) that would likely be required to observe acute expansion of large clonal families.

There are several caveats to our findings. Because individuals in our control group did not exhibit increased relative levels of IgA+ plasmablasts, we were not able to sort enough of these cells to generate a meaningful population with which to sequence and compare IgG/IgA antibody repertoires with those in the antibody-positive at-risk group. Thus, we did not compare cross-isotype clonality between controls and antibody-positive at-risk subjects. We have previously observed cross-isotype families in Lyme disease, pulmonary arterial hypertension, and following influenza vaccination (Blum LK, Robinson WH: unpublished observations); the ability to capture developing clones in the process of class switching makes sense in the context of sequencing plasmablasts, cells that represent nascent immune responses rather than established memory.

It is also possible that vaccinations or illnesses influenced the presence of circulating plasmablasts in our study subjects. In particular, the timing of standard influenza vaccinations (October to March) coincided with the collection of samples from subjects who had increased plasmablast levels. We did not collect vaccination information, nor did we exclude individuals who had recently had any infection. However, a critical finding was that there was a significant increase in the relative frequency of IgA+ plasmablasts in antibody-positive at-risk subjects. This increase was not limited to individuals who had increased plasmablast levels and was thus likely to be independent of any potential vaccination or illness that might have caused a general increase in plasmablasts.

Another caveat concerning our findings is that the antibodies of interest may be responding to as-yet-undetermined pathogens, though an immune response to these pathogens may be the initial response that leads down the path to autoimmunity and eventual clinical disease.

The findings of these studies enhance our understanding of potentially pathogenic immune responses in the preclinical stages of RA. By using barcode-enabled antibody sequencing to study this unique population of at-risk individuals, we were able to find potentially pathogenic autoantibodies that could be implicated in the development of clinical disease. The discovery of these antibodies and their cognate antigens may lead to the development of tolerizing therapies for at-risk individuals. Study of synovial fluid and tissue samples from individuals with early and longstanding RA may allow us to determine whether cells from mucosal tissues drive local joint inflammation. Future studies will focus on longitudinal analyses of plasmablast evolution in preclinical RA to the development of classifiable RA, sequencing of plasmablasts that stain positive with citrullinated antigen multimers in order to identify specific antigen reactivity, characterization of plasmablasts at mucosal sites, analysis of recombinant expression and characterization of sequence-derived antibodies, and further bioinformatic analyses of antibody sequence characteristics that may be shared across multiple subjects.

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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. Holers 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. Deane, Demoruelle, Norris, Robinson, Holers.

Acquisition of data. Kinslow, Blum, Parish, Kongpachith, Lahey.

Analysis and interpretation of data. Kinslow, Blum, Okamoto.

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