Science Translational Medicine
AAAS

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Supplementary Materials for

Persistently activated, proliferative memory autoreactive B cells promote inflammation in rheumatoid arthritis

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Published 18 November 2020, *Sci. Transl. Med.* **12**, eaaz5327 (2020)
DOI: 10.1126/scitranslmed.aaz5327

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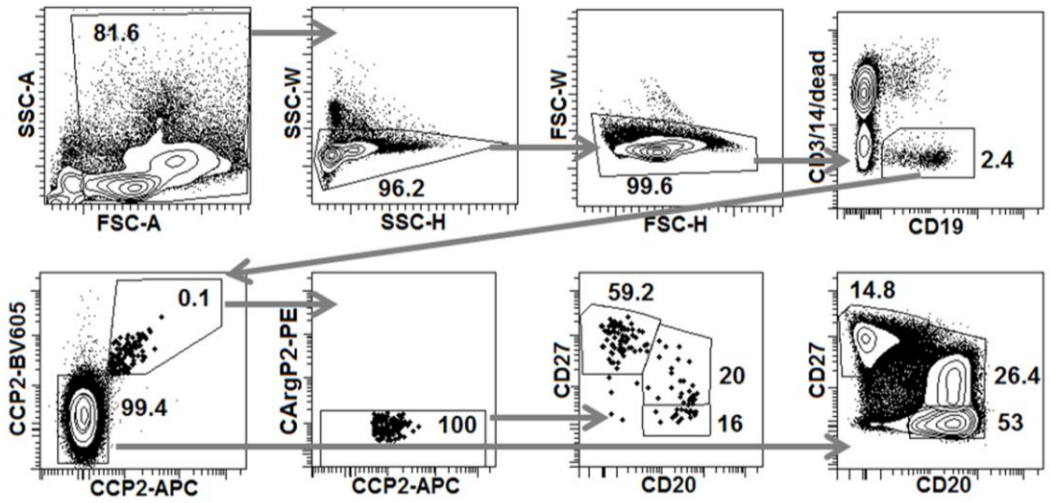

Fig. S1. Subsets of ACPA-positive B cells in synovial fluid of inflamed joints from patients with RA. Gating strategy for the identification and subset characterization of ACPA-positive B cells. ACPA-positive B cells were identified using differentially labelled CCP2 and CArgP2 streptavidin tetramers. B cell subsets were delineated based on the differential abundance of CD20 and CD27.

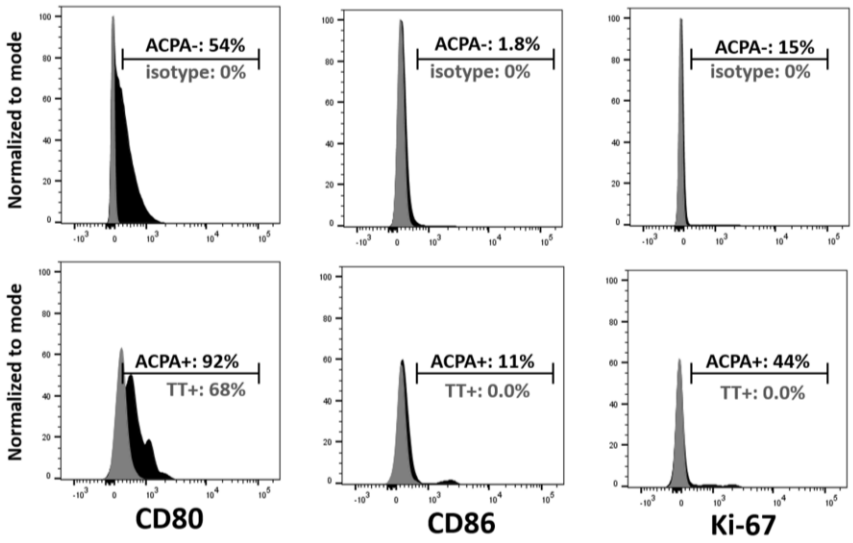

Fig. S2. Histogram plots depicting the positivity for CD80, CD86, or Ki-67 in ACPA-positive ($ACPA^+$), TT-specific (TT^+), and bulk ($ACPA^-$) MBC populations. Data depict results from one representative donor (for combined data see Figure 2B).

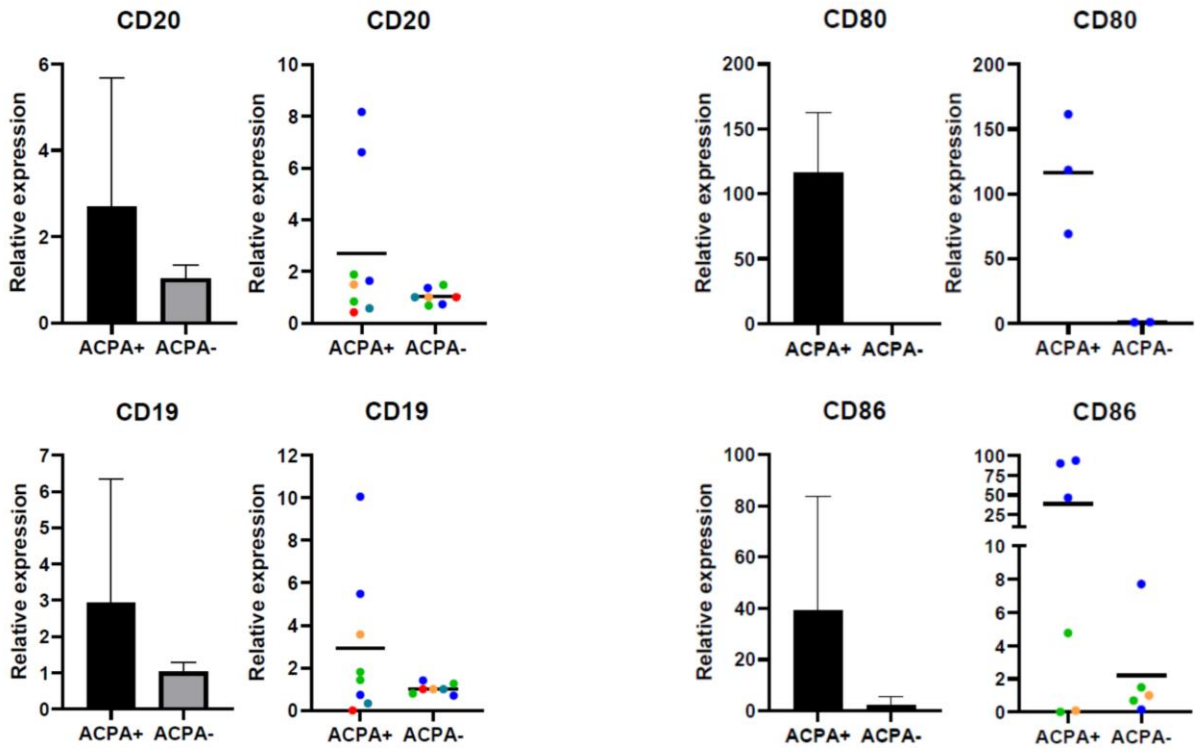

Fig. S3. Relative gene expression of B cell phenotypic markers in ACPA-positive and non-citrulline-reactive MBCs. Transcript abundance was determined by RT-PCR using mRNA derived from ACPA-positive (ACPA+) B cells and non-citrulline reactive (ACPA-) B cells**.** Each dot corresponds to a pool of 8 – 30 cells, colors represent individual patients (n = 5). Expression is presented relative to the expression in the ACPA- samples and corrected for the expression of beta-2 microglobulin (B2M) within donors. ACPA+ cells [CD19+CD20+CD27+ (CCP2+ or CArgP2+)] and ACPA- cells [CD19+CD20+CD27+(CCP2- or CArgP2-)] were obtained by FACS from patients with ACPA-positive RA.

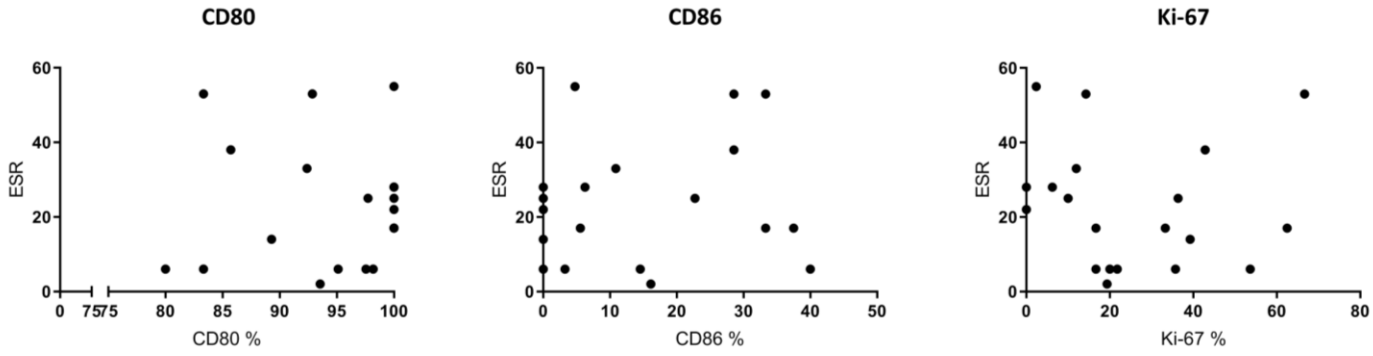

Fig. S4. Correlation analysis between systemic inflammation and the characteristics of ACPA-positive MBCs. Data are presented as percentage of ACPA-positive MBCs that are also positive for the indicated marker in relation to the erythrocyte sedimentation rate (ESR) (n = 20).

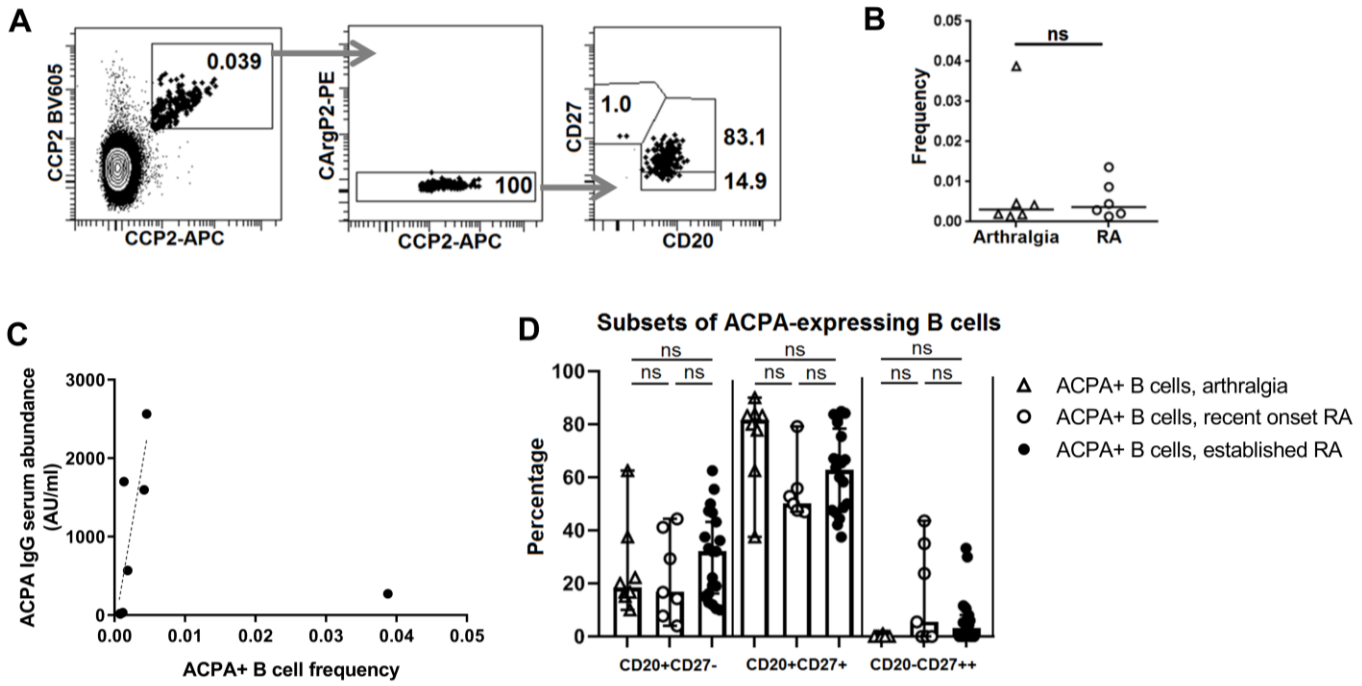

Fig. S5. Characteristics of ACPA-positive B cells in individuals with ACPA$^+$ arthralgia. (A) ACPA-positive B cells in individuals with arthralgia in PBMCs. **(B)** Frequency of ACPA-positive B cells in PBMCs from individuals with arthralgia compared to the frequency in RA patients with matched ACPA IgG plasma abundance (available for n = 6). **(C)** Correlation between the frequency of ACPA-positive B cells in PBMCs from individuals with arthralgia and serum ACPA IgG abundance (n = 8). The dotted line depicts the correlation if the one outlying donor with a high frequency of ACPA-positive B cells is removed from the analysis (Pearson R 0.81, P 0.02) **(D)** B cell subsets identified by the abundance of CD20 and CD27 on ACPA-positive B cells from individuals with arthralgia (n = 8), patients with early onset, untreated RA (n = 7), and patients with established, treated RA (n = 20). ns, non-significant P > 0.05. Two-tailed Mann-Whitney test in B; Pearson correlation in C; one-way ANOVA with Dunn's multiple comparison test in D. (n = number of donors)

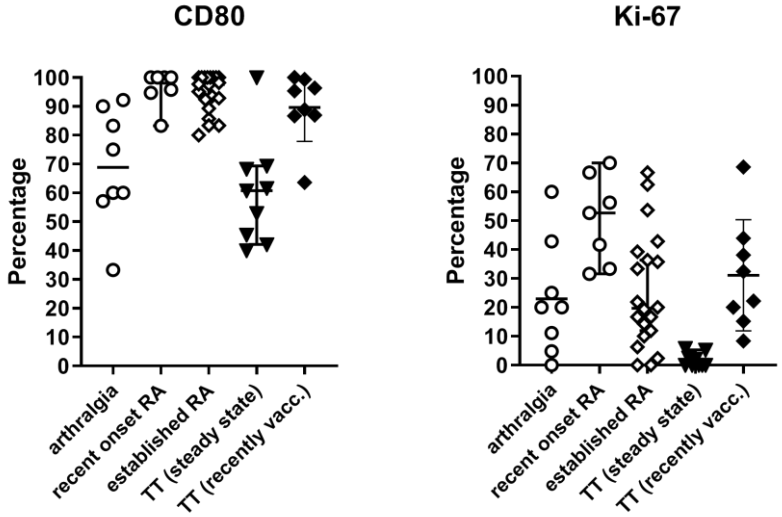

Fig. S6. Phenotype of ACPA-positive B cells in different disease phases compared to TT-specific B cells in the steady state and upon recent vaccination in patients with RA. ACPA-positive B cells (open symbols), TT specific B cells (closed symbols). Recently vaccinated (recently vacc.) indicates analysis 28 days after booster injection.

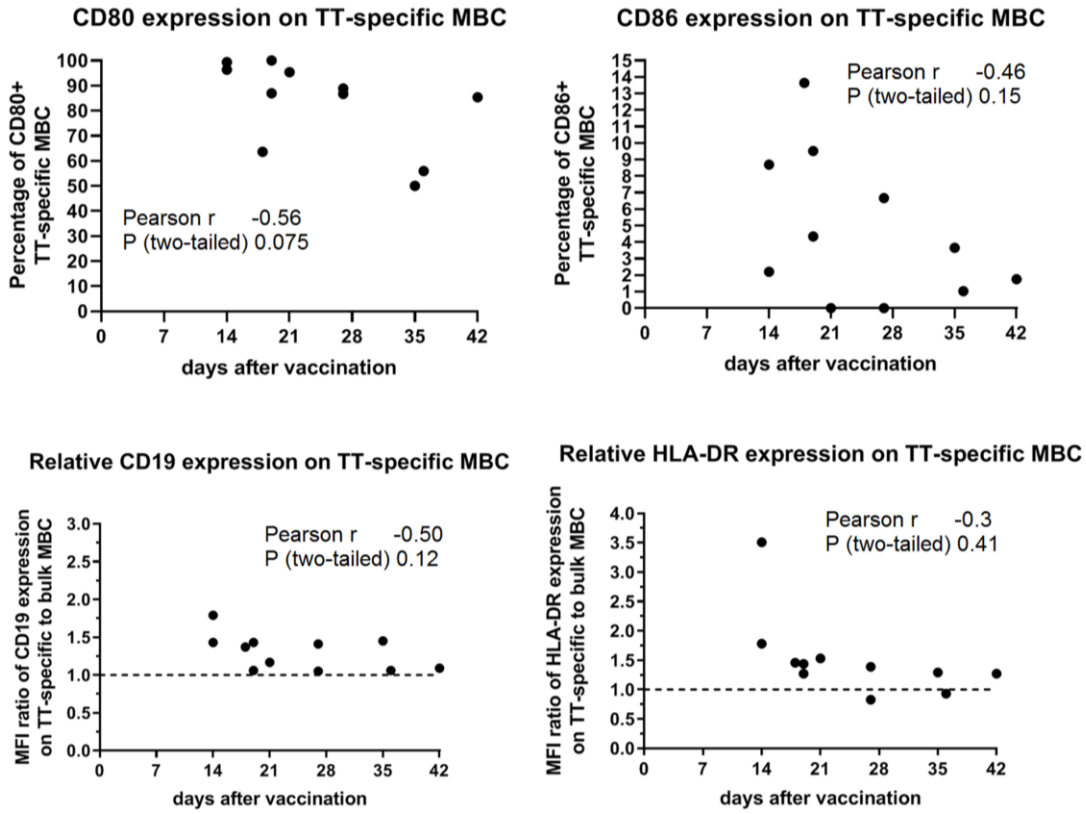

Fig. S7. Correlation between the proportion of TT-specific MBCs positive for CD80 or CD86, and abundance of CD19 or HLA-DR and time after TT vaccination in untreated patients with RA. (n=11)

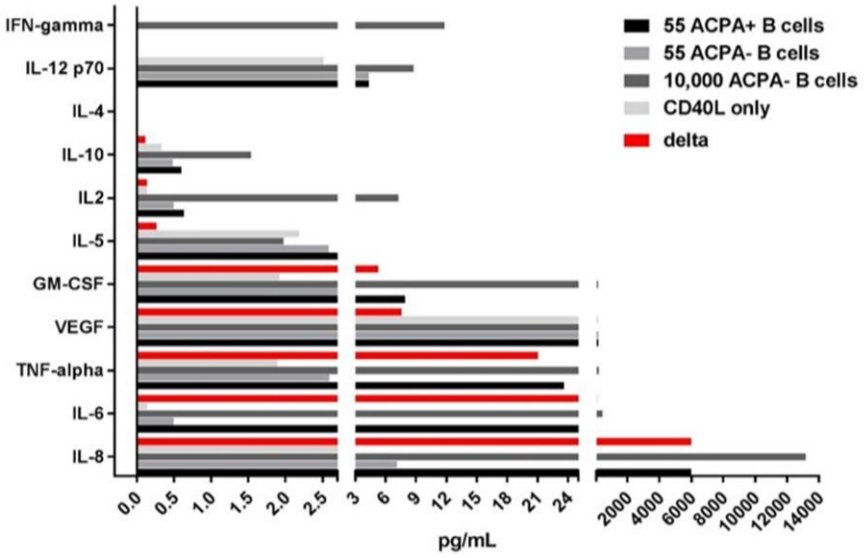

Fig. S8. Cytokine secretion by ACPA-positive MBCs. Representative graph of 11 cytokines secreted by sorted, CD20+CD27+ ACPA-positive and ACPA-negative B cells after 7 days of CD40 and BCR stimulation. Cytokines are ranked from bottom to top based on the difference in secretion between ACPA-positive and ACPA-negative B cells (red, delta). As a positive control, 10,000 CD20+CD27+ ACPA-negative B cells were treated with the same stimulants (dark grey). As a negative control, irradiated CD40L-positive cells were treated with anti-IgG/IgM Fab2 fragments in the absence of B cells (light grey).

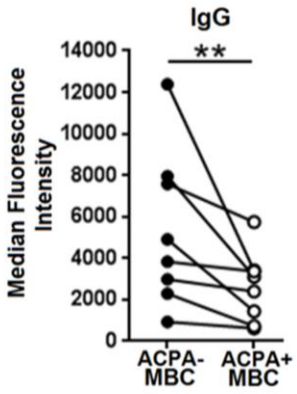

Fig. S9. Abundance of IgG on CD20^{+}CD27^{+} ACPA-positive and ACPA-negative B cells from RA patient peripheral blood. Each dot represents an individual donor (n = 8). Connected dots depict data from individual patient samples. ** $p \leq 0.01$. Two-tailed Wilcoxon signed rank test.

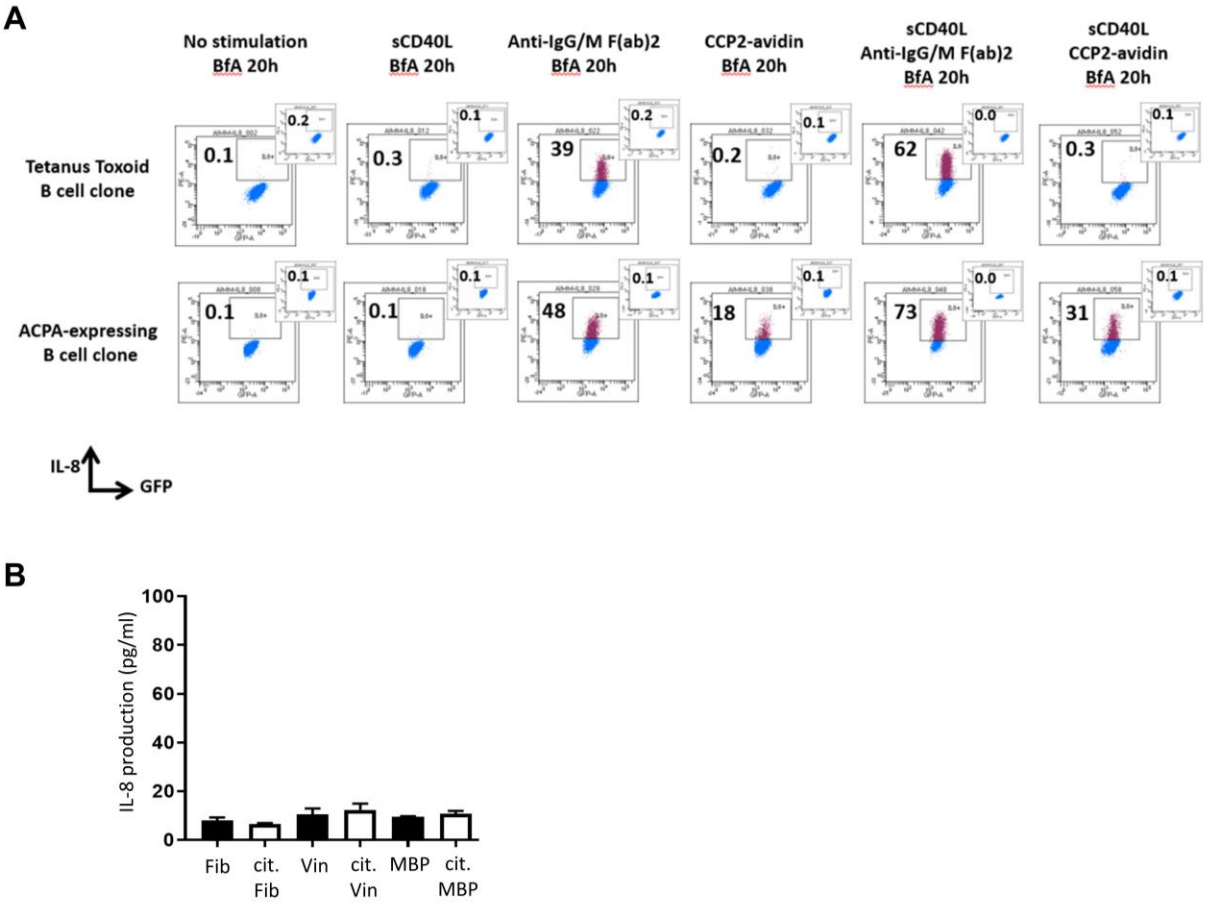

Fig. S10. Production of IL-8 by TT-specific and ACPA-positive, immortalized B cell clones triggered by various stimulants. (**A**) Data obtained by flow cytometry. Transduced cells are visualized by the expression of GFP. Data are representative of 1 of 3 experiments. Large graph shows staining with anti-IL-8 PE; small popout indicates isotype control staining for IL-8. (**B**) Data obtained by ELISA upon stimulation of the TT-specific B cell clone with different citrullinated proteins or their native variants.

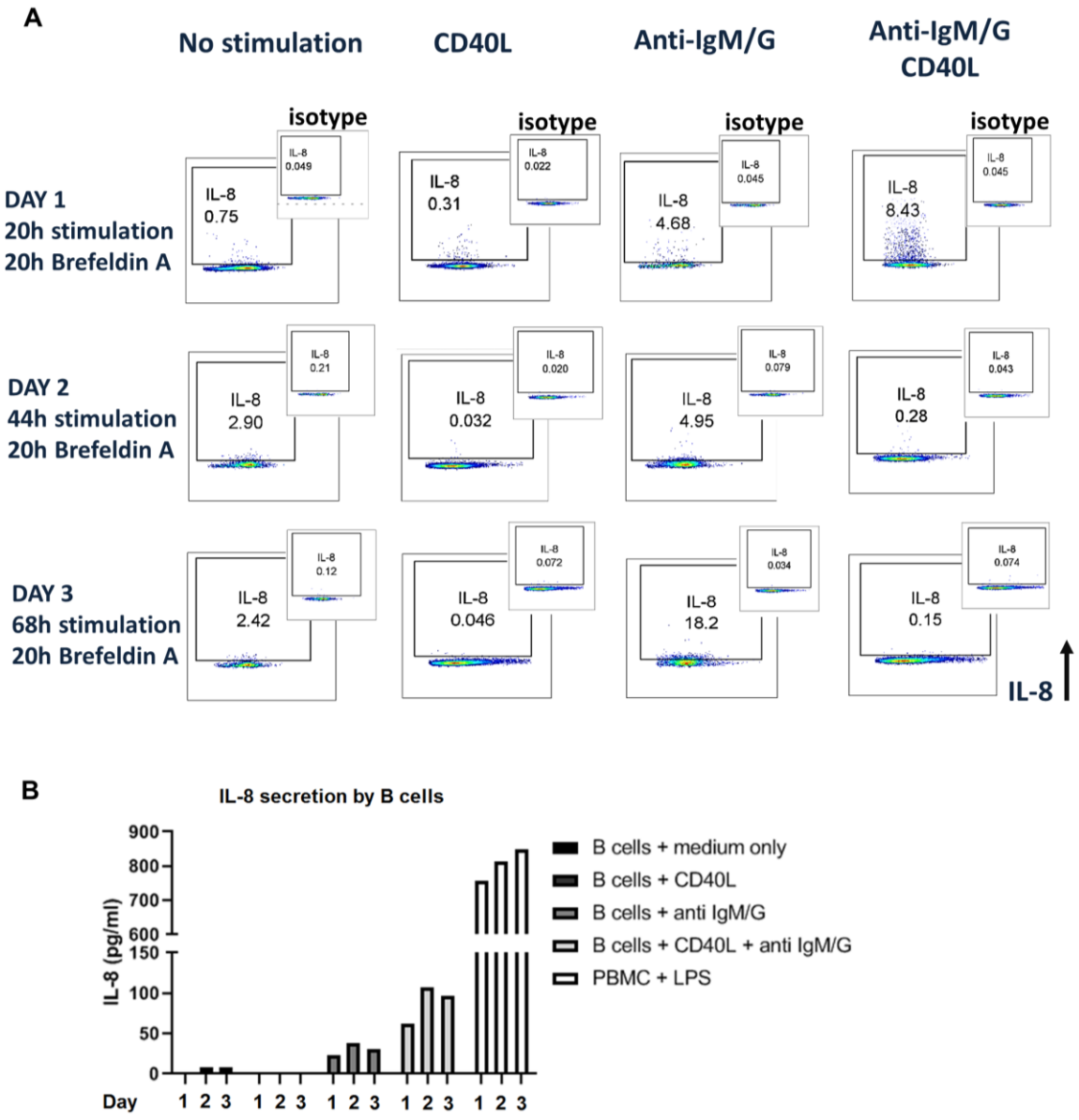

Fig. S11. Production of IL-8 by B cells from healthy individuals under different stimulation conditions and at different time points after stimulation. (A) Data obtained by flow cytometry. Data are representative of 1 of 3 experiments. Large graph shows staining with anti-IL-8 PE; small popout indicates isotype control stainings. **(B)** ELISA analysis of IL-8 secretion by the indicated cells stimulated as indicated. Supernatant was collected on day 1, day 2, and day 3 after stimulation. LPS, lipopolysaccharide

Data file S1. Subject-level data for experiments with *n* < 20 donors or replicates.
Figure 2A.

No	Patient code	FSC (ratio to ACPA- MBC)	
		TT+ MBC	ACPA+ MBC
1	RL29	0,94	1,08
2	RL44	1,11	1,18
3	RL45	0,89	0,94
4	RL47	0,90	1,15
5	RL48	0,94	1,11
6	RL49	0,96	1,03
7	RL50	1,03	1,03
8	RL51	1,06	1,05
9	RL52	1,00	1,10

No	Patient code	CD19 (MFI)			HLA-DR (MFI)		
		ACPA- MBC	TT+ MBC	ACPA+ MBC	ACPA- MBC	TT+ MBC	ACPA+ MBC
1	RL17	380	432				
2	RL18	339	438	714	342	358	1748
3	RL19	417	483	556	352	463	662
4	RL20	385	311	482	268	321	449
5	RL21	371	402	585	219	230	366
6	RL23	600	438	789	527	681	1333
7	RL24	497	442	818	376	317	358
8	RL25	490	478	1095	644	666	720
9	RL26	508	463	832	814	596	1209
10	RL22	529	402	767	nd	nd	nd
11	RL29	526	629	811	nd	nd	nd
12	RL44	471	529	753	nd	nd	nd
13	RL45	413	466	588	nd	nd	nd

nd = no data

Figure 2B.

No	Patient code	CD80 (%)			CD86 (%)			Ki-67 (%)		
		ACPA- MBC	TT+ MBC	ACPA+ MBC	ACPA- MBC	TT+ MBC	ACPA+ MBC	ACPA- MBC	TT+ MBC	ACPA+ MBC
1	RL18	49,15	42,11	83,33	4,77	0,00	33,33	7,24	5,26	66,67
2	RL19	53,52	69,35	97,73	1,76	1,61	22,73	14,65	0,00	36,36
3	RL20	51,74	52,94	80,00	3,84	0,00	40,00	3,37	5,88	20,00
4	RL21	59,24	68,33	92,39	5,09	0,00	10,87	7,58	0,00	11,96
5	RL23	73,37	100,00	85,71	14,06	12,50	28,57	9,33	0,00	42,86
6	RL24	59,93	61,62	97,56	2,15	0,54	3,25	5,67	2,16	35,77
7	RL25	41,90	40,00	89,29	1,24	0,00	0,00	6,15	0,00	39,29
8	RL26	43,01	60,76	83,33	2,27	1,27	0,00	6,28	2,53	16,67

Figure 2C, Figure 3A (ACPA+ MBC, established RA), Fig. S4.

No	Patient code	CD19 (MFI ratio to bulk MBC)	HLA-DR (MFI ratio to bulk MBC)	CD80 (%)	CD86 (%)	Ki-67 (%)	DAS44 (3v)	Tender joint count	Swollen joint count	ESR (mm/1h)
1	RL5	1,45	1,46	100,00	37,50	62,50	1,16	0	0	17
2	RL6	1,88	0,71	100,00	33,33	33,33	2,05	2	2	17
3	RL7	1,87	1,40	93,55	16,13	19,35	nd	nd	nd	2
4	RL8	2,26	1,92	100,00	0,00	0,00	1,85	1	1	22
5	RL9	1,73	1,43	100,00	0,00	0,00	nd	nd	nd	28
6	RL10	1,50	1,28	100,00	6,25	6,25	2,79	4	6	28
7	RL11	1,49	1,38	100,00	5,56	16,67	nd	nd	nd	17
8	RL12	2,06	0,95	92,86	28,57	14,29	nd	nd	nd	53
9	RL13	1,54	1,32	100,00	4,76	2,38	3,8	12	6	55
10	RL14	1,45	1,37	98,18	14,55	21,82	0,82	0	0	6
11	RL15	1,37	2,14	95,12	0,00	53,66	nd	nd	nd	6
12	RL16	1,71	1,36	100,00	0,00	10,00	1,83	1	0	25
13	RL18	2,11	5,11	83,33	33,33	66,67	3,74	13	4	53
14	RL19	1,33	1,88	97,73	22,73	36,36	nd	nd	nd	25
15	RL20	1,25	1,68	80,00	40,00	20,00	0,82	0	0	6
16	RL21	1,58	1,67	92,39	10,87	11,96	1,44	0	1	33
17	RL23	1,32	2,53	85,71	28,57	42,86	1,42	0	0	38
18	RL24	1,65	0,95	97,56	3,25	35,77	3,04	17	0	6
19	RL25	2,23	1,12	89,29	0,00	39,29	1,86	2	0	14
20	RL26	1,64	1,49	83,33	0,00	16,67	nd	nd	nd	6

nd, no data
bulk MBC, ACPA-negative MBC

Figure 3A.

ACPA+ MBC, arthralgia

No	Patient code	CD19 (MFI ratio to bulk MBC)	HLA-DR (MFI ratio to bulk MBC)	CD80 (%)	CD86 (%)	Ki-67 (%)
1	RL35	1,46	1,36	90,00	10,00	20,00
2	RL36	1,14	1,08	83,33	0,00	11,11
3	RL37	1,09	1,34	92,22	0,60	4,79
4	RL38	1,46	0,86	75,00	0,00	25,00
5	RL39	0,74	0,91	33,33	0,00	0,00
6	RL40	0,97	1,08	60,00	0,00	20,00
7	RL42	1,11	2,24	57,14	14,29	42,86
8	RL43	1,49	0,60	60,00	0,00	60,00

bulk MBC, ACPA-negative MBC

ACPA+ MBC, recent onset RA

No	Patient code	CD19 (MFI ratio to bulk MBC)	HLA-DR (MFI ratio to bulk MBC)	CD80 (%)	CD86 (%)	Ki-67 (%)
1	RL62	1,49	2,41	100	44,44	66,67
2	RL64	1,86	1,58	95,85	12,50	56,25
3	RL65	1,40	2,32	100	0	33,33
4	RL67	1,98	2,17	100	10,53	31,58
5	RL68	1,87	2,07	94,74	21,05	52,63
6	RL69	1,89	1,72	83,33	12,5	41,67
7	RL71	1,36	3,17	100	50	70

bulk MBC, ACPA-negative MBC

Figure 3B.

TT+ MBC, steady state

No	Patient code	CD19 (MFI ratio to bulk MBC)	HLA-DR (MFI ratio to bulk MBC)	CD80 (%)	CD86 (%)	Ki-67 (%)
1	RL17	1,14	1,02	45,45	3,6	0
2	RL18	1,29	1,05	42,11	0,00	5,26
3	RL19	1,16	1,32	69,35	1,61	0,00
4	RL20	0,81	1,20	52,94	0,00	5,88
5	RL21	1,08	1,05	68,33	0,00	0,00
6	RL23	0,73	1,29	100,00	12,50	0,00
7	RL24	0,89	0,84	61,62	0,54	2,16
8	RL25	0,98	1,03	40,00	0,00	0,00
9	RL26	0,91	0,73	60,76	1,27	2,53
10	RL22	0,76	nd	nd	nd	nd
11	RL29	1,20	nd	nd	nd	nd
12	RL44	1,12	nd	nd	nd	nd
13	RL45	1,13	nd	nd	nd	nd

nd = no data

bulk MBC, ACPA-negative MBC

TT+MBC, Recently vaccinated

No	Patient code	CD19 (MFI ratio to bulk MBC)	HLA-DR (MFI ratio to bulk MBC)	CD80 (%)	CD86 (%)	Ki-67 (%)
1	RL60	1,41	0,83	86,67	6,67	20,00
2	RL61	1,43	1,44	100,00	9,52	38,10
3	RL62	1,17	1,53	95,37	0,00	8,33
4	RL63	1,05	1,39	88,89	0,00	22,22
5	RL65	1,43	1,78	96,34	2,20	68,54
6	RL66	1,06	1,27	86,96	4,35	15,22
7	RL69	1,37	1,46	63,64	13,64	43,94
8	RL70	1,79	3,51	99,41	8,70	32,46

bulk MBC, ACPA-negative MBC

Figure 3C and Fig. S7.

No	Patient code	Days after booster tetanus vaccination	CD19 (MFI ratio to bulk MBC)	HLA-DR (MFI ratio to bulk MBC)	CD80 (%)	CD86 (%)	Ki-67 (%)
1	RL65	14	1,43	1,78	96,34	2,2	68,54
2	RL70	14	1,79	3,51	99,41	8,7	32,46
3	RL69	18	1,37	1,46	63,64	13,64	43,94
4	RL61	19	1,43	1,44	100	9,52	38,1
5	RL66	19	1,06	1,27	86,96	4,35	15,22
6	RL62	21	1,17	1,53	95,37	0	8,33
7	RL60	27	1,41	0,83	86,67	6,67	20
8	RL63	27	1,05	1,39	88,89	0	22,22
9	RL68	35	1,45	1,29	50	3,65	4,17
10	RL67	36	1,06	0,93	55,93	1,03	1,8
11	RL64	42	1,09	1,27	85,38	1,75	6,43

bulk MBC, ACPA-negative MBC

Figure 4A.

No	Patient code	ACPA- MBC (in MFI)	TT+ MBC (in MFI)	ACPA+ MBC (in MFI)
1	RL44	1094	1424	1056
2	RL47	1619	1660	844
3	RL48	1317	1961	5
4	RL49	1674	2109	107
5	RL50	1977	925	499
6	RL51	1227	1817	227
7	RL52	1713	3112	518

Figure 4B.

No	Patient code	ACPA- MBC (in MFI)	ACPA+ MBC (in MFI)
1	RL56	159	82
2	RL57	132	8
3	RL58	107	-64
4	RL59	59	15

Figure 5A.

No	Patient code	ACPA- MBC			ACPA+ MBC		
		IL-8	IL-6	TNFa	IL-8	IL-6	TNFa
1	RL31	0,25	0,03	0,12	1,63	0,03	0,12
2	RL32	0,87	0,10	0,22	11,81	0,46	0,40
3	RL33	0,13	0,01	0,05	108,78	1,36	0,43
4	RL34	0,66	0,01	0,21	239,73	2,75	1,43
5	RL41	0,06	0,00	0,04	51,59	0,58	0,58

Cytokine concentrations in pg/ml.

Figure 5B.

No	Patient code	ACPA- B cells			ACPA+ B cells		
		IL-8	IL-6	TNFa	IL-8	IL-6	TNFa
1	RL46	85	3	7	225	14	5
2	RL53	174	14	7	1140	57	220
3	RL54	60	3	18	87	388	128
4	RL55	25	-2	7	79	-13	46

Values presented as delta MFI

Figure 5C.

ACPA-positive B cell clone

Well number	Fib	cit-Fib	Vin	cit-Vin	MBP	cit-MBP
1	2,34	10,25	2,44	8,26	2,93	160,16
2	2,83	6,87	4,31	8,16	3,23	149,82
3	3,52	8,85	5,98	10,25	4,01	148,88

Concentrations presented in pg/ml.

Figure 5D.

Well number	TT+ clone	ACPA+ clone
1	28,84	847,13
2	27,66	779,44
3	29,60	865,43

Figure 5E.

Well number	Medium only	100ng/ml IL-8	IL-8 + Anti-IL-8 Ab	ACPA+ supernatant	ACPA+ supernatant + Anti-IL-8 Ab	Tetanus supernatant	Tetanus supernatant + Anti-IL-8 Ab
1	61187	172278	95183	102504	55395	65341	59419
2	67711	178138	84913	103369	72969	80545	63041
3				101998	68238	70022	57808

Figure S5B.

No	Arthralgia			Established RA		
	Patient code	ACPA concentration (AU/ml)	ACPA+ B cell frequency (%)	Patient code	ACPA concentration (AU/ml)	ACPA+ B cell frequency (%)
1	RL35	2563	0,0046	RL23	2671	0,0029
2	RL36	1597	0,0042	RL10	1279	0,0045
3	RL37	272	0,0388	RL15	222	0,0087
4	RL38	1701	0,0014	RL26	1297	0,0013
5	RL40	569	0,0019	RL9	765	0,0021
6	RL43	31	0,0016	RL5	101	0,0021

Figure S5C.

No	Patient code	ACPA+ B cell frequency (in %)	ACPA IgG serum concentration (AU/ml)
1	RL35	0,0046	2563
2	RL36	0,0042	1597
3	RL37	0,0388	272
4	RL38	0,0014	1701
5	RL39	0,0008	14
6	RL40	0,0019	569
7	RL42	0,0008	19
8	RL43	0,0016	31

Figure S5D.

ACPA+ B cells, arthralgia

No	Patient code	CD20+ CD27- frequency (in %)	CD20+ CD27+ frequency (in %)	CD20- CD27++ frequency (in %)
1	RL35	17	83	0
2	RL36	10	86	0
3	RL37	15	84	1
4	RL38	20	80	0
5	RL39	63	38	0
6	RL40	38	63	0
7	RL42	22	78	0
8	RL43	17	83	0

ACPA+ B cells, recent onset RA

No	Patient code	CD20+ CD27- frequency (in %)	CD20+ CD27+ frequency (in %)	CD20- CD27++ frequency (in %)
1	RL62	29	53	0
2	RL64	4	48	44
3	RL65	44	50	1
4	RL67	17	79	0
5	RL68	41	56	0
6	RL69	8	47	35
7	RL71	14	48	24

ACPA+ B cells, established RA

No	Patient code	CD20+ CD27- frequency (in %)	CD20+ CD27+ frequency (in %)	CD20- CD27++ frequency (in %)
1	RL5	47	42	11
2	RL6	22	67	11
3	RL7	16	84	0
4	RL8	63	38	0
5	RL9	56	44	0
6	RL10	11	84	5
7	RL11	43	49	8
8	RL12	38	58	4
9	RL13	19	81	0
10	RL14	32	65	2
11	RL15	12	82	6
12	RL16	19	48	33
13	RL18	10	60	30
14	RL19	36	64	0
15	RL20	50	50	0
16	RL21	32	67	1
17	RL23	47	47	7
18	RL24	13	75	12
19	RL25	15	85	0
20	RL26	33	67	0

Figure S9.

No	Patient code	ACPA- MBC (in MFI)	ACPA+ MBC (in MFI)
1	RL1	7950	3107
2	RL2	7573	5741
3	RL3	930	624
4	RL4	12382	3414
5	RL27	2975	2402
6	RL28	4905	1442
7	RL29	3828	3360
8	RL30	2288	725

Figure S10.

TT-specific B cell clone

Well number	Fib	cit-Fib	Vin	cit-Vin	MBP	cit-MBP
1	6,67	6,77	11,24	9,75	9,35	10,15
2	8,46	6,67	7,57	14,95	9,85	9,95
3	9,05	5,88	12,44	12,34	9,45	12,14

Concentrations presented in pg/ml.