Supporting Information

Kishi et al. 10.1073/pnas.1204509109

SI Materials and Methods

Mice. C57BL/6, BALB/c, and MRL/lpr mice were purchased from Sankyo Labo Service. The conventional Tg mice expressing the H chain of anti-DNA antibody 56R or 3H9 on the BALB/c background (1, 2) and CD40LTg mice on the C57BL/6 background (3) were described previously. 56R Tg and CD40L/56R double Tg mice on the (BALB/c × C57BL/6) F1 background were used through out this study except for the experiment shown in Fig. 4, where 56R Tg mice on the BALB/c background were used. Mice were maintained under specific pathogen-free (SPF) conditions in our animal facility. All procedures followed the guidelines of the Tokyo Medical and Dental University for animal research and were approved by the animal ethics committee.

Flow Cytometry. Cells were stained with the following antibodies and reagents: FITC-conjugated goat anti-mouse IgM (Southern Biotechnology), Alexa Fluor 647-conjugated anti-mouse B220, APC-conjugated rat anti-mouse CD21 (BD Pharmingen, BD Biosciences), PE-conjugated rat anti-mouse CD23 (eBioscience), PerCP-conjugated streptavidin (BD Pharmingen), Alexa Fluor 488, or Pacific Blue-conjugated anti-56R/Vκ21D (IgG1), Alexa Fluor 488 or Pacific Blue-conjugated anti-56R/Vκ38C (IgG2b), and FITC-conjugated rat anti-mouse CD5 (eBioscience). Cells were analyzed on a CyAn ADP flow cytometer (Beckman Coulter).

ELISA. Total serum IgM was measured by standard sandwich ELISA analysis using goat anti-mouse IgM (Southern Biotechnology). dsDNA was generated by treating bovine thymus DNA (Nacalai Tesque) with S1 nuclease (Takara) (4), followed by dialysis with PBS. ssDNA was prepared by boiling PBS containing dsDNA for 5 min, followed by ice-bath immersion. ELISA plates were coated with 10 μg/mL ssDNA or 10 μg/mL histone type II-S from calf thymus (Sigma) at 4 °C. Alternatively, ELISA plates were coated with 10 μg/mL dsDNA in the presence of poly L-lysine (Sigma). ELISA plates coated with Sm/RNP were prepared by incubating the plates with 10 U/mL Sm/RNP (ImmunoVision) or purchased from Orgentec. ELISA plates coated with the components of Sm/RNP were prepared by incubating the plates with 10 μg/mL recombinant U1-RNP-70kD, U1-RNP-A, U1-RNP-C or SmB/B′ proteins (gifts from T. Hachiya, MBL, Ina, Nagano, Japan), or the SmD1(83-119) peptide synthesized as free peptides according to the protocol described previously (5) and purified by reverse-phase chromatography. Plates were blocked with PBS containing 0.5% FCS and 0.05% NaN_3 at room temperature for 2 h, and sequentially diluted sera or culture supernatants were incubated in the plates at room temperature for 30 min. After further incubation with alkaline phosphatase-conjugated goat anti-mouse IgM (Southern Biotechnology) at room temperature for 1 h, plates were developed by phosphatase substrate (Sigma). Concentrations of anti-ssDNA antibodies and anti-Sm/RNP antibodies were determined relative to standard curves of anti-DNA monoclonal antibody BW28-20 (IgM; a gift from S. Hirose, Juntendo University, Tokyo, Japan) and pooled MRL/lpr sera, respectively. The absorbance was measured by an ELISA reader V_{max} (Molecular Devices) at 405 nm and analyzed with SoftMax Pro-4.8 (Molecular Devices).

ANA Test. Cos-7 cells were placed on slide glass and fixed by immersion in 85% (vol/vol) ethanol/15% (vol/vol) acetate at room temperature for 30 min. After blocking with blocking buffer (PBS containing 0.5% FCS, 0.05% NaN_3), hybridoma culture supernatants or mouse sera were applied neat and incubated at room temperature for 30 min. Slides were then washed twice in PBS at room temperature and incubated with 20 μg/mL FITC-conjugated goat anti-mouse IgM (Southern Biotechnology) together with 4′,6-diamino-2-phenylindole (DAPI) (Sigma) for staining nuclei at room temperature for 20 min and observed under a fluorescence microscope (Leica) or a confocal laser-scanning microscope LSM510 (Carl Zeiss).

Immunohistochemistry. Tissues were embedded in Tissue-Tek O.C.T. compound (Sakura), snap-frozen in liquid nitrogen, and stored at −80 °C. Cryostat sections of 5 μm in thickness were mounted onto slide glass, air dried, and fixed in acetone at room temperature for 20 min. The sections were incubated with blocking buffer (PBS containing 2.0% (wt/vol) BSA, 0.05% NaN_3) for 30 min and were stained at room temperature for 60 min with Alexa Fluor 647-conjugated rat anti-mouse IgD (BioLegend), biotin-conjugated rat anti-mouse MOMA-1 (BMA Biomedicals), Alexa Fluor 594-conjugated streptavidin (Invitrogen), Alexa Fluor 488-conjugated anti-56R/Vκ21D or anti-56R/Vκ38C, Pacific Blue-conjugated anti-56R/Vκ21D or anti-56R/Vκ38C, and Alexa Fluor 647-conjugated rat anti-mouse B220. For detection of apoptosis, the sections were fixed in acetone at room temperature for 5 min, incubated with blocking buffer (PBS containing 2.0% (wt/vol) BSA, 0.05% NaN_3) for 30 min, and then stained for 60 min with Alexa Fluor 488 or Pacific Blue-conjugated anti-56R/Vκ38C and biotin-conjugated rat anti-human IgG1 (BD Pharmingen) or biotin-conjugated rat anti-mouse MOMA-1. After washing with PBS, the sections were treated with 4% (wt/vol) paraformaldehyde at room temperature for 5 min, and were permeabilized in 0.1% Triton X-100 /0.1% sodium citrate for 2 min on ice. The sections were then stained at room temperature for 60 min with rabbit anti-human/mouse active caspase 3 (R&D Systems), Alexa Fluor 647-conjugated goat anti-rabbit IgG (Invitrogen), Alexa Fluor 594-conjugated streptavidin, FITC-conjugated goat anti-mouse lambda (Southern Biotechnology), and Pacific Blue-conjugated rat anti-mouse B220. The sections were analyzed with a confocal laser-scanning microscope LSM510 (Carl Zeiss).

Generation of Anti-Idiotype Antibody to 56R/Vκ38C and 56R/Vκ21D. BALB/c mice were immunized with keyhole limpet hemocyanin (KLH) (Sigma) conjugated with the purified antibodies from the hybridoma D568 expressing the 56R H chain and Vκ38C and the hybridoma 1-1-7 expressing the 56R H chain and Vκ21D generated in this study from 56R and CD40L/56R mice, respectively. Spleen cells were fused with the PAI myeloma cell line (6), and hybridomas secreting anti-idiotype antibodies were screened by solid-phase ELISA. ELISA plates were coated with 10 μg/mL 1-1-7 and D568 antibodies at room temperature for 1 h, and hybridoma supernatants were added. Binding was detected using alkaline phosphatase-conjugated goat anti-mouse IgG antibody (Southern Biotechnology). For determining the specificity of anti-idiotype antibody, ELISA plates were coated with 10 μg/mL purified antibodies from positive hybrids and then incubated with supernatants from hybridomas expressing various Vκ genes generated in this study (Table S1). Binding was detected using alkaline phosphatase-conjugated goat anti-mouse IgM antibody (Southern Biotechnology). Anti-idiotype antibodies were conjugated with Alexa Fluor 488 or Pacific Blue using labeling kits (Invitrogen).

Preparation of Clodronate Liposomes. Clodronate liposomes were prepared as described previously (7). In brief, phosphatidylcholine from soy (SPC; Avanti Polar Lipids) and cholesterol (Sigma) were dissolved in chloroform in a glass tube, followed by evaporation of the chloroform using argon gas, resulting in a thin layer film. The tube containing the film was dried in a desiccator overnight. Clodronate disodium (LKT Laboratories) dissolved in PBS was added to the tube containing the film, and then liposomes were generated by vortexing. The liposome-containing solution was frozen and thawed three times, and subsequently passed through an extruder (Avanti) with a 400-nm membrane. Clodronate liposomes were washed three times by suspending in sterilized PBS followed by centrifugation at 100,000 × *g* for 30 min.

1. Chen C, et al. (1994) Deletion and editing of B cells that express antibodies to DNA. *J Immunol* 152:1970–1982.
2. Erikson J, et al. (1991) Expression of anti-DNA immunoglobulin transgenes in non-autoimmune mice. *Nature* 349:331–334.
3. Higuchi T, et al. (2002) Cutting Edge: Ectopic expression of CD40 ligand on B cells induces lupus-like autoimmune disease. *J Immunol* 168:9–12.
4. Shishido K, Ando T (1972) Estimation of the double-helical content in various single-stranded nucleic acids by treatment with a single strand-specific nuclease. *Biochim Biophys Acta* 287:477–484.
5. E. Atherton R C S. (1989) *Solid phase peptide synthesis: A practical approach* (IRL Press, Oxford).
6. Stocker JW, et al. (1982) Generation of two new mouse myeloma cell lines 'PAI' and 'PAI-O' for hybridoma production. *Hoffmann-LaRoche Research Disclosure* 21713: 155–157.
7. Van Rooijen N, Sanders A (1994) Liposome mediated depletion of macrophages: Mechanism of action, preparation of liposomes and applications. *J Immunol Methods* 174:83–93.

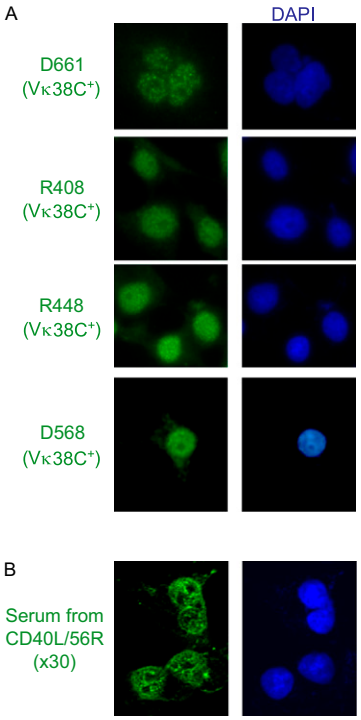

Fig. S1. ANA staining pattern of supernatants from Vκ38C+ hybridomas and sera from CD40L/56R mice. Supernatants from the Vκ38C+ hybridomas R408 and R448 from 56R mice and D568 and D661 from CD40L/56R mice (*A*) and sera from 15 wk-old CD40L/56R mice diluted for 30 times (*B*) were examined by ANA test. Staining pattern was observed under a fluorescent (*A*) or a confocal microscope (*B*).

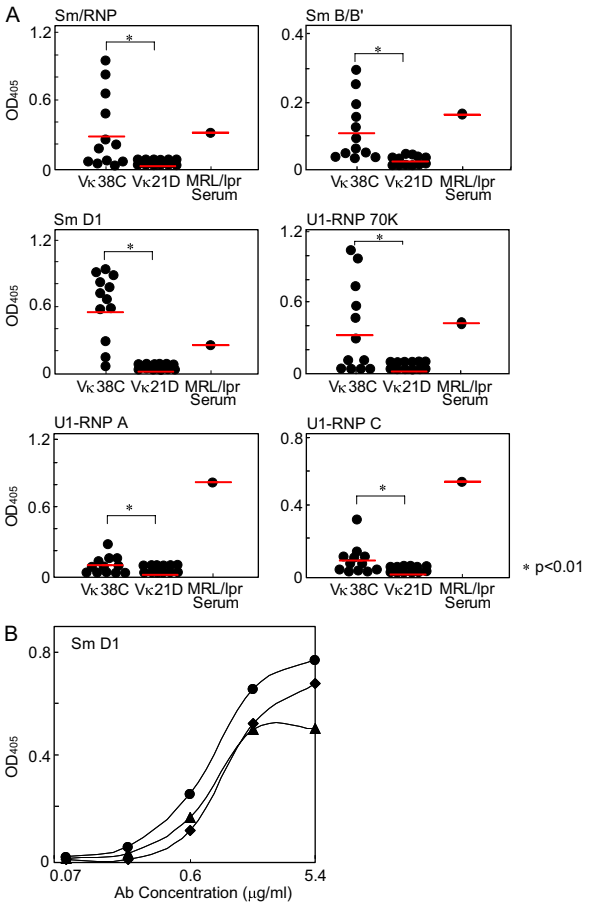

Fig. S2. Binding specificities of Vκ38C$^+$ antibodies to the components of Sm/RNP antibodies. Twelve Vκ38C$^+$ and 12 Vκ21D$^+$ hybridomas were randomly selected from hybridomas obtained from 56R and CD40L/56R. Antibodies from these hybridomas were diluted to 2.3 μg/mL and analyzed by ELISA using Sm/RNP, U1-RNP-70kD, U1-RNP-A, U1-RNP-C, and SmB/B′ proteins and SmD1 peptide-containing residues 89–113 as antigens (*A*). Sera from MRL mice diluted for 100 times was used as a control. Horizontal red lines represent mean values. Dose–response curves of ELISA for binding to SmD1 peptide are shown for three Vk38C$^+$ antibodies that most strongly bind to the SmD1 peptide (*B*).

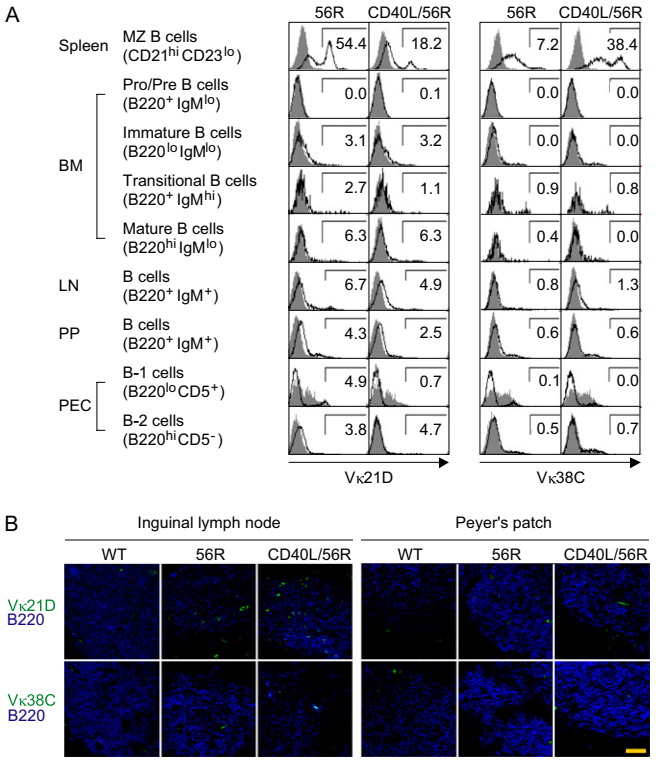

Fig. S3. Analysis of various lymphoid organs from 56R and CD40L/56R mice. (*A*) Flow cytometric analysis. Cells from bone marrow (BM), inguinal lymph node (LN), Peyer patch (PP), and peritoneal exudate (PEC) obtained from 20- to 24-wk-old mice were stained with Alexa Fluor 488 (LN, PP, and PEC) or Pacific Blue (BM)-conjugated anti-56R/Vκ21D or anti-56R/Vκ38C antibody (open histograms) together with Alexa Fluor 647-conjugated rat anti-mouse B220 antibody and either FITC-conjugated rat anti-mouse CD5 antibody (PEC), the combination of biotin-conjugated goat anti-mouse IgM antibody and PerCP-conjugated streptavidin (LN and PP), or FITC-conjugated goat anti-mouse IgM antibody (BM). Histograms of cells from each organ of WT (C57BL/6 × BALB/c) F1 mice are shown as negative controls (shaded histograms), and those of spleen MZ B cells from 56R and CD40L/56R mice stained as in Fig. 3*B* are shown as positive controls. Note that anti-idiotype antibodies show weak reactivity to non-Vκ21D non-Vκ38C B cells in 56R mice (see Fig. 3*B*). Percentages of Vκ21D$^+$ and Vκ38C$^+$ cells in corresponding B-cell populations are indicated. Representative data of three experiments are shown. (*B*) Immunohistological analysis. Sections of inguinal lymph nodes and intestine from 16- to 24-wk-old mice were stained for 56R/Vκ21D or 56R/Vκ38C idiotypes together with B220. [Scale bar (yellow line): 50 μm.] Representative data of three experiments are shown.

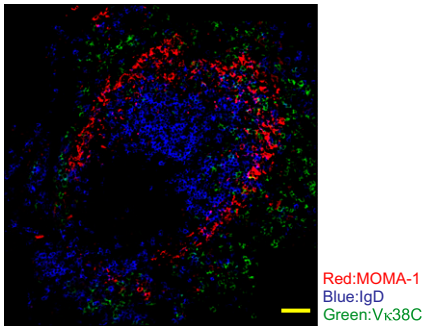

Fig. S4. Vκ38C$^+$ B cells are not located in T/B boundary in spleen. Spleen sections from 16- to 20-wk-old 56R mice were stained for MOMA-1 (red), IgD (blue) and Vκ38C (green), and analyzed by confocal microscopy. Note that the image is a composite figure. [Scale bar (yellow line): 50 μm.]

Table S1. Use of Ig L chain and Vκ in hybridomas from 56R and CD40L/56R mice

Mice	κ Chain					λ Chain	Total
	Vκ21D	Vκ38C	Vκ20	Vκ12/13	Others		
56R (age)							
8 wk	27 (0)	2 (2)	3 (0)	1 (0)	4 (1)	4 (0)	41 (3)
33 wk	15 (0)	1 (1)	0 (0)	1 (0)	2 (0)	0 (0)	19 (1)
CD40L/56R (age)							
8 wk	29 (0)	7 (7)	6 (1)	0 (0)	6 (4)	4 (1)	52 (13)
33 wk	13 (0)	10 (10)	2 (0)	0 (0)	3 (3)	2 (2)	30 (15)

Data represent number of hybridomas. Numbers in parenthesis indicate the numbers of hybridomas secreting anti-dsDNA antibody. Others, other Vκ.

Table S2. Specificity of anti-idiotype antibodies that recognizes Vκ21D or Vκ38C together with 56R H chain

L chain	No. of hybridomas	Reactivity	
		Anti-56R/Vκ21D	Anti-56R/Vκ38C
Vκ2	1	0 (0)	0 (0)
Vκ12/13	1	0 (0)	0 (0)
Vκ19	2	1 (50)	0 (0)
Vκ20	6	0 (0)	0 (0)
Vκ21D	47	47 (100)	0 (0)
Vκ38C	14	0 (0)	14 (100)
λ	6	0 (0)	0 (0)
Others	9	2 (22)	0 (0)

Data represent number of hybridomas. Numbers in parenthesis indicate percentages. Others, all other light chains.

Table S3. Primer list

Primer	Sequence	Reference
VH		
Sense		
VH3H9/56R-F	CTG TCA GGA ACT GCA GGT AAG G	1
Antisense		
VH3H9/56R-R	CAT AAC ATA GGA ATA TTT ACT CCT CGC	1
Vκ		
Sense		
Vκ12/13	CGA GCA AGT GAG AAT ATT TAC AGT AAT TTA GC	2
Vκ20	ACC AGC ACT GAT ATT GAT GAT	3
Vκ21D	GCC AGC CAA AGT GTT GAT TAT G	3
Vκ38C	AAG GCA AGC CAA GAC ATT AAC AAG TAT ATA GCT	3
Vκs	GGC TGC AGS TTC AGT GGC AGT GGR TCW GGR AC	4
Leader 5	CCA GAT GTG AGC TCG TGA TGA CCC AGA CTC CA	5
Antisense		
Jκ2	GGT TAG ACT TAG TGA ACA AGA GTT GAG AA	6
Jκ4	TTC CAA CTT TGT CCC CGA GCC GAA CGT G	2
Jκ5	TGC CAC GTC AAC TGA TAA TGA GCC CTC TC	6
Cκ Deletion		
Sense		
Vκs	GGC TGC AGS TTC AGT GGC AGT GGR TCW GGR AC	4
Antisense		
RS101	ACA TGG AAG TTT TCC CGG GAG AAT ATG	7

1. Erikson J, et al. (1991) Expression of anti-DNA immunoglobulin transgenes in non-autoimmune mice. *Nature* 349:331–334.
2. Prak EL, Trounstine M, Huszar D, Weigert M (1994) Light chain editing in kappa-deficient animals: A potential mechanism of B cell tolerance. *J Exp Med* 180:1805–1815.
3. Li H, Jiang Y, Prak EL, Radic M, Weigert M (2001) Editors and editing of anti-DNA receptors. *Immunity* 15:947–957.
4. Schlissel MS, Baltimore D (1989) Activation of immunoglobulin kappa gene rearrangement correlates with induction of germline kappa gene transcription. *Cell* 58:1001–1007.
5. Huse WD, et al. (1989) Generation of a large combinatorial library of the immunoglobulin repertoire in phage lambda. *Science* 246:1275–1281.
6. Ramsden DA, Paige CJ, Wu GE (1994) Kappa light chain rearrangement in mouse fetal liver. *J Immunol* 153:1150–1160.
7. Retter MW, Nemazee D (1998) Receptor editing occurs frequently during normal B cell development. *J Exp Med* 188:1231–1238.