Ultrasound Modulates the Splenic Neuroimmune Axis in Attenuating AKI

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ABSTRACT

We showed previously that prior exposure to a modified ultrasound regimen prevents kidney ischemia-reperfusion injury (IRI) likely via the splenic cholinergic anti-inflammatory pathway (CAP) and α7 nicotinic acetylcholine receptors (α7nAChR). However, it is unclear how ultrasound stimulates the splenic CAP. Further investigating the role of the spleen in ischemic injury, we found that prior splenectomy (7d) or chemical sympathectomy of the spleen with 6-hydroxydopamine (6OHDA; 14d) exacerbated injury after subthreshold (24-minute ischemia) IRI. 6-OHDA-induced splenic denervation also prevented ultrasound-induced protection of kidneys from moderate (26-minute ischemia) IRI. Ultrasound-induced protection required hematopoietic but not parenchymal α7nAChRs, as shown by experiments in bone marrow chimeras generated with wild-type and α7nAChR−/− mice. Ultrasound protection was associated with reduced expression of circulating and kidney-derived cytokines. However, splenocytes isolated from mice 24 hours after ultrasound treatment released more IL-6 ex vivo in response to LPS than splenocytes from sham mice. Adoptive transfer of splenocytes from ultrasound-treated (but not sham) mice to naïve mice was sufficient to protect kidneys of recipient mice from IRI. Ultrasound treatment 24 hours before cecal ligation puncture–induced sepsis was effective in reducing plasma creatinine in this model of AKI. Thus, splenocytes of ultrasound-treated mice are capable of modulating IRI in vivo, supporting our ongoing hypothesis that a modified ultrasound regimen has therapeutic potential for AKI and other inflammatory conditions.


AKI is a major health burden without major pharmacologic advances in its prevention or treatment. To improve outcomes in AKI, a better understanding of the pathophysiology of AKI is necessary to develop novel therapies in well designed clinical trials. Much work has been done on the intrinsic cellular and molecular mechanisms of AKI, but extrarenal factors are key modulators of AKI. Kidney ischemia-reperfusion injury (IRI), a form of AKI, results in a systemic inflammatory response that affects multiple organ systems. Multiorgan dysfunction in response to AKI likely accounts for the high mortality associated with this disorder. Inflammatory molecules are found in both the arterial supply to and venous outflow from the kidney as soon as 1 minute after AKI. These data suggest that the kidney is a source of inflammatory molecules after AKI, but they also suggest that the release of inflammatory mediators from other unidentified peripheral tissues immediately after AKI could modulate kidney injury. The contribution of renal and nonrenal sources of inflammatory mediators in the development of AKI remains unclear.

Previous studies have suggested an important role of the spleen in AKI. Splenectomy before kidney...
IRI exacerbated lung injury through proinflammatory mechanisms that may involve removal of protective splenic IL-10. Splenectomy also reduced the protective efficacy of the anti-inflammatory agent, chloroquine, in AKI. These results suggest that the spleen has an important role in modulating inflammation after AKI. Not all studies, however, have shown a protective role of the spleen in ischemic organ injury. Splenectomy reduces ischemic damage in both the liver and brain. These studies demonstrate the need to better understand the function of the spleen in kidney IRI.

Recently, we reported that a simple ultrasound-based protocol reduced tissue inflammation and prevented kidney IRI in mice. This effect was dependent on an intact spleen and functional α7 nicotinic acetylcholine receptors (α7nAChRs), which is consistent with the hypothesis that ultrasound stimulates the splenic cholinergic anti-inflammatory pathway. As described by Tracey, the cholinergic anti-inflammatory pathway is initiated via activation of the adrenergic splenic nerve and culminates with the activation of α7nAChRs. However, it is still unclear how ultrasound initiates this pathway and protects kidneys from AKI. In this study we further explored the importance of the spleen and the splenic cholinergic anti-inflammatory pathway in modulating kidney IRI. We now report that the protective effect of ultrasound depends on sympathetic innervation of the spleen and hematopoietic α7nAChRs and that ultrasound-induced modulation of splenocyte function is sufficient to confer protection from IRI.

RESULTS

**Splenectomy or Splenic Denervation Exacerbates Injury in a Subthreshold Ischemia-Reperfusion Model**

Our data suggest that the spleen is an important target in mediating the protective effect of ultrasound. Others have demonstrated that splenectomy before kidney IRI increased lung injury but not kidney injury. It is possible that an immune modulatory function of the spleen in protecting the kidney from IRI may be missed if the injury is too severe. Therefore, in the studies on splenectomy we used a reduced ischemic time of 24 minutes (subthreshold ischemia-reperfusion injury [subIRI]) to determine if splenectomy exacerbates AKI. Subthreshold IRI was not sufficient to alter plasma creatinine or cause acute tubular necrosis in mice with sham splenectomy; however, mice that were splenectomized 1 week before subIRI had significantly higher plasma creatinine (1.2 mg/dl, *P*<0.001), extensive acute tubular necrosis (Figure 1), and increased renal inflammation (Figure 2). The percentage of CD45+ leukocytes and 7/4+ neutrophils and the level of expression of chemokines/cytokines CXCL1 and IL-6 was greater in mice splenectomized 1 week before subIRI (Figure 2). Interestingly, similar increases in plasma creatinine were observed in *Rag1−/−* mice. Plasma creatinine in *Rag1−/−* mice exposed to sham splenectomy and subIRI was 1.1±0.08 mg/dl, a value significantly lower than those splenectomized a week before (1.7±0.07 mg/dl, *P*<0.001, *n*=7–8). Combined, these data suggest that prior splenectomy exacerbates IRI, and this effect is independent of splenic T and B lymphocytes.

**Figure 1.** SPLX exacerbates kidney IRI. Mice underwent SPLX or sham surgery 7 days before a subIRI (24 minutes of ischemia, 24 hours of reperfusion), which was not sufficient alone to cause AKI. Prior SPLX exacerbated subthreshold injury and resulted in elevated plasma creatinine (A) and robust ATN (B) (scored from hematoxylin and eosin samples) (C). ATN, acute tubular necrosis; SPLX, splenectomy; subIRI, subthreshold ischemia-reperfusion injury. n=6–13. **P**<0.001.

Activation of the cholinergic anti-inflammatory pathway requires catecholaminergic input to the spleen. To explore the role of this input in splenic modulation of AKI, the spleen was chemically denervated by locally injecting 6-hydroxydopamine (6-OHDA), a neurotoxin that destroys catecholaminergic neurons. Similar to splenectomy, splenic 6-OHDA, as little as 15 μg injected 14 days before subIRI, exacerbated kidney injury as shown by increased plasma creatinine (*P*<0.001) and acute tubular necrosis compared with mice with splenic vehicle (saline) injections before subIRI (Figure 3).

**Protective Effect of Ultrasound is Dependent on the Splenic Nerve and Hematopoietic α7nAChRs**

We have shown that prior exposure to a modified ultrasound regimen (up to 48 hours before ischemia) prevents AKI and the subsequent development of renal fibrosis in mice exposed to renal IRI. Here, the same ultrasound regimen 24 hours before our standard bilateral kidney IRI (26 minutes of ischemia) reduced kidney injury as assessed by plasma creatinine (*P*=0.002; data not shown) and significantly (*P*=0.004) improved mouse survival (Figure 4).

Our previous data suggest that ultrasound prevents IRI by stimulating the cholinergic anti-inflammatory pathway, which can be initiated by activation of the splenic nerve.

To determine whether the splenic nerve is needed for the protection from IRI, we injected mice with saline (vehicle) or 6-OHDA at different times before subIRI. The percentage of CD45+ leukocytes and 7/4+ neutrophils and the level of expression of chemokines/cytokines CXCL1 and IL-6 was greater in mice splenectomized 1 week before subIRI (Figure 2). Interestingly, similar increases in plasma creatinine were observed in *Rag1−/−* mice. Plasma creatinine in *Rag1−/−* mice exposed to sham splenectomy and subIRI was 1.1±0.08 mg/dl, a value significantly lower than those splenectomized a week before (1.7±0.07 mg/dl, *P*<0.001, *n*=7–8). Combined, these data suggest that prior splenectomy exacerbates IRI, and this effect is independent of splenic T and B lymphocytes.
The protective effect of ultrasound treatment, the spleen was chemically sympathectomized by direct injection with 6-OHDA 14 days before IRI. Prior splenic 6-OHDA treatment abolished the protective effect of ultrasound after IRI compared with vehicle-treated mice (Figure 5).

Although the splenic nerve is believed to stimulate the cholinergic anti-inflammatory pathway, binding of acetylcholine to the α7nAChR is considered the key step in the pathway.13 We showed that α7nAChR agonists prevent kidney IRI, and the protective effect of ultrasound is dependent on α7nAChRs.12 Nicotine reduces inflammation and protects the kidney from IRI in an α7nAChR-dependant manner, but the contribution of hematopoietic versus parenchymal α7nAChRs was not examined.16 To determine whether hematopoietic or parenchymal α7nAChRs mediate the protective effect of ultrasound, we generated bone marrow chimeras with wild-type and α7nAChR−/− mice. Ultrasound pretreatment was protective only in mice with bone marrow cells from wild-type mice regardless of the parenchymal genotype, therefore pointing to the essential role of hematopoietic α7nAChRs in ultrasound protection from IRI (Figure 6).

Ultrasound Reduces Circulating and Kidney Cytokines after IRI
Inflammation is a key component of IRI, and stimulation of the cholinergic anti-inflammatory pathway has been shown to reduce systemic cytokine production16 Therefore, the protective effect of ultrasound may involve modulation of systemic inflammatory pathways. IRI-induced increases in circulating IL-6, IL-10, LIF, IL-15, MCP-1, MIP-2, and TNFα concentrations (Table 1) and kidney IL-6 and MIP2 mRNA expression (Figure 7) were reduced by ultrasound treatment 24 hours before IRI.

Ultrasound Modulates Splenocyte Function: ex vivo and in vivo Adoptive Transfer Studies
To determine whether splenocytes from ultrasound-treated mice respond differently to inflammatory stimuli, splenocytes were isolated from ultrasound- or sham-treated animals 24 hours after treatment. Cells were then incubated ex vivo with increasing concentrations of LPS, and IL-6 production was measured 16 hours later. IL-6 is known to have both pro- and anti-inflammatory effects.17 In contrast with the decreases in circulating IL-6 and kidney mRNA levels in ultrasound-treated animals previously described, IL-6 production ex vivo in response to LPS concentrations of 0.1–10 μg/ml was greater in splenocytes from ultrasound-treated mice compared with cells from sham mice (Figure 8). These results demonstrate functionally that splenocytes behave differently when exposed to ultrasound, therefore supporting either a direct or indirect effect of ultrasound.

Figure 2. Prior SPLX increases renal inflammation after subIRI. (A) Leukocyte infiltration (CD45+ cells as a percent of total kidney cells) measured in kidneys by flow cytometry 24 hours after subIRI was higher in mice with prior SPLX. (B) Neutrophil infiltration was highest in the outer medulla (upper left quadrant of large panel) and sparse in the inner medulla (lower right region) and other areas of kidney after subIRI. White box indicates area sampled for photographs in smaller panels at right. Neutrophils (green, FITC-7/4 immunofluorescence) were densely sequestered in the outer medulla of kidneys from mice with prior SPLX. Blue, DAPI-labeled nuclei. Scale bar is 50 μm. (C) Renal mRNA expression of CXCL1 and IL-6 was significantly greater in mice with SPLX plus subIRI compared with subIRI alone. Sham, sham ischemia-reperfusion injury surgery; SPLX, splenectomy; SPLX+subIRI, splenectomy plus subthreshold ischemia-reperfusion injury; subIRI, sham splenectomy plus subthreshold ischemia-reperfusion injury. n=4–11. *P<0.001; **P=0.003.

ultrasound treatment on splenocytes. The result is different from what we observed in vivo likely because of the simple artificial culture conditions relative to the complex microenvironment of the spleen and the difference in stimulus (IRI versus LPS).

To determine if ultrasound modulates splenocyte response to inflammatory stimuli in vivo, splenocytes were isolated from mice 24 hours after ultrasound (or sham) treatment and were adoptively transferred (2×10^5 or 1×10^6 cells, intravenously) to naïve recipient mice 24 hours before IRI. Ultrasound did not appear to change the relative composition of populations of splenocytes, as determined by flow cytometry; the proportions of Ly6C+, CD3+, CD4+, and CD11b+ cells were similar in spleens from control and ultrasound-treated mice. However, adoptive transfer of splenocytes from ultrasound-treated mice produced a dose-dependent decrease in plasma creatinine (Figure 9) in recipient mice after IRI compared with transfer of splenocytes from sham-treated animals.

**Prior Ultrasound Treatment Reduces Severity of AKI in Sepsis**

A critical issue in sepsis is the pathologic dysregulated response to bacterial infection leading to a hyperinflammatory cytokine storm response,\(^{18}\) and the lack of homeostasis from dysfunction of the neuroendocrine and immune systems leads to multiorgan dysfunction and early death.\(^{19}\) Using the cecal ligation puncture (CLP) model of sepsis,\(^{20}\) the rise in plasma creatinine (Figure 10A) and circulating cytokines (Table 2) in WT mice subjected to CLP was reduced significantly by pretreatment with ultrasound. Subtle histologic changes observed in kidneys of CLP mice, consistent with existing literature,\(^{21}\) were indistinguishable in CLP mice pretreated with ultrasound (Figure 10B). Furthermore, prior ultrasound treatment did not reduce plasma creatinine in \(\alpha 7nAChR^{−/−}\) mice (Figure 10A), suggesting that, like our prior results with IRI, ultrasound protection from sepsis-induced AKI requires the cholinergic anti-inflammatory pathway. Ultrasound pretreatment reduced Kim-1 expression by approximately 25% (Figure 10C), but there was no significant difference from CLP alone (\(P=0.09\)). These results suggest that CLP induces marked functional changes and mild damage consistent with prior studies,\(^{22}\) and ultrasound blocks these effects in an \(\alpha 7nAChR\)-dependent manner.

**DISCUSSION**

We have shown that the spleen plays a critical role both in modulating the inflammatory response and subsequent kidney injury that occurs after IRI and in ultrasound-mediated protection from IRI. Our results suggest that ultrasound-mediated protection from IRI requires the splenic nerve and hematopoietic, but not parenchymal, \(\alpha 7nAChRs\). Ultrasound altered the profile of circulating and renal inflammatory mediators after IRI and the response of splenocytes to proinflammatory stimulation. Transfer of splenocytes from ultrasound-treated mice to naïve mice was sufficient to reduce kidney IRI. Combined, these data support our ongoing hypothesis that ultrasound reduces ischemic AKI via stimulation of the splenic cholinergic anti-inflammatory pathway. In addition to IRI, ultrasound was protective in another model of AKI (i.e., CLP-induced sepsis).

**Splenic Control of Inflammation and IRI**

The spleen is a peripheral lymphoid organ that participates in innate and adaptive immunity through its unique organization.
There are distinct areas of B and T cells, macrophages, dendritic cells, and stromal cells. The spleen removes older erythrocytes, bloodborne micro-organisms, and cellular debris from the circulation. Before the concept of the cholinergic anti-inflammatory pathway developed, the spleen had generally been considered a source of inflammatory mediators capable of propagating injury. Some work demonstrated a protective effect of prior splenectomy in animal models of organ injury. In contrast, others demonstrated that prior splenectomy had deleterious effects. Prophylactic treatments for AKI were ineffective in previously splenectomized animals, including the ultrasound treatment described here. Faubel's group demonstrated that splenectomy did not exacerbate AKI, but there was an increase in lung permeability. The severity of kidney injury may have precluded demonstrating an effect of splenectomy on AKI, which we uncovered by using a subthreshold IRI model. However, the authors found that splenic IL-10 production was increased in lymphocytes after kidney IRI, suggesting that the spleen contributed to the anti-inflammatory response and reduction of lung injury. Not all studies have demonstrated a protective effect in organ injury. In particular, splenectomy was associated with worse hepatic IRI; however, other factors may contribute, including reduction in pressure in the portal vein, which might increase the arterial blood supply to the liver, promoting hepatic recovery after IRI. Our results are consistent with studies demonstrating the potential role of the spleen in modulating the inflammatory response to organ injury. Given that the cholinergic anti-inflammatory pathway appears to be an intrinsic pathway that is initiated by the splenic nerve, we tested and confirmed that the deleterious effect of splenectomy could be recapitulated with splenic denervation alone. Therefore, our data suggest that the spleen performs an inherent anti-inflammatory function in AKI that is mediated by the splenic nerve.

**Neural Control of Inflammation**

The nervous and immune systems interact in complex ways to maintain homeostasis and respond to stress or injury. Clinical evidence of this interaction has been documented in times of stress and in athletes subjected to extreme conditions. Studies in animal models and human blood cells demonstrate a relationship between the autonomic nervous system and inflammation. Norepinephrine inhibits LPS-induced TNFα and IL-6 production through stimulation of

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**Figure 5.** Protective effect of prior ultrasound exposure is abolished by splenic sympathectomy. Mice received splenic injections of saline or 6-OHDA (as in Figure 3) 14 days before the ultrasound (US) and IRI (26 minutes of ischemia) treatments. Splenic injections of 6-OHDA abolished the protective effects of US as determined by (A) plasma creatinine and (B) histologic assessment (hematoxylin and eosin) of acute tubular necrosis. Scale bar is 100 μm in the main panel and 50 μm in the inset. n=5. *P<0.001.

**Figure 6.** Hematopoietic α7nAChRs are required for the protective effects of prior ultrasound (US) exposure. α7nAChR−/− or WT mice were lethally irradiated, reconstituted with bone marrow from either WT or α7nAChR−/− (KO) mice, and 8–10 weeks later were exposed to ultrasound and IRI (26 minutes of ischemia). Only mice with WT bone marrow reconstitution (WT → WT or WT → KO), regardless of the parenchymal phenotype, benefited from the ultrasound treatment, as determined by (A) plasma creatinine and (B) hematoxylin and eosin analysis of morphology. Scale bar is 100 μm in the main panel and 50 μm in the inset. n=4–7. *P<0.001.
The spleen appears to be a critical site for the neural control of immune-mediated diseases. Early work demonstrated that vagal nerve stimulation increased release of acetylcholine from the spleen. However, because choline acetyltransferase, the enzyme necessary to produce acetylcholine, had not been demonstrated in splenic nerve fibers, it was difficult to reconcile the finding of splenic production of acetylcholine in response to vagal stimulation. Seminal studies by Rosas-Ballina and colleagues determined that a subset of lymphocytes produced acetylcholine in response to vagal stimulation. Activation of the splenic nerve resulted in the release of norepinephrine that binds β-adrenergic receptors in human leukocytes, and epinephrine signaling through protein kinase A induces IL-10 synthesis in human mononuclear cells.

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Nicotinic acetylcholine receptors belong to a family of ligand-gated pentameric ion channels. Acetylcholine binding to α7nAChRs can induce cellular effects through activation of canonical ion channel and calcium channel fluxes, increased activation of PI3K and phospholipase C–induced intracellular calcium release, or phosphorylation of STAT3 through Jak2,45 which may mediate the anti-inflammatory effects of α7nAChR stimulation.46 Nicotine, a nicotinic cholinergic agonist, inhibits endotoxin- and TNFα-induced HMGB1 release by preventing activation of the NF-κB pathway.47 Nicotine reduced the production of Th1 (TNFα and IFN-γ) and Th17 cytokines (IL-17, IL-17F, IL-21, and IL-22) but increased IL-4 production, thereby inducing a shift from Th1 cytokines to Th2 cytokines.48

Activation of α7nAChRs to reduce inflammation may be mediated through hematopoietic (T and B lymphocytes, macrophages, neutrophils, or dendritic cells) or nonhematopoietic cells.45 Previously we showed using both pharmacologic blockade and genetically α7nAChR-deficient mice that ultrasound-induced protection from AKI is dependent on α7nAChRs.12 In this study we used bone marrow chimeras to isolate α7nAChR deficiency to either bone marrow–derived cells or parenchymal cells and further showed that hematopoietic, but not parenchymal cell, α7nAChRs are responsible for mediating the protective effect of ultrasound, thereby supporting the original hypothesis that the cholinergic anti-inflammatory pathway culminates with the activation of α7nAChRs on leukocytes.

Ultrasound, Splenocytes, and Cytokines in IRI
Although we have shown how deficiencies (pharmacologic blockade and α7nAChR-deficient mice) in the cholinergic anti-inflammatory pathway prevent the protective effect of ultrasound, there has been little evidence linking these splenic processes to a specific cell type. Furthermore, the details of how these splenic processes interact with the end organ are still unclear. Here, we show that adoptive transfer of splenocytes isolated from ultrasound-treated mice conferred protection from IRI in the recipient mice, suggesting that ultrasound produced changes in splenocyte phenotype. Our flow cytometry analysis revealed that this does not appear to be caused by differences in splenocyte composition because the proportions of Ly6C+, CD3+, CD4+, and CD11b+ cells were similar in spleens from control and ultrasound-treated mice. It will be important to define splenocyte subsets and function after ultrasound treatment in vivo.

As a first step in exploring functional changes, we found that splenocytes isolated from ultrasound-treated mice responded differently to inflammatory signaling and produced more IL-6 in response to LPS incubation ex vivo. Increased IL-6 may seem paradoxical given our results that ultrasound pretreatment reduced circulating levels and kidney mRNA expression of IL-6 after IRI and the prevailing view that IL-6 has a proinflammatory role in various injury models, including kidney IRI.49 For example, we showed previously that IL-6 increased in kidney monocyte/macrophage populations after IRI.50 In humans, systemic IL-6 levels predict survival in dialysis patients.51,52 However, these roles may not apply in all cases because IL-6 can be pro- or anti-inflammatory.17 Furthermore, the complexity of the kidney and spleen microenvironment after ultrasound and IRI differs markedly from culture conditions of splenocytes examined ex vivo. Although future studies will be needed to understand our findings, they clearly demonstrate a functional change in splenocytes in response to ultrasound and in conjunction with prior work, also showing increased production of IL-6 and TNFα by splenocytes.
previously cultured with nicotine, further support the involvement of the cholinergic pathway in the protective effect of ultrasound. This does not preclude the possibility that other mechanisms are involved in the protective effect of transferred splenocytes.

In summary, our studies reveal mechanistic insight on the spleen-dependent protective effect of ultrasound and demonstrate that the spleen is capable of antagonizing inflammation and tissue injury in kidney IRI. Ultrasound-induced protection requires hematopoietic \( \alpha_7 \text{nAChRs} \) and sympathetic innervation of the spleen, consistent with stimulation of the cholinergic anti-inflammatory pathway. Adoptive transfer of splenocytes from mice previously exposed to ultrasound is sufficient to protect recipient mice from kidney IRI, suggesting that ultrasound alone alters splenocyte function through mechanisms not yet identified and requiring additional investigation. These studies broaden the therapeutic potential for ultrasound in ischemic and sepsis-induced kidney injury. Given the possibility of off-target side effects of pharmacologic agents, the nonpharmacologic approach using therapeutic ultrasound within the spectrum of use currently approved for humans provides an attractive alternative therapy for this devastating disorder.

**CONCISE METHODS**

**Mice and Reagents**

Male mice (8–12 weeks of age) were used for all experiments. Wild-type C57/Bl6 mice were purchased from the National Cancer Institute (Frederick, MD), and the \( \alpha_7 \text{nAChR}^{-/-} \) mice (B6.129S7-Chrtna7tm1Bay/J) were obtained from The Jackson Laboratory (Bar Harbor, ME). The 6-OHDA (Sigma-Aldrich, St. Louis, MO) was used for denervation studies.

**Ultrasound Application**

All experiments were performed in accordance with the National Institutes of Health and Institutional Animal Care and Use Guidelines. The Animal Care and Use Committee of the University of Virginia approved all procedures and protocols. For ultrasound exposure, mice were anesthetized with an i.p. injection of a mixture of ketamine (90 mg/kg), xylazine (9 mg/kg), and atropine (0.18 mg/kg). Fur was shaved and removed using a depilatory. Mice were then placed on a modified microscope stage, which was positioned under an ultrasound transducer held in place with a ring clamp. Prewarmed ultrasound gel was then placed on the depilated skin for ultrasound application. Mouse body temperature was monitored via rectal probe (Fine Science Tools, Foster City, CA) and maintained at 36 ± 0.5°C with a heating pad and heat lamp.

A clinical Sequoia 512 ultrasound machine with a 15L8w transducer (Acuson, Malvern, PA) was used for ultrasound application. Once the animal’s body temperature was stabilized, the left kidney was localized in real time using conventional B-mode imaging with a frequency of 14 MHz and an on-screen imaging mechanical index of 0.99. Cadence imaging began with a frequency of 7 MHz and an imaging mechanical index of 0.16. The ultrasound treatment consisted of ultrasound pulses
administered with a bursting mechanical index of 1.2. Ultrasound pulses were 1 second in duration and were applied once every 6 seconds for 2 minutes (20 seconds of exposure in total per kidney). After 2 minutes, conventional B-mode imaging was used to localize the right kidney for ultrasound pulse application. Control animals underwent the same preparation procedures but were not exposed to ultrasound. Total ultrasound exposure was approximately 5 minutes, with small variations in the time required to stabilize body temperature and localize the kidneys. After ultrasound treatment, animals were allowed to recover from the anesthetic in a temperature-controlled incubator.

**Surgical Procedures**

**IRI**

Mice underwent renal IRI 24 hours after ultrasound or sham ultrasound treatments. Mice were anesthetized with an i.p. injection of ketamine (120 mg/kg), xylazine (12 mg/kg), and atropine (0.324 mg/kg). Bilateral renal IRI was performed through flank incisions by clamping the renal pedicles for 26 minutes. Severity of IRI was modulated by reducing the ischemic time from 26 (IRI) to 24 minutes (subIRI) in the splenectomy experiments only. The clamps were then removed, and the wound was sutured after restoration of blood flow to kidney (reperfusion) was visually observed. Sham-operated mice underwent the same procedure except that the renal pedicles were not clamped. For splenectomy, mice were anesthetized with an i.p. injection of ketamine (120 mg/kg), xylazine (12 mg/kg), and atropine (0.324 mg/kg). The splenic vessels were then ligated, and the spleen was removed through a small flank incision. Sham mice underwent the same procedure with the exception of splenic artery ligation and removal of the spleen. Chemical splenic denervation was performed by injecting 6-OHDA (60–120 µg, Sigma-Aldrich) or saline into the exteriorized spleen. Buprenorphine (0.15 mg/kg) was administered as a postoperative analgesic for both IRI and splenectomy. A single dose of 10 mg/kg of buprenorphine was administered as a postoperative analgesic for both IRI and splenectomy. Mice were monitored every 6 hours.

**Plasma Creatinine and Stereological Analysis of Tissue Morphology**

Plasma was prepared by centrifuging heparinized blood at 7000 rpm for 5 minutes. Plasma creatinine (mg/dl) was determined via colorimetric analysis following the manufacturer’s protocol (Sigma-Aldrich). Kidneys were dissected, and the capsule was removed. A center transverse section was cut and placed in 4% paraformaldehyde/1.4% DL-lysine/0.2% sodium periodate in 0.1 M sodium phosphate buffer (pH 7.4) for 24 hours and then stored in 70% EtOH until paraffin embedding. Then 5 µm paraffin sections were cut and stained with hematoxylin and eosin.

The extent of acute tubular necrosis was assessed in an unbiased, systematic manner using design-based stereology to achieve statistically accurate random sampling of kidney sections and yielding the percentage of total area of the section occupied by injured tubules or fibrotic tissue. The investigator was blinded to the experimental identity of the sections. Sections were imaged using a Carl Zeiss Axio Imager Z1/Apoptome Microscope fitted with motorized focus drives and motorized XYZ microscope stage and integrated to a workstation running Stereo Investigator software (MBF Bioscience, Williston, VT). The area fraction fractionator probe was used for stereological analysis of the fractional area of the section occupied by tubular necrosis. The following parameters were defined: counting frame (250×250 mm), sample grid (600×600 mm), and grid spacing (50 mm). These values were defined empirically such that adequate numbers of sample sites were visited and adequate numbers of markers (indicating injured tubules or extracellular deposition of collagen) were acquired, in keeping with accepted counting rules for stereology. Injured tubules were identified on the basis of the presence of cast formation, tubule dilation, and/or tubular epithelial denudation.

**Flow Cytometry and Immunofluorescence Microscopy**

To determine splenocyte composition after ultrasound treatment, spleen single-cell suspensions were generated and stained with the following antibodies (clone, source): PE-Cy7-labeled anti-CD45 (30-F11; BioLegend, San Diego, CA), FITC-labeled anti-Ly6C (AL-21; BD Biosciences, San Jose, CA), Alexa-Fluor 647-labeled anti-CD3e (17A2; eBioscience, San Diego, CA), V500-labeled anti-CD4 (RM4-5; Becton-Dickinson), V500-labeled anti-CD8 (53-67.2; Becton-Dickinson), PE-Cy7-labeled anti-CD11b (M1/70; BioLegend, San Diego, CA), PE-labeled anti-CD19 (6D5; BD Biosciences, San Jose, CA), and PE-Cy7-labeled anti-CD49d (DX5; BioLegend, San Diego, CA). Flow cytometric analysis was performed using the FACSCanto II (BD Biosciences, San Jose, CA).
Cell pellets were diluted, and 2 \times 10^7 cells were then reconstituted with bone marrow (70% RPMI, 10% FBS, 1% glutamine, 1% penicillin-streptomycin, and 1% heparin) 24 hours before IRI or transferred to tissue culture plates with filtrates into 1% BSA/PBS solution. The cell pellet was collected by centrifugation, the resulting supernatant (7nAChR, WT or a7nAChR--/--) were then stained with FITC-labeled anti-7/4 antibody (CLB9993F, 7 µg/ml; CedarLane). Samples were covered with Prolong Gold antifade reagent with DAPI (Invitrogen) to label cell nuclei, coverslips were applied, and samples were visualized using a Carl Zeiss Axiosvert 200M microscope with ApoTome imaging and AxioVision software (Carl Zeiss).

For the adoptive transfer of splenocytes, spleens were harvested from ultrasound- or sham-treated animals 24 hours after treatment. Single-cell suspensions were generated by passing whole spleen through 40 µm filters into 1% BSA/PBS solution. The cell pellet was collected by centrifugation and then treated with ACK red blood cell lysis buffer (Life Technologies). The samples were then again centrifuged, the resulting cell pellet was diluted, and 2 \times 10^5 or 1 \times 10^6 cells were injected via tail vein 24 hours before IRI or transferred to tissue culture plates with RPMI, 10% FBS, 1% glutamine, 1% penicillin-streptomycin, and 1% β-mercaptoethanol for ex vivo stimulation.

Bone Marrow Chimeras and Adoptive Transfer Studies
Chimeric mice were generated as described previously. Briefly, WT or a7nAChR--/-- mice were lethally irradiated twice at 550 rad. Mice were then reconstituted with bone marrow (7-10 \times 10^5) from either WT or a7nAChR--/-- donor mice. Chimeric mice were housed in microisolators for 10 weeks before experimentation to allow complete reconstitution and were fed autoclaved food and water containing 5 mM sulfamethoxazole and 0.86 mM trimethoprim.

For the adoptive transfer of splenocytes, spleens were harvested from ultrasound- or sham-treated animals 24 hours after treatment. Single-cell suspensions were generated by passing whole spleen through 40 µm filters into 1% BSA/PBS solution. The cell pellet was collected by centrifugation, the resulting cell pellet was diluted, and 2 \times 10^5 or 1 \times 10^6 cells were injected via tail vein 24 hours before IRI or transferred to tissue culture plates with RPMI, 10% FBS, 1% glutamine, 1% penicillin-streptomycin, and 1% β-mercaptoethanol for ex vivo stimulation.

Real-Time PCR and Cytokine Analysis
Renal mRNA was isolated after the ethanol-precipitation method, and RNA concentration was determined on the basis of spectrophotometric determination of 260:280 ratio. cDNA was generated from the resultant tissue RNA using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA) as described by the manufacturer. Resultant cDNA was then used to determine relative mRNA expression of IL-6, IL-1β, MCP1 (fwd: TCA CCT GCT GCT ACT CAT TCA CCA, (rev): TAC AGC TTC TTT GGG ACA CCT GCT; Kim-1 (fwd): ACA TTG ATT GAT GCA CCA TTT GGG ACA CCT GCT; GGA ATC ACA ACA AC, (rev): ACT GCT TTG ATG ATA GGT GAC A; glyceraldehyde 3-phosphate dehydrogenase (fwd): ACG GCA AAT TCA ACG GCA CAG TCA, (rev): TGG GGG CAT CGG CAG AAG G; CXCL2/MIP2 (fwd): ACATCCCAACCCCCACAGTGAAAGA, (rev): TCCTTCATGAAAGCCATCAGACT; IL1β (fwd): AT-GAGCTTTTTTGAAGTTGAC, (rev): GTGATCTGCTGGCT-GAA; and IL-10 (fwd): TGCAGCTAAGAGGCCAACAAGCAG, (rev): TGCAGTTATTGTCTTCCCGGTGT.

A panel of serum cytokines and chemokines from ultrasound and IRI experiments was assessed using Mouse Cytokine/Chemokine Magnetic Bead Multiplex Assay (EMD Millipore, MA) as described by the manufacturer. Plasma samples were analyzed as recommended by the manufacturer using a Luminex 100 IS system (UVA Flow Cytometry Core Facility). In the ex vivo stimulation studies, supernatant IL-6 was determined by ELISA following the manufacturer’s protocol (eBiosciences).

Statistical Analyses
All animal studies were conducted using a complete randomized design. Data were analyzed using one-way or two-way ANOVA, with a significant difference defined as P<0.05. Repeated experiments were analyzed as a randomized complete block design. Animals receiving sham treatments were included in the experiments for reference only and were not included in the statistical analysis. Means were compared by post hoc multiple comparison test (Tukey), and all values are presented as mean±SEM. The survival analysis was performed using log-rank Kaplan–Meier survival analysis. All statistical analyses were performed using SigmaPlot 11.0 software (Systat, Chicago, IL).

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DISCLOSURES
None.

REFERENCES


