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Supplementary Materials for

The ion transporter Na^+-K^+-ATPase enables pathological B cell survival in the kidney microenvironment of lupus nephritis

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This PDF file includes:

Supplementary Materials

Supplementary Materials and Methods

In vitro B cell cultures

Splenic B cells from C57BL/6, $MRL^{+/+}$, MRL^{lpr} and $NZBWF_1$ mice were isolated and purified by magnetic negative selection kit (STEMCELL Technologies). IgD^+ versus IgD^- B cells were isolated following the negative selection using a biotinylated IgD antibody and a positive selection biotin kit (STEMCELL Technologies). Cells were plated at 1-2x10^6/mL of RPMI 1640 media supplemented with 10% FBS (fetal bovine serum), 2mM L-glutamine and 55nM 2-mercaptoethanol with 5μg/ml lipopolysaccharide (LPS) added for B cell stimulation. NaCl, NaGluconate, $MgCl_2$, mannitol and ouabain were added to the media at the indicated concentrations. Cells were analyzed by flow cytometry or prepared for RT-PCR analysis 72 hours later. Calculation of "% live" was determined by gating the fraction of cells negative for the eBioscience™ fixable viability dye eFluor™ 780 (a membrane-integrity dye that stains intracellular amines).

Cell Death Assays

Splenic B cells from C57BL/6, $MRL^{+/+}$, MRL^{lpr} mice were isolated and cultured with varying NaCl concentrations as above. At 24, 48 and 72 hours, media were collected and cells were either prepared for flow cytometry or used for RNA isolation. For caspase-3 studies, CaspGlow™ system from Invitrogen (cat# 88-7004-42) which detects active caspase-3 was used in conjunction with an eBioscience™ fixable viability dye. Flow cytometry Annexin-V studies were performed using a kit from Biolegend (cat# 640928). Gasdermin D detection was performed via enzyme-linked immunoassay (ELISA) from Adipogen (cat# AG-45B-0011). Interleukin 1β (IL1β) protein detection was performed via ELISA from R&D Systems (cat# DY401). For apoptosis studies in the *ex vivo* mouse kidney, lymphocytes were isolated as previously described and stained for flow cytometry with surface markers, fixable viability dye and active caspase-3 per commercially available protocol (CaspGlow™, Invitrogen).

Retroviral Transduction

LMP vectors were cloned with short hairpin ribonucleic acid (shRNA) 97-mer sequences targeting *Fxyd2*, the gene for γNa^+-K^+-ATPase (**Suppl. Table 2)**, using In-Fusion® HD Cloning Kit (TaKaRa, 639650). For retroviral transduction, 1 mg of LMP, or LMP cloned with the above-mentioned shRNA vector with 0.5 mg of EcoHelp plasmid were transfected in HEK293T cells using X-tremeGENE 9 DNA transfection reagent for 24 hours as we previously described[51]. After replacing the culture media, the green fluorescence protein positive (GFP^+) virus was grown for another 24 hours before applying to 2 x 10^6 purified cultured B cells by spin transduction. After 48 hours the B cell media was changed to media supplemented with NaCl and incubated for another 96 hours prior to analysis.

Flow Cytometry and Cell Sorting

Flow cytometry data were acquired by BD LSRII at Flow Cytometry Facility, Yale University, and analyzed by FlowJo 10.0.7. Fluorescence-activated cell sorting (FACS) of specific populations was performed on a FACSAria (BD Biosciences).

Microscopy

From formalin-fixed paraffin-embedded (FFPE) human lupus nephritis biopsy samples, sections were cut at 3-μm and placed onto coated glass slides (SuperFrost Plus, Fisher Scientific), and stored at room temperature until used. Slides were baked at 60°C for 30 minutes then dewaxed with xylene, rinsed in a graded alcohol series then rehydrated in distilled water. Antigen retrieval was done at 96°C for 20 min in citrate buffer, pH 6.0 (Spring Bioscience). Slides were then blocked in 1x TBS containing 0.2% Triton X-100, 10% normal donkey serum and Human BD Fc block (2.5 mg/ml) (TBST+) for 1 hour at room temperature. Primary antibodies were applied overnight in TBST+ at 4°C. Slides were then washed 3 x 5 minutes in 1x TBS, followed by the appropriate conjugated secondary antibodies for 1 hour at room temperature. After washing, DAPI was applied for 10 min, washed, then the slides mounted in SlowFade Gold (ThermoFisher) and imaged on a Leica Stellaris 8 Laser Scanning Confocal Microscope using a Leica 63X HCX-APO oil immersion objective.

Mice were perfused with 4% paraformaldehyde (PFA) to fix the tissues and kidneys and spleens harvested. Organs were next incubated in 30% sucrose solution prior to being snap frozen in OCT tissue-freezing solution

and stored at −80°C. 8μm sections were cut and blocked with 5% rat serum, 3% BSA and, 0.1% Tween. Sections were stained with anti-B220 (B cell marker) and anti- Na^+-K^+-ATPase antibodies discussed below or anti-mouse FITC-conjugated C3 (MP Biomedicals) for 16 to 18 hours in a dark, humidified chamber at 4°C. Cellular nuclei were stained with nuclear dye 4',6-diamidino-2-phenylindole (DAPI) for 20 minutes at room temperature (25°C). Images were obtained on an ECHO Revolve microscope with 10-40x magnification. ImageJ software (NIH) was used for image analysis. List of antibodies used can be found in **Suppl. Table 1**.

Na^+-K^+-ATPase Antibodies

Antibodies against the α and γ subunits of Na^+-K^+-ATPase were detected using an anti-rabbit secondary (eBioscience, A32795). The same anti-Na^+-K^+-ATPase α subunit antibody was used for all flow cytometry and human and mouse microscopy studies (Abcam, ab58475). The γ subunit antibody used for microscopy was kindly provided by Elena Arystarkhova of Massachusetts General Hospital[41]. For the γ subunit of Na^+-K^+-ATPase the following antibodies were unsuccessfully trialed for flow cytometry: Invitrogen PA5-66670, Abbexa abx112610, clone 2004 provided by Elena Arystarkhova referenced above.

Real-time PCR

RNA was isolated using RNeasy Micro kit (Qiagen) for sorted cells or the RNeasy Mini kit (Qiagen) for *in vitro* grown cells. cDNA was synthesized with the iScript™ cDNA Synthesis kit (Bio-Rad). Real-time PCR was set up using Brilliant II SYBR Green Master Mix™ and performed on an MX4005P Thermal Cycler™ (Agilent Technologies). Primers used are listed in **Suppl. Table 2**. Expression was calculated with the ΔΔxp method normalized to βActin with all measurements performed in quadruplicate.

Histology and H&E staining

One kidney from each experimental mouse was cut and incubated in a cassette in 10% paraformaldehyde (PFA) for 24 hours prior to submission to Yale Pathology Tissue Services. Tissue was cut into 7μm sections and stained with hematoxylin and eosin. O.H., a renal pathologist, blindly scored the sections based on the NIH lupus activity score.

BUN, Creatinine, [Na^+], Proteinuria measurements

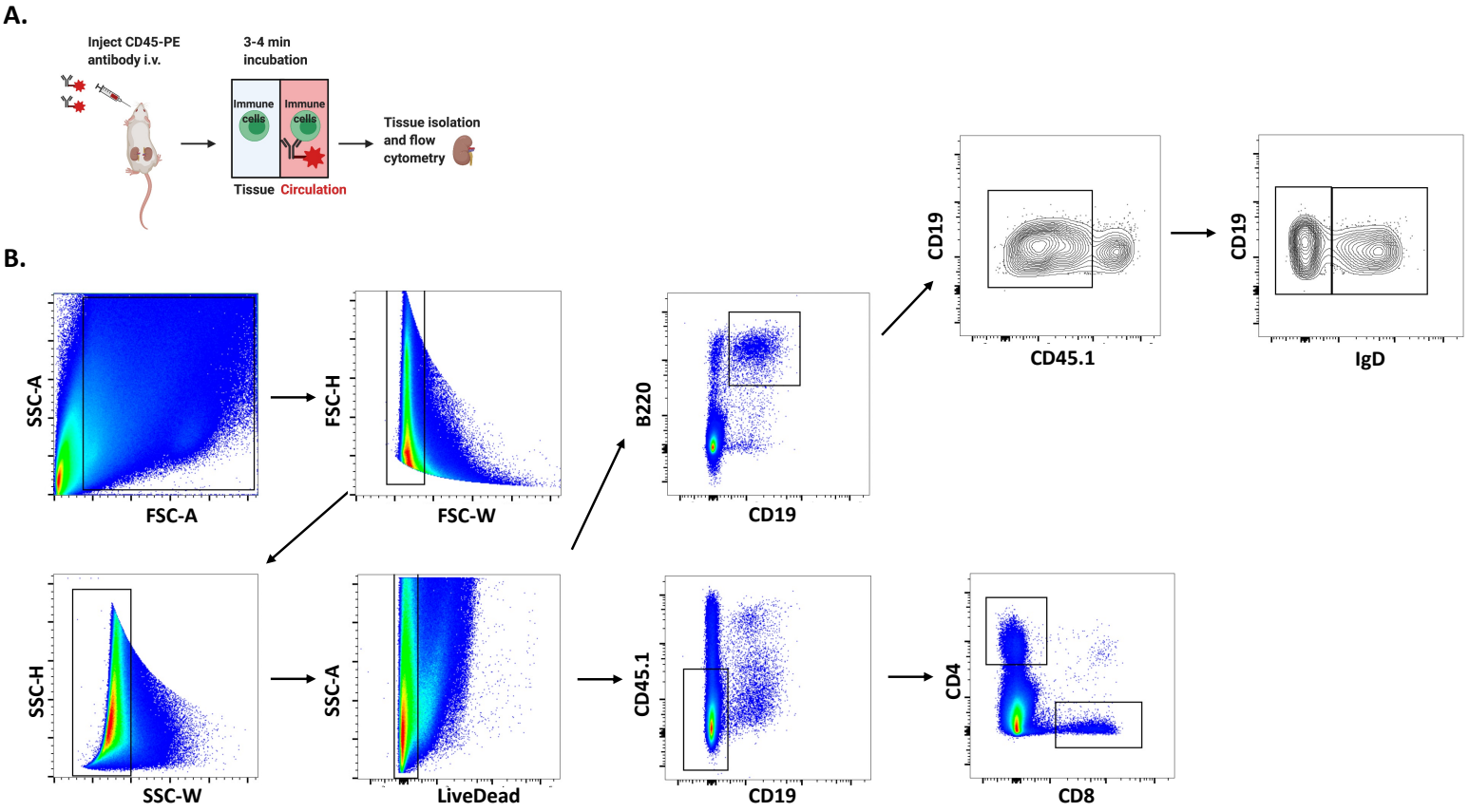
Blood urea nitrogen (BUN) and serum [Na^+] were quantitated using established quantitative colorimetric methods (Stanbio Procedures 0580 and 0140, respectively) on an Excel Chemistry Analyzer (Stanbio Laboratory, Boerne, Texas) by the George M. O'Brien Kidney Center at Yale. Serum creatinine was measured using a targeted Liquid Chromatography-Multiple Reaction Monitoringworkflow on a 4000 QTRAP mass spectrometer by the George M. O'Brien Kidney Center at Yale. Semi-quantitative proteinuria was measured using Uristix® (SIEMENS, 2184). Urine albumin and creatinine were quantitated using the BioAssay Systems QuantiChrom™ BCG Albumin Assay Kit (DIAG-250) and QuantiChrom™ Creatinine Assay Kit (DICT-500), respectively. Urine albumin and creatinine samples were analyzed using SpectraMax Microplate Reader at 620nm and 510nm, respectively.

ELISAs

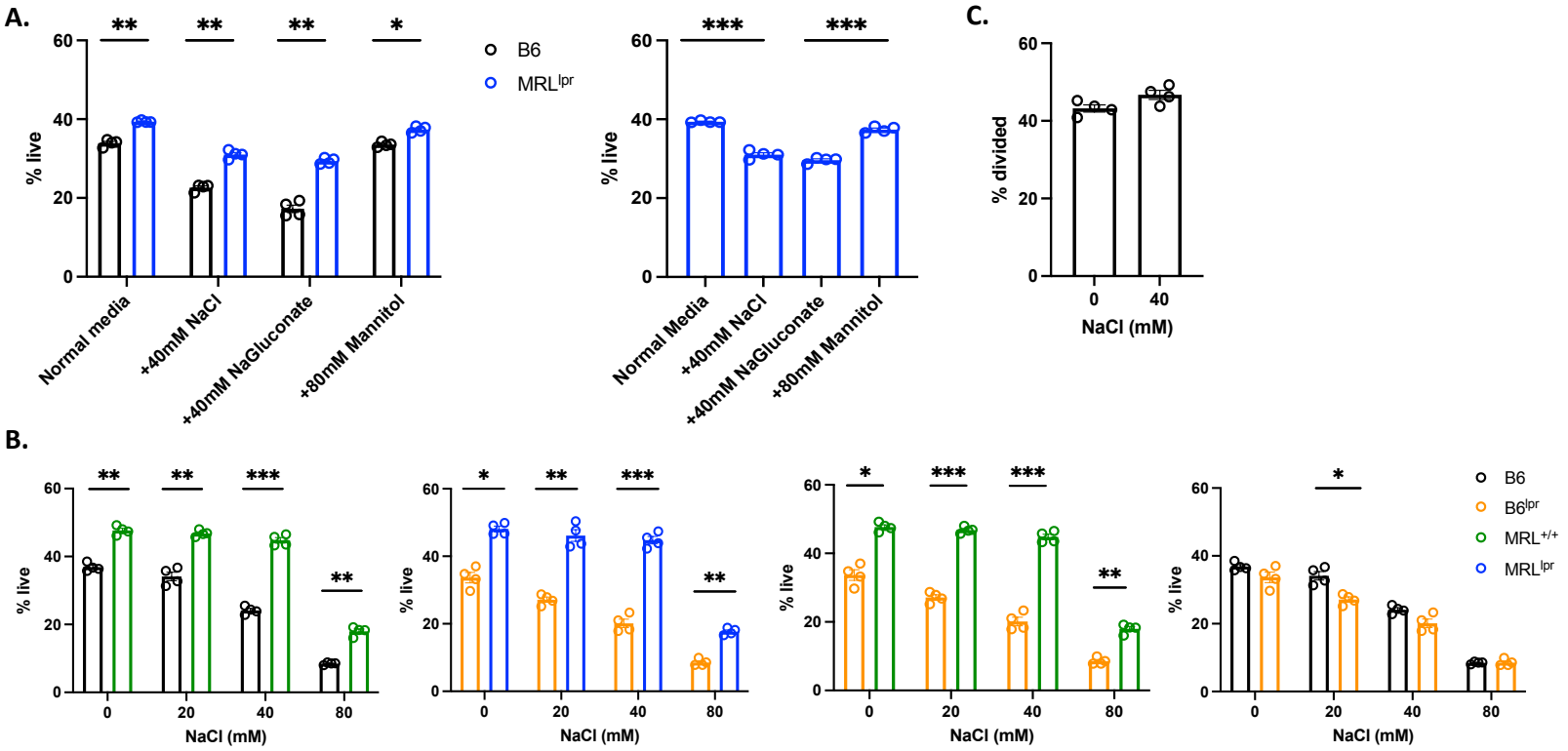
Anti-dsDNA antibodies in mouse sera were measured on Nunc PolySorp 96 well plates (Thermo Fisher) coated with calf thymus DNA (Sigma, D4522) in Reacti-Bind™ buffer (Thermo Scientific, 17250). Alkaline phosphatase-conjugated anti-mouse IgG and IgM antibodies (Southern Biotech) and phosphatase substrate (Sigma) were used for detection. ODs were read at 405nm on a SpectraMax Microplate Reader (Molecular Devices).

Graphical Illustration

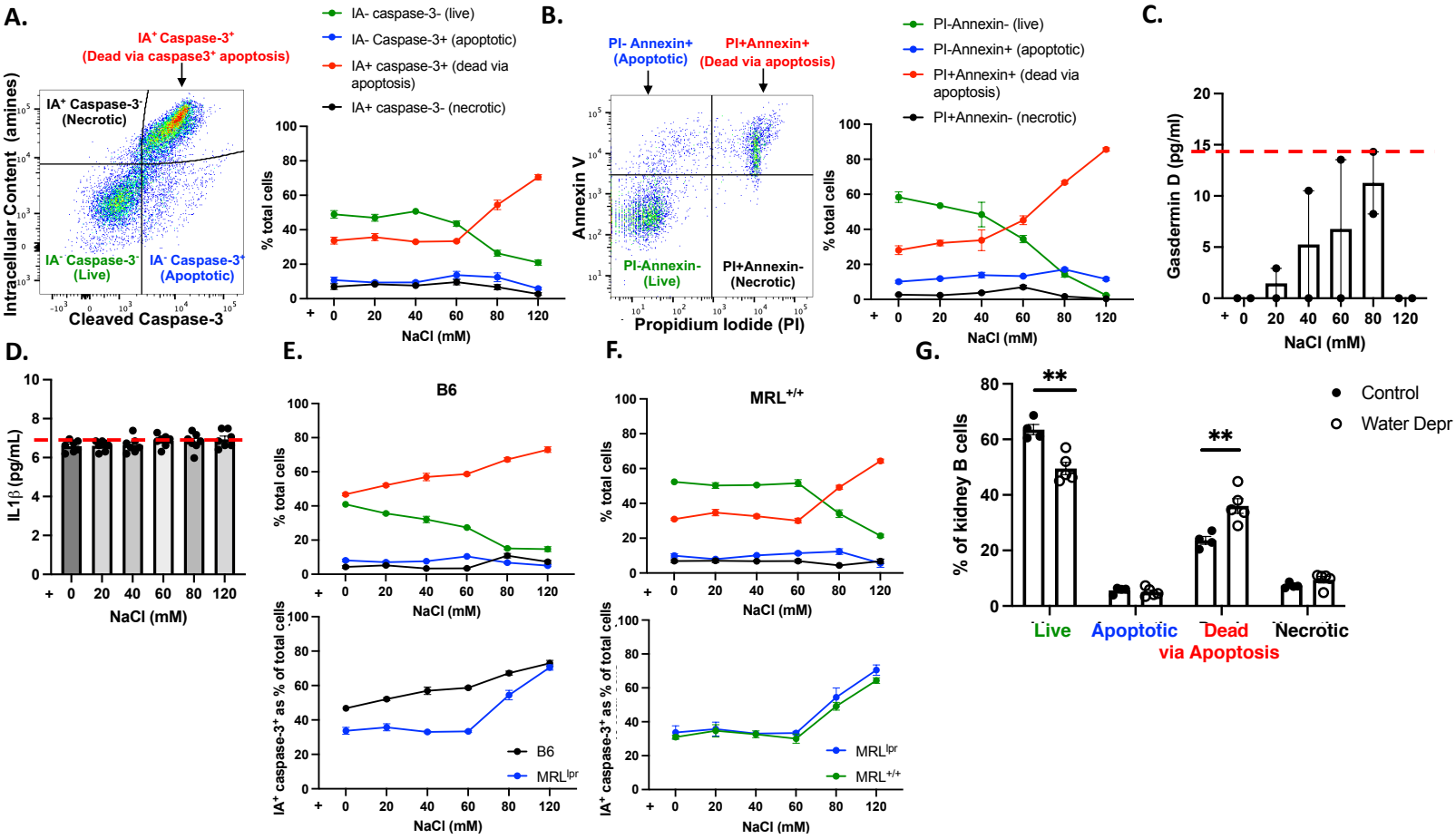
Graphical illustrations were created using Biorender.com.



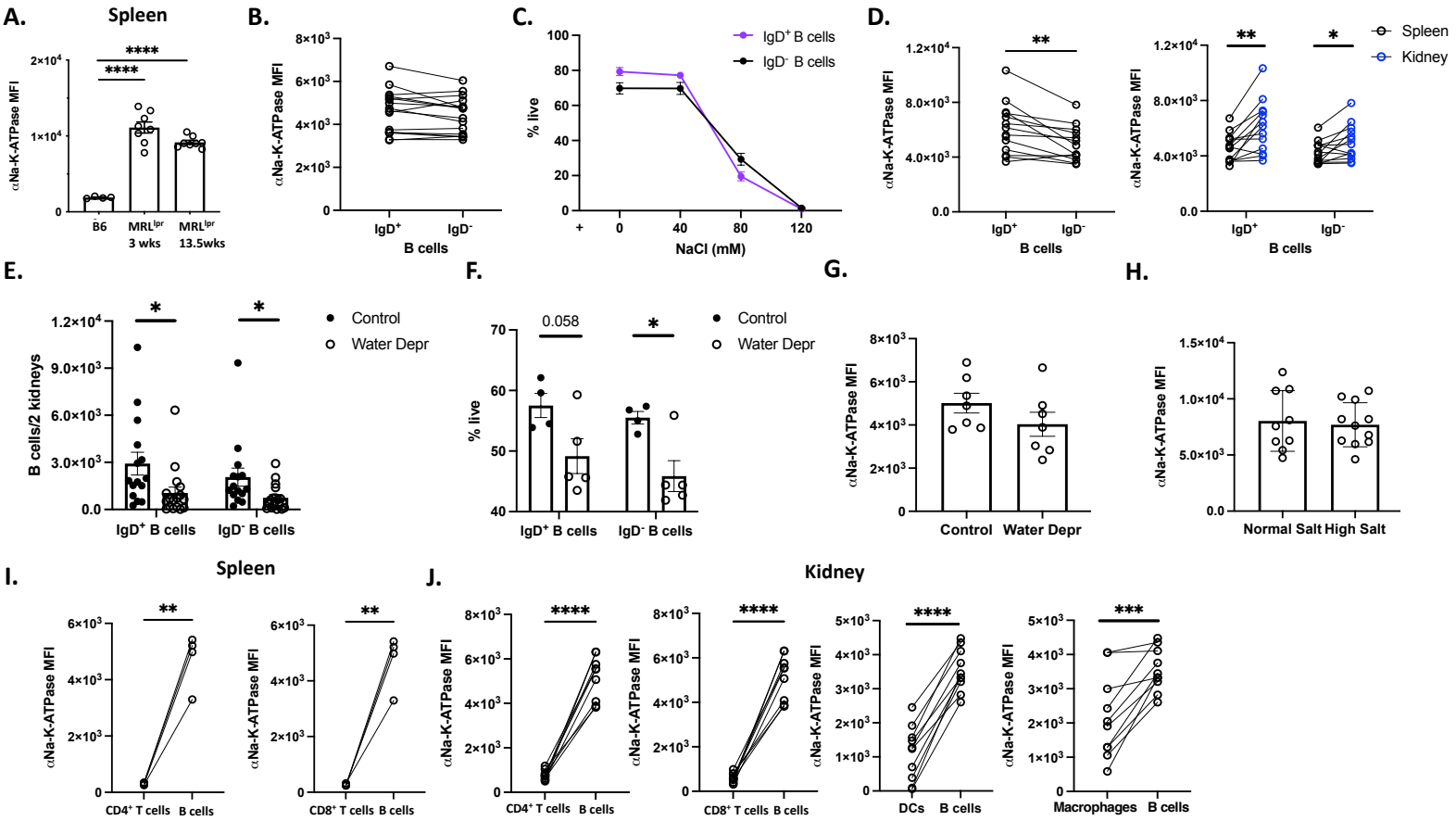
SFig.1. Representative kidney flow cytometry. **A.** Strategy for identifying tissue-resident lymphocytes, created with BioRender.com. **B.** Representative flow plots for identifying kidney-resident B cells and T cells – B cells are LiveCD19^{+}B220^{+}CD45.1^{-}IgD$^{+/-}$, plasma cells are LiveIgD^{-}CD138^{hi}CD45.1^{-}, CD4^{+} T cells are LiveCD19^{-}CD8^{-}CD4^{+}CD45.1^{-} and CD8^{+} T cells are LiveCD19^{-}CD4^{-}CD8^{+}CD45.1^{-}.



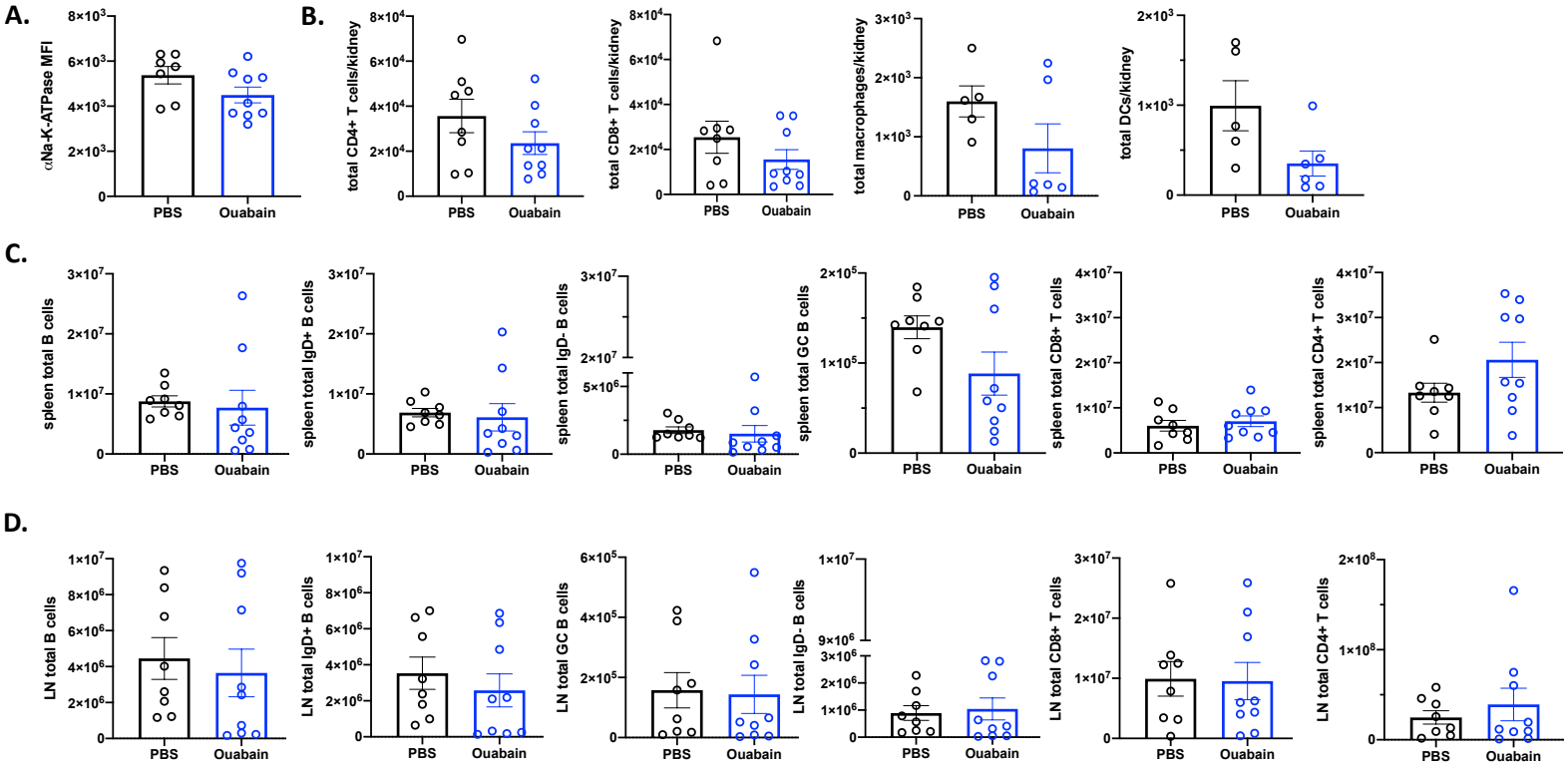
SFig.2. Enhanced survival of MRL^{lpr} B cells in high salt conditions is independent of the lymphoproliferative mutation (lpr) and osmolality. (A) B cells from B6 and MRL^{lpr} mice were cultured with NaCl, NaGluconate and mannitol as indicated. **(B)** B cells from B6, B6^{lpr}, MRL$^{+/+}$ and MRL^{lpr} mice were cultured as in Fig 1C. **(C)** B cells were cultured with high salt in the presence of CellTraceViolet proliferation dye; representative of >2 independent experiments. p value *<0.05, **<0.01, ***<0.001, results not significant unless otherwise indicated.



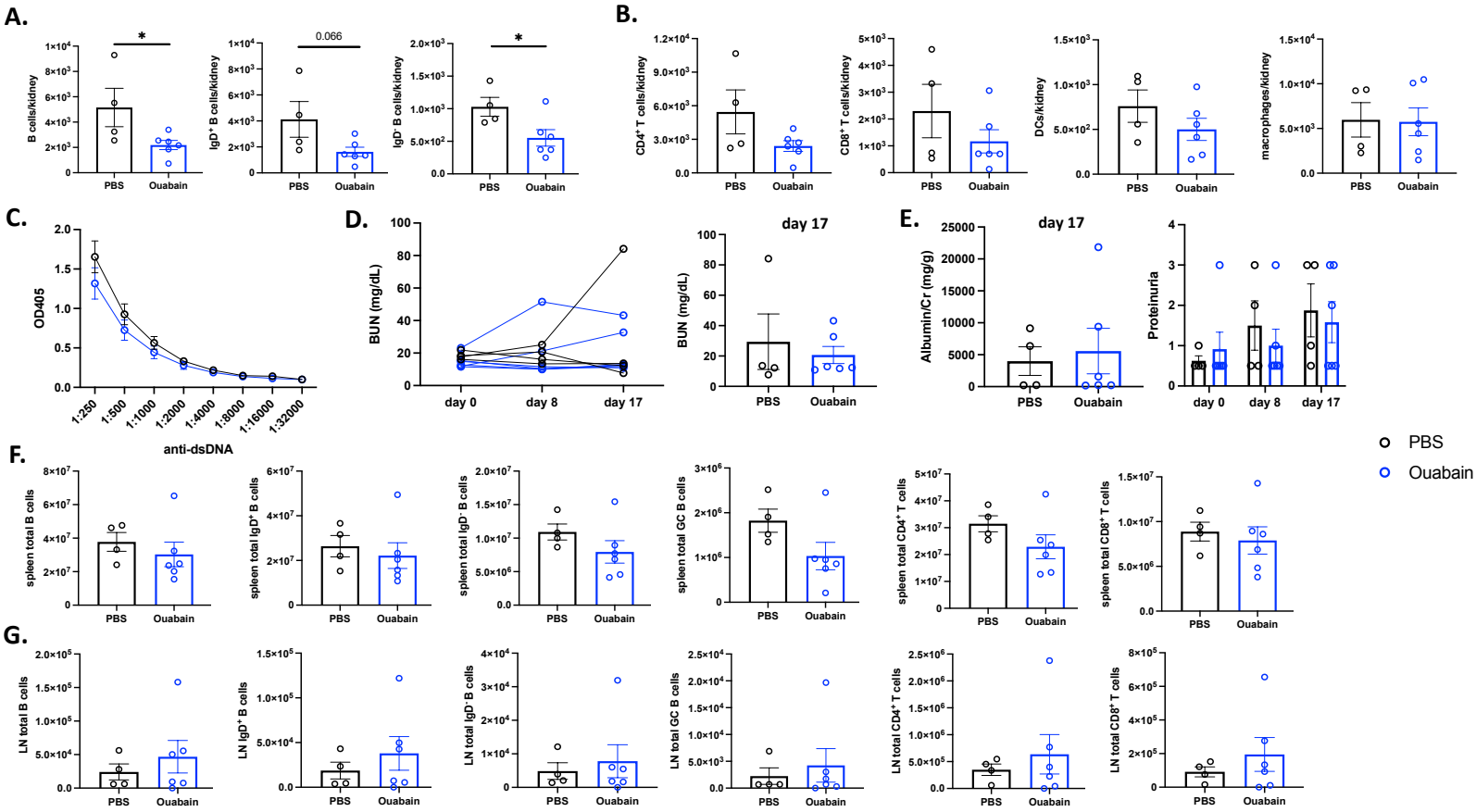
SFig.3. B cells undergo apoptotic cell death in response to high [Na$^+$]. **(A)** B cells from MRL^{lpr} mice were cultured as previously described and stained for cleaved caspase-3 and intracellular amines (IA). Representative flow cytometry plot is shown. **(B)** MRL^{lpr} B cells were cultured as in (**A**) and assayed for apoptotic marker Annexin-V and intracellular marker propidium iodide (PI). Representative flow cytometry plot is shown. **(C)** Supernatants from (**A**) were assayed for gasdermin D via ELISA; dashed line represents limit of detection of the assay. **(D)** Supernatants from (**A**) was assayed for IL1β expression via ELISA; dashed line represents limit of detection of the assay (media alone). **(E)** B cells from C57BL/6 mice were cultured in various NaCl concentrations and assayed as in (**A**); figure legend in (**A**) is applicable for (**E**) and (**F**). Bottom panel compares IA$^+$ caspase-3$^+$ cells from B6 and MRL^{lpr} mice. **(F)** B cells from MRL$^{+/+}$ mice were assayed as in (**E**); bottom panel compares IA$^+$ caspase-3$^+$ cells from MRL$^{+/+}$ and MRL^{lpr} mice. **(G)** B cells were isolated from kidneys of MRL^{lpr} mice that were either given water *ad libidum* (control) or water deprived for 48 hours (water depr) as described in **Fig. 1J**. Cells were stained *ex vivo* for surface markers, IA and caspase-3; colors correspond to figure legend in (**A**). All data representative of ≥2 independent experiments. IA= intracellular amine, PI = propidium iodide.



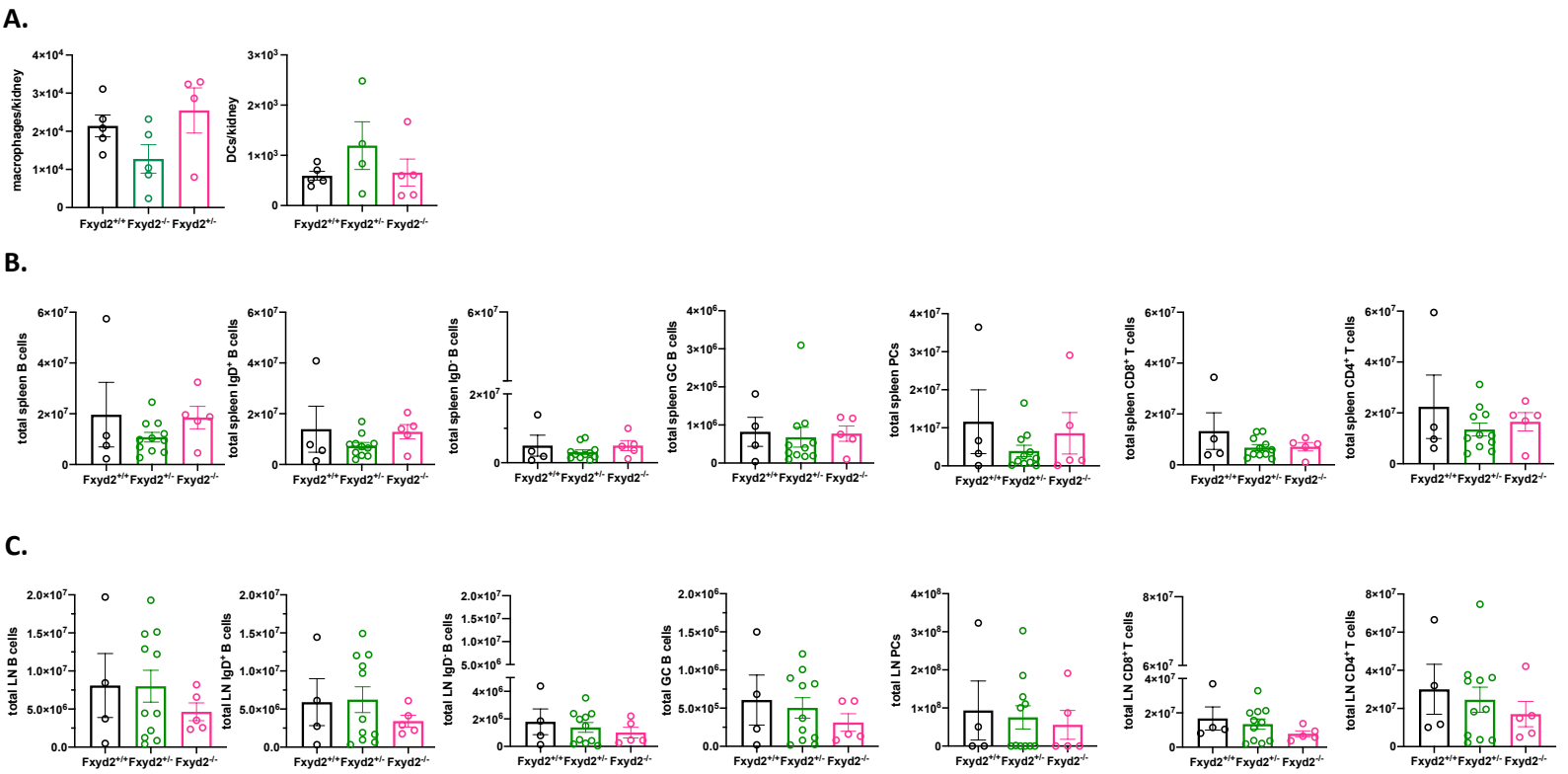
SFig. 4. MRL^{lpr} B cells have high αNa$^+$-K$^+$-ATPase expression. **(A)** αNa$^+$-K$^+$-ATPase is highly expressed on splenic B cells of newly weaned 3 week-old MRL^{lpr} mice (note: there are no kidney B cells at this timepoint). **(B)** αNa$^+$-K$^+$-ATPase expression on IgD$^+$ versus IgD$^-$ splenic B cells from 3 combined experiments. **(C)** IgD$^+$ versus IgD$^-$ B cells were isolated from spleens of MRL^{lpr} mice and cultured as indicated and viability measured as in Fig. 1C. **(D)** αNa$^+$-K$^+$-ATPase expression on IgD$^+$ versus IgD$^-$ B cells compared in kidney and spleen from 3 combined experiments. **(E)** IgD$^+$ and IgD$^-$ B cells were isolated from kidneys of water deprived and control MRL^{lpr} mice as previously described; data from 3 combined experiments are shown. **(F)** IgD$^+$ and IgD$^-$ kidney B cells were analyzed for apoptosis markers from mice described in **Supp. Fig. 3G**. **(G)** αNa$^+$-K$^+$-ATPase expression in mice from representative high salt experiment (Fig. 1E-I). **(H)** αNa$^+$-K$^+$-ATPase expression in mice from representative water deprivation experiment (Fig. 1J-N). **(I and J)** αNa$^+$-K$^+$-ATPase expression in MRL^{lpr} mice on B cells, CD4$^+$ and CD8$^+$ T cells in spleens (**I**) and on B cells, CD4$^+$ (LiveCD45.1$^-$CD8$^-$CD4$^+$) and CD8$^+$ (LiveCD45.1$^-$CD4$^-$CD8$^+$) T cells, dendritic cells (LiveCD45.1$^-$F4/80$^-$B220$^-$CD11c^{hi}MHCII^{hi}) and macrophages (LiveCD3$^-$MHCII$^+$F4/80$^+$) in kidneys (**J**). p value *<0.01, **<0.001, ***<0.0001, results not significant unless otherwise indicated.



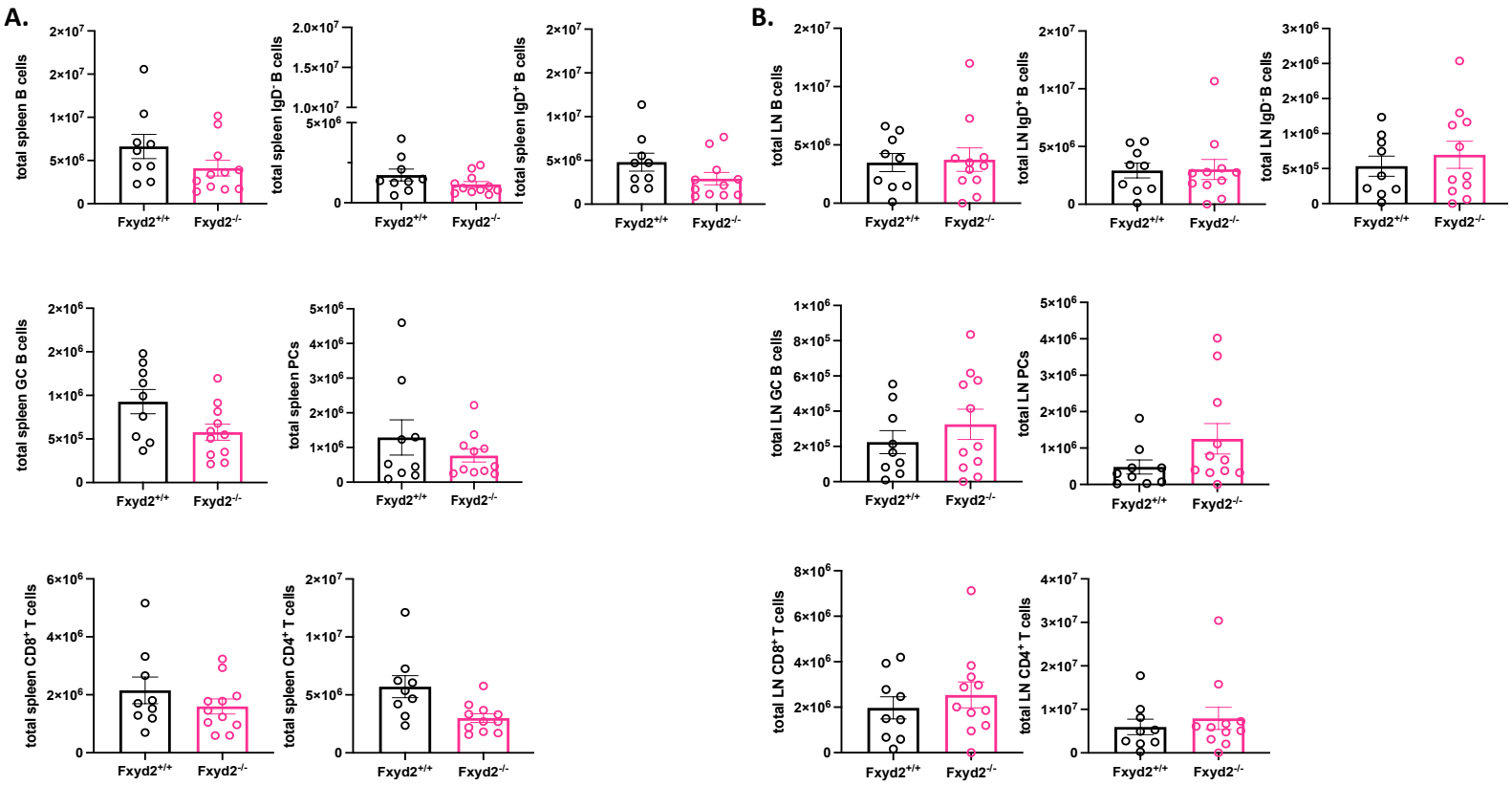
SFig. 5. Kidney T cells and innate cells and spleen and lymph node lymphocytes are unchanged by pharmacologic Na^+-K^+-ATPase blockade. **(A)** αNa-K-ATPase expression on kidney B cells following ouabain treatment. **(B)** $CD4^+$ T cells, $CD8^+$ T cells, dendritic cells and macrophages were quantified in the kidneys of untreated and ouabain-treated MRL^{lpr} mice as in **Supp. Fig. 4J**. **(C and D)** Splenic **(C)** and mesenteric and popliteal LN **(D)** lymphocyte subsets quantified by flow cytometry. IgD^+ and IgD^- B cell subsets are $LiveCD19^+B220^+IgD^{+/-}$, GC B cells are $LiveCD19^+B220^+IgD^-GL7^+$, T follicular helper cells are $LiveTCR\beta^+CD4^+CD44^{hi}PD1^{hi}CXCR5^{hi}$. All comparisons with p value >0.05.



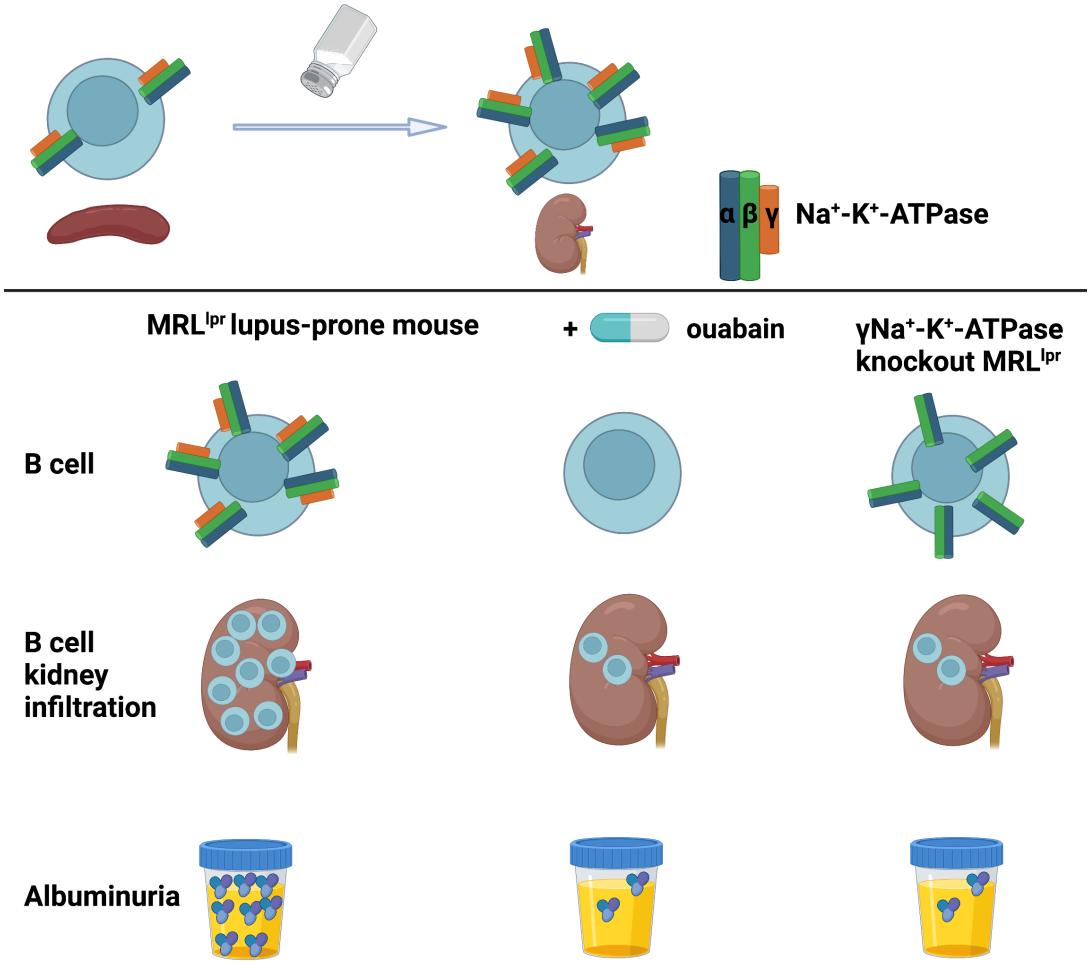
SFig.6. Pharmacological blockade of Na^+-K^+-ATPase decreases kidney B cells in NZW/BF_1 lupus-prone mice. NZW/BF_1 female mice were treated with PBS (control) or ouabain (Na^+-K^+-ATPase inhibitor) daily for 16 days and then sacrificed at 34 weeks of age. **(A)** Kidney interstitial B cells were quantified utilizing flow cytometry, gating strategy as in Supp Fig 1. **(B)** $CD4^+$ T cells, $CD8^+$ T cells, dendritic cells and macrophages were quantified in the kidneys of untreated and ouabain-treated MRL^{lpr} mice as in **Supp. Fig. 4J**. **(C)** Sera anti-dsDNA antibodies as measured by ELISA from control and ouabain-treated mice at d17 post-treatment. **(D)** Blood urea nitrogen (BUN) in mg/dL. **(E)** Semiquantitative urine dipstick analysis for proteinuria and colorimetric urine albumin/Cr measurement. **(F and G)** Splenic **(F)** and mesenteric and popliteal LN **(G)** lymphocyte subsets quantified by flow cytometry as in **Supp. Fig 5**. p value *<0.05, results not significant unless otherwise indicated.



SFig.7. Kidney innate cells and spleen and lymph node lymphocytes are unaffected in MRL^{lpr}.*Fxyd2*$^{+/-}$ and MRL^{lpr}.*Fxyd2*$^{-/-}$ animals. Kidney innate cells and spleen and LN lymphocytes were examined in MRL^{lpr}.*Fxyd2*$^{+/+}$, MRL^{lpr}.*Fxyd2*$^{+/-}$, and MRL^{lpr}.*Fxyd2*$^{-/-}$ mice as described in **Supp. Fig. 4**. **(A)** Kidney dendritic cells and macrophages were quantified as described. **(B)** Spleen lymphocyte subsets quantified by flow cytometry. **(C)** Mesenteric and popliteal LN subsets quantified by flow cytometry. All comparisons with p value >0.05.



SFig.8. Spleen and lymph node B cell and T cell subsets are unchanged in MRL^{lpr}.*Fxyd2*$^{-/-}$ chimeras as compared to controls. Subset gating strategy as outlined in Supp. Fig. 4. **(A)** Spleen lymphocyte subsets quantified by flow cytometry. **(B)** Mesenteric and popliteal LN subsets quantified by flow cytometry. All comparisons with p value >0.05.



SFig.9. Model. Created with Biorender.com.

Patient	1	2	3	4
Age at biopsy (years)	24	59	28	33
Sex	Female	Female	Female	Female
Race (self-reported)	Black	Black	Black	Black
Serum Creatinine (mg/dL)	2.3	8.5	2.2	1.3
eGFR (mL/min/1.73m^2)	27	5	31	60
ESRD on dialysis	Yes	Yes	No	No
Lupus Nephritis Class	4,5	4	4,5	4,5
Activity Index	5	4	14	3
Chronicity Index	6	9	0	7
Crescents (%)	Yes (9-15%)	No	Yes (60%)	No
Urine Pr/Cr	3.2	8.5		2.7
24hr urine protein (mg/24h)	5670		10570	1936
Dipstick proteinuria	3+	3+	3+	1+
C3 complement (mg/dL)	87	39		93
C4 complement (mg/dL)	15	7.4		21
SLEDAI 2K Score	16	12	12	4
Hypertension	No	Yes	No	Yes
Post-biopsy disease-modifying medications	Prednisone 20mg, MMF, hydroxychloroquine	Prednisone 60mg, MMF, hydroxychloroquine	IV pulse steroids, prednisone 40mg, induction and maintenance cyclophosphamide, hydroxychloroquine	MMF (induction only)

Supp. Table 1. Demographic information for lupus nephritis kidney biopsy patients.

Antibody	Company	Clone	Catalog #	Dilution
GL7-FITC	BD Pharmingen™		553666	1:200
TCRβ-FITC	BD Pharmingen™	H57-597	553170	1:200
donkey anti-rabbit AF488™	Invitrogen™		A-21206	1:400
CD45.1-PE	Invitrogen™	A20	12-0453-83	N/A
CD19-PECy7	Invitrogen™	1D3	25-0193-82	1:200
PD1-PECy7	BioLegend®	BMP1-30	109110	1:200
B220-PE-CF594	BD Horizon™	RA3-6B2	562290	1:500
CD44-PE/Dazzle™594	BioLegend®	IM7	103056	1:200
goat anti-rabbit AF647	Invitrogen™		A-21244	1:400
CD4-PacBlu	BD Pharmingen™	BM4-5	558107	1:200
CD138-BV421	BD Horizon™	281-2	562610	1:200
IgD-BV605	BioLegend®	11-26c2a	405727	1:500
CXCR5-BV605	BioLegend®	L138D7	145513	1:200
CD19-BV711	BioLegend®	6D5	115555	1:200
CD8α-BV711	BioLegend®	53-6.7	100748	1:200
CD95-BV711	BD Optibuild™	Jo2	740716	1:200
CD16/32 purified	BD Pharmingen™	2.4G2	553142	N/A
Fixable Viability Dye eFluor™780	eBioscience™		65-0865-18	1:1000

Supp. Table 2. List of antibodies used for flow cytometry and microscopy.

sgRNA		
Name	**Forward**	**Reverse**
Fxyd2 – guide 1	5’-caccgACCGGCTTACCGTACTCGAA-3’	5’-aaacTTCGAGTACGGTAAGCCGGTc-3’
Fxyd2 – guide 2	5’-caccgTATGAAACCGTCCCGCAAAGG-3’	5’-aaacCCTTTGCGGACGGTTTCATAc-3’
Fxyd2 – guide 3	5’-caccgGATGAGGAGGCCCACGACGA-3’	5’-aaacTCGTCGTGGGCCTCCTCATCc-3’
Fxyd2 – guide 4	5’-caccgCCCACTTACTGAGAATGATG-3’	5’-aaacCATCATTCTCAGTAAGTGGGc-3’
RT- PCR		
Name	**Forward**	**Reverse**
Fxyd2	5’-ACTATGAAACCGTCCGCAAA-3’	5’-TTCTTACCGCCCCCACAG-3’
βActin	5’-TGTCCACCTTCCAGCAGATGT-3’	5’-AGCTCAGTAACAGTCCGCCTAG-3’

Supp. Table 3. Primers used for CRISPR and qPCR experiments