Supplementary Methods

Affinity Maturation Drives Epitope Spreading and Generation of Pro-inflammatory Anti-Citrullinated Protein Antibodies in Rheumatoid Arthritis

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Single-cell sorting of plasmablasts

Plasmablasts were sorted into 96-well plates containing lysis buffer (10 m*M* Tris with 1 unit/µl of RiboLock RNase inhibitor) by flow cytometry using a FACSAria (BD), with plasmablasts defined as CD19+CD3-IgD-CD14-CD20-CD27+CD38++. IgG-producing plasmablasts were defined by the absence of IgA and IgM staining, since these cells possess low surface expression of B cell receptors. Single plasmablasts were stored in lysis buffer at -80°C until ready for reverse transcription (RT).

To generate the multimeric citrullinated peptide sort reagent, each biotinylated peptide (Supplementary Table 2) was individually incubated overnight at 4°C with streptavidin (SA) conjugated to allophycocyanin (APC) or R-phycoerythrin (R-PE) (LifeTechnologies), and purified using Bio-Spin P-30 Gel Columns (Bio-Rad). Each cit-peptide:SA-fluorophore complex (28 total) was pooled into the final staining mixture. Initial samples were pre-sorted to identify plasmablasts staining positive with citrullinated peptide sort reagent. Once available, cells were index sorted to track staining of individual, sorted cells and identify ACPA-producing plasmablasts within the total population without pre-sorting.

Cell barcode-enabled sequencing of the antibody repertoire

Sequencing of immunoglobulin genes from single cells was performed as previously described [1,2] with minor modifications. During RT, a unique well-specific DNA barcode was added to cDNA by template switching with Maxima RT (ThermoFisher Scientific) using TruGrade oligonucleotides (IDT). The HC (alpha, gamma, and mu) and LC (kappa and lambda) were amplified using nested gene-specific PCR primers while adding on the Illumina-based adapter and

sequencing regions. Prior to September 2015, samples were prepared for sequencing using two rounds of PCR [2]. All other samples underwent three rounds of PCR for gene-specific amplification and addition of Illumina adapters and sequencing regions (Supplementary Table 3). TruSeq i7 index sequences were added to distinguish each sort plate. Cell-barcoded DNA from up to 48 individual sort plates was pooled followed by gel-purification and AMPure XP beads (Beckman Coulter), and paired-end sequencing (2×330) was performed by Illumina MiSeq using the excess reagent of 2x300 cartridges.

Bioinformatic analysis of immunoglobulin sequences

Sequence data was processed as previously described [1–3], with some modifications. Briefly, FASTQ files de-multiplexed by TruSeq i7 plate ID indices using the MiSeq onboard GenerateFASTQ workflow were input to a custom data analysis pipeline. Poor-quality reads and bases were trimmed (Trimmomatic-0.32) [4], and the remaining paired reads were stitched together (Flash-1.2.10) [5]. Using Python, these stitched sequencing reads were distinguished by the cell barcode applied during RT to create a separate FASTQ file for each cell. A consensus sequence for HC and LC genes was identified by clustering the reads into operational taxonomic units (OTU) [6]. Isotypes expressed by individual cells were defined by isotype-specific constant region sequences from the IMGT database [7].

A complete FASTA file consisting of all OTUs identified from each cell was submitted to ImMunoGeneTics (IMGT) HighV-Quest for alignment of HC VDJ and LC VJ chain segments [8]. The IMGT files were input to a post-IMGT analysis pipeline, which distinguished the dominant, productive OTUs. The dominant, consensus HC and LC sequences were concatenated

and used to construct phylogenetic trees. Sequences were separated by HC V gene, aligned by MUSCLE [9], and clustered using PhyML [10]. Trees were visualized with ETE [11], anchored by the IMGT HC V gene assignments. A custom analysis pipeline assigned clonal families and persistent lineages based on shared, IMGT-based assignments of VJ gene usage for HC and LC as well as 60% identity within the HC and LC CDR3 regions (Levenshtein distance [12]). The identity threshold was set by examining the bimodal distribution of the nearest distances for the HC and LC CDR3 sequences [13,14].

Monoclonal antibody expression

A subset of subject-derived plasmablast antibody sequences was selected for production either in-house or by LakePharma, Inc. (San Carlos, CA). Antibodies were selected as representatives from clonal families observed at a specific timepoint or from persistent lineages. Additionally, singleton antibodies that stained positive for the cit-specific sort reagent were selected for expression. Different antibodies derived from subjects without RA from ongoing studies or previously isolated to target influenza [15] were selected as negative controls. An antibody that targets desmoglein 3 was expressed based on the published sequence [16]. In-house production was done as previously described [17]. Briefly, constructs including the HC and LC variable region sequences were synthesized as gBlock gene fragments (IDT) for cloning into pFUSE antibody plasmids (Invivogen) using the Cold Fusion Cloning Kit (System Biosciences). We utilized pFUSEss-CHIg-hIg1 for gamma, pFUSE2ss-CLIg-hK for kappa, and pFUSE2ss-CLIg-hL2 for lambda. The Expi293 Expression System (ThermoFisher Scientific) was used for transient transfections, and harvested culture supernatants were purified using Pierce™ Protein A Plus Agarose

(ThermoFisher Scientific). Antibody concentration was determined with the Human IgG ELISA Quantitation set (Bethyl).

Characterizing antibody binding specificities

Candidate targets of antibodies belonging to persistent lineages were analyzed for binding by ELISA. To coat ELISA plates, full-length proteins (1-5 µg/mL) and peptides (10-15 µg/mL) were diluted in bicarbonate/carbonate buffer (pH 9.5). A variety of conditions were tested to identify the final conditions for each set of lineage members evaluated by ELISA. For rAb65 and rAb66, coating was done with full-length proteins (1 µg/mL) and peptides (10 µg/mL) and antibodies were added to the wells at 12.5 µg/mL for proteins and 25 µg/mL for peptides. In both cases, a biotinylated anti-human IgG secondary (1:200,000; Jackson Immunoresearch) followed by a streptavidin-HRP (1:15,000; ThermoFisher) reagent was used for detection. For rAb81 and rAb82, coating was done with full-length proteins (2.5 µg/mL) and peptides (15 µg/mL), antibodies were added to the wells at 50 µg/mL, and binding detection utilized an anti-human IgG antibody directly conjugated to HRP (1:2000; Sigma). For rAb71 and rAb72, these recombinant antibodies (50 µg/mL) were added to wells coated with full-length proteins (1 µg/mL) or peptides (15 µg/mL), and the HRP-conjugated anti-human IgG (1:2000) used to detect binding. For rAb127-129, wells coated with full-length proteins (1 µg/mL), probed with antibodies (6.25 µg/mL), and binding detected with the biotinylated anti-human IgG secondary (1:200,000) followed by a streptavidin-HRP (1:15,000). Finally, for rAb130-133, antibodies (50 µg/mL) were added to wells coated with full-length proteins (5 µg/mL) and peptides (10 µg/mL) and detected with the HRP-conjugated anti-human IgG (1:2500). All ELISAs used 1-Step Ultra TMB-ELISA Substrate (ThermoFisher)

for HRP-based detection. Similar trends were observed in independent experiments while optimizing these conditions.

In vitro citrullination of proteins was carried out using PAD isolated from rabbit skeletal muscle (Sigma) or recombinant PAD4. For rabbit-isolated enzyme, 40 units PAD/mg of the protein to be citrullinated was added to citrullination buffer (100 mM Tris-HCl, 5 mM $CaCl_2$, 5 mM DTT) and incubated with the protein for 2 hours at 37°C. Recombinant PAD4 was added at a 1:10 (PAD4:protein) molar ratio into 100 mM Tris-HCl, 10 mM $CaCl_2$, 40 mM DTT and again incubated with protein 2 hours at 37°C.

Macrophage stimulation assays

Monocytes were isolated from plated PBMCs by adhesion, and cultured at 37°C in complete RPMI with 10% FBS and 1× Penicillin-Streptomycin-Glutamine (ThermoFisher). After initial overnight culture, 30 ng/mL of human macrophage stimulating factor (hMCSF, Peprotech) was added to the media. The cells were cultured for seven days with an additional media change on day 3 and 5.

To form plate-bound IC, plates were coated with 50 µL of protein (20 µg/mL) diluted in PBS and incubated (4°C). Plates were washed three times with PBS and blocked with 150 µL of 1% low-endotoxin BSA in PBS for one hour (room temperature). After washes, recombinant, subject-derived antibody (50 µg/mL) was added to each well for IC formation. After further washes, differentiated macrophages were added (50,000 cells/well) in media (5% FBS, without hMCSF). To block the FcγRII and/or TLR4, cells were pre-incubated (37°C) for one hour with anti-CD32 (Stemcell Technologies, clone IV.3) and/or InSolution™ TLR4 Inhibitor, TAK-242 (EMD

Millipore) and washed prior to plate addition. Lipopolysaccharide (50 ng/mL; LPS, Sigma-Aldrich) was added to the media for select wells of macrophages as a positive control. All conditions included at least duplicate wells. After 24-hour stimulation at 37°C, supernatants were harvested, and the level of TNF-α production was tested by ELISA (Peprotech).

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Supplementary Table 1. Peptides and sequences for generating citrullinated peptide tetramer sort reagent

Peptides	Form	Sequences	MW
FibA 616-635 Cit3 sm Cyclic	cyclic	Biotin-Ahx-THSTK[Cit]CHAKS[Cit]PV[Cit]GIHTSC-$CONH_2$	2758.22
CFC 48-65 Cit2 Cyclic	cyclic	Biotin-Ahx-CTIHAHPGSR[Cit]GG[Cit]HGYHHC-$CONH_2$	2615.97
Clusterin 231-250 Cit3 sm1 Cyclic	cyclic	Biotin-Ahx-CHFS[Cit]ASSCIDELFQD[Cit]FFT[Cit]-$CONH_2$	2961.36
FibA 556-575 Cit sm Cyclic	cyclic	Biotin-Ahx-NTKESSSHHPGCAEFPS[Cit]GKC-$CONH_2$	2616.93
Vim 58-77 Cit3 sm1 Cyclic	cyclic	Biotin-Ahx-GGCVYAT[Cit]SSACV[Cit]L[Cit]SSVPGV-$CONH_2$	2621.07
H2A/a 1-20 Cit3 sm2 Cyclic	cyclic	Biotin-Ahx-MSG[Cit]GKQGCKA[Cit]AKAKT[Cit]SSC-$CONH_2$	2607.12
CF 48-65 Cit2	linear	Biotin-Ahx-TIHAHPGSR[Cit]GG[Cit]HGYHH-$CONH_2$	2409.69
FibA 616-635 Cit3	linear	Biotin-Ahx-THSTK[Cit]GHAKS[Cit]PV[Cit]GIHTS	2609.97
Clusterin 231-250 Cit3	linear	Biotin-Ahx-HFS[Cit]ASSIIDELFQD[Cit]FFT[Cit]-$CONH_2$	2868.23
Fibronectin 1029-1042 Cit2	linear	Biotin-Ahx-LTVGLT[Cit][Cit]GQPRQY	2022.37
FibA 556-575 Cit	linear	Biotin-Ahx-NTKESSSHHPGIAEFPS[Cit]GK-$CONH_2$	2523.8
H2A/a-2 1-20 Cit3	linear	Biotin-Ahx-MSG[Cit]GKQGGKA[Cit]AKAKT[Cit]SS-$CONH_2$	2457.88
Vim 58-77 Cit3	linear	Biotin-Ahx-GGVYAT[Cit]SSAV[Cit]L[Cit]SSVPGV-$CONH_2$	2414.79
CFC1	linear	Biotin-Ahx-SHQEST[Cit]GRSRGRSGRSGS	2403.58

Supplementary Table 2. Primers used for gene-specific PCR amplification of HC and LC

Primer	Sequence
PCR1	
IgK_GSP1	TCTCCCCTGTTGAAGCTCTTTGTG
IgL_GSP1	TYTGTGGGACTTCCACTGCTC
IgG_GSP1	TCTTGTCCACCTTGGTGTTGCTG
IgA_GSP1	ATTCGTGTAGTGCTTCACGTG
IgM_GSP1	CTCTCAGGACTGATGGGAAGC
PCR1_Fwd	CACGACCGGTGCTCGATTTAG
PCR2	
IgK_GSP2	TCCCTACACGACGCTCTTCCGATCTGAAGACAGATGGTGCAGCCACAG
IgL_GSP2	TCCCTACACGACGCTCTTCCGATCTAGAGGAGGGYGGGAACAGAGTGA
IgG_GSP2	TCCCTACACGACGCTCTTCCGATCTAAGACSGATGGGCCCTTGGTGGA
IgA_GSP2	TCCCTACACGACGCTCTTCCGATCTGAAGACCTTGGGGCTGGTCG
IgM_GSP2	TCCCTACACGACGCTCTTCCGATCTGAGACGAGGGGGAAAAGGGTT
PCR2_Fwd	GACTGGAGTTCAGACGTGTGCTCTTCCGATCCACGACCGGTGCTCGATTTAG
PCR3	
PCR3_Rev	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTT
PCR3_Fwd_MID[a]	CAAGCAGAAGACGGCATACGAGATNNNNNNGTGACTGGAGTTCAGACGTG

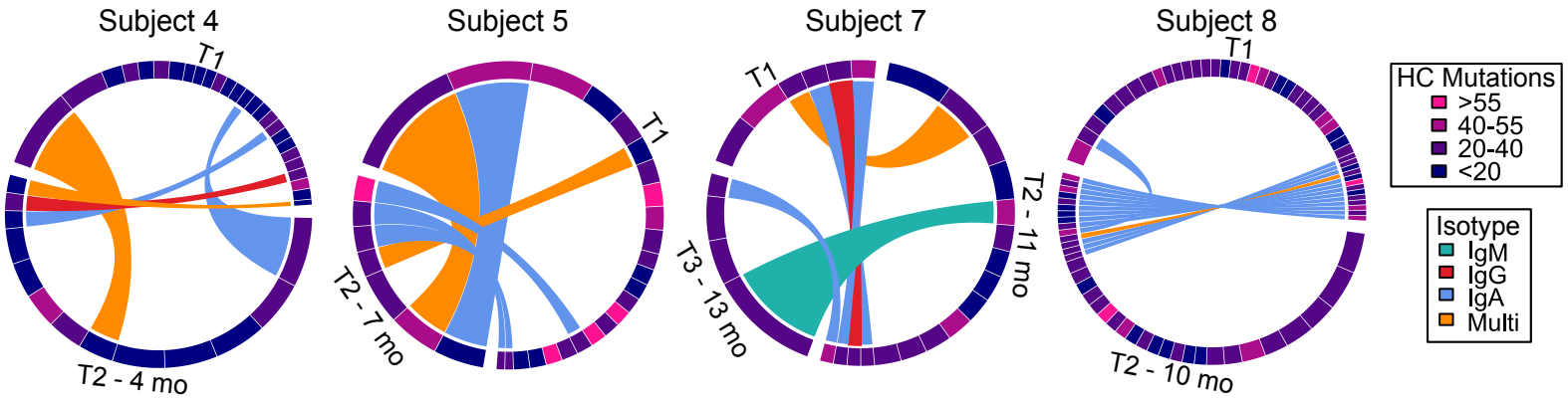
[a]48 different primers were used with defined 6 bp sequences as indexes indicated here as NNNNNN

Supplementary Table 3: Expressed recombinant antibodies

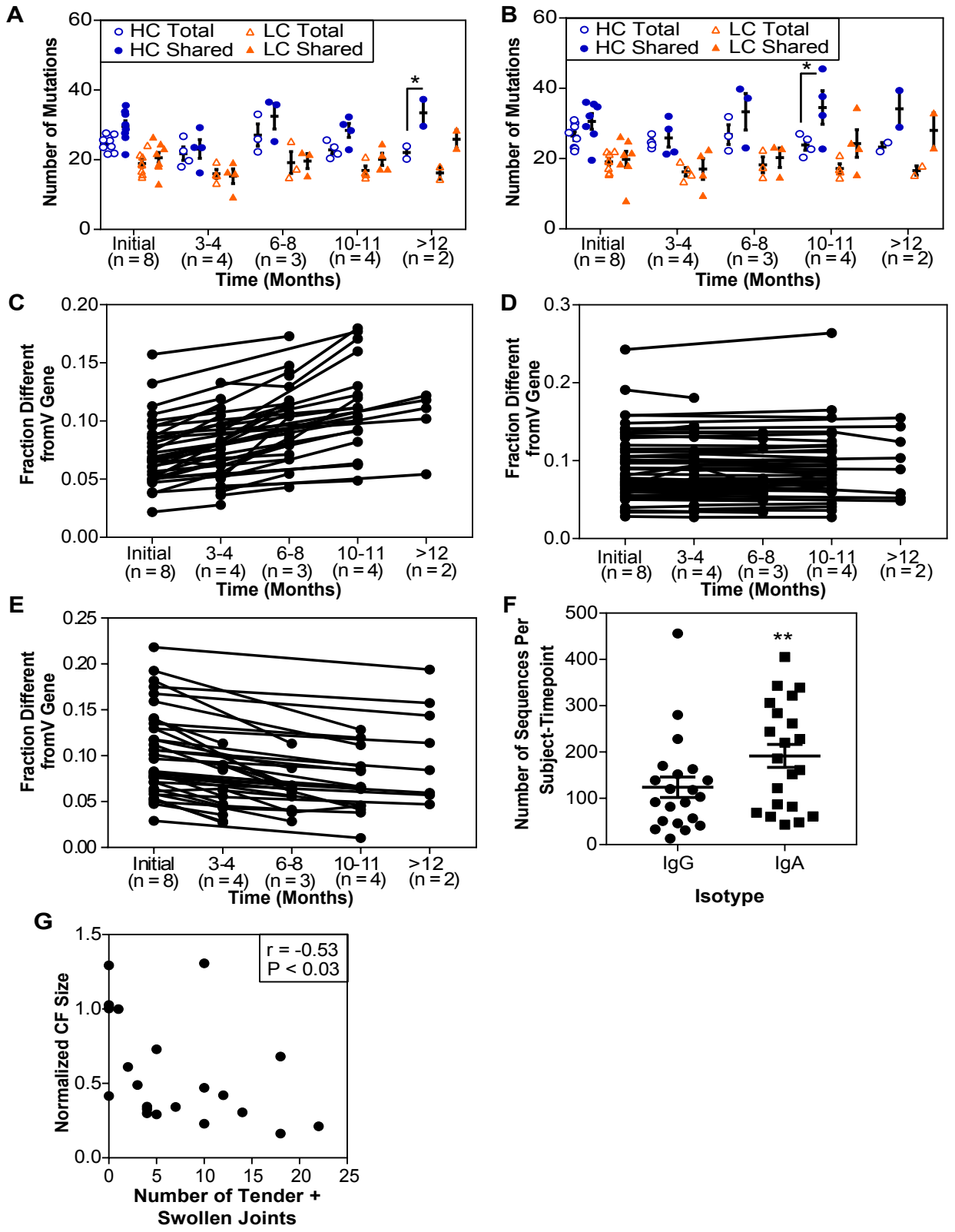
Name	Subject	CF[a]	Timepoint	Persistent	Cit-Tet+	Members[b]
rAb63	Subject1	1	T1	Yes	Yes	50
rAb64	Subject1	42	T2	No	Yes	2
rAb65	Subject1	8	T1	Yes	No	4
rAb66	Subject1	8	T3	Yes	No	4
rAb67	Subject1	53	T2	Yes	Yes	4
rAb68	Subject1	26	T3	Yes	Yes	4
rAb69	Subject2	19	T3	Yes	No	2
rAb70	Subject2	NA	T2	No	Yes	NA
rAb71	Subject3	77	T2	Yes	No	2
rAb72	Subject3	77	T4	Yes	Yes	2
rAb73	Subject3	72	T3	Yes	No	30
rAb74	Subject3	104	T3	No	Yes	39
rAb75	Subject4	24	T1	No	Yes	2
rAb76	Subject5	7	T1	Yes	Yes	18
rAb77	Subject5	7	T2	Yes	Yes	18
rAb78	Subject6	10	T2	No	Yes	2
rAb79	Subject7	10	T2	Yes	No	7
rAb80	Subject7	10	T3	Yes	No	7
rAb81	Subject8	28	T1	Yes	No	2
rAb82	Subject8	28	T2	Yes	Yes	2
rAb83	Subject8	38	T1	No	Yes	3
rAb84	Subject8	21	T2	No	Yes	3
rAb85	Subject1	53	T3	Yes	No	4
rAb86	Subject1	12	T1	Yes	No	3
rAb87	Subject1	12	T2	Yes	No	3
rAb88	Subject1	12	T3	Yes	No	3
rAb89	Subject1	27	T1	Yes	Yes	4
rAb90	Subject3	7	T1	Yes	No	13
rAb91	Subject3	7	T2	Yes	No	13
rAb92	Subject3	7	T3	Yes	No	13
rAb93	Subject3	7	T4	Yes	No	13
rAb94	Subject3	28	T1	Yes	No	5
rAb95	Subject3	28	T2	Yes	No	5
rAb96	Subject3	28	T3	Yes	No	5
rAb97	Subject3	28	T4	Yes	No	5
rAb98	Subject3	17	T1	Yes	No	4
rAb99	Subject3	17	T3	Yes	Yes	4
rAb100	Subject4	2	T1	Yes	No	18
rAb101	Subject4	2	T2	Yes	No	18
rAb102	Subject5	6	T1	Yes	No	13
rAb103	Subject5	6	T2	Yes	Yes	13
rAb104	Subject6	17	T1	Yes	No	7
rAb105	Subject6	17	T2	Yes	No	7
rAb106	Subject7	1	T1	Yes	No	5
rAb107	Subject7	1	T2	Yes	No	5
rAb108	Subject7	15	T2	Yes	No	2
rAb109	Subject7	15	T3	Yes	No	2
rAb112	Subject3	158	T3	Yes	No	2
rAb113	Subject3	158	T4	Yes	No	2
rAb114	Subject5	NA	T2	No	Yes	NA
rAb115	Subject1	28	T1	Yes	Yes	2
rAb116	Subject1	28	T3	Yes	No	2
rAb117	Subject3	53	T1	Yes	No	3
rAb118	Subject3	53	T3	Yes	No	3

rAb119	Subject2	12	T1	Yes	No	2
rAb120	Subject8	18	T1	Yes	No	2
rAb121	Subject8	18	T2	Yes	No	2
rAb122	Subject6	47	T1	Yes	No	3
rAb123	Subject6	47	T2	Yes	No	3
rAb124	Subject1	52	T3	Yes	No	2
rAb125	Subject6	48	T1	Yes	No	2
rAb126	Subject6	48	T2	Yes	No	2
rAb127	Subject1	30	T1	Yes	No	2
rAb128	Subject3	27	T1	Yes	No	2
rAb129	Subject3	27	T4	Yes	No	2
rAb130	Subject3	4	T1	Yes	No	2
rAb131	Subject3	4	T3	Yes	No	2
rAb132	Subject6	39	T1	Yes	No	2
rAb133	Subject6	39	T2	Yes	No	2
rAb134	Subject2	27	T2	Yes	No	3
rAb135	Subject7	NA	T2	No	Yes	NA

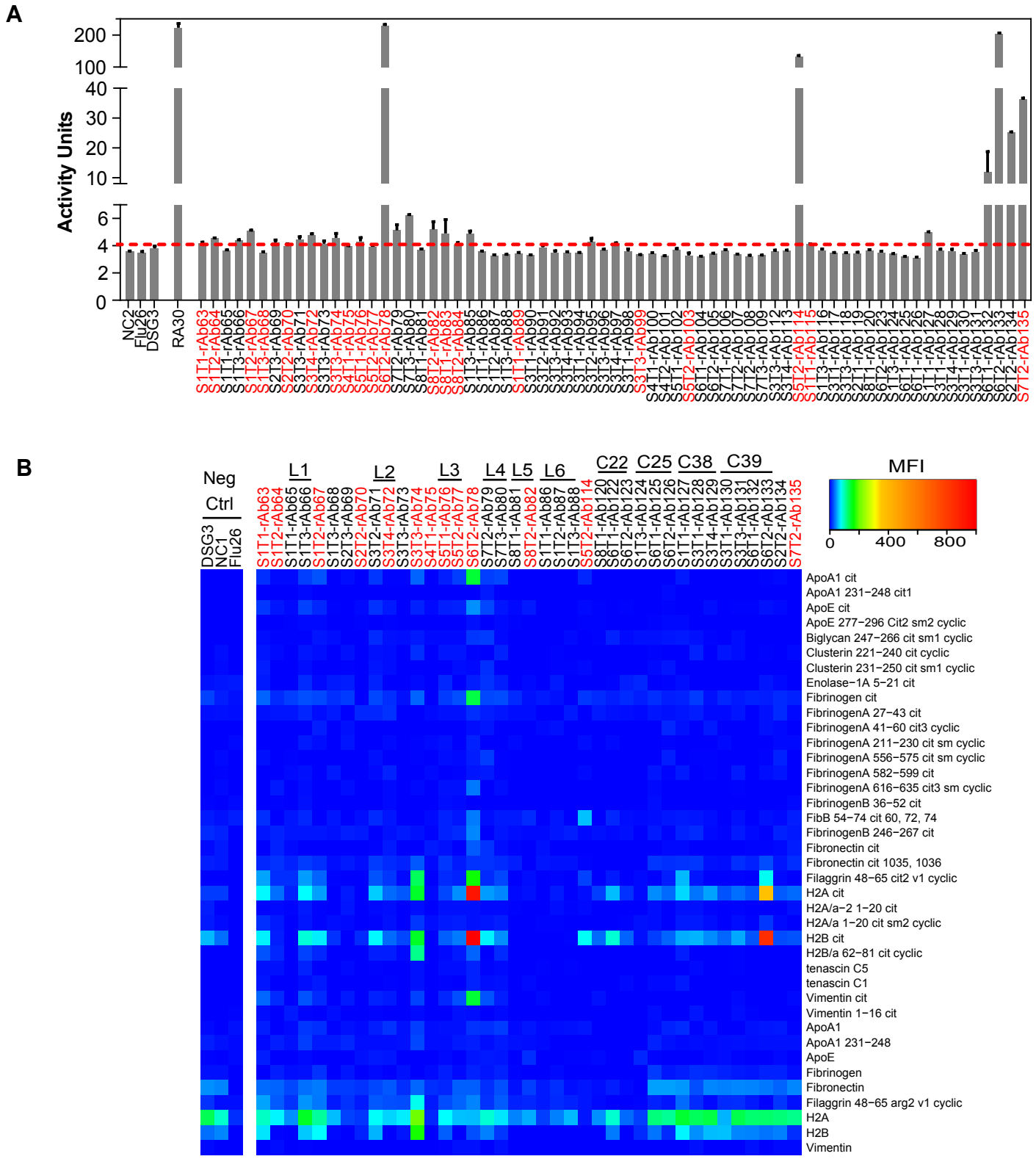
[a]Clonal family number, if applicable; [b]Number of members in family/lineage



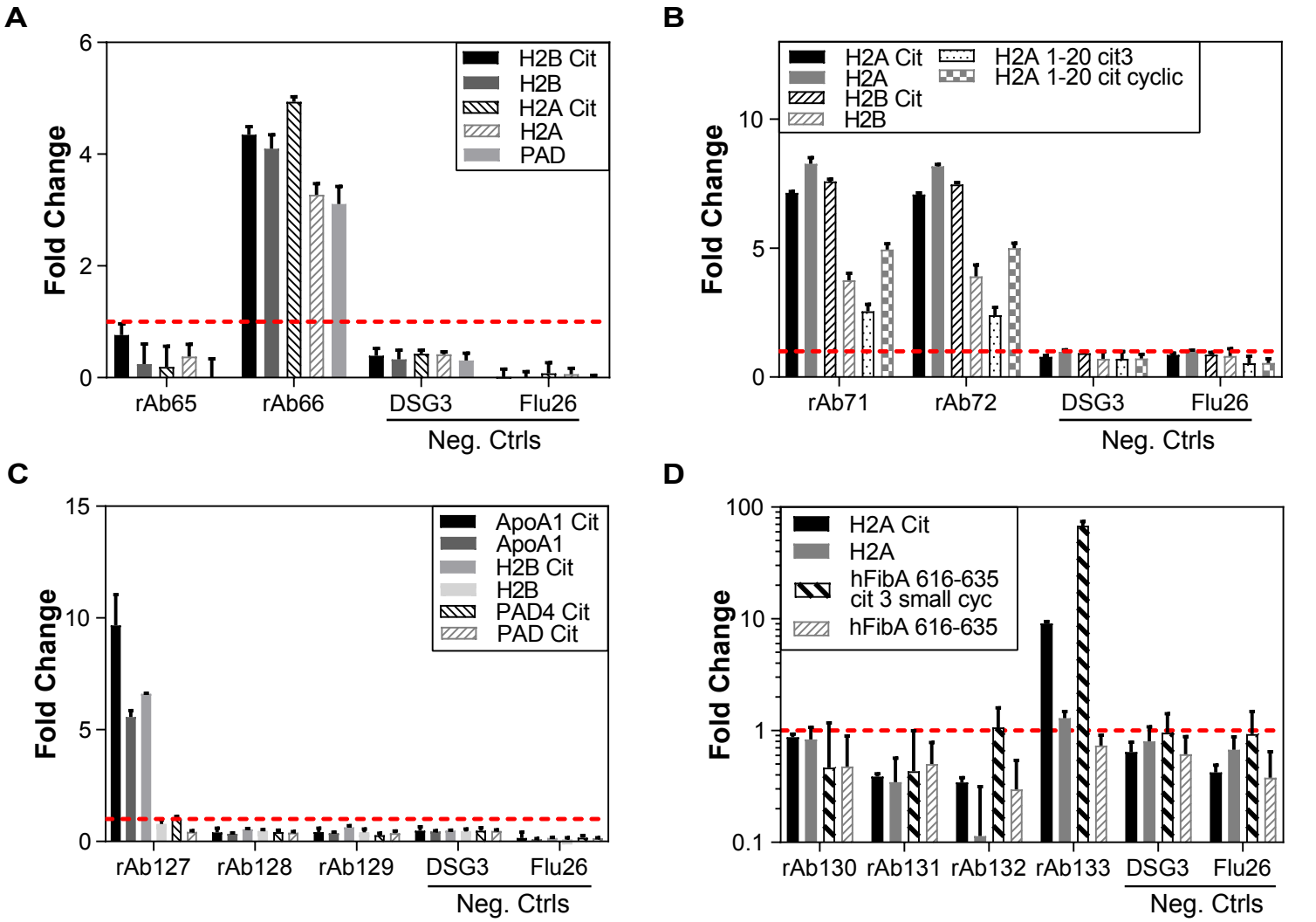
Supplementary Figure 1. Plasmablast clonal families persist and evolve over time in individuals with RA. Antibody repertoires were generated using a cell-barcoding method from blood plasmablasts isolated from serial PBMC samples obtained ≥2 months apart. To further characterize the evolution of clonal lineages, plasmablast antibody sequences were analyzed to identify related members at other timepoints (T) and to evaluate the relative size of lineage expansions at each timepoint. The number of months (mo) from the initial timepoint (T1) is indicated. Clonally related plasmablasts express antibodies with shared HC and LC V and J gene usage as well as CDR3 sequences with ≥60% identity according to Levenshtein distance. The presented chord diagrams depict clonal families observed at the indicated serial timepoints for four additional anti-CCP+ individuals with established RA. Sector width represents the size of the clonal expansion within the timepoint. Sector colors indicate the average number of mutations for the given family or persistent lineage at each timepoint. Connections between timepoints are colored by isotype.



Supplementary Figure 2: Overview of mutational analysis and isotypes of sequenced antibody repertoire. A, The number of mutations in the HC and LC among the entire population of sequences compared to the subset that belong to a persistent lineage. **B,** A similar analysis was performed as in A but only including the sequences for which the full-length V region was identified. * $P < 0.05$ by two-way ANOVA followed by Tukey. The average change in the SHM level normalized by the sequence length was calculated for the 170 different persistent lineages. These were then categorized as **C,** increasing (>1.1 fold change on average), **D,** staying the same, or **E,** decreasing (<0.9 fold change on average). **F,** For each subject at each timepoint, we compared the number of IgG and IgA sequences captured. ** $P < 0.01$ by Wilcoxon matched-pairs. **G,** The relationship between average normalized size of clonal expansions and number of tender and swollen joints observed at each timepoint was evaluated by Spearman correlation (r).



Supplementary Figure 3: Binding specificities of recombinant subject-derived antibodies. A, Expressed recombinant antibodies were evaluated for citrulline reactivity using the CCP3 ELISA. A cutoff of three standard deviations above the negative control antibodies is indicated (red dotted line). **B,** Recombinant antibodies were evaluated for their epitope specificity using an RA antigen bead-based microarray. Bead microarrays were probed with recombinant antibodies representing the identified clonal lineages/families and singletons that bind citrullinated-peptide tetramers. A fluorophore-conjugated anti-human IgG secondary was used to detect antibody binding and the heatmap depicts median fluorescent intensities. The specific subject and timepoint from which each antibody was identified is indicated, and antibodies derived from cells that stained with the citrullinated peptide tetramers are marked in red.



Supplementary Figure 4. Clonal lineage-derived recombinant antibodies bind citrullinated antigens by ELISA. ELISA analysis was performed to measure the binding of representative recombinant antibodies from different persistent lineages, including those from different CDR3 clusters. Using an activity cutoff of three standard deviations above the negative control antibodies, the fold change above this cutoff was calculated. **A,** Subject 1 lineage 1 recombinant antibody binding to full-length citrullinated or native H2A, H2B, and PAD. **B,** Subject 3 lineage recombinant antibody binding to full-length citrullinated or native H2A and H2B and an H2A peptide. **C,** Binding activity of antibodies belonging to persistent lineages from Subject 1 (rAb127) and Subject 3 (rAb128 and rAb129) with CDR3 sequences that cluster together. **D,** Binding activity of antibodies belonging to persistent lineages from Subject 3 (rAb130 and rAb131) and Subject 6 (rAb132 and rAb133) with CDR3 sequences that cluster together. Data presented as mean ± SEM from duplicates.