IMMUNOLOGY

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

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The kidney is a comparatively hostile microenvironment characterized by highsodium concentrations; however, lymphocytes infiltrate and survive therein in autoimmune diseases such as lupus. The effects of sodium-lymphocyte interactions on tissue injury in autoimmune diseases and the mechanisms used by infiltrating lymphocytes to survive the highsodium environment of the kidney are not known. Here, we show that kidney-infiltrating B cells in lupus adapt to elevated sodium concentrations and that expression of sodium potassium adenosine triphosphatase (Na^+-K^+-ATPase) correlates with the ability of infiltrating cells to survive. Pharmacological inhibition of Na^+-K^+-ATPase and genetic knockout of Na^+-K^+-ATPase γ subunit resulted in reduced B cell infiltration into kidneys and amelioration of proteinuria. B cells in human lupus nephritis biopsies also had high expression of Na^+-K^+-ATPase. Our study reveals that kidney-infiltrating B cells in lupus initiate a tissue adaption program in response to sodium stress and identifies Na^+-K^+-ATPase as an organ-specific therapeutic target.

INTRODUCTION

Systemic lupus erythematosus (SLE; lupus) is a multiorgan autoimmune disease characterized by antibody deposition in target tissues (*1*). Lupus nephritis is a leading cause of morbidity and mortality with 10% of those afflicted progressing to end-stage renal disease (*1*). Kidneys of patients with lupus are characterized by immune cell infiltration including T and B cells with the degree of infiltrate correlating with tissue damage, disease severity, and renal survival. The presence of B cell infiltrates in human kidney biopsies is correlated with higher creatinine, blood urea nitrogen (BUN), and urine protein as compared to biopsies without B cells (*2*–*7*). However, the extent to which tissue injury is mediated by systemic versus in situlymphocytes remains uncertain. While B cell depletion is a mainstay of treatment for many patients with SLE, it targets both systemic and tissue B lymphocytes and is associated with whole body immunosuppressive side effects (*8*). Thus, specifically targeting kidney lymphocytes presents an attractive approach for the treatment of lupus nephritis. However, little is known about how pathogenic immune cells survive and function in the renal microenvironment.

The renal microenvironment presents unique challenges for infiltrating lymphocytes that may shape their phenotype and function. We have recently demonstrated that reduced oxygen tension, a feature common to the microenvironment of the kidney in comparison to that of the blood, promotes T cell–mediated tissue damage in lupus nephritis (*9*). Another feature of the kidney microenvironment essential to its function is the marked axial concentration gradient of sodium (Na^+), up to twofold higher than serum, resulting in an environment where the cells are bathed in "a variable but hypertonic interstitial solution" (*10*). This Na^+ gradient is maintained under homeostatic, diuretic, and water deprivation conditions (*10*). The mechanisms and pathways used by immune cells to adapt to the highsodium concentration (high [Na^+], hypernatremic) environment have not been fully characterized.

A potential role for Na^+ in modulating autoimmunity has recently garnered attention. In lupus, increased Na^+ content in the muscle and skin, in comparison to that in the serum, has been correlated with increased disease activity as assessed by the SLE Disease Activity Index score and higher circulating concentrations of the cytokine interleukin-10 (IL-10) (*11*). Exposure in vitro or in vivo to elevated [Na^+] beyond that found in plasma promotes differentiation of potentially pathogenic T helper 17 (T_H17) cells in autoimmunity (*12*, *13*) leading to an enhanced disease phenotype in a mouse model of multiple sclerosis (*12*). Lupus-prone mice fed a high-salt diet display increased numbers of splenic T_H1 and T_H17 cells and more severe renal disease and increased mortality compared to control animals (*14*). Sodium-driven differentiation and activation of dendritic cells and T lineage lymphocytes have also been implicated in increased disease severity in lupus-prone mice (*15*–*17*). Increased [Na^+] decreases survival and differentiation of B cells in vitro (*18*); however, its effect on potentially pathogenic, kidney-infiltrating B cells in SLE is not known (*4*–*6*).

The pathways mediating cell survival in high [Na^+] remain incompletely characterized in most renal cell types, including lymphocytes, although some insights can be gained from the kidney epithelial cell literature. Kidney epithelial cells up-regulate sodium potassium adenosine triphosphatase (Na^+-K^+-ATPase), a constitutively expressed transporter moving three Na^+ molecules extracellularly and two potassium (K^+) molecules intracellularly against their concentration gradients, to promote survival under hyperosmolar conditions (*19*, *20*). Expression of the γ subunit (encoded by *Fxyd2*) of Na^+-K^+-ATPase (γNa^+-K^+-ATPase) is essential for epithelial cell survival upon adaptation to highsalt conditions, with

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disruption of *Fxyd2* via interfering RNA leading to cell death in hypertonic medium (*21*). *Fxyd2* overexpression has the opposite, protective effect (*22*).

We now demonstrate that B cells from several strains of lupus-prone mice exhibit enhanced survival in high [Na^+] compared to nonautoimmune C57BL/6 (B6) mice, a phenotype mediated via increased Na^+-K^+-ATPase expression. B cells further demonstrated high expression of γNa^+-K^+-ATPase, not previously known to be expressed in immune cells. Pharmacologic blockade and genetic manipulation of Na^+-K^+-ATPase reduced renal B cell infiltration and improved lupus nephritis disease parameters in an organ-specific manner. Last, we demonstrate expression of Na^+-K^+-ATPase, including γNa^+-K^+-ATPase, in kidney B cells in biopsies of patients with SLE, positing modulation of Na^+-K^+-ATPase as a therapeutic target for lupus nephritis.

RESULTS

B cells from lupus-prone mice exhibit enhanced survival in high [Na^+]

We isolated B cells from nephritic kidneys of 14- to 15-week-old lupus-prone MRL/MpJ-*Fas^{lpr}*/J (MRL^{lpr}) and wild-type age-matched B6 mice and quantified them using flow cytometry as described previously (fig. S1) (*9*). The nephritic kidneys of MRL^{lpr} animals were characterized by a lymphocytic infiltrate consisting of T cells (*3*) and B cells, compared to control B6 mice with minimal lymphocytic infiltrates (Fig. 1A). We next dissected nephritic kidneys into anatomical subsections and showed that intrarenal B cells are found throughout the cortex and outer and inner medullas (Fig. 1B), the latter of which experiences [Na^+] up to twofold higher than serum (*10*). Most intrarenal B cells were found in the hypernatremic inner medulla (Fig. 1B). To better understand how B cells can tolerate this extreme [Na^+], we exposed activated splenic B cells from MRL^{lpr} and B6 mice to increasing sodium chloride (NaCl) concentrations, as numbers of renal B cells were limiting for in vitro experiments. While B cells from MRL^{lpr} mice had enhanced survival in isotonic medium, this phenotype was further augmented in NaCl-supplemented medium when compared to cells from B6 animals (Fig. 1C). To determine whether the MRL^{lpr} B cell survival advantage was due to [Na^+] rather than tonicity, we cultured cells with NaCl, Na gluconate (Na^+ control) and mannitol (tonicity control). While MRL^{lpr} B cells better endured an elevated mannitol concentration than B6 controls, likely reflecting their enhanced comparative viability, mannitol had no effect on survival compared to that observed in normal medium (fig. S2A). Meanwhile, Na^+ gluconate recapitulated the effects of NaCl in both B6 and MRL^{lpr} B cells, pointing toward a Na^+-mediated effect on survival (fig. S2A). The increased viability of MRL^{lpr} B cells was not due to the mutation in the Fas death receptor that is responsible for their lymphoproliferative (lpr) phenotype as B cells from age-matched Fas-intact MRL/MpJ (MRL$^{+/+}$) mice evidenced the same enhanced survival in Na^+-supplemented medium as those from Fas-mutated MRL^{lpr} animals (Fig. 1D and fig. S2B). Although survival was diminished as [Na^+] increased, cell division in MRL^{lpr} B cells was unchanged, suggesting that elevated [Na^+] affects survival but not cellular activation programs (fig. S2C).

We next investigated the mode of cell death that B cells undergo in response to high [Na^+]. Flow cytometric staining for two markers of apoptosis, annexin V (*23*, *24*) and cleaved caspase-3 (*25*, *26*), demonstrated that nearly all B cells from MRL^{lpr} lupus-prone animals undergo apoptotic cell death when exposed to high [Na^+] (fig. S3, A and B). Moreover, there was no evidence of secretion of classic markers of pyroptosis such as cleaved gasdermin D or IL-1β protein (fig. S3, C and D). While B cells from both wild-type B6 animals and MRL^{lpr} lupus-prone mice underwent apoptotic cell death in response to high [Na^+], we observed a higher percentage of caspase-3/intracellular amine double-positive B6 B cells, suggesting that increased Na^+-induced apoptosis is responsible for the survival disadvantage in the B6 strain (fig. S3E). Meanwhile, B cells from MRL$^{+/+}$ mice again phenocopied their MRL^{lpr} counterparts, demonstrating that this Na^+-induced death pathway was Fas independent (fig. S3F).

While at an advantage compared to B6 B cells, MRL^{lpr} B cells nonetheless had reduced survival at very high [Na^+] in vitro, suggesting that increased in vivo [Na^+] may have a similar effect on intrarenal B cells in lupus. To address this experimentally, we increased interstitial renal [Na^+] in two ways. First, we placed MRL^{lpr} animals on a highsodium diet (8% NaCl in chow and 1% NaCl in tap water) as previously described (Fig. 1E) (*27*). Intrarenal B cell numbers were decreased in the highsalt group as compared to littermate controls given normal chow and ad libitum tap water (Fig. 1F). This depletion of intrarenal B cells was accompanied by a decrease in BUN. While serum [Na^+] and proteinuria were unchanged as expected (Fig. 1, G and H), serum creatinine was elevated in the highsalt group (Fig. 1I) as has been previously described in rodent models with decreased renal reserve (*28*, *29*). Second, we increased renal interstitial [Na^+] with water deprivation (Fig. 1I). Intrarenal B cell numbers were decreased in animals after 48 hours of water deprivation compared to those with ad libitum water access (Fig. 1, J and K), consistent with the findings in the high-sodium diet experiments. Moreover, a higher percentage of kidney B cells in the water-deprived animals were positive for cleaved caspase-3 and an intracellular amine, suggesting that kidney B cells undergo Na^+-induced caspase-3–mediated cell death in vivo similarly to their splenic counterparts in vitro (fig. S3G). Serum [Na^+] was increased in the water deprivation group as expected (Fig. 1L). There was a trend toward an increase in proteinuria in the water deprivation group that is likely due to the known limitation of the assay in concentrated urine (Fig. 1M) (*30*). Similarly, the increase in creatinine was difficult to interpret given that water deprivation can lead to kidney injury and concomitant rise in BUN in a lupus-independent manner (Fig. 1N) (*31*). Together, these in vivo approaches showed that elevated kidney [Na^+] leads to decreased intrarenal B cell numbers and raised questions about the mechanisms infiltrating B cells use to handle the hypernatremic kidney environment under homeostatic conditions.

Na^+-K^+-ATPase mediates in vitro survival of B cells from lupus-prone mice in high [Na^+] and is up-regulated on intrarenal B cells

Renal epithelial cells up-regulate Na^+-K^+-ATPase (Fig. 2A) as one mechanism to handle hyperosmolar stress (*21*, *22*), which led us to question whether intrarenal lymphocytes use a similar program. When B cells from MRL^{lpr} mice were cultured in high [Na^+], surface expression of the α subunit of Na^+-K^+-ATPase (αNa^+-K^+-ATPase) was up-regulated (Fig. 2B). To test whether the enhanced survival in high [Na^+] was due to Na^+-K^+-ATPase, we cultured B

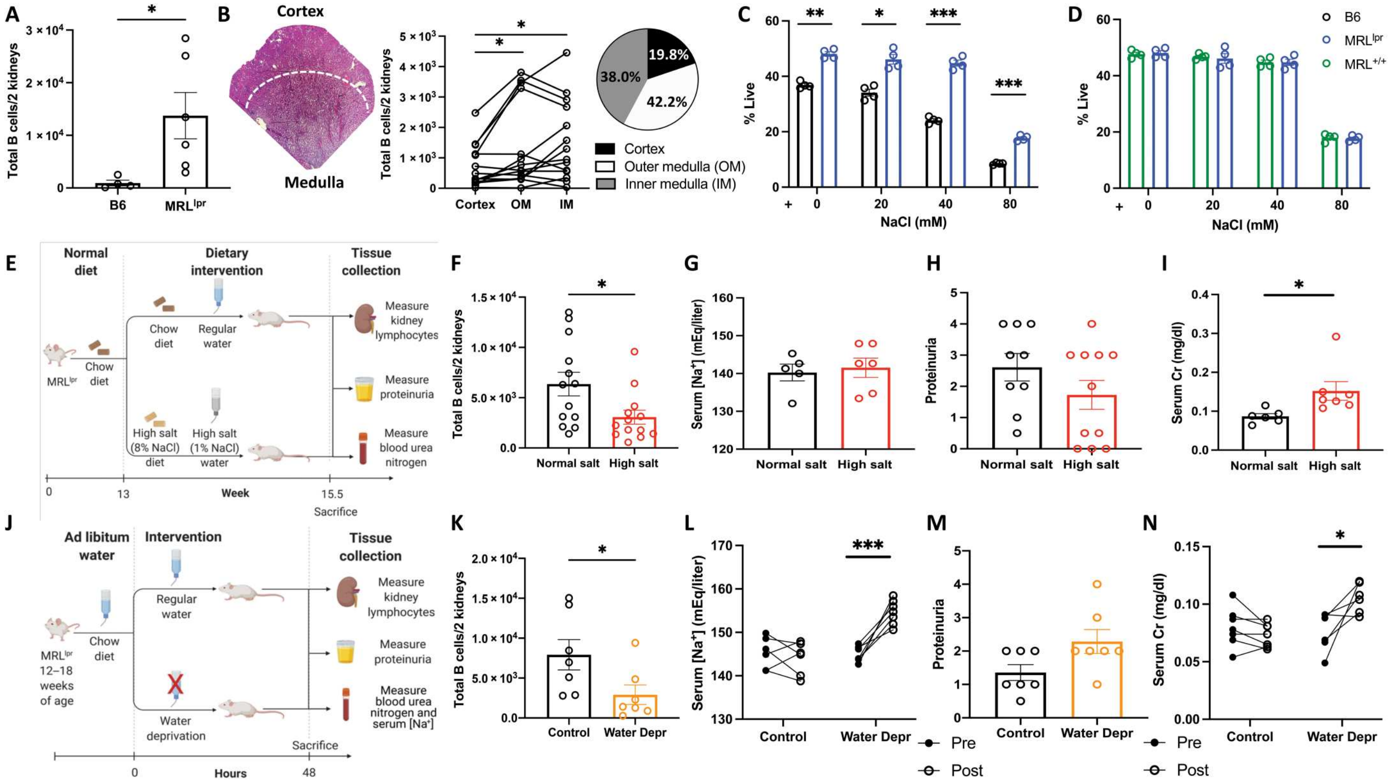

Fig. 1. B cells from lupus-prone mice exhibit enhanced survival under hypernatremic conditions compared to wild-type lymphocytes but are unable to sustain the advantage at very high [Na⁺]. (**A**) Tissue-resident B cells from kidneys from B6 and MRL^{lpr} mice were quantified via flow cytometry. (**B**) MRL^{lpr} kidneys were separated into cortex, outer and inner medulla, and B cells quantified as in (A); combined from four independent experiments. Representative histology is shown. (**C**) Splenic B cells from B6 and MRL^{lpr} mice were cultured in NaCl-supplemented medium with lipopolysaccharide (5 μg/ml) in quadruplicate; representative of >3 independent experiments. (**D**) B cells from MRL$^{+/+}$ and MRL^{lpr} mice were cultured as in (C). (**E**) Study design for high-salt diet in MRL^{lpr} mice; created with BioRender.com. (**F**) Kidney B cells of control and high salt–treated MRL^{lpr} mice were quantified as in (A); combined from two independent experiments. (**G**) Serum [Na^+] (in milliequivalents/liter) on a subset of mice shown in (F). (**H**) Semiquantitative dipstick proteinuria for mice in (F). (**I**) Serum creatinine (Cr; in milligrams per deciliter) on a subset of mice shown in (F). (**J**) Study design for water deprivation in MRL^{lpr} mice; created with BioRender.com. "Pre" and "post" measurements were obtained at $t = 0$ and $t = 48$ hours, respectively. (**K**) Kidney B cells quantified as in (A); representative of >2 independent experiments. (**L**) Serum [Na^+] (in milliequivalents per liter) for mice shown in (K). (**M**) Semiquantitative dipstick proteinuria for mice in (K). (**N**) Serum creatinine (in milligrams per deciliter) for mice shown in (K). *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$; results are not significant unless indicated.

cells in the presence or absence of its inhibitor, ouabain. MRL^{lpr} B cells cultured in high [Na^+] in the presence of ouabain had significantly fewer live cells compared to cells in high [Na^+] or ouabain alone (Fig. 2C). This synergistic effect was not as robust in B cells from control B6 mice (Fig. 2C). B cells from control B6 animals also expressed substantially less αNa^+-K^+-ATPase at baseline than their MRL^{lpr} counterparts (Fig. 2D). Together, these data suggested that the viability advantage under high [Na^+] conditions observed in MRL^{lpr} B cells was mediated via Na^+-K^+-ATPase. Consistent with this notion, intrarenal B cells evidenced high αNa^+-K^+-ATPase expression that was persistently higher than splenic B cells in the same animal (Fig. 2, D to F). High αNa^+-K^+-ATPase expression appeared to be independent of autoimmune activation as MRL^{lpr} B cells had high αNa^+-K^+-ATPase at weaning, before the manifestation of autoimmune disease (fig. S4A). Furthermore, splenic naïve [immunoglobulin D^+ (IgD^+)] and activated (IgD^-) B cells had equivalent αNa^+-K^+-ATPase expression (fig. S4B) and exhibited similar survival in high [Na^+] in vitro (fig. S4C). In contrast, IgD^- kidney B cells expressed less αNa^+-K^+-ATPase compared to IgD^+ B cells, although both renal B cells subsets evidenced higher αNa^+-K^+-ATPase expression than their splenic counterparts (fig. S4D). Analogous to the in vitro splenic data, the higher expression on IgD^+ kidney B cells did not appear to be additionally protective as both naïve and activated B cells were depleted under high kidney [Na^+] conditions in vivo (fig. S4, E and F). We also did not observe further up-regulation of αNa^+-K^+-ATPase as renal interstitial [Na^+] was increased, such as in the inner medulla or under conditions of high-salt diet or water deprivation, suggesting an upper limit to αNa^+-K^+-ATPase regulation by elevated [Na^+] (Fig. 2G and fig. S4, G and H). Last, MRL^{lpr} B cells expressed higher αNa^+-K^+-ATPase than $CD4^+$ or $CD8^+$ T cells, dendritic cells, or macrophages in the same animals (Fig. 2D and fig. S4, I and J), suggesting that B cells are unique among immune cells in up-regulating Na^+-K^+-ATPase for survival in high [Na^+].

We next probed whether αNa^+-K^+-ATPase–mediated enhanced survival in high salt was at play in other mouse models of lupus, including B6.*Fas^{lpr}* (B6^{lpr}), Fas-intact MRL$^{+/+}$, and (NZB × NZW)F_1 (NZBWF_1). These various models recapitulate different aspects of lupus, with B6^{lpr} and MRL$^{+/+}$ mice developing relatively mild lupus nephritis at >9 months of age, while NZBWF_1 animals

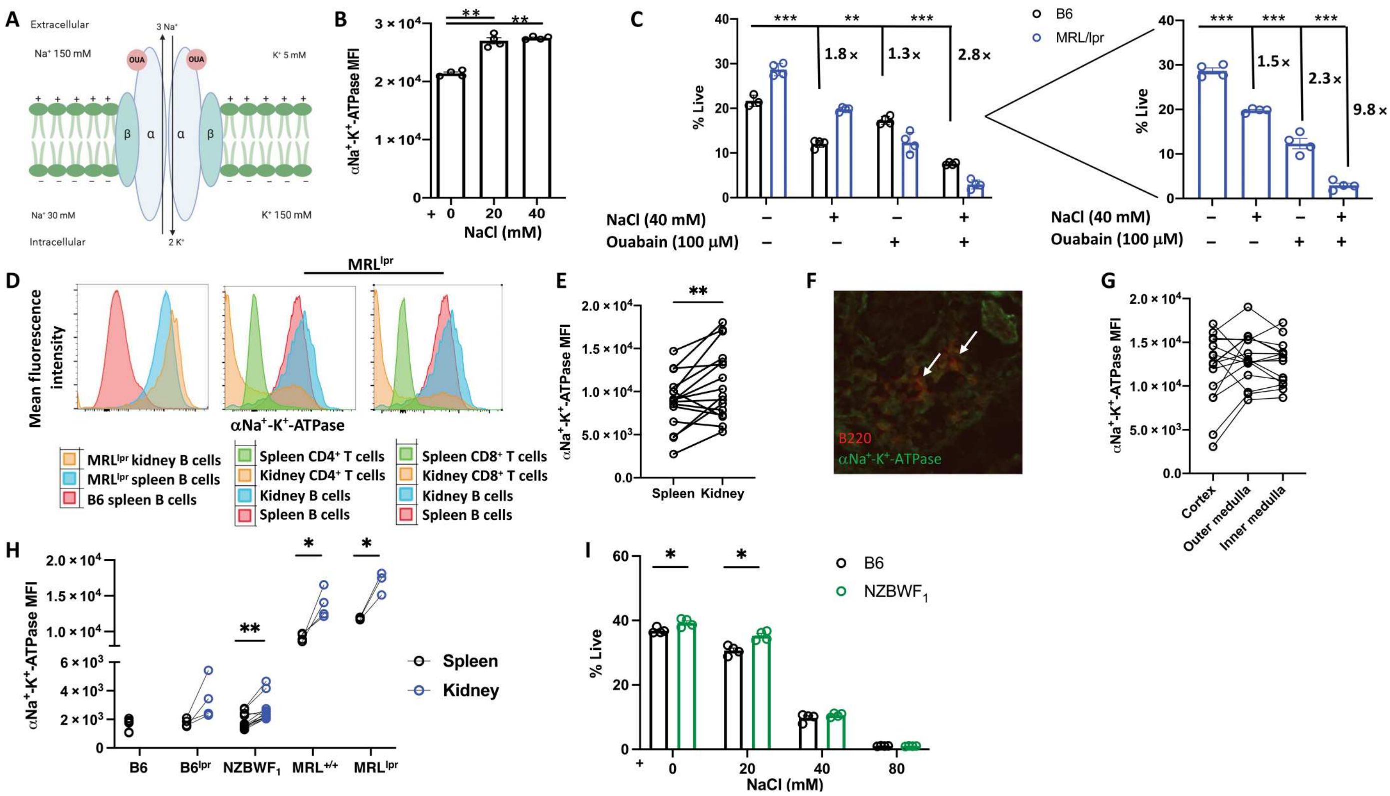

Fig. 2. Na⁺-K⁺-ATPase is up-regulated on B cells in lupus-prone kidneys and mediates the enhanced survival under highNaCl conditions. Lymphocytes isolated from spleens and kidneys of the indicated mouse strains were assayed by flow cytometry. Gating strategy in fig. S1. (**A**) Structure of Na^+-K^+-ATPase; oua, ouabain (small-molecule inhibitor of Na^+-K^+-ATPase); created with BioRender.com. (**B**) αNa^+-K^+-ATPase quantified on MRL^{lpr} B cells cultured under highNaCl conditions as determined by flow cytometry. (**C**) B6 and MRL^{lpr} B cells were cultured with +40 mM NaCl, +100 μM ouabain, or both. Left panel shows MRL^{lpr} data and right panel shows comparison B6 data. Fold change compared to control normal medium is shown; representative of three independent experiments. Asterisks indicate comparison to normal medium condition. (**D**) Representative flow cytometric staining of αNa^+-K^+-ATPase in B6 and MRL^{lpr} mice. (**E**) αNa^+-K^+-ATPase expression quantified on spleens and kidneys of MRL^{lpr} mice; combined from three independent experiments. (**F**) Representative immunofluorescence microscopy of αNa^+-K^+-ATPase on renal B cells of MRL^{lpr} mice; representative of three mice. (**G**) αNa^+-K^+-ATPase expression across different kidney compartments; combined from three independent experiments. (**H**) αNa^+-K^+-ATPase expression in the spleen and kidney of lupus-prone strains compared to B6 controls: $B6^{lpr}$, $MRL^{+/+}$, MRL^{lpr}, and $NZBWF_1$. (**I**) Splenic B cells from $NZBWF_1$ mice were cultured as in Fig. 1C; representative of three independent experiments. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$; results are not significant unless otherwise indicated. MFI, mean fluorescence intensity

develop severe renal disease, albeit at later ages than the MRL^{lpr} strain (*32*–*36*). αNa^+-K^+-ATPase was up-regulated in kidney-infiltrating B cells, as compared to the spleen, in each of these lupus-prone strains, although the expression, as measured by mean fluorescence intensity, differed with high baseline expression correlating with the MRL background and not the lpr mutation (Fig. 2H). In vitro examination of B cells from $NZBWF_1$ mice, a strain with relatively low splenic αNa^+-K^+-ATPase expression, demonstrated decreased B cell survival in high [Na^+], largely phenocopying the control B6 phenotype and further pointing toward αNa^+-K^+-ATPase expression and not strain background as a key factor facilitating survival under highsalinity conditions (Fig. 2I). It is tempting to speculate that B cells from strains with modest up-regulation of αNa^+-K^+-ATPase are disadvantaged from infiltrating the hypernatremic kidney environment, but direct interstrain comparisons are challenging due to stochastic and temporally varying disease onset.

Na^+-K^+-ATPase blockade ablates intrarenal B cells

Given the expression pattern of Na^+-K^+-ATPase and its apparent role in B cell survival under conditions of increased [Na^+], we next sought to investigate its role on B cells in vivo. Genetic knockouts of αNa^+-K^+-ATPase are embryonic lethal (*37*), making pharmacologic blockade a preferred approach. Because Na^+-K^+-ATPase mediates B cell survival in high [Na^+] in vitro, we hypothesized that in vivo Na^+-K^+-ATPase blockade would lead to kidney B cell ablation and possible amelioration of impaired renal function in lupus nephritis while circumventing systemic immunosuppression. To this end, we intravenously administered ouabain, a Na^+-K^+-ATPase small-molecule inhibitor, to MRL^{lpr} mice for 16 days. Numbers of intrarenal B cells were significantly decreased in the ouabain-treated animals, with a more significant decrease in the activated IgD^- B cell pool (Fig. 3A). Notably, ouabain treatment did not alter Na^+-K^+-ATPase surface expression on renal B cells (fig. S5A). Numbers of intrarenal plasma cells, $CD4^+$ and $CD8^+$ T cells, macrophages and dendritic cells were largely unaffected by ouabain treatment, suggesting that they are less sensitive to Na^+-K^+-ATPase inhibition and possibly use alternative survival

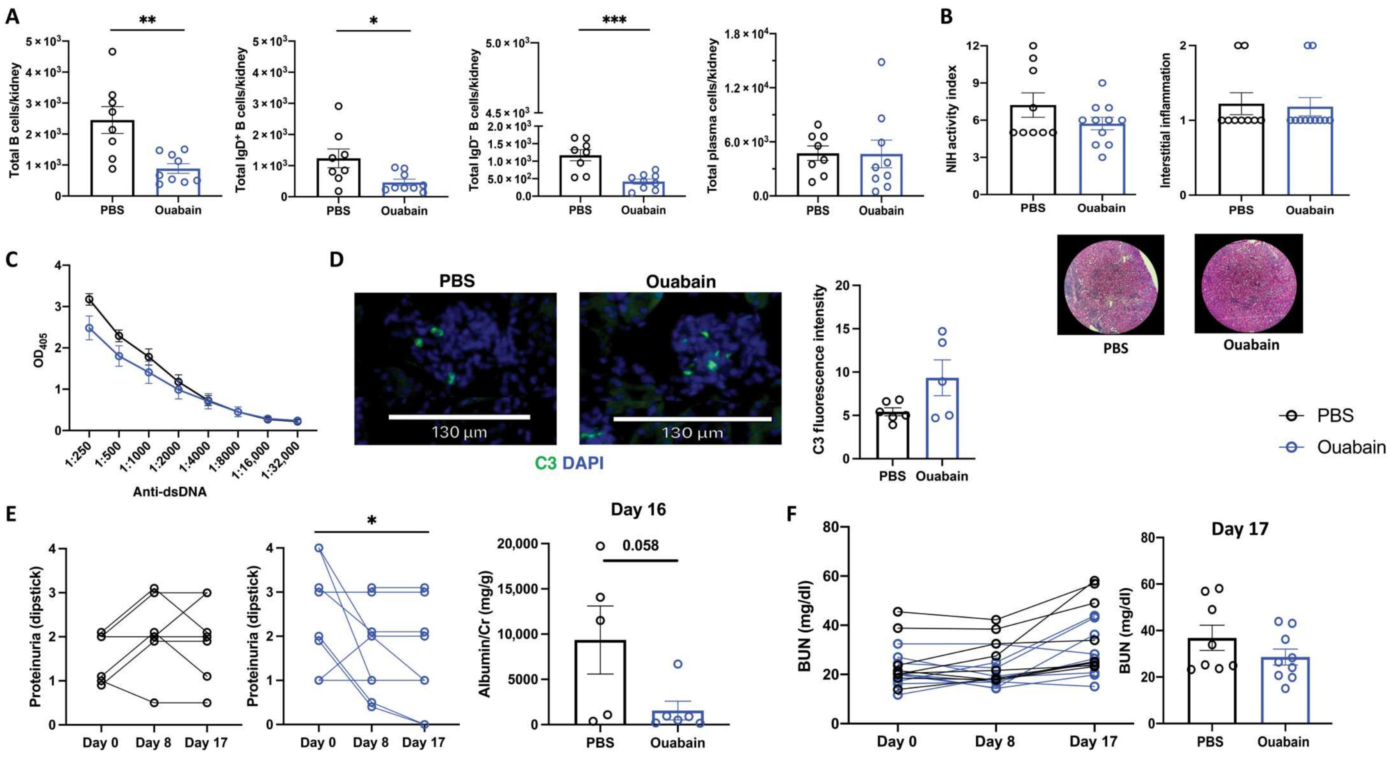

Fig. 3. Pharmacological blockade of Na^+-K^+-ATPase ablates kidney B cells and improves proteinuria. MRL^{lpr} female mice were treated with phosphate-buffered saline (PBS; control) or ouabain (Na^+-K^+-ATPase inhibitor) daily for 16 days and then euthanized at 19 to 22 weeks of age. Data from two combined experiments are shown, of five total experiments. (**A**) Kidney interstitial B cells were quantified using flow cytometry, gating strategy as in fig. S1. (**B**) Representative histology from control and treated mice and quantitated pathological scores based on the National Institutes of Health (NIH) activity scale. (**C**) Sera anti-dsDNA antibodies as measured by enzyme-linked immunosorbent assay (ELISA) from control and ouabain-treated mice at day 17 after treatment. OD_{405}, optical density at 405 nm. (**D**) C3 (green) and 4′,6-diamidino-2-phenylindole (DAPI; blue) immunofluorescent staining in glomeruli of control and ouabain-treated mice, fluorescence intensity quantified across multiple glomeruli and multiple animals using ImageJ software. (**E**) Semiquantitative urine dipstick analysis for proteinuria and colorimetric urine albumin/creatinine measurement. (**F**) BUN in milligrams per deciliter. $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$; results are not significant unless otherwise indicated.

mechanisms in the kidney (Fig. 3A and fig. S4B). Consistent with preserved T cell numbers, histological renal inflammation, as measured by quantification of interstitial immune cells on hematoxylin and eosin (H&E)–stained fixed sections, was not different between the ouabain-treated and untreated control MRL^{lpr} groups (Fig. 3B) (*38*, *39*). B cell and T cell subsets in the spleen and lymph nodes were also unchanged by ouabain treatment, consistent with the notion that it is specifically the B cells in the hypernatremic kidney environment that are sensitive to Na^+-K^+-ATPase inhibition (fig. S5, C and D). Consistent with this lack of systemic B cell effect, autoantibodies to double-stranded DNA (dsDNA) and antibody deposition in the kidney, as measured by complement 3 (C3) staining, were unaffected by ouabain treatment (Fig. 3, C and D). However, proteinuria in the treated group was significantly improved even within the short treatment period (Fig. 3E). Serum BUN was unchanged (Fig. 3F). Ouabain treatment in the NZBWF$_1$ lupus model demonstrated a similar effect on kidney B cells (fig. S6). The lack of an effect on systemic B cells, BUN, and autoantibodies and the correlation between improved proteinuria and decreased intrarenal B cell numbers suggest differential contributions of systemic versus kidney B cells to renal injury, although effects of ouabain on epithelial cells cannot be eliminated as a contributing factor.

γNa^+-K^+-ATPase regulates [Na^+]-dependent B cell survival

While Na^+-K^+-ATPase is typically composed of two α and two β subunits (Fig. 2A), at least five auxiliary subunits of Na^+-K^+-ATPase have been described to regulate its activity in a tissue- and isoform-specific way (*40*). γNa^+-K^+-ATPase, the γ subunit of the Na^+-K^+-ATPase encoded by *Fxyd2*, is up-regulated by kidney tubule cells in response to hypertonicity (Fig. 4A) (*21*, *22*); however, its expression has not previously been described in immune cells. Here, we show that γNa^+-K^+-ATPase expression was up-regulated when B cells were cultured in high [Na^+] (Fig. 4B). This up-regulation likely has functional significance, as CRISPR-mediated knockout of *Fxyd2* reduced survival of MRL^{lpr} B cells grown in Na^+-supplemented medium (Fig. 4C). Consistent with γNa^+-K^+-ATPase up-regulation in increased [Na^+], B cells in kidneys from lupus-prone mice demonstrated high *Fxyd2* expression compared to their splenic counterparts (Fig. 4D). We next generated γNa^+-K^+-ATPase–deficient (*Fxyd2*-deficient) MRL^{lpr} mice via marker directed backcrossing (*41*), breeding the mutation onto the lupus-prone MRL^{lpr} background (N6, >99% MRL^{lpr} background confirmed with marker-assisted genotyping; see Materials and Methods). This allowed us to confirm γNa^+-K^+-ATPase protein expression via immunofluorescence microscopy (Fig. 4E), although we were unable to validate the existing antibodies for flow cytometry. Overall, these data phenocopied our findings with

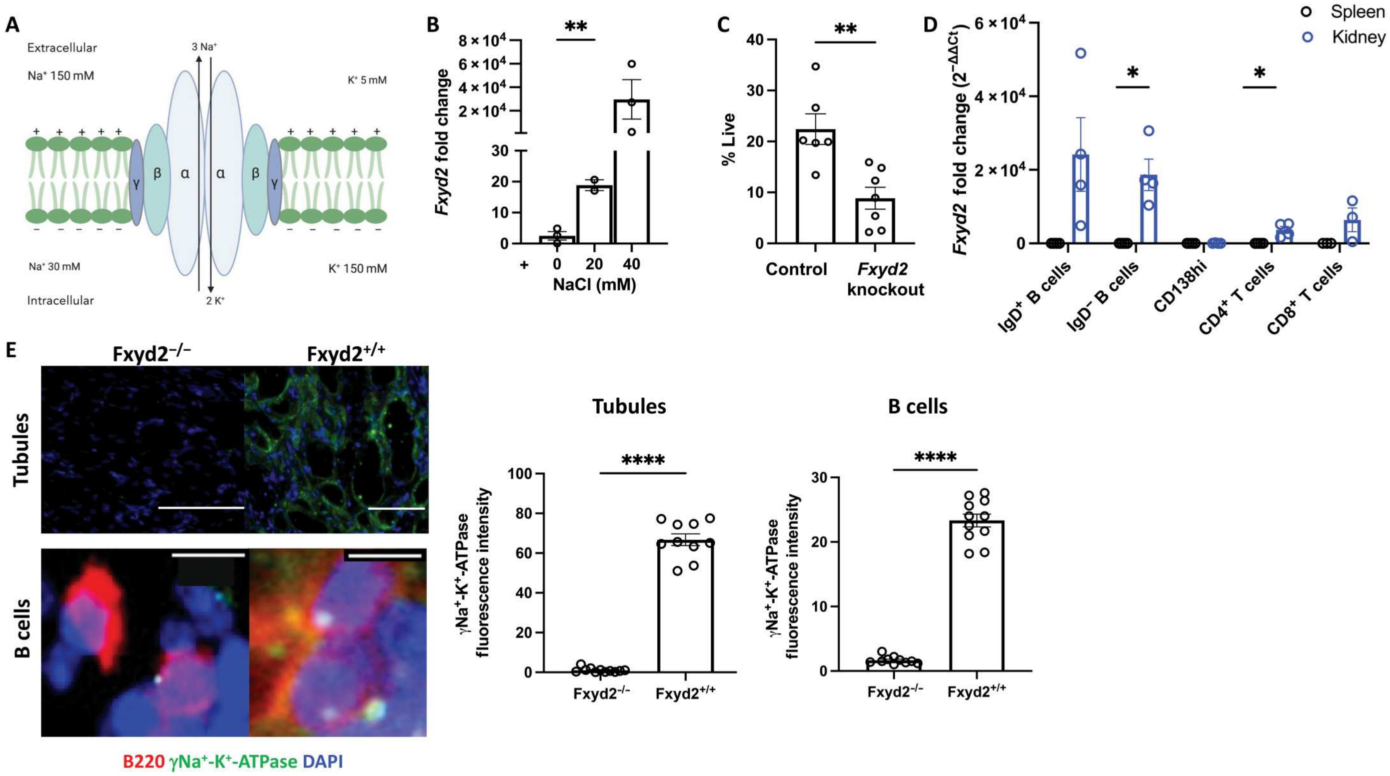

Fig. 4. Hypertonicity-responsive γNa⁺-K⁺-ATPase is up-regulated on kidney B cells in lupus-prone mice and partially mediates B cell survival in high salinity. (A) Structure of γ-containing Na⁺-K⁺-ATPase; created with BioRender.com. (B) *Fxyd2* expression from MRL^{lpr} B cells cultured as in Fig. 1C; representative of three independent experiments. (C) Splenic B cells from MRL^{lpr}-Cas9⁺ mice were transduced with green fluorescent protein–positive (GFP⁺) virus containing guide RNA against γNa⁺-K⁺-ATPase versus control guide RNA and then switched to highsalt medium 48 hours later (see Materials and Methods). GFP⁺ B cells were then analyzed at day 6 of culture for survival via flow cytometry; each condition was done in triplicate, and combined data from two independent experiments are shown. (D) B cell and T cell subsets were sorted from spleens and kidneys of MRL^{lpr} mice and queried for *Fxyd2* (γNa⁺-K⁺-ATPase gene) expression via real-time polymerase chain reaction (RT-PCR) in quadruplicate; data from four independent animals are shown. (E) Immunofluorescence microscopy of γNa⁺-K⁺-ATPase on renal tubules (scale bars, 40 μM) and B cells (scale bars, 5 μM) of MRL^{lpr}.*Fxyd2*$^{+/+}$ and MRL^{lpr}.*Fxyd2*$^{-/-}$ mice; representative of three mice per group. Fluorescence intensity of individual cells was determined using ImageJ software. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, and ****$P < 0.0001$; results are not significant unless otherwise indicated.

αNa⁺-K⁺-ATPase and identify γNa⁺-K⁺-ATPase as a previously unknown regulator of ionic stress in B cells, suggesting a coordinated up-regulation of a functional, three-component Na⁺-K⁺-ATPase by B cells in the lupus kidney environment.

Genetic deletion of γNa⁺-K⁺-ATPase ablates intrarenal B cells in lupus-prone mice and improves proteinuria

Given our in vitro findings and γNa⁺-K⁺-ATPase expression pattern in B cells from lupus-prone MRL^{lpr} mice, we hypothesized that γNa⁺-K⁺-ATPase is an important mediator of intrarenal B cell survival. We thus examined the kidney, spleen, and lymph node lymphocyte compartments in lupus-prone MRL^{lpr} mice that were wild-type, heterozygote, or homozygote for the *Fxyd2* mutation (MRL^{lpr}.*Fxyd2*$^{+/+}$, MRL^{lpr}.*Fxyd2*$^{+/-}$, and MRL^{lpr}.*Fxyd2*$^{-/-}$ mice, respectively). Intrarenal B cells were decreased in MRL^{lpr}.*Fxyd2*$^{-/-}$ animals compared to *Fxyd2*-intact controls (Fig. 5A), similar to our findings with ouabain treatment. There also was a trend toward decrease in intrarenal plasma cells and CD4⁺ and CD8⁺ T cells in homozygous mutant mice (Fig. 5A), which we had not observed with pharmacologic blockade, with these differences becoming significant when we examined MRL^{lpr}.*Fxyd2*$^{+/+}$ mice compared to MRL^{lpr}.*Fxyd2*$^{+/-}$ heterozygotes, suggesting haploinsufficiency of the phenotype (Fig. 5A). Kidney dendritic cell and macrophage numbers were not affected (fig. S7A). Analogous to data from ouabain-treated mice, metrics including autoantibody production (anti-dsDNA antibodies), C3 complement deposition in the kidney, BUN, and histology scores were not changed (Fig. 5, B to E), while proteinuria was significantly improved in mice lacking either one or both copies of the γNa⁺-K⁺-ATPase (Fig. 5F). Numbers and phenotypes of splenic and lymph node B cells and T cells were unaffected in homozygous or heterozygous mutant mice (fig. S7, B and C). In sum, intrarenal B cells and proteinuria are decreased in *Fxyd2*-deficient animals, providing genetic confirmation of the role of Na⁺-K⁺-ATPase as a possible kidney-specific target in lupus nephritis.

γNa⁺-K⁺-ATPase deletion effect is specific to the hematopoietic lineage

To investigate whether the observed effects on intrarenal B cells and proteinuria in γNa⁺-K⁺-ATPase knockout mice were due to a mutation in the hematopoietic lineage, we produced bone marrow (BM) chimeras in which the MRL^{lpr}.*Fxyd2* mutation was isolated to these cells. MRL^{lpr} mice were irradiated at 9 weeks of age and

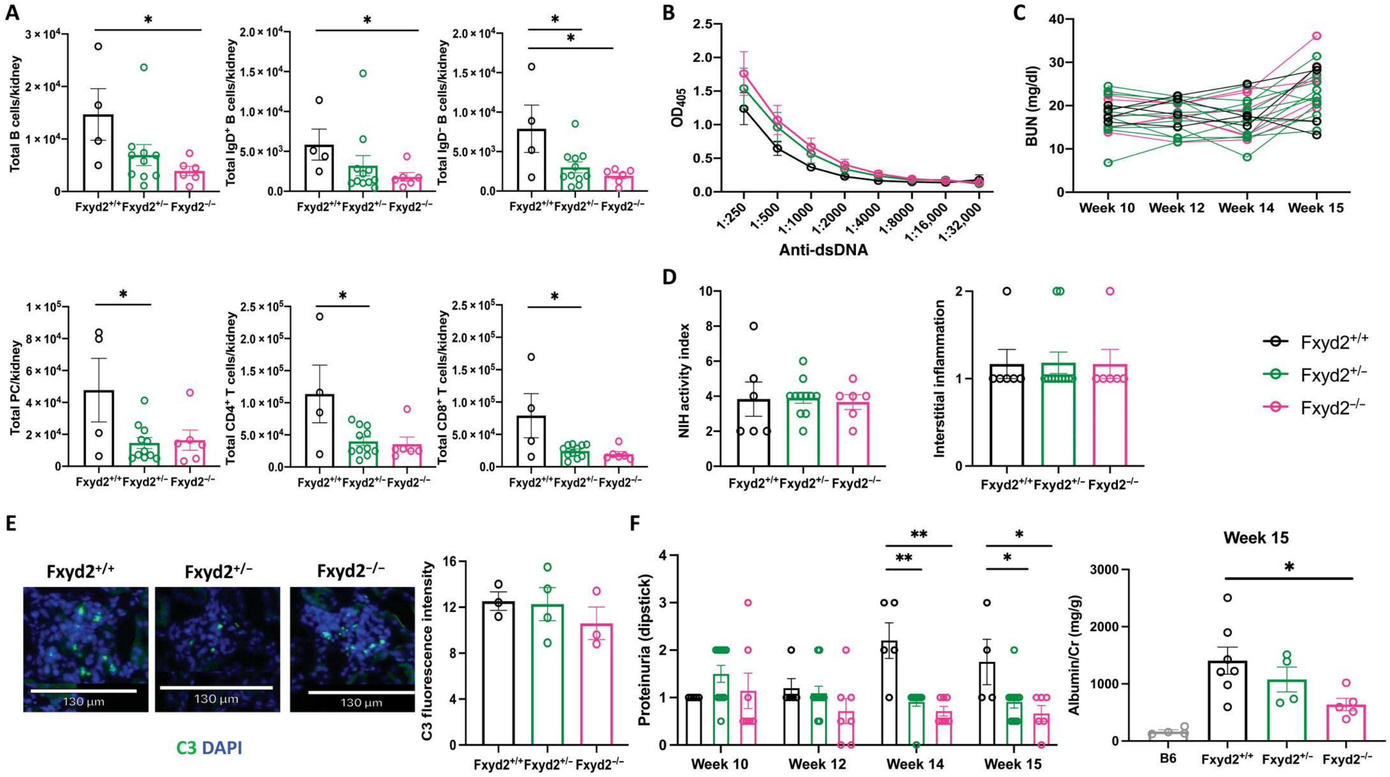

Fig. 5. Lupus-prone mice lacking the γNa⁺-K⁺-ATPase have fewer kidney B cells and have decreased proteinuria compared to wild-type littermates. MRL^{lpr}.*Fxyd2*$^{+/+}$, MRL^{lpr}.*Fxyd2*$^{+/-}$, and MRL^{lpr}.*Fxyd2*$^{-/-}$ littermate control mice were analyzed longitudinally and euthanized at 15 weeks of age. Combined data from two experiments are shown, of four total experiments. (**A**) Kidney interstitial lymphocytes were quantified using flow cytometry, gating strategy as in fig. S1. (**B**) Sera anti-dsDNA antibodies as measured by ELISA at week 15. (**C**) BUN in milligrams per deciliter. (**D**) Quantitated pathological scores based on the NIH activity scale. (**E**) C3 (green) and DAPI (blue) immunofluorescent staining in glomeruli of indicated mice; fluorescence intensity quantified across multiple glomeruli and multiple animals using ImageJ software. (**F**) Semiquantitative urine dipstick analysis for proteinuria and colorimetric urine albumin/creatinine measurement. *$P < 0.05$ and **$P < 0.01$; results are not significant unless otherwise indicated.

reconstituted with either MRL^{lpr}.*Fxyd2*$^{+/+}$ or MRL^{lpr}.*Fxyd2*$^{-/-}$ BM and then followed longitudinally until 18 to 21 weeks of age. We confirmed reconstitution and the genetic identity of our chimeras with real-time polymerase chain reaction (RT-PCR; see Materials and Methods). Animals reconstituted with *Fxyd2*$^{-/-}$ BM had fewer intrarenal B cells by comparison to MRL^{lpr}.*Fxyd2*$^{+/+}$ reconstituted animals, while intrarenal T cells were unchanged (Fig. 6A). As seen previously in whole-body knockout animals, splenic B cell and T cell subsets were unaffected (fig. S8). Just as in whole-body knockouts, proteinuria was consistently lower in the animals in which hematopoietic cells lacked γNa⁺-K⁺-ATPase (Fig. 6B), although as whole-body knockouts, BUN, anti-dsDNA antibodies, and histology scores were unchanged between groups (Fig. 6, C to E). Together, these data identify hematopoietic-intrinsic expression of γNa⁺-K⁺-ATPase as integral to the cells′ ability to persist in the SLE kidney environment and drive proteinuria.

αNa⁺-K⁺-ATPase and γNa⁺-K⁺-ATPase are expressed in B cells in human lupus nephritis

Our mouse studies have identified B cell Na⁺-K⁺-ATPase up-regulation as a critical step facilitating B cell infiltration of kidneys in lupus models with proteinuria, leading us to wonder about the relevance of this mechanism to human lupus nephritis. To this end, we confirmed expression of the α and γ subunits in renal-infiltrating B cells in biopsies of patients with class IV and V lupus nephritis and proteinuria using immunofluorescence microscopy (Fig. 7, A and B, and table S1). As the inner medulla is infrequently sampled in human biopsies, we were unable to examine the differences in expression between cortical and medullary B cells; we were also unable to detect immune cell γNa⁺-K⁺-ATPase transcript in a known human lupus nephritis dataset, possibly due to read depth issues (*3*). Both αNa⁺-K⁺-ATPase and γNa⁺-K⁺-ATPase were highly expressed on kidney-infiltrating B cells consistent with our mouse data. While the constitutive α subunit was expressed in both B and T cells albeit with higher intensity in B cells, γ subunit expression was specific to kidney B cells with minimal staining in CD4⁺ or CD8⁺ T cells. Accordingly, B cell expression confirmed
Na⁺-K⁺-ATPase and especially its γ subunit as potential kidney B cell–specific therapeutic targets in human lupus nephritis.

DISCUSSION

The tissue adaptation programs used by kidney-infiltrating lymphocytes have not been fully elucidated nor have the relative contributions of systemic and tissue-dwelling B cells to the pathogenesis of lupus nephritis. We demonstrated that B cells in lupus-prone mice exhibit enhanced survival under high[Na⁺] conditions, including

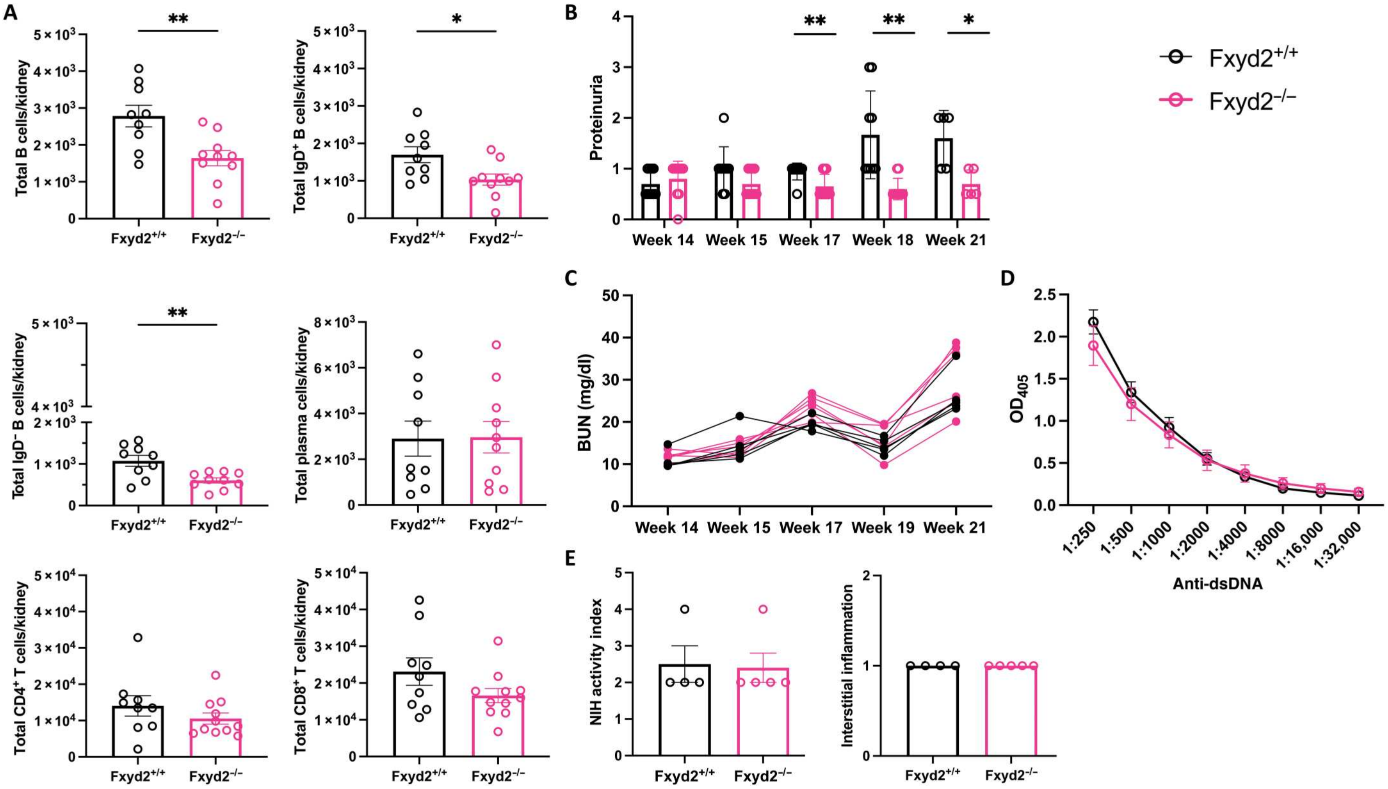

Fig. 6. γNa⁺-K⁺-ATPase modulates intrarenal B cell numbers and proteinuria via its function in the hematopoietic lineage. MRL^{lpr}.*Fxyd2*$^{+/+}$ and MRL^{lpr}.*Fxyd2*$^{-/-}$ BM chimeras were generated as described in Materials and Methods and analyzed longitudinally until 18 to 21 weeks of age. Combined data from two experiments are shown. (**A**) Kidney interstitial lymphocytes were quantified using flow cytometry, gating strategy as in fig. S1. (**B**) Semiquantitative urine dipstick analysis for proteinuria. (**C**) BUN in milligrams per deciliter for a subset of the mice. (**D**) Sera anti-dsDNA antibodies as measured by ELISA at time of euthanasia (weeks 18 to 21). (**E**) Quantitated pathological scores based on the NIH activity scale for a subset of the same mice. *$P < 0.05$, **$P < 0.01$; results are not significant unless otherwise indicated.

the renal microenvironment that they infiltrate. This is mediated by increased expression of αNa^+-K^+-ATPase and γNa^+-K^+-ATPase, as short-term pharmacological inhibition of this Na^+-K^+ exchanger and genetic deletion of its γ subunit led to an ablation of intrarenal B cells (fig. S9). Whether this was due to in situ apoptotic cell death, analogous to the water deprivation experiments, or migration out of the kidney remains unknown; however, we suspect the effect is in the kidney given the high extracellular [Na^+] there as compared to lymphoid tissue. The specific loss of B cells infiltrating the kidney, rather than those in secondary lymphoid organs, was associated with significant amelioration of proteinuria. These observations point toward a role of kidney-resident B cells in locally driving pathologic proteinuria, consistent with correlations observed in several, although not all, human biopsy studies (*3*, *6*, *7*). Specific depletion of intrarenal B cells may thus be particularly advantageous in proteinuria-predominant forms of SLE, such as the difficult-to-treat World Health Organization class V lupus nephritis included in our study (*1*). Therapy with the Na^+-K^+-ATPase inhibitor ouabain presents a particularly attractive option given that ouabain is already approved by the Food and Drug Administration, while small molecules targeting γNa^+-K^+-ATPase, which we show is highly expressed on B cells in class V lupus nephritis, have the potential to provide increased tissue selectivity.

Tissue-infiltrating lymphocytes encounter the dual challenges of maintaining both viability and functionality in a new inimical environment such as the Na^+-rich kidney with lupus nephritis presenting an excellent model to investigate lymphocyte survival in the unique kidney microenvironment. We found that B cell viability declined significantly under conditions of high [Na^+], while up-regulation of Na^+-K^+-ATPase facilitated partial adaptation to this stressor. In contrast, human T cells did not exhibit survival defects when exposed to an additional 40 mM NaCl, a concentration at which we reliably saw a decrease in B cell survival in non-autoimmune mice (*12*). T cells, compared to B cells, from mice and humans with lupus nephritis expressed less αNa^+-K^+-ATPase and γNa^+-K^+-ATPase and were not affected by ouabain treatment, suggesting that Na^+-K^+-ATPase is not a main player mediating T cell survival under conditions of elevated [Na^+]. Thus, T and B cells differentially regulate their response to tissue [Na^+] and likely use distinct tissue adaptation programs to mediate survival upon ionic stress. Whether B cells use similar mechanisms to facilitate survival in the kidney in settings other than lupus nephritis, such as infection or other autoimmune diseases, is unknown and presents a future area of study. Furthermore, the effects of Na^+ on B cell functionality remain underexplored. Unlike the effect of Na^+ on promoting T cell differentiation (*12*, *42*), the survival effects we observed in B cells were similar upon various subsets, including naïve (IgD^+) and in vivo activated (IgD^-) B cells. While it remains unclear how the loss of these B cells mediates proteinuria in lupus, work in another proteinuric kidney disease has identified

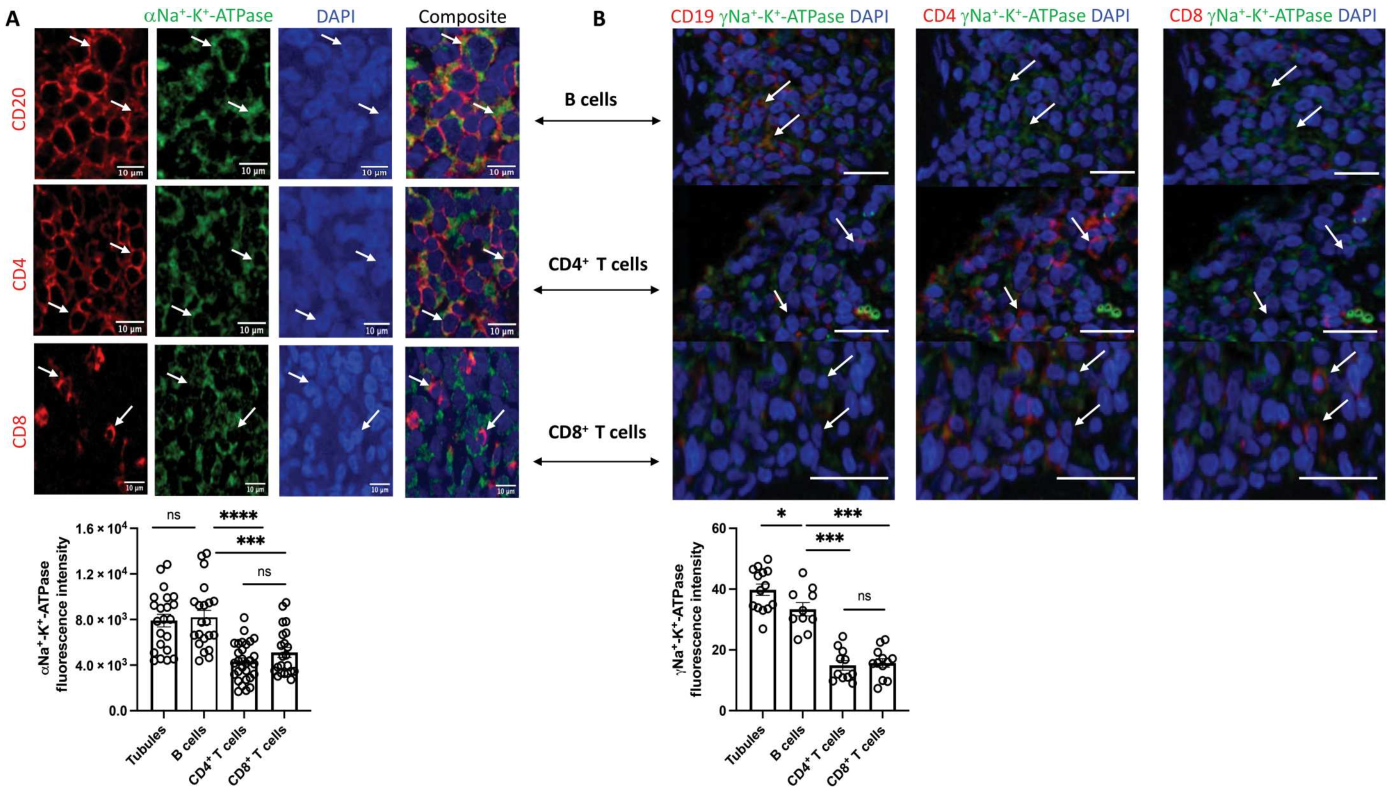

Fig. 7. αNa$^+$-K$^+$-ATPase and γNa$^+$-K$^+$-ATPase are highly expressed on B cells in human lupus nephritis. Representative sections from human lupus kidney. Merged immunofluorescence staining images of DAPI, αNa$^+$-K$^+$-ATPase (**A**), or γNa$^+$-K$^+$-ATPase (**B**) with either CD19/CD20, CD4, or CD8. Arrows point to the same B cells and CD4$^+$ or CD8$^+$ T cells in each serial image. Fluorescence intensity of individual cells was determined using ImageJ software. Arrows point to some representative cells in each image. Scale bars, 10 μm (A) and 20 μm (B). Representative of three to four sections; *$P < 0.05$, ***$P < 0.001$, and ****$P < 0.0001$. ns = not statistically significant.

the B cell–derived cytokine IL-4 as an inducer of podocyte foot process effacement and proteinuria (*43*). Therefore, it is tempting to speculate that the decrease in B cell–derived intrarenal IL-4 may be responsible for the amelioration of proteinuria we observe (*43*). Whether Na$^+$ has additional direct effects on functionality of B cells, such as IL-4 production, remains unknown.

The role of Na$^+$ in regulating Na$^+$-K$^+$-ATPase expression as part of a B cell tissue adaptation program was another intriguing aspect of our work, yet questions remain. While Na$^+$-K$^+$-ATPase was up-regulated with increasing [Na$^+$] in vitro, the ex vivo situation was more complex. Although we did see higher expression in the kidney as compared to splenic B cells, we did not appreciate increased Na$^+$-K$^+$-ATPase expression when [Na$^+$] was further elevated—such as in cells taken from the kidney medulla versus cortex nor in animals fed a highNaCl diet or subjected to water deprivation. We note one caveat with the high-salt diet experiment with recent work demonstrating that [Na$^+$] of the kidney medulla may not be reliably increased under these dietary conditions (*44*); however, these limitations do not arise with water deprivation (*45*). The effect of high-sodium diet was also unlikely to be mediated via changes in blood pressure (BP) as prior studies have indicated no BP changes in mice placed on short-term highsalt diet (*12*). Comparisons of B cells from the cortex to the medulla were done under homeostatic conditions (when differences in [Na$^+$] between cortex and medulla may be modest), while we examined wholekidneys in the highNaCl diet and the water deprivation experiments. Thus, a focused analysis of the B cells under the most hypernatremic conditions, e.g., the inner medulla of mice subjected to water deprivation, might demonstrate increased Na$^+$-K$^+$-ATPase expression; unfortunately, these studies were not feasible given small cell numbers. Alternatively, there may be a limit to the influence of sodium on Na$^+$-K$^+$-ATPase up-regulation, explaining its higher expression in the kidney as compared to splenic B cells in multiple lupus-prone strains but no further up-regulation as kidney interstitial sodium was increased. We also showed that the higher expression of Na$^+$-K$^+$-ATPase on IgD$^+$ B cells, as compared to IgD$^-$ B cells in the same animal, does not confer an additional survival advantage under high-[Na$^+$] conditions in vivo, further suggesting that high Na$^+$-K$^+$-ATPase expression is advantageous within a narrow range of values. Sodium-independent Na$^+$-K$^+$-ATPase regulation is also likely as suggested by the highly variable Na$^+$-K$^+$-ATPase expression in splenic B cells among mouse strains, the mechanisms underlying which remain uncharacterized.

Another intriguing aspect of Na$^+$-K$^+$-ATPase regulation is the unique contribution of the different subunits. While ouabain and γNa$^+$-K$^+$-ATPase deletion appear to phenocopy each other regarding the in vivo B cell phenotype, this effect may be achieved in distinct ways. By blocking the exchanger functionality of the Na$^+$-K$^+$ pump, ouabain leads to intracellular [Na$^+$] accumulation. This alteration in the electrochemical gradient of the cell results in increased intracellular calcium concentration ([Ca^{++}]), possibly via changes in sodium calcium antiporter (Na$^+$-Ca^{++} antiporter) activity (*46*). In contrast, γNa^+-K^+-ATPase appears to lower the transporter's affinity for Na^+ (*41*, *47*, *48*), suggesting that γ-deficient cells would have decreased intracellular [Ca^{++}], although this has not been experimentally demonstrated. Concomitantly, γNa^+-K^+-ATPase has been reported to increase the pump's affinity for adenosine triphosphate (ATP) (*49*, *50*), a potentially important advantage for a cell located in the hypertonic and hypoxic renal medulla. It is tempting to speculate that the phenotype observed in *Fxyd2*-deficient mice, including on kidney T cells, is due to an effect on Na^+-K^+-ATPase's affinity for ATP. Further dissecting these physiological pathways will enhance our understanding of lymphocyte tissue adaptation and enable us to design cell- and tissue-specific therapies.

Last, our data raise questions about how lymphocytes handle Na^+ stress outside of the context of the lupus kidney. While the kidney is the organ most central to Na^+ homeostasis and thus ideal to study the effect of Na^+ on lymphocytes, other work has investigated how modulating its concentration affects T cells in nonrenal autoimmune conditions (*12*, *42*). Moreover, recent work in SLE suggested that organs not classically thought to be involved in Na^+ homeostasis, including the muscle and skin, may serve as Na^+ depots (*11*). Whether this has any effect on immune cells remains to be studied but the list of organs and diseases potentially affected by sodium-immune cell interactions is expanding. Our work represents the first investigation into the effect of Na^+ on B cells in lupus, with further work warranted to investigate sodium's role on lymphocyte survival under other organs and autoimmune conditions.

MATERIALS AND METHODS

Study design

We characterized spleen and kidney B cells from nonautoimmune B6 mice and lupus-prone $MRLl^{pr}$ mice under in vitro and in vivo high [Na^+] conditions. We identified the αNa^+-K^+-ATPase and γNa^+-K^+-ATPase as regulators of B cell survival under high-sodium conditions, particularly in the kidney. We used pharmacologic and genetic blockade of Na^+-K^+-ATPase to demonstrate that this ionic exchanger promoted survival of renal-infiltrating B cells, with blockade therapeutically improving proteinuria. All experiments were performed at least twice, preceded by pilot experiments to determine numbers of replicates needed to reach statistical significance. The therapeutic potential of Na^+-K^+-ATPase blockade in patients with SLE was demonstrated by analysis of these markers in human lupus nephritis biopsies.

Mice

Mice were housed in the pathogen-free facility in Yale Animal Resources Center (Yale University, New Haven, CT). Animal handling and experimental protocols were approved by the Yale Institutional Animal Care and Use Committee. B6 mice were purchased from Charles River Laboratories, and MRL^{lpr} and NZBWF1/J mice were purchased from the Jackson Laboratory. MRL^{lpr}-$Cas9^+$ (MRL^{lpr} mice expressing CRISPR-associated protein 9) mice were generated via breeding in our laboratory. To generate MRL.$Fxyd2^{+/-}$ and MRL.$Fxyd2^{-/-}$ mice, we obtained frozen sperm from *Fxyd2* knockout mice ($Fxyd2^{tm1Kdr}$)[41] from the Mutant Mouse Resource and Research Centers (MMRRC: 011562-MU). Female MRL^{lpr} mice were successfully impregnated via in vitro fertilization and produced live offspring; animals have been backcrossed onto the lupus-prone MRL^{lpr} background for six generations with 99% MRL^{lpr} identity confirmed by marker-assisted genotyping performed by the Jackson Laboratory services. Mice of both sexes were used unless otherwise indicated. Wild-type, heterozygous, or homozygous status of the MRL.*Fxyd2* mice was confirmed by RT-PCR at time of terminal experiments. Blood collection for all experiments was performed retroorbitally, with ~100 to 150 μl of blood collected. A total of 8% NaCl chow was manufactured on request by TestDiet. For water deprivation experiments, blood was collected at $t = 0$ ("pre") and $t = 48$ hours ("post") after water deprivation or control conditions were initiated.

BM chimeras

Recipient 9-week-old male MRL^{lpr} mice were irradiated with a single dose of 950 rad (radiation absorbed dose units) and reconstituted 6 hours later with 10×10^6 BM cells from 8-week-old MRL.$Fxyd2^{+/+}$ or MRL.$Fxyd2^{-/-}$ littermate donors via retroorbital injection. Recipients were maintained on an antibiotic diet for 2 weeks after transfer. Genetics of the reconstituted blood lymphocytes were confirmed 6 weeks later via RT-PCR for the *Fxyd2* gene. Serial urine and serum collections were performed at 2-week intervals starting at 13 weeks of age.

Human nephritis biopsy samples

Formalin-fixed paraffin-embedded biopsy samples from deidentified patients with lupus nephritis were obtained from the University of Chicago Human Tissue Resource Center and stained in accordance with Institutional Review Board (IRB) protocol no. 15065B-CR0009.

Isolation of renal-infiltrating lymphocytes

Intravascular antibody injection of anti-CD45.1 (clone A20, eBioscience, 12-0453-82) was administered to mice 3 min before euthanasia, allowing us to distinguish intravascular and tissue-infiltrating cells as previously described (*51*). After organ harvest and removal of renal capsule, renal tissues were crushed into small pieces and incubated in RPMI 1640 with 10% fetal bovine serum, collagenase D (100 IU/ml) with $CaCl_2$ (2 mM), and $MgCl_2$ (2 mM) at 37°C for 45 min. After enzymatic digestion, gentleMACS was used to disrupt remaining tissue. Cell suspensions were filtered through 70-μm strainer and resuspended in phosphate-buffered saline (PBS). Mononuclear cells were isolated after passing the single cell suspension through Ficoll density gradient centrifugation.

Pharmacologic ouabain treatment

To block Na^+-K^+-ATPase in vivo, we administered ouabain octahydrate (Sigma-Aldrich, O3125) resuspended in PBS at 0.6 mg/kg to MRL^{lpr} females daily for 16 days. Control MRL^{lpr} mice were treated with corresponding volume of PBS. Treatment started at 17 to 20 weeks of age, after onset of renal dysfunction.

Statistics

Statistical analysis was performed using GraphPad Prism version 8. Bar graphs in all figures indicate the mean, and the error bars represent the SEM. To compare data between two groups, a two-tailed *t* test was performed. Statistical significance was defined as $P < 0.05$ with specific values as indicated in each figure legend.

Supplementary Materials

This PDF file includes:
Supplementary Materials and Methods
Figs. S1 to S9
Tables S1 to S3

View/request a protocol for this paper from *Bio-protocol*.

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