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Supplemental Information

Complement C4A Regulates Autoreactive B Cells in Murine Lupus

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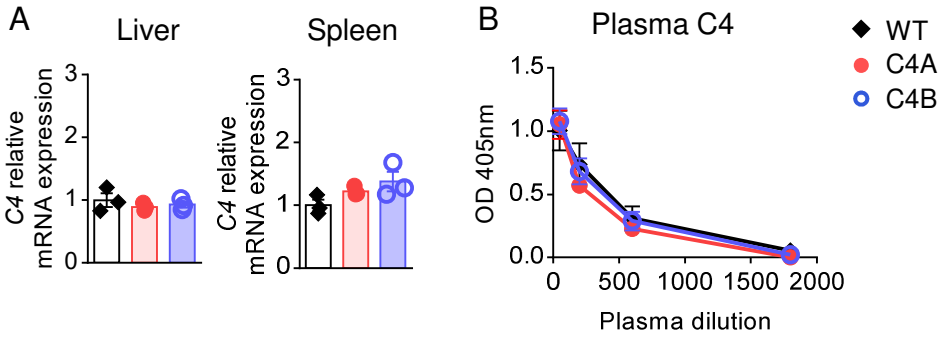

Figure S1*:* C4A and C4B strains present physiological levels of C4, Related to Figure 1.

(A) *C4* mRNA expression quantification by ddPCR. Each sample was normalized to the endogenous control *eif4h* and relative to *C4* expression in WT. Mean ± SEM, one dot represents one animal. n=3 for each strain.
(B) C4 protein level measured in plasma from WT, C4A and C4B mice by ELISA. One dot represents the average value ± SEM of n=4 for each strain.

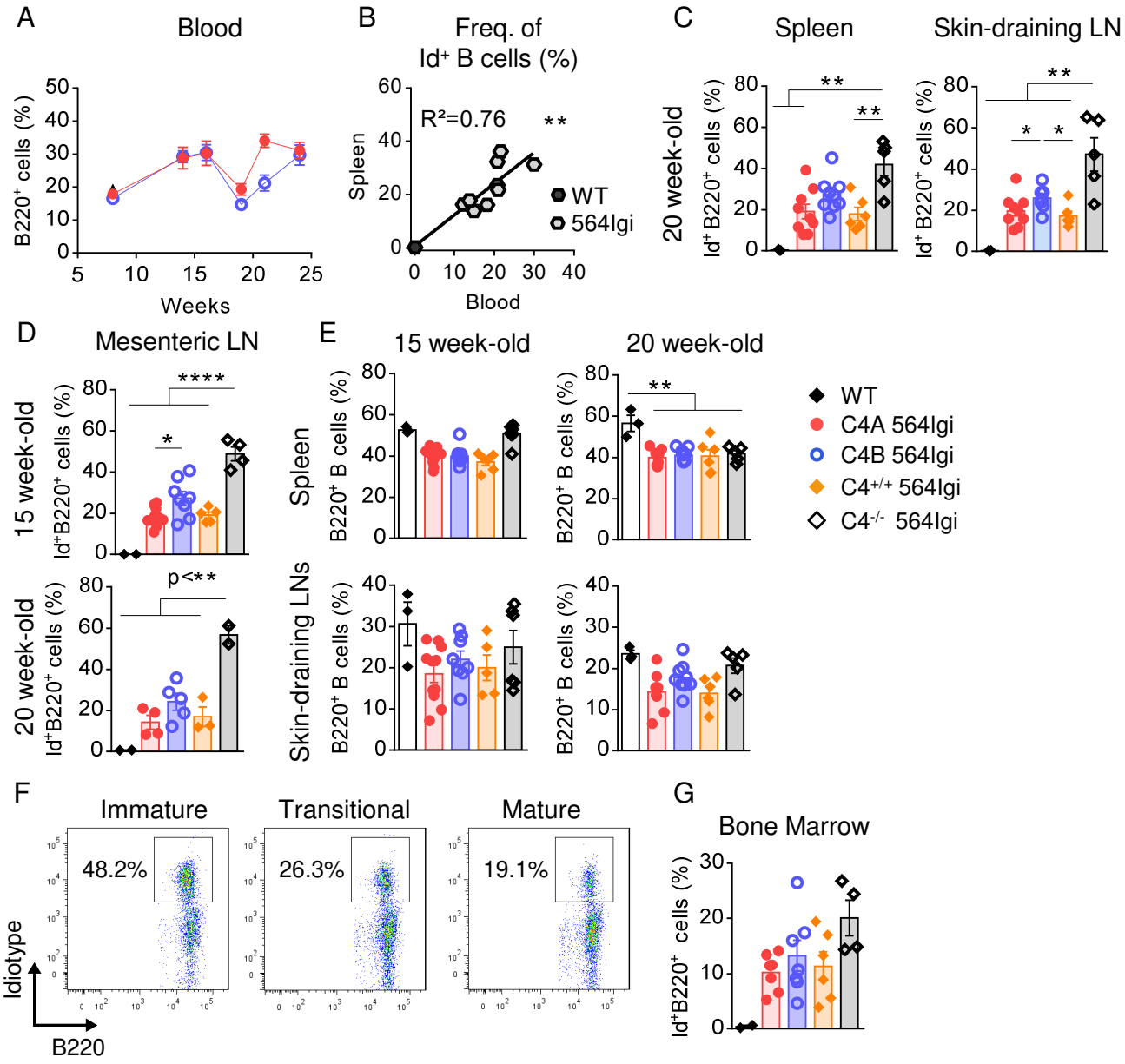

Figure S2: Analysis of B220⁺ B cells and Id⁺ B cells at 8, 15 and 20 wo in periphery and in BM, correlation of Id⁺ B cells in blood vs spleen, Related to Figure 2.

(A) Analysis of B220⁺ cells percentages in blood circulation by flow cytometry. Mean ± SEM, one dot represents the average percentage of n=5 mice for each strain.
(B) Correlation of Id⁺ B cells in spleen vs blood by flow cytometry in 564Igi animals. One dot represents one animal, Spearman test, n=9.
(C) Frequency of Id⁺ B cell proportions in spleen and skin-draining LN at 20 wo determined by flow cytometry. Mean ± SEM, one dot represents one animal, n>5. One-way ANOVA with Tukey's test.
(D) Same as (C) in mesenteric LN at 15 wo and 20 wo, n>4.
(E) Same as (A) in spleen and skin-draining LN at 15 wo and 20 wo, n>5. One-way ANOVA with Tukey's test.
(F) Representative dot plots of Id⁺ B220⁺ cell population (gated on B220⁺ cells) within immature (B220⁺ AA4.1⁺), transitional (B220⁺ AA4.1^{int}) and mature (B220⁺ AA4.1⁻) subpopulations of 15 wo 564Igi animals.
(G) Same as (C) in bone marrow at 15 wo, n>4.
(*<0.05, **<0.01, ***< 0.001, ****<0.0001)

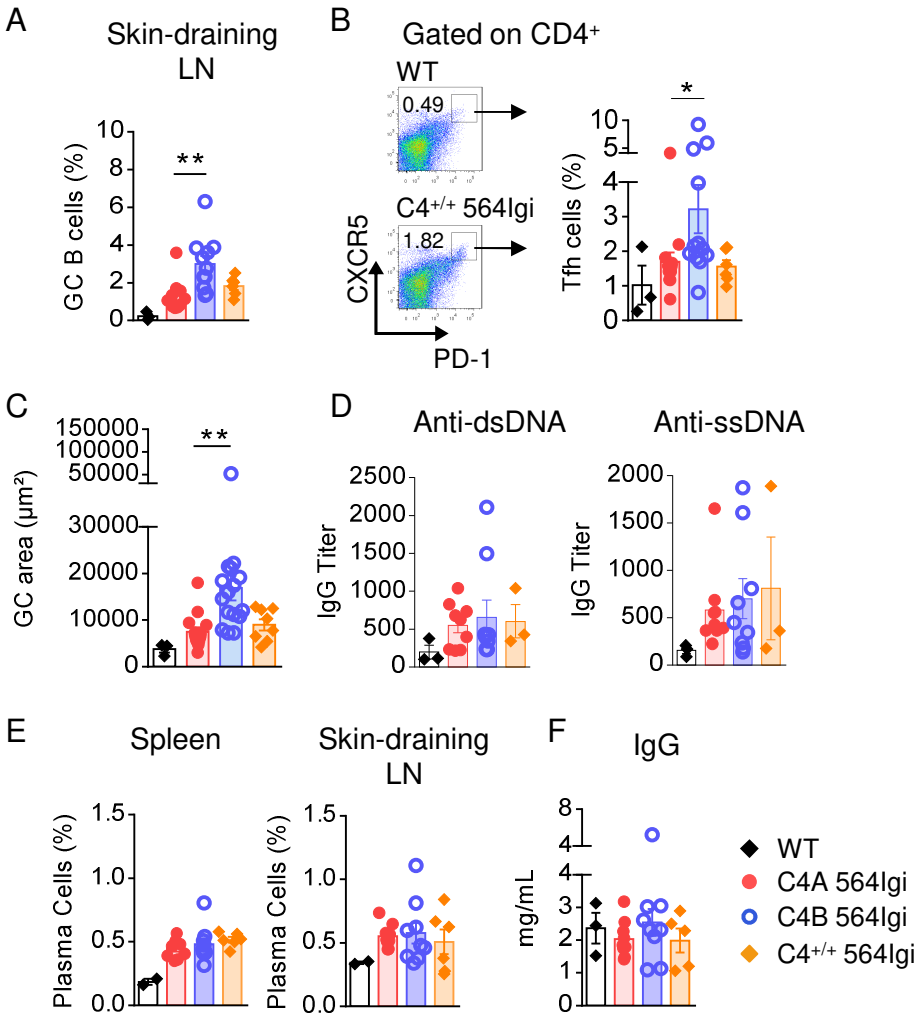

Figure S3: Analysis of GC and PC, measurement of anti-DNA autoantibody titers and IgG levels, Related to Figure 3.
(A) GC B cell population (B220^{+} CD38$^{dim/-}$ GL7^{high}) analyzed by flow cytometry in skin-draining LN from 15 wo C4A, C4B and C4$^{+/+}$564Igi. Mean ± SEM, one dot represents one mouse, n>6. One-way ANOVA with Tukey's test.
(B) Splenic Tfh cells (CD4^{+} PD-1^{high} CXCR5^{+}) from 15 wo C4A, C4B and C4$^{+/+}$ 564Igi mice, analyzed by flow cytometry. Mean ± SEM, one dot represents one mouse, n>6. One-way ANOVA with Tukey's test.
(C) Splenic GL7^{+} area quantification. Spleens were stained for marginal zone macrophages: CD169, B cell zone: B220, T cell zone: CD3 and GC: GL7. Individual GL7^{+} areas were measured using ImageJ software. Mean ± SEM, one dot represents the average value obtained for one mouse, n>8. One-way ANOVA with Tukey's test.
(D) Serum anti-dsDNA and -ssDNA IgG titers measured by ELISA. Mean ± SEM, one dot represents one mouse, n>3.
(E) Plasma cells (B220^{-} CD138^{+}) from 15 wo C4A, C4B and C4$^{+/+}$564Igi mice (spleen (left) and skin-draining LNs (right)) analyzed by flow cytometry. Mean ± SEM, one dot represents one mouse, n>6 .
(F) Serum IgG levels measured by ELISA. Mean ± SEM, one dot represents one mouse, n>5.

(*<0.05, **<0.01)

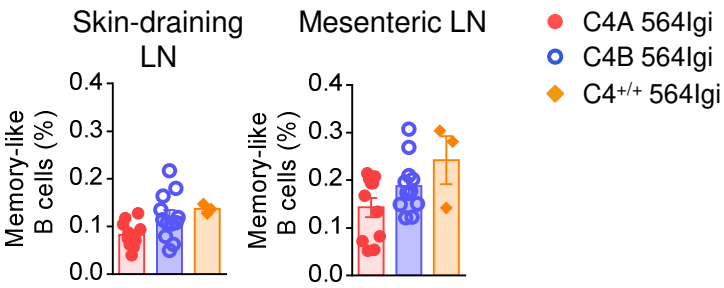

Figure S4: Analysis of autoreactive memory-like B cells, Related to Figure 4.
Analysis of autoreactive memory-like B cell population ($B220^+$ YFP^+ $CD138^-$ $GL7^-$) at 15 wo by flow cytometry. Mean ± SEM, one dot represents one mouse, n>3.

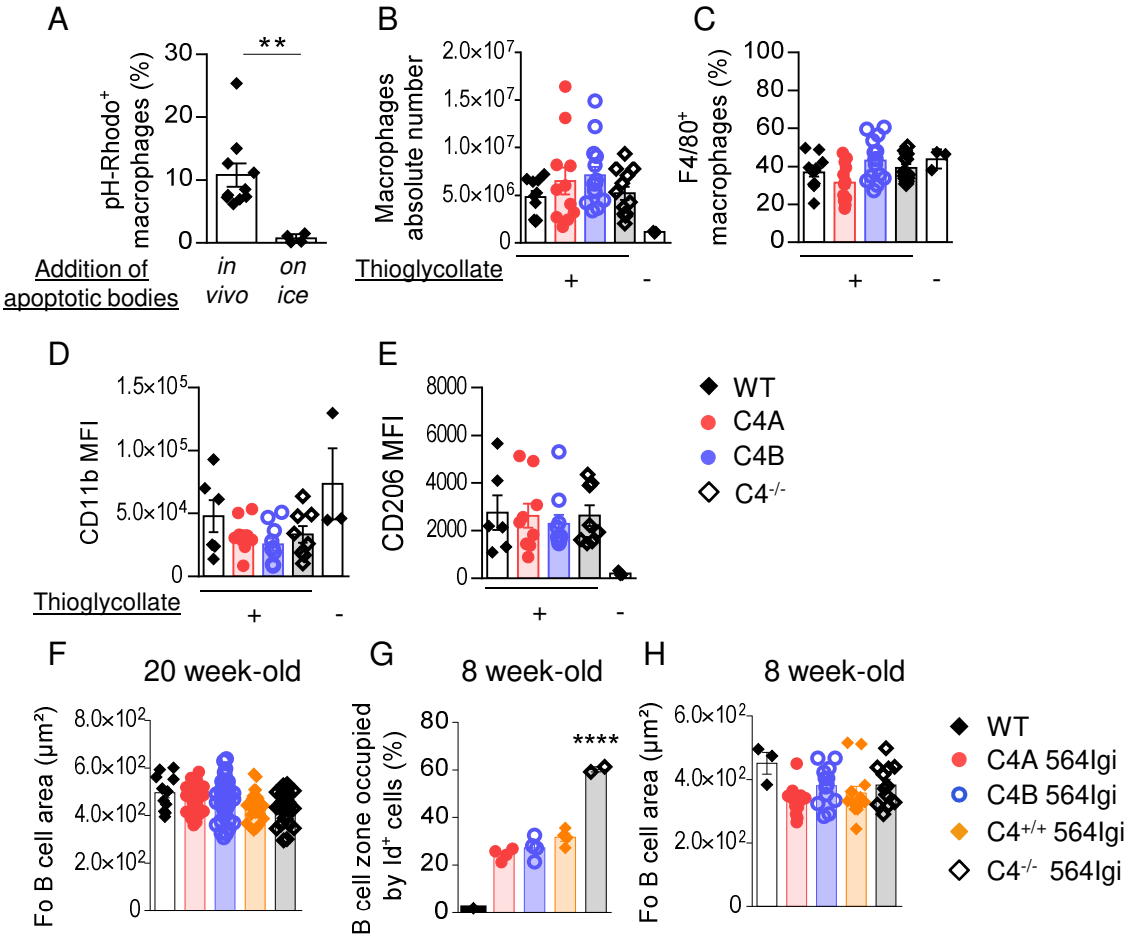

Figure S5: Elicited peritoneal macrophages phenotype and follicular exclusion of Id⁺ B cells analysis, Related to Figure 5.

(A) Assessment of phagocytosis activity on ice. Macrophages were incubated on ice for 3 hours (time needed to perform the experiment) with apoptotic cells and then analyzed for apoptotic cell uptake. Mean ± SEM, one dot represents one mouse, n>4. Mann and Whitney test, **< 0.005.

(B-E) Characterization of elicited macrophages. The absolute numbers of F4/80⁺ peritoneal macrophages (B), their frequency (C), and the expression of CD11b (D) and CD206 (E) at the surface were analyzed by flow cytometry. Mean ± SEM, one dot represents one mouse, n>10.

(F) Follicular B cell zone area analyzed per field of view of splenic sections from 20 wo mice. Mean ± SEM, each dot represents one field of view, n>31 per strain.

(G-H) Analysis of Id⁺ B cell follicular exclusion at 8 wo using cell profiler software. (G) Quantification of follicular exclusion expressed as % of follicular B cell zone occupied by Id⁺ B cells. Mean ± SEM, each dot represents one mouse, n=4 for all groups except n=2 for C4$^{-/-}$ 564Igi. One-way ANOVA with Tukey's test, ****< 0.001; (G) Follicular B cell zone area analyzed per field of view of splenic sections from 8 wo mice. Mean ± SEM, each dot represents one field of view, n>12.

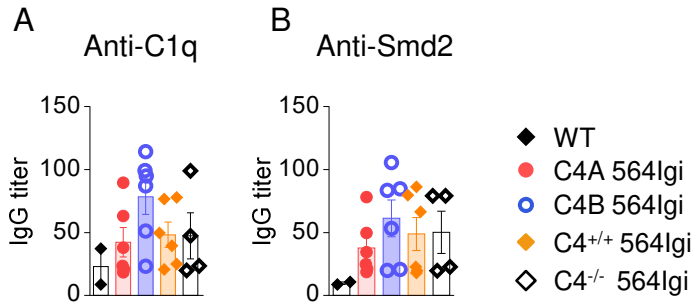

Figure S6: Measurement of anti-C1q and anti-Smd2 IgG titers, Related to Figure 6.
(A-B) Dosage of anti-C1q and anti-Smd2 IgG titers in serum of 15-20 wo $C4^{-/-}$, C4A, C4B and $C4^{+/+}$ 564Igi by ELISA. Mean ± SEM, one dot represents one mouse, n>4. One-way ANOVA with Tukey's test.

Table S1. Cas9 DNA templates used to generate C4A and C4B gene-edited strains, Related to STAR Methods.

Cas9 DNA template	
C4A	ccaggaacaggtgggcaactccccggagaagctgcaggagacggctagctggctgctggcccagcagctgggtgatggctcctttcaggatccctgtccagtgttagacaggagcatgc aggtgtgaacacgctggaggcacgtgtaggatgtcagcaggaagacgggctcctcacaactctggcctgacagcctctctt
C4B	gagaagctgcaggagacggctagctggctgctggcccagcagctgggtgatggctcctttcaggacctctctccggtgatacataggagcatgcaggtgtgaacacgctggaggcacgt gtaggatgtcagcaggaagacgg gctcctca

Table S2. Primer sequences and reaction conditions used for PCR genotyping, Related to STAR Methods.

Trans-genes	Primers (IDT)	Sequences (5'-3')	Amplification program					Fragment size (bp)
			I. 94°C	II. Cycles		III. 72°C	IV. 8°C	
				Description	Number			
564 Light Chain	564k	CCAGTGCAGATTTTCAGCTTC	4 min	94°C 1min 55°C 1min 72°C 1min	40 cycles	5 min	∞	724 bp
	Jk5	CAGCTTGGTCCCAGCACCGAA						
564 Heavy Chain	VH4emu	CACAGATTCTTAGTTTTTCAA	4 min	94°C 1min 50°C 1min 72°C 1min	40 cycles	5 min		542 bp
	VH564	TGGAGCTATATCATCCTCTTT						
C4 KO	C4 23F	ATAACTGGGTCGGACTTTGG	4 min	94°C 1min 56°C 1min 72°C 1min	30 cycles	10 min		Mutant: 800 bp WT: 600 bp
	C4 25R	TCTTCCGAAACTGCTGGATCC						
	C4 29R	TACCTGGGTACTGCGGAATGC						
	Neo4483	AAGCCGGTCTTGTCGATCAG						
C4Crispr_PCR1	3' mC4 ex22-28	GTCGACCAAGTCCCAGGTGCTCTGCC	2 min	95°C 30sec 65°C 30sec 72°C 1min30	35 cycles	5 min		N/A
	5'mC4 ex22-28	GTCGACCTGGCCTTGAACTTCAGTAATC						
C4Crispr_PCR2	C4BF1	TCACCTGAAGTCCATGGACCCTG	2 min	95°C 30sec 65°C 30sec 72°C 2min	35 cycles	5 min		978 bp
	C4BR1	CATCGTATCCTGGAAGACGTCCAA						

Table S3. Post-PCR digestion conditions to genotype C4A and C4B mouse strains, Related to STAR Methods.

Transgenes	Fragment size before digestion	Restriction enzyme	Digestion conditions	Fragment size after digestion
C4A	978 bp	BamHI	2 hours – 37°C	WT: 515 + 463 bp Mut: 293 + 222 + 463 bp
C4B	978 bp	BsAWI	2 hours – 60°C	WT: 978bp Mut: 765 + 213 bp

Table S4. Primer sequences and reaction conditions used to measure *C4* mRNA expression by ddPCR, Related to STAR Methods.

Transgenes	Primers	Sequences (5'-3')	Amplification program				
			I. 94°C	II. Cycles		III. 72°C	IV. 8°C
				Description	Number		
C4	Forward	AGCGTGTTTCCAGCTCAAAG	4 min	94°C 1min 55°C 1min 72°C 1min	40 cycles	5 min	∞
	Reverse	GTCCTAAGGCCTCACACCTG					
eif4h	Forward	TGCAGCTTGCTTGGTAGC		94°C 1min 50°C 1min 72°C 1min			
	Reverse	GTAAATTGCCGAGACCTTGC					

Table S5. ELISA conditions, Related to STAR Methods**.**

Measurement	Coating conditions	Sample dilutions in PBS-T 0.1%	Standard	Detection
C4 level	1.5µg/mL rat anti-mouse C4 (gift of E. Kremmer, Munich, Germany)	1/50 to 1/5000	N/A	4µg/mL of rabbit anti-human C4c (Dako) + goat anti-Rabbit-AP (Southern biotech, 1/1000)
IgG level	1.5µg/mL goat anti-mouse IgG (southern Biotech)	1/500 to 1/250 000	Mouse IgG (sigma) from 1000ng/mL to 1.9ng/mL	Goat anti-mouse IgG-AP (Southern biotech, 1/1000)
IgG2a level	1.5µg/mL goat anti-mouse IgG 2a (southern Biotech)	1/500 to 1/250 000	Purified mouse IgG2a (southern biotech) from 1000ng/mL to 1.9ng/mL	Goat anti-mouse IgG2a-AP (Southern biotech, 1/1000)
Anti-nucleoli IgG titers	100µL of purified nucleoli per 96-well plate, spun 10min at 1300g	1/25 to 1/200	N/A	Goat anti-mouse IgG-AP (Southern biotech, 1/10000)
Anti-Ro60 IgG titers	1.5µg/mL Ro60 protein (Diarect)	1/20 to 1/1280	N/A	Goat anti-mouse IgG-HRP (Southern biotech, 1/10000)
Anti-BPI IgG titers	1.5µg/mL BPI protein (Diarect)			
Anti-CTGF IgG titers	1.5µg/mL CTGF protein (R&D system)			
Anti-Smd2 IgG titers	1µg/mL Smd2 protein (Diarect)			
Anti-C1q IgG titers	1µg/mL C1q protein (Meridian Life Science)			
Anti-APRIL IgG titers	1.5µg/mL APRIL protein (PEPROTECH)			

Table S6. List of self-antigens and controls tested to measure sera autoreactivity, Related to Figure 6 and STAR Methods.

Antigens
Human IgG from serum
Anti-Human IgG Fc fragment Specific
Anti-Human IgG (H+L)
Anti-Human IgG F(ab') fragment specific
La/SSB
Ro 52/SSA
CENP B
EBV EBNA-1
EGFR
FBL (Fibrillarin/U3RNP)
Hepatitis B Surface Antigen HBSAg
U11/U12
Ku, p70/p80
Nucleolin
PDC-E2
PM/Scl 100
POLR3H
Pyruvate dehydrogenase (PDH)
Ro 60/SSA (bovine)
Ro 60/SSA (recombinant)
RPP14 (Th/To)
Scl-70, full-length
Scl-70, truncated
Sm/RNP (SRC-3000)
Smith (SMA-3000)
ssDNA
TG
TPO (Thyroperoxidase)
U1-snRNP 68
U1-snRNP A
U1-snRNP C
RPP25 (Th/To)
MDC
TNF-B
GBM
APRIL
CTGF
IFN-g
IL17-A
Catalase
SSA/Ro60
BPI
Smd2
TPO
OVA
C1q