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Supplementary Materials for

Identification of IgA autoantibodies targeting mesangial cells redefines the pathogenesis of IgA nephropathy

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The PDF file includes:

Other Supplementary Material for this manuscript includes the following:

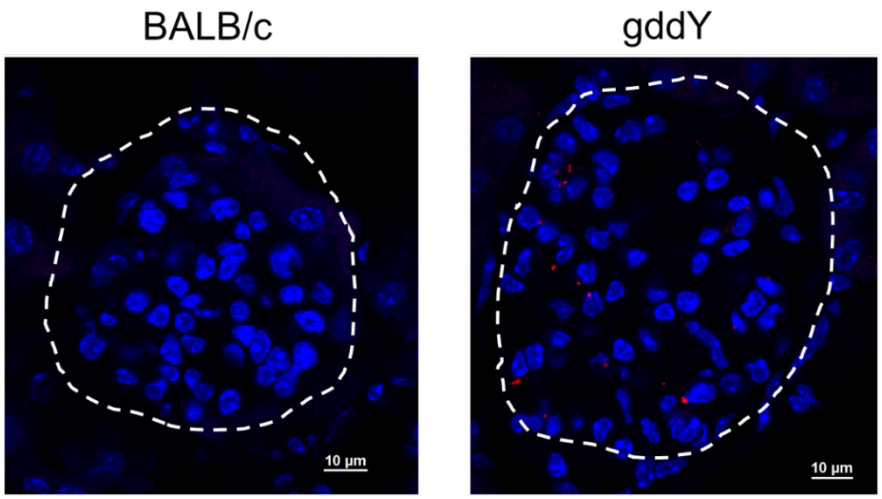

Fig. S1. Reactivity to glomeruli of purified serum IgA from gddY mice

Serum IgA was purified from 20- week-old (wo) BALB/c or gddY mice. Kidney sections from AID-knockout mice were stained with the purified IgA (10 μg/ml) from BALB/c (left) or gddY (right) mice, and secondarily with PE-anti-IgA Ab (red) with DAPI (blue). Dashed circles indicate areas of glomeruli.

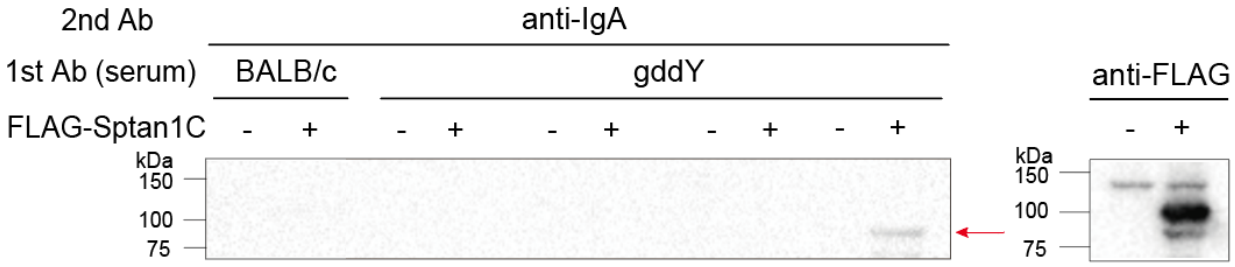

Fig. S2. Reactivity to αII-spectrin by the serum IgA of gddY mice

A representative WB of HEK293T cells transfected with a mock (-) or FLAG-tagged Sptan1C (+) vectors and probed with pooled sera from four 16-wo BALB/c mice or sera from gddY mice (n=4) followed by anti-IgA Ab (left). The red arrow indicates a band of Sptan1C, as confirmed with an anti-FLAG Ab (right). Shown is a representative of four independent experiments with different samples.

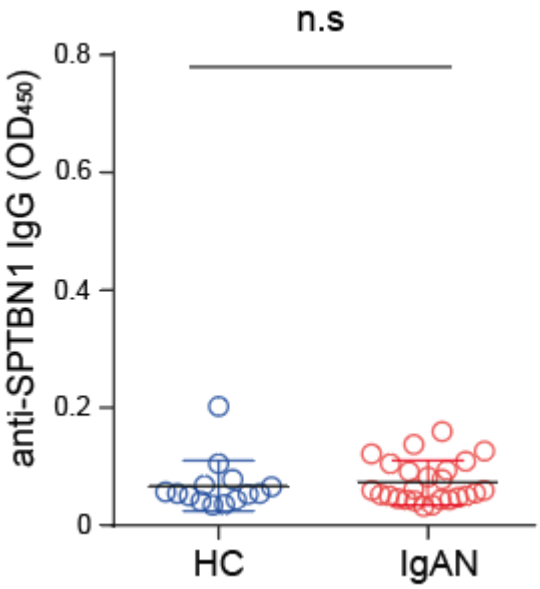

Fig. S3. Serum anti-SPTBN1 IgG levels in HC and IgAN patients

ELISA determination of anti-SPTBN1 IgG Abs in the same serum samples as in Fig. 2E were determined by ELISA. Small horizontal lines indicate the mean (black) ± s.d. (colored) of each group. n. s: not significant.

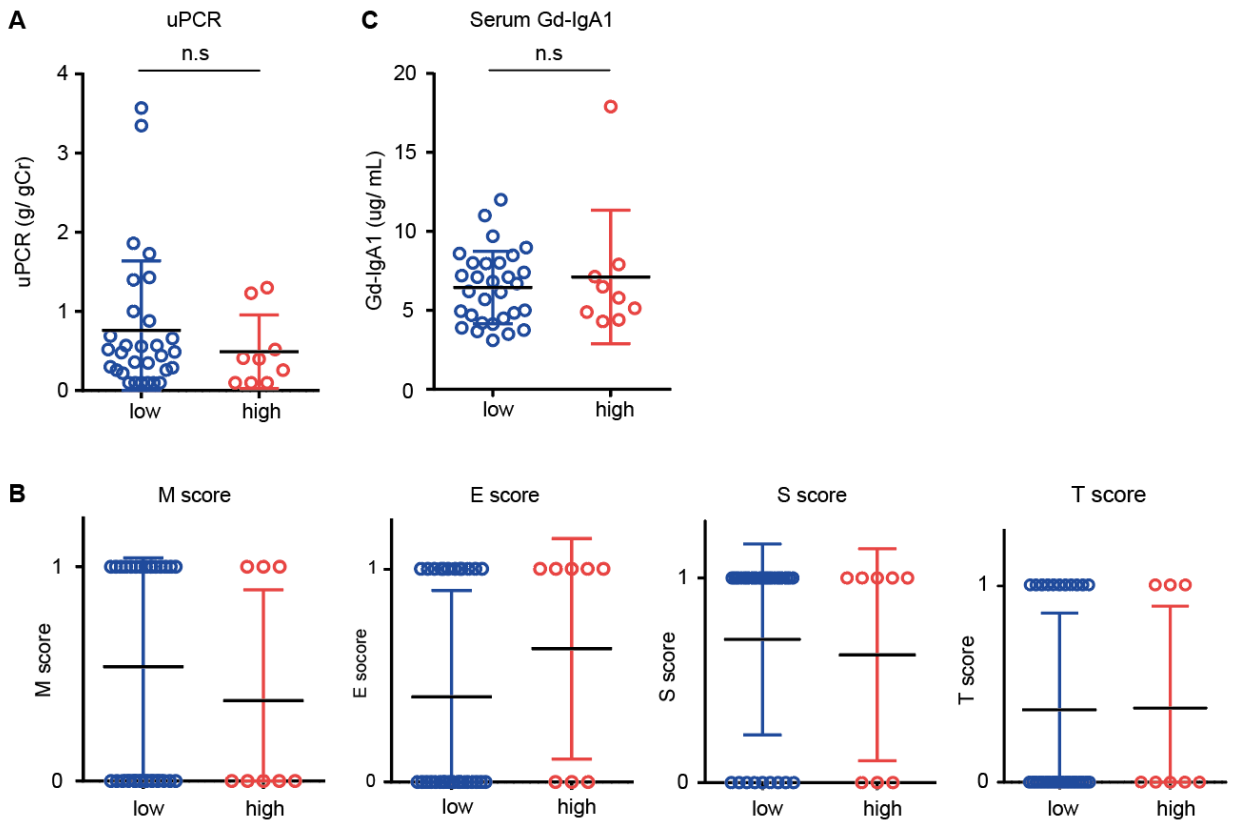

Fig. S4. Comparison of clinical and pathological parameters between anti-SPTBN1 IgA low and high groups of IgAN patients

(A to C) The levels of proteinuria (uPCR: urinary protein-to-creatinine ratio; A), MEST pathological scores (B) or serum Gd-IgA1 levels (C) were compared between the anti-SPTBN1 IgA 'low' (low) and 'high' (high) groups of IgAN patients. Small horizontal lines indicate the mean (black) ± s.d. (colored) of each group. n. s: not significant.

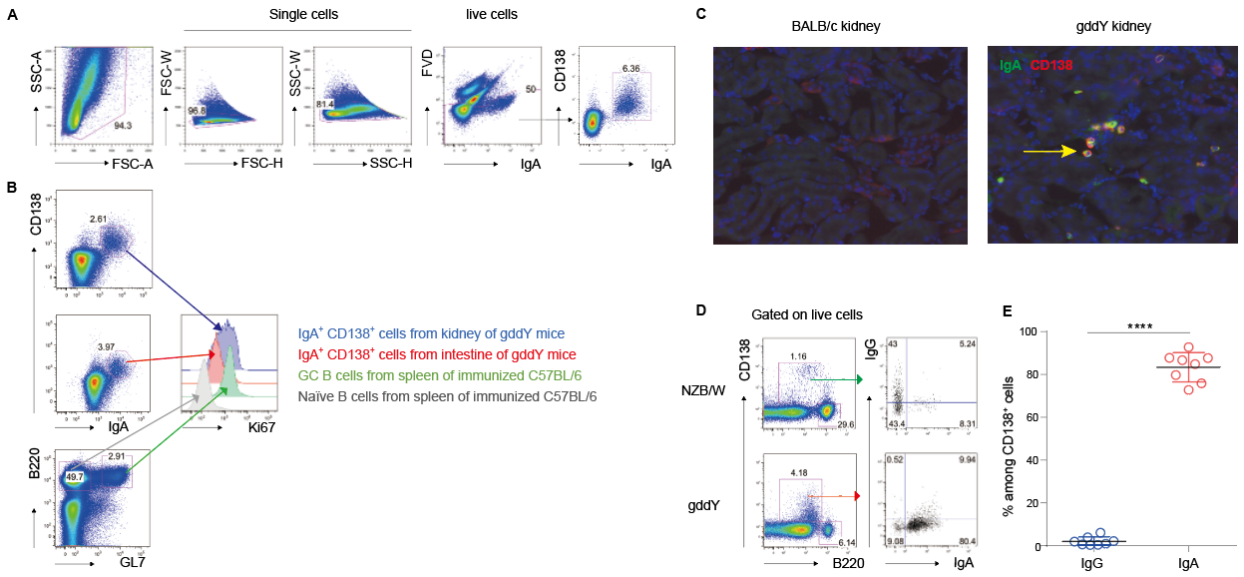

Fig. S5. IgA$^+$ plasmablast accumulation in the kidney of gddY mice

(A) Gating strategy of IgA$^+$ CD138$^+$ plasmablasts (PBs) in the kidney of gddY mice.

(B) Flow cytometric analysis of Ki67 expression in the cells in the indicated gates. Shown here are histograms of IgA$^+$ CD138$^+$ cells in the kidney (blue) or the small intestine (red) of gddY mice, or B220$^+$ GL7$^+$ GC B cells (green) and B220$^+$ GL7$^-$ naïve B cells (gray) from the spleen of C57BL/6 mice immunized with NP-CGG in alum six days previously.

(C) Immunofluorescence microscopy of kidney sections of BALB/c or gddY mice stained for IgA (green) and CD138 (red), and with DAPI (blue). The arrow indicates one of the IgA$^+$ CD138$^+$ cells.

(D) Flow cytometric analysis of leukocytes from the kidneys of NZB/W F1 (top) and gddY (bottom) mice. CD138$^+$ B220^{low} cells (left) were further analyzed for intracellular staining with anti-IgA and anti-IgG antibodies (right).

(E) The frequency of IgG$^+$ (blue) or IgA$^+$ (red) cells among the CD138$^+$ B220^{low} cells in the kidneys of gddY mice as determined in (D) (n=8). Small horizontal lines indicate the mean (black) ± s.d. (colored) of each group. ****$P < 0.0001$ (Student's *t*-test). The numbers in the panels indicate the percentage of cells within the adjacent gates among live cells (A, B and D) or within the quadrants (D).

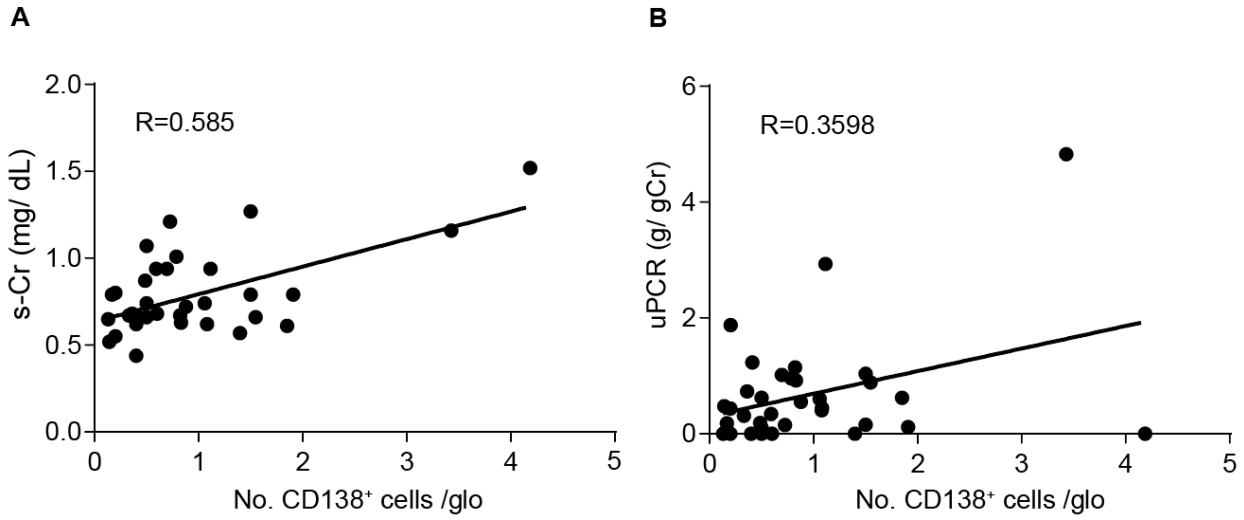

Fig. S6. Correlation between the frequency of IgA$^+$ ASCs in the kidneys and disease severity in IgAN patients

(A and B) Serum creatinine levels (s-Cr; A) and the levels of proteinuria (uPCR; B) of the patients are plotted against the average number of CD138$^+$ cells per glomeruli (CD138$^+$ cells /glo) in biopsy samples of the same patients.

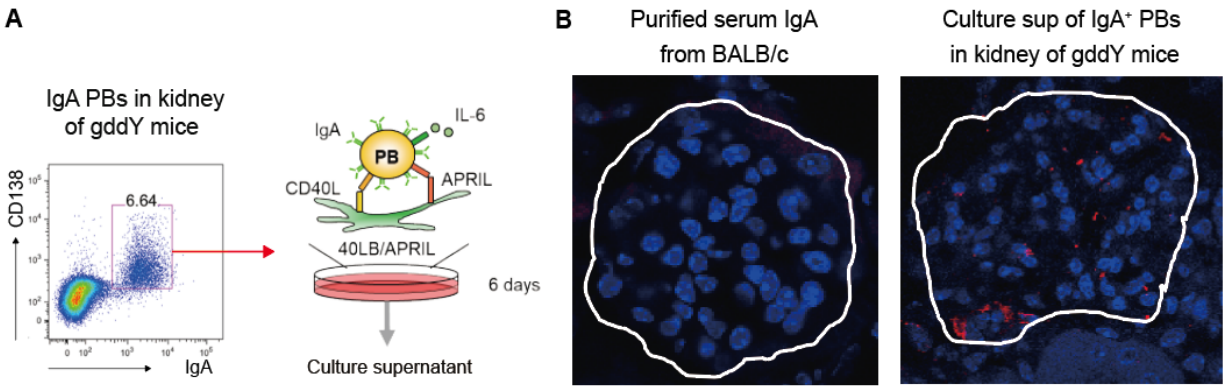

Fig. S7. Reactivity to glomerulus of IgA secreted by IgA$^+$ PBs of the kidney in gddY mice

(A) Schematic representation of the culture system to produce Ab from PBs. IgA$^+$ PBs sorted from the kidneys of gddY mice were cultured with IL-6 on 40LB/APRIL feeder cells for six days.

(B) Immunofluorescence microscopy of kidney sections of an AID-knockout mouse stained with serum IgA from 12-week-old BALB/c mice (control) or the supernatant of the culture described in (A), followed by PE-anti-IgA Ab (red) and DAPI (blue). White lines represent areas of the glomeruli.

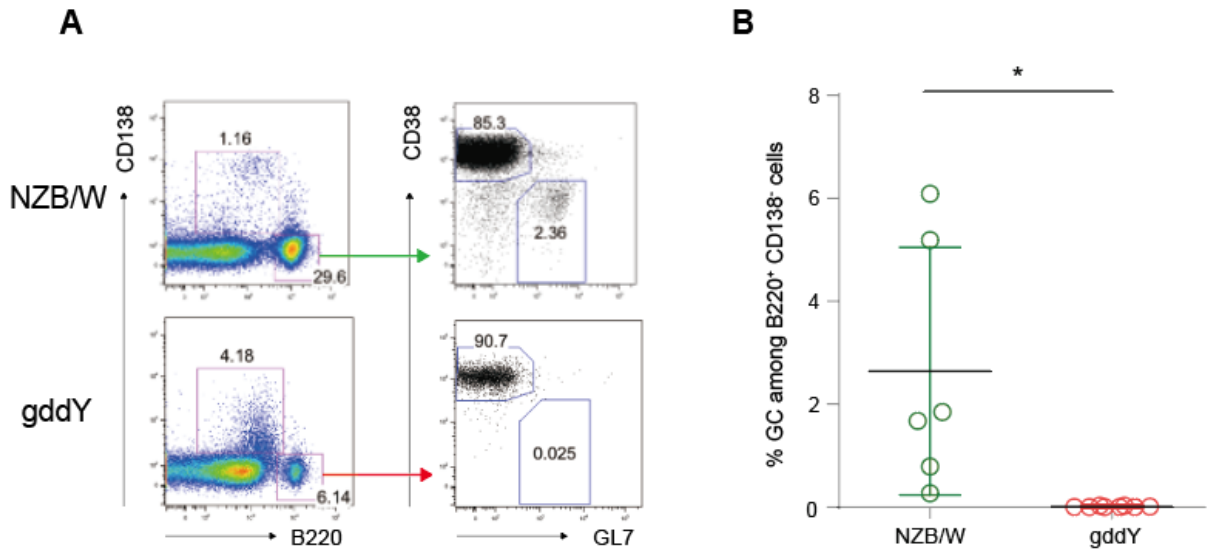

Fig. S8. Absence of GC B cells in the kidneys of 8-week-old gddY mice

(A) Flow cytometric analysis for the expression of GL7 and CD38 (right panels) on $B220^{+}$ $CD138^{-}$ cells (left panels) in the same samples as in fig. S5D.

(B) The frequency of $GL7^{+}$ $CD38^{-}$ (GC) B cells among $B220^{+}$ $CD138^{-}$ cells as determined in (A) (n=6 for NZB/W F1, n=8 for gddY). Small horizontal lines indicate the mean (black) ± s.d. (colored) of each group. $*P < 0.05$ (Student's *t* test). Data are representative of three independent experiments.

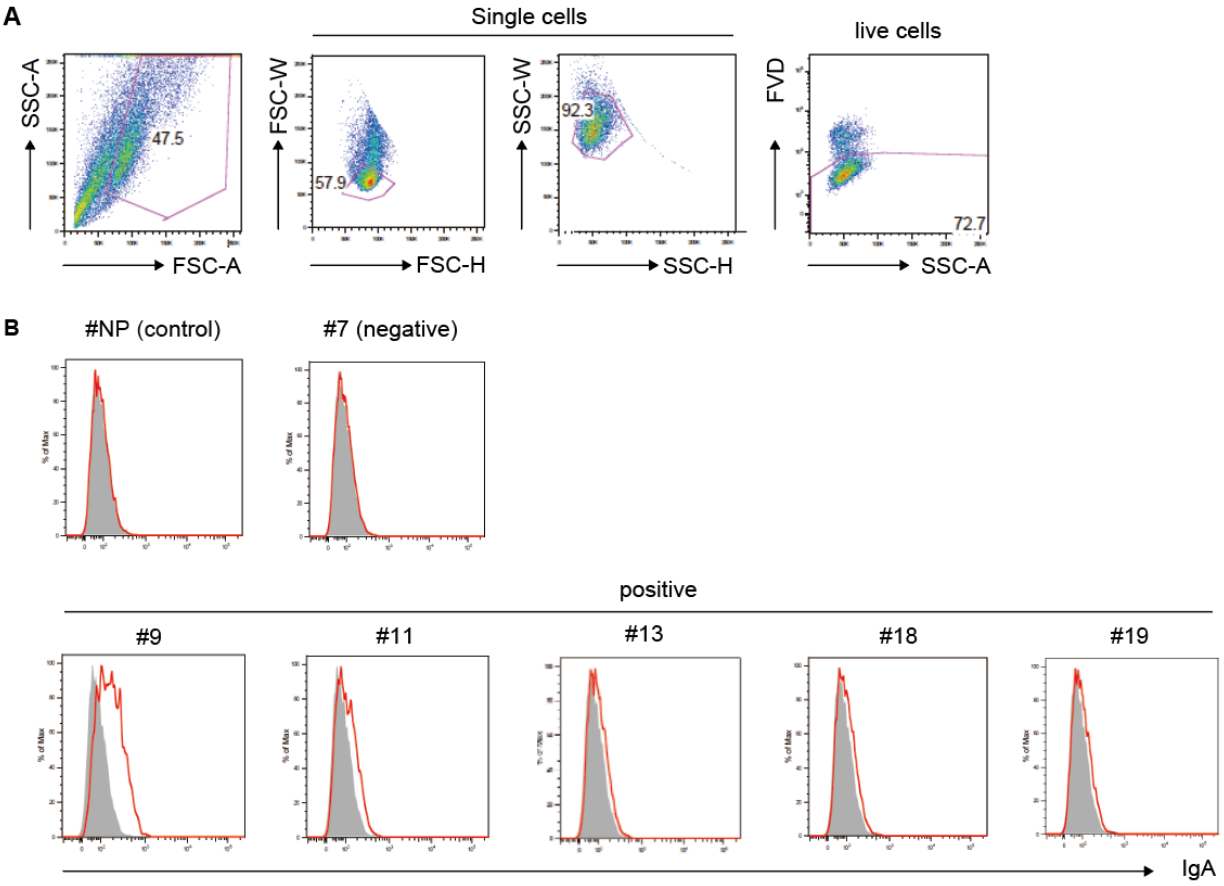

Fig. S9. MC binding of rIgAs generated from kidney IgA$^+$ PBs of gddY mice

(A) Gating strategy for the flow cytometric analysis (Fig. 5B and fig. S9B) of primary-cultured mouse MCs.

(B) The MCs were intracellularly stained with the indicated rIgA Abs derived from IgA$^+$ PBs in gddY mouse kidneys or anti-NP rIgA (#NP) (red line), or without the primary Ab (shaded), followed by anti-IgA Ab. Data are from the same experiment as shown in Fig. 5B.

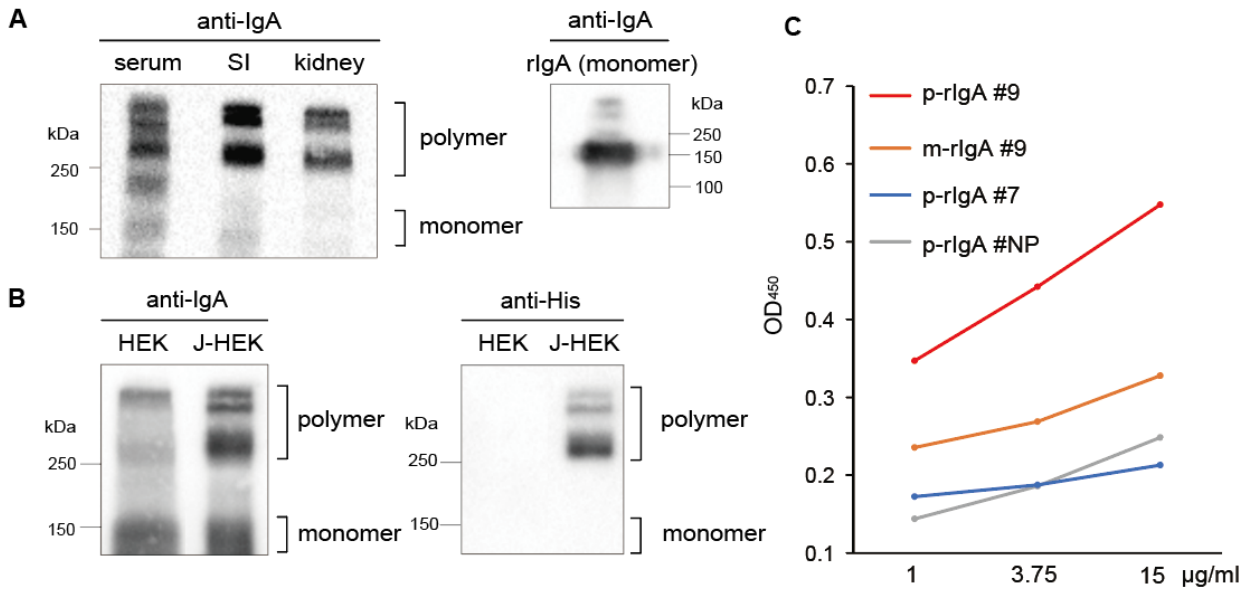

Fig. S10. Generation of polymeric rIgA derived from kidney IgA$^+$ PBs of gddY mice

(A) Pooled sera from three gddY mice (serum) or supernatants of IgA$^+$ ASCs from the small intestine (SI) or the kidney (kidney) of the same mice cultured for one day without any cytokines (left panel) and rIgA #9 (right panel) were subjected to SDS-PAGE under non-reducing conditions and probed with anti-IgA.

(B) Recombinant IgAs secreted from HEK293T cells (HEK) or those expressing His-tagged J chain (J-HEK) were analyzed as in (A) (left panel) and re-probed with anti-His-tag Ab (right panel).

(C) Reactivity of the indicated monomeric (m-) or polymeric (p-) rIgAs with FL-Sptbn1 was evaluated by ELISA. OD_{450} values of each rIgA at the indicated concentrations are shown.

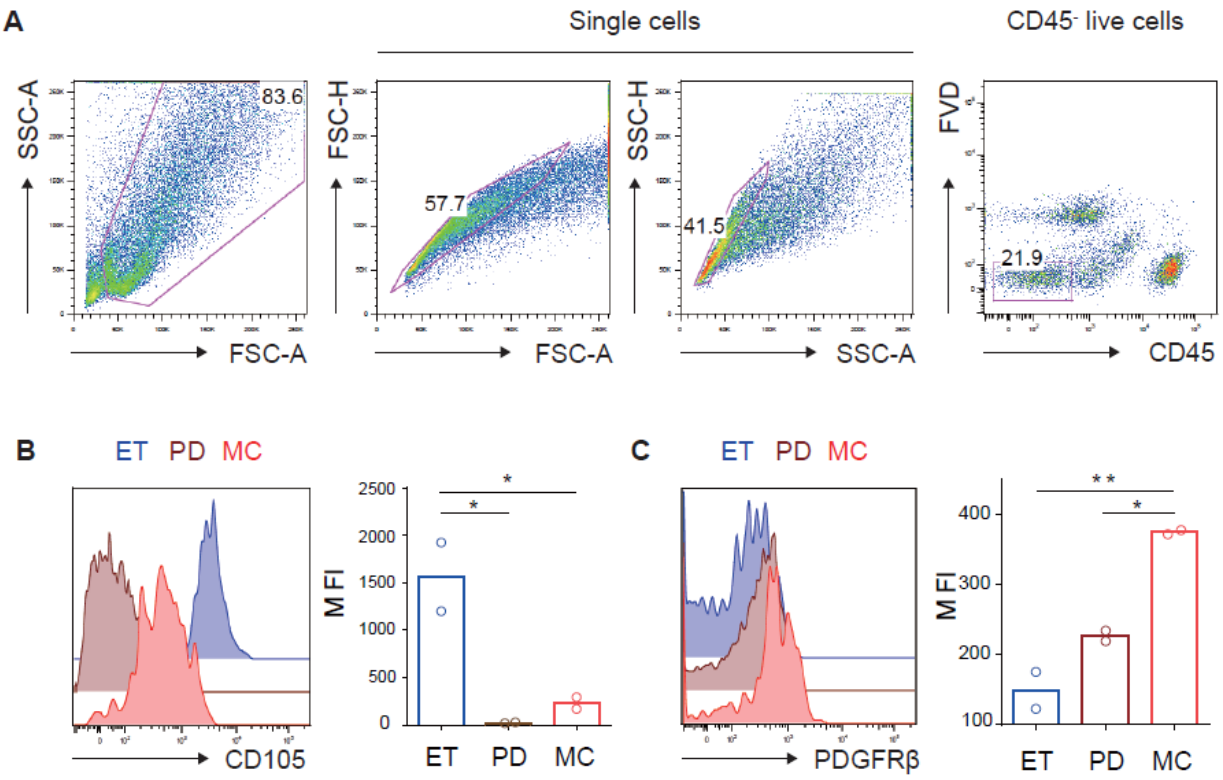

Fig. S11. Flow cytometric analysis of purified glomerular cells

(A to C) Flow cytometric analysis of combined glomerular cells collected from the kidneys of eight BALB/c mice.

(A) Gating strategy of non-leukocytes ($CD45^{-}$) for (B and C) and Fig. 6A-C. The numbers indicate the percentage of gated cells (outlined) among the total cells plotted.

(B and C) Histograms showing CD105 (B) and PDGFRβ (C) expression on the same ETs (blue), PDs (brown) or MCs (red) as shown in Fig. 6A-C. Average MFIs of CD105 or PDGFRβ are shown as bar graphs (n=2). $*P < 0.05$, $**P < 0.01$ (one-way ANOVA with multiple comparison test).

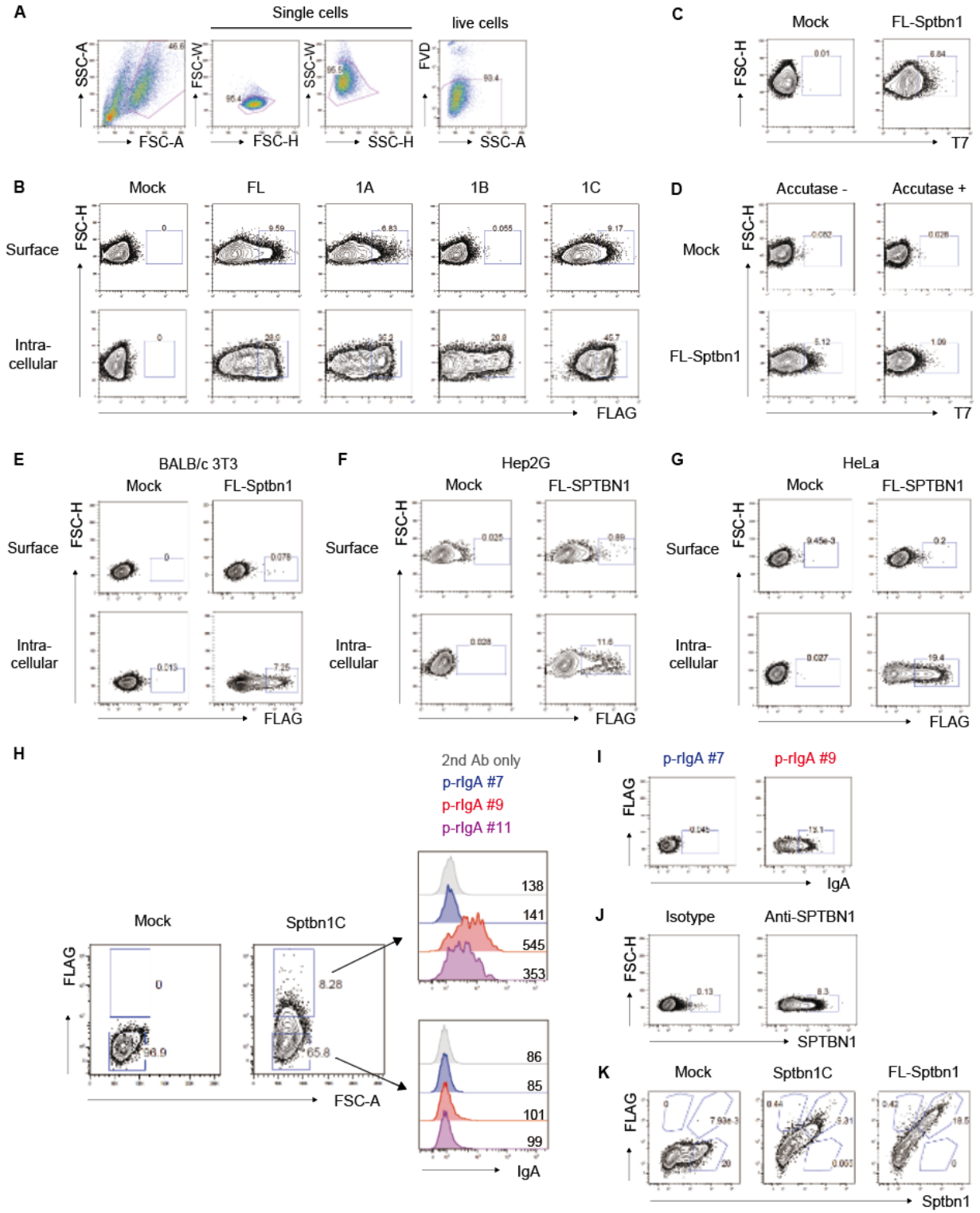
A
Single cells
live cells
SSC-A
FSC-A
FSC-W
FSC-H
SSC-W
SSC-H
FVD
SSC-A
B
Mock
FL
1A
1B
1C
Surface
Intra-cellular
FSC-H
FLAG
C
Mock
FL-Sptbn1
FSC-H
T7
D
Accutase -
Accutase +
Mock
FL-Sptbn1
FSC-H
T7
E
BALB/c 3T3
Mock
FL-Sptbn1
Surface
Intra-cellular
FSC-H
FLAG
F
Hep2G
Mock
FL-SPTBN1
Surface
Intra-cellular
FSC-H
FLAG
G
HeLa
Mock
FL-SPTBN1
Surface
Intra-cellular
FSC-H
FLAG
H
2nd Ab only
p-rIgA #7
p-rIgA #9
p-rIgA #11
Mock
Sptbn1C
FLAG
FSC-A
8.28
65.8
138
141
545
353
86
85
101
99
IgA
I
p-rIgA #7
p-rIgA #9
FLAG
IgA
J
Isotype
Anti-SPTBN1
FSC-H
SPTBN1
K
Mock
Sptbn1C
FL-Sptbn1
FLAG
Sptbn1

Fig. S12. Cell surface expression of βII-spectrin and its recognition by IgA auto-Abs of gddY mice

(A) Gating strategy for live cells analyzed by flow cytometry in (B to K) and Fig. 6D and E. The numbers indicate the percentage of the cells within the adjacent gates (outlined) among the total cells plotted.

(B to K) Flow cytometric analysis of the indicated cells transfected, or not, with the indicated expression vectors. The numbers indicate percentages of the cells within the gates among live cells.

(B) HEK293T cells transfected with mock, FLAG-tagged FL-Sptbn1 or Sptbn1A, 1B, or 1C vectors were stained with anti-FLAG Ab on the surface (top) or intracellularly (bottom).

(C) HEK293T cells transfected with mock or T7-tagged FL-Sptbn1 vectors were surface stained with anti-T7 Ab.

(D) HEK293T cells transfected as in (C) were treated with (right) or without (left) accutase for 10 min and then surface stained with anti-T7 Ab.

(E to G) BALB/c 3T3 cells transfected with mock or FLAG-tagged FL-Sptbn1 vectors (E), and Hep2G cells (F) or HeLa cells (G) transfected with mock or FLAG-tagged FL-SPTBN1 vectors, were analyzed as in (B).

(H) HEK293T cells transfected with mock or FLAG-Sptbn1C vectors were stained with anti-FLAG Ab and with or without the indicated p-rIgA Ab followed by the secondary anti-mouse IgA Ab. Surface binding of the secondary Ab on $FLAG^+$ (upper gate) or $FLAG^-$ (lower gate) live cells is shown as histograms with MFIs.

(I) HEK293T cells transfected with a mock vector were surface stained with p-rIgA #7 or #9, followed by anti-mouse IgA Ab.

(J) Untransfected HEK293T cells were surface stained with rabbit anti-βII-Spectrin Ab (right) or isotype-matched control Ab (left) followed by BV421-labelled anti-rabbit IgG Ab.

(K) HEK293T cells transfected with mock, FLAG-tagged Sptbn1C or FL-Sptbn1 vectors were surface stained with anti-FLAG and anti-βII-Spectrin Abs. All data are representative of two or three independent experiments.

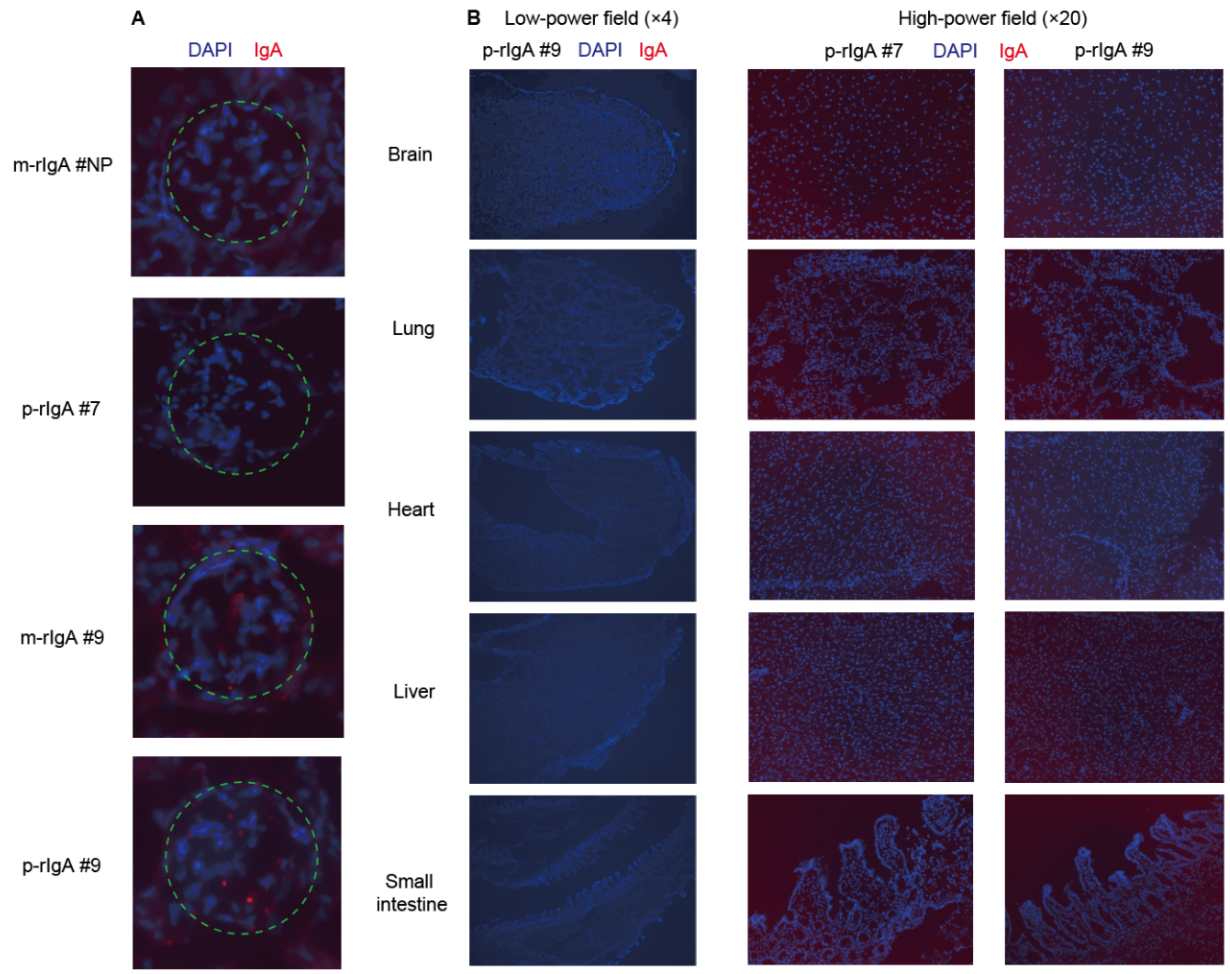

Fig. S13. Tissue specificity of *in vivo* binding by p-rIgA #9

(A) Immunofluorescence microscopy of sections of an AID-knockout mouse kidney, first stained with the indicated m-rIgA or p-rIgA Abs followed by PE-anti-IgA Ab (red) and DAPI (blue). The dashed circles represent areas of glomeruli.

(B) Sections of the indicated tissues from the same mice used in Fig. 6F and G (the mice injected with p-rIgA #7 or #9 as indicated) were stained with PE-anti-IgA Ab (red) and DAPI (blue). Data is one of two independent experiments with similar results.

rAb ID	V region (H)	D region (H)	J region (H)	Specific PCR primer (H, Fw)	Specific PCR primer (H, Rv)	V region (L)	J region (L)	Specific PCR primer (L, Fw)	Specific PCR primer (L, Rv)	No. amino acid mutation (H)	No. amino acid mutation (L)
1	IGHV1-19*01	IGHD5-2	IGHJ1*01	mVH8 Fw XhoI	mJH1 Rv NotI	IGKV5-39*01	IGKJ5*01	mVK11 Fw XhoI	mJK04 Rv NotI	6	2
2	IGHV1-18*01	IGHD2-1	IGHJ2*01	mVH8 Fw XhoI	mJH2 Rv NotI	IGKV6-13*01	IGKJ5*01	mVK11 Fw XhoI	mJK04 Rv NotI	9	6
3	IGHV5-17*01	IGHD2-4	IGHJ4*01	mVH6 Fw XhoI	mJH4 Rv NotI	IGKV4-61*01	IGKJ5*01	mVK3 Fw XhoI	mJK04 Rv NotI	4	10
4	IGHV5-12*01	IGHD2-14	IGHJ3*01	mVH19 Fw XhoI	mJH3 Rv NotI	IGKV6-15*01	IGKJ2*01	mVK11 Fw XhoI	mJK02 Rv NotI	4	14
5	IGHV1-12*01	IGHD4-1	IGHJ2*01	mVH23 Fw XhoI	mJH2 Rv NotI	IGKV1-110*02	IGKJ1*01	mVK15 Fw XhoI	mJK01 Rv NotI	1	4
6	IGHV6-3*01	IGHD1-1	IGHJ3*01	mVH5 Fw XhoI	mJH3 Rv NotI	IGKV4-54*01	IGKJ2*01	mVK3 Fw XhoI	mJK02 Rv NotI	4	13
7	IGHV1-82*01	IGHD1-1	IGHJ2*01	mVH2 Fw XhoI	mJH2 Rv NotI	IGKV4-68*01	IGKJ2*01	mVK3 Fw XhoI	mJK02 Rv NotI	5	5
8	IGHV1-18*01	IGHD2-2	IGHJ2*01	mVH8 Fw XhoI	mJH2 Rv NotI	IGKV12-38*01	IGKJ1*01	mVK10 Fw XhoI	mJK01 Rv NotI	8	10
9	IGHV5-6*01	IGHD1-1	IGHJ2*01	mVH6 Fw XhoI	mJH2 Rv NotI	IGKV10-96*02	IGKJ1*01	mVK14 Fw XhoI	mJK01 Rv NotI	4	2
10	IGHV1-19*01	IGHD2-4*01	IGHJ4*01	mVH8 Fw XhoI	mJH4 Rv NotI	IGKV1-110*02	IGKJ1*01	mVK15 Fw XhoI	mJK01 Rv NotI	0	2
11	IGHV5-9*01	IGHD2-14*01	IGHJ4*01	mVH19 Fw XhoI	mJH4 Rv NotI	IGKV6-29*01	IGKJ2*01	mVK22 Fw XhoI	mJK02 Rv NotI	6	3
12	IGHV1-18*01	IGHD2-14*01	IGHJ3*01	mVH8 Fw XhoI	mJH3 Rv NotI	IGKV14-126*01	IGKJ2*01	mVK8 Fw XhoI	mJK02 Rv NotI	4	0
13	IGHV1-72*01	IGHD2-3*01	IGHJ4*01	mVH1 Fw XhoI	mJH4 Rv NotI	IGKV10-96*02	IGKJ1*01	mVK14 Fw XhoI	mJK01 Rv NotI	4	1
14	IGHV3-1*01	IGHD2-3*01	IGHJ2*02	mVH15 Fw XhoI	mJH2 Rv NotI	IGKV4-68*01	IGKJ5*01	mVK3 Fw XhoI	mJK04 Rv NotI	5	5
15	IGHV5-17*01	IGHD2-4*01	IGHJ4*01	mVH6 Fw XhoI	mJH4 Rv NotI	IGKV4-68*01	IGKJ5*01	mVK3 Fw XhoI	mJK04 Rv NotI	4	11
16	IGHV1-52*01	IGHD2-14*01	IGHJ4*01	mVH6 Fw XhoI	mJH4 Rv NotI	IGKV6-13*01	IGKJ5*01	mVK11 Fw XhoI	mJK04 Rv NotI	6	7
17	IGHV1-58*01	IGHD2-13*01	IGHJ2*01	mVH8 Fw XhoI	mJH2 Rv NotI	IGKV14-100*01	IGKJ1*01	mVK19 Fw XhoI	mJK01 Rv NotI	3	3
18	IGHV1-18*01	IGHD2-1*01	IGHJ2*01	mVH8 Fw XhoI	mJH2 Rv NotI	IGKV6-13*01	IGKJ2*01	mVK11 Fw XhoI	mJK02 Rv NotI	0	3
19	IGHV5-17*01	IGHD1-1*01	IGHJ1*01	mVH6 Fw XhoI	mJH1 Rv NotI	IGKV1-117*02	IGKJ4*01	mVK23 Fw XhoI	mJK03 Rv NotI	0	5
20	IGHV1-19*01	IGHD2-4*01	IGHJ4*01	mVH8 Fw XhoI	mJH4 Rv NotI	IGKV6-13*01	IGKJ4*01	mVK11 Fw XhoI	mJK03 Rv NotI	5	2

Table S1. Characteristics of rIgA Abs generated from the kidneys of gddY mice

Identities of the germline V, (D) and J segments constituting the variable regions of heavy (H) and light (L) chains. Specific primers used for PCR amplification of the variable regions and the number of amino-acid mutations in the translated variable regions of the H and L chains, are outlined for each rIgA (rAb ID).

	HC (n=14)	IgAN (n=45) for detecting serum auto-Abs	IgAN (n=35) for detecting IgA ASCs in kidney samples
Age	33.3【27-35】	35.2【18-65】	36.9 【20-68】
Gender (M/F)	8/6	21/24	16/19
Serum IgA (mg/dl)	151【41-260】	313.0【150-533】	319【174-533】
Gd-IgA1(μg/ml)	4.2 【1.7-8.3】	6.74 【3.1-18.0】	6.2【2.9-11.9】
eGFR (ml/min/1.73m²)	n. m	86.2 【35.8-117.6】	84.8【26.8-138.2】
Proteinuria (g/gCr)	n. d	0.62 【0.1-3.57】	0.67【0.3-4.83】

Table S2. Clinical characteristics of the participants

Clinical information of the healthy control individuals (HC) and the patients with IgAN involved in this study. M/F: male/female; Gd-IgA1: Galactose-deficient IgA1; eGFR: estimated glomerular filtration rate; n. m: not measured; n. d: not detected.

For generation of rAb		
PCR step	**Name**	**5'-3' sequence**
Igh 1st PCR	Igh 1st PCR Fw	GGGAATTCGAGGTGCAGCTGCAGGAGTCTGG
	Igh 1st PCR Rv (Cα)	CCTATATTGGTGGCACTTGAAAG
Igh 2nd PCR	Igh 2nd PCR Fw	GGGAATTCGAGGTGCAGCTGCAGGAGTCTGG
	Igh 2nd PCR Rv (Cα)	TAAGTGCTAATGAAGGGAAAGCCGT
Igκ 1st PCR	Igκ 1st PCR Fw (mix)	TGCTGCTGCTCTGGGTTCCAG
		ATTWTCAGCTTCCTGCTAATC
		TTTTGCTTTTCTGGATTYCAG
		TCGTGTTKCTSTGGTTGTCTG
		ATGGAATCACAGRCYCWGGT
		TCTTGTTGCTCTGGTTYCCAG
		CAGTTCCTGGGGCTCTTGTTGTTC
		CTCACTAGCTCTTCTCCTC
	Igκ 1st PCR Rv (Cκ)	TTGACATAGGTAGAAGGGTGGTAG
Igκ 2nd PCR	Igκ 2nd PCR Fw	GAYATTGTGMTSACMCARWCTMCA
	Igκ 2nd PCR Rv (IgJκ 1,2,4,5)	GTGGTTCGACCTTTAGTTCGCCGGCGTAATA
		CTGGTTCGACCTTTATTTCGCCGGCGTAATA
		CTGGTTCGACCTCGACTTCGCCGGCGTAATA
		CTGTTTCAACCTTTATTTCGCCGGCGTAATA
gddY Cα	Cα Fw NotI	ATAATGCGGCCGCGAGAAATCCCACCATCTACCC
	Cα Rv NheI	ATAATGCTAGCTCAGTAGCAGATGCCATCTCCCT
Cκ	Igκ Fw NotI	ATAATGCGGCCGCATGCTGCACCAACTGTATCCA
	Igκ Rv NheI	ATAATGCTAGCCTAACACTCATTCCTGTTGAAGC
Specific PCR	mVH1 Fw XhoI	ATAATCTCGAGTCAGGTGCAGCTGCAGCAGCCTGG
	mVH2 Fw XhoI	ATAATCTCGAGTCAGGTGCAGCTGCAGCAGTCTGG
	mVH5 Fw XhoI	ATAATCTCGAGTGAGGTGAAGCTGGAGGAGTCTGG
	mVH6 Fw XhoI	ATAATCTCGAGTGAGGTGCAGCTGGTGGAGTCTGG
	mVH8 Fw XhoI	ATAATCTCGAGTGAGGTGCAGCTGCAGCAGTCTGG
	mVH15 Fw XhoI	ATAATCTCGAGTGATGTACAGCTTCAGGAGTCAGG
	mVH19 Fw XhoI	ATAATCTCGAGTGAAGTGATGCTGGTGGAGTCTGG
	mVH23 Fw XhoI	ATAATCTCGAGTGAGTTCCAGCTGCAGCAGTCTGG
	mJH1 Rv NotI	ATAATGCGGCCGCTGAGGAGACGGTGACCGTGG
	mJH2 Rv NotI	ATAATGCGGCCGCTGAGGAGACTGTGAGAGTGG
	mJH3 Rv NotI	ATAATGCGGCCGCTGCAGAGACAGTGACCAGAG
	mJH4 Rv NotI	ATAATGCGGCCGCTGAGGAGACGGTGACTGAGG
	mVκ3 Fw XhoI	ATAATCTCGAGTCAAATTGTTCTCACCCAGTCTCCA
	mVκ8 Fw XhoI	ATAATCTCGAGTGACATCAAGATGACCCAGTCTCCA
	mVκ10 Fw XhoI	ATAATCTCGAGTGACATCCAGATGACTCAGTCTCCA
	mVκ11 Fw XhoI	ATAATCTCGAGTGACATTGTGATGACTCAGTCTC
	mVκ14 Fw XhoI	ATAATCTCGAGTGATATCCAGATGACACAGACTACA
	mVκ15 Fw XhoI	ATAATCTCGAGTGATGTTGTGATGACCCAAACTCCA
	mVκ19 Fw XhoI	ATAATCTCGAGTGACATCCTGATGACCCAATCTCCA
	mVκ22 Fw XhoI	ATAATCTCGAGTAACATTGTAATGACCCAATCTCCC
	mVκ23 Fw XhoI	ATAATCTCGAGTGATGTTTTGATGACCCAAACTCCA
	mJκ01 Rv NotI	ATAATGCGGCCGCTTGATTTCCAGCTTGGTG
	mJκ02 Rv NotI	ATAATGCGGCCGCTTTATTTCCAGCTTGGTC
	mJκ03 Rv NotI	ATAATGCGGCCGCTTTATTTCCAACTTTGTC
	mJκ04 Rv NotI	ATAATGCGGCCGCTTCAGCTCCAGCTTGGTC

For generation of Sptbn1		
FLAG-Sptbn1A	Sptbn1A Fw EcoRI	ATAATGAATTCAACGACCACGGTAGCCACAGACT
	Sptbn1A Rv XbaI	ATAATTCTAGATCAATCTTTATGCTTCTTGACCA
FLAG-Sptbn1B	Sptbn1B Fw EcoRI	ATAATGAATTCAGTAGCAGAAGAGATCACCAACT
	Sptbn1B Rv XbaI	ATAATTCTAGATCAGAGCCCCCACAGCTGCTTCA
FLAG-Sptbn1C	Sptbn1C Fw EcoRI	ATAATGAATTCACTCATTGAGGAAACTGAGAAAC
	Sptb1C Rv XbaI	ATAATTCTAGATCACTTCTTCTTGCCGAAAAGGC
T7-FL-Sptbn1	Sptbn1 FL Fw NotI	ATAATGCGGCCGCAACGACCACGGTAGCCACAGA
	Sptbn1 FL Rv EcoRI	ATAATGAATTCTCACTTCTTCTTGCCGAAAAGGC
FLAG-Sptan1C	Sptan1C Fw NotI	ATAATGCGGCCGCGGCTGAGAAGAGCCAGAAGCT
	Sptan1C Rv XbaI	ATAATTCTAGATCAATTCACAAAGAGTGAGCGGG

For generation of J-chain		
His tagged J chain	J chain Fw EcoRI	ATAATGAATTCGCCACCATGAAGACCCACCTGCT
	J chain Rv XhoI	ATAATCTCGAGTCAGTGGTGATGATGGTGGTGGTCAGGGTAGCAAGA

Table S3. Primer information

Primer information for generating rIgA Abs, FLAG-tagged Sptbn1A-C and FL-Sptbn1, and a His-tagged mouse J chain.

Supplemental auxiliary files:

Raw data for the figures of this article (an Excel file).