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Renal tubular cell spliced X-box binding protein-1 (Xbp1s) has a unique role in sepsis-induced acute kidney injury and inflammation

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Abstract

Sepsis is a systemic inflammatory state in response to infection, and concomitant acute kidney injury (AKI) increases mortality significantly. Endoplasmic reticulum stress is activated in many cell types upon microbial infection and modulates inflammation. The role of endoplasmic reticulum signaling in the kidney during septic AKI is unknown. Here we tested the role of the spliced X-box binding protein 1 (Xbp1s), a key component of the endoplasmic reticulum stress-activated pathways, in the renal response to sepsis in the lipopolysaccharide (LPS) model. Xbp1s was increased in the kidneys of mice treated with LPS but not in other models of AKI, or several chronic kidney disease models. The functional significance of *Xbp1s* induction was examined by genetic manipulation in renal tubules. Renal tubule-specific overexpression of *Xbp1s* caused severe tubule dilation and vacuolation with expression of the injury markers *Kim1* and *Ngal,* the pro-inflammatory molecules interleukin-6 *(Il6)* and Toll-like receptor 4 *(Tlr4),* decreased kidney

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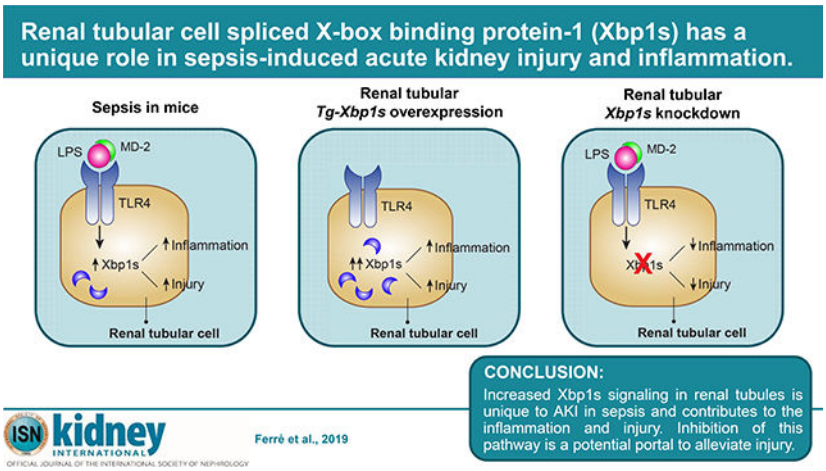
DISCLOSURE

None.

Supplementary information is available at Kidney International's website

function and 50% mortality in five days. Renal tubule-specific genetic ablation of *Xbp1* had no phenotype at baseline. However, after LPS, *Xbp1* knockdown mice displayed lower renal NGAL, pro-apoptotic factor CHOP, serum creatinine levels, and a tendency towards lower *Tlr4* compared to LPS-treated mice with intact *Xbp1s.* LPS treatment in *Xbp1s*-overexpressing mice caused a mild increase in NGAL and CHOP compared to LPS-treated mice without genetic *Xbp1s* overexpression. Thus, increased Xbp1s signaling in renal tubules is unique to sepsis- induced AKI and contributes to renal inflammation and injury. Inhibition of this pathway may be a potential portal to alleviate injury.

Graphical Abstract



Keywords

ER stress; Xbp1s; AKI; sepsis; inflammation

INTRODUCTION

Sepsis is a systemic inflammatory response elicited by microbial infection. It is frequently encountered in the intensive care unit (ICU) and is associated with significant morbidity and mortality.[1,2] Acute kidney injury (AKI) is a frequent complication in critically ill patients occurring in about 45% of septic patients and 60% of those with septic shock.[3–5] The combination of sepsis and AKI synergistically increases mortality to beyond 50%.[1]

The pathophysiology of AKI in sepsis is complex, but results in part from systemic inflammation, nephrotoxins, and hemodynamic alterations.[6,7] Systemic endotoxin, or lipopolysaccharide (LPS), released from gram-negative bacteria binds to the Toll-like receptor 4 (TLR4)-myeloid differentiation factor 2 (MD-2) complex on immune cells followed by secretion of various cytokines, such as interleukin (IL)-1, tumor necrosis factor (TNF)-α, and IL-6, resulting in a cytokine storm, hemodynamic instability, and eventually septic shock.[6,8,9] LPS can cause direct injury through interactions with epithelial and endothelial TLR4-MD-2 complex in the kidney. Both LPS and TNF-α have direct pro-

inflammatory effects on renal epithelial cells,[10,11] and can induce renal tubular production of cytokines.[12–14]

Endoplasmic reticulum (ER) stress and the unfolded protein response (UPR) are induced upon viral, bacterial, or parasitic infection.[15,16] UPR is a complex network of intracellular signaling pathways that restores ER homeostasis towards normality, or activates pro-apoptotic towards cellular demise, depending on cell types and severity of the stress.[17,18] UPR activation is associated with production of many pro-inflammatory molecules that contribute to the pathogenesis and/or progression of conditions such as diabetes, obesity, inflammatory bowel disease, and cancer.[19] However, little is known about the role of the UPR in the renal response to sepsis *in vivo.*

Among the three UPR signaling cascades, the inositol-requiring enzyme 1α (IRE1α) pathway is the most conserved across species.[20] Activated IRE1α has endoribonuclease activity, which cleaves its primary target, *X-box-binding protein 1* (*Xbp1*) mRNA. The specific excision of 26 nucleotides from the *Xbp1* mRNA causes a frameshift, generating the spliced *Xbp1* form (referred to as *Xbp1s*), which is translated into a potent transcription factor.[17] *Xbp1s* transcript levels were increased in the lungs of mice treated with LPS and in primary bronchial epithelial cells treated with *Pseudomonas aeruginosa* virulence factors.[21,22] In macrophages, the spliced Xbp1s protein is required downstream of TLR4 for sustained transcription and optimal production of immune mediators such as IL-6.[23] Deletion of *Xbp1s* in macrophages and other hematopoietic cells increases bacterial burden in animals infected with the TLR2-activating human pathogen *Francisella tularensis*,[23] and specific disruption of *Xbp1s* in the intestinal epithelium results in inability to respond appropriately to inflammatory signals and increased susceptibility to colitis.[24] These studies suggest Xbp1s expression may be beneficial in these inflammatory states.

Thus far, studies on the role of Xbp1s in the kidney have mainly focused on podocytes, and Xbp1s response to ER stress secondary to metabolic signals or accumulation of immature proteins. Disruption of *Xbp1s* in podocytes is not pathogenic,[25] but when coupled with persistent hyperglycemia, podocyte *Xbp1s* deficiency aggravates glucose-induced cellular injury, in part by increased oxidative stress, and exacerbates diabetic nephropathy.[26,27] Simultaneous genetic ablation of *Xbp1* and *Sec63,* a heat shock protein-40 chaperone required for protein folding in the ER, in podocytes results in foot process effacement and albuminuria, suggesting that intact *Xbp1* is essential for the maintenance of a normal glomerular filtration barrier.[25] Concomitant inactivation of both *Sec63* and *Xbp1* in distal tubules in a polycystic kidney disease model, decreases maturation of polycystin-1 and exacerbated the disease,[28] while in the collecting ducts causes chronic tubulointerstitial injury.[29] However, the role of Xbp1s in the tubular epithelium in the context of sepsis is unknown.

In the present study, we examined the response of native Xbp1s in response to LPS and used renal tubule-specific *Xbp1s*-overexpression or deletion to examine the functional role of Xbp1s on kidney injury during LPS-mediated sepsis.

RESULTS

Xbp1s is activated in the mouse kidney upon LPS-induced sepsis

In the kidneys of control mice, Xbp1s expression was detected in the nucleus of LTA (proximal convoluted) and NCC (distal convoluted) positive tubules in the renal cortex, whereas Xbp1s expression in the renal medulla was modest (Figure 1C and Supplementary Figure 1). After acute kidney injury (AKI), kidney *Xbp1s* mRNA was increased exclusively after LPS injection, but not after folic acid-induced or ischemia-reperfusion AKI (Figure 1A and Supplementary Table 1). The increase of Xbp1s nuclear expression in response to LPS treatment occurred in the nucleus of all renal tubules, both in the renal cortex and medulla (Figure 1C and Supplementary Figure 1). *Xbp1s* was also increased in the kidneys of mice that developed sepsis after cecal ligation puncture (CLP), a condition that shares pathophysiologic features with LPS (Supplementary Figure 2). Our findings were confirmed by RT-PCR and qPCR using *Xbp1s*-specific primers that selectively amplify the spliced form of the transcript (Primer set 3, Supplementary Figure 3). Primers that anneal to the *Xbp1* mRNA outside the cleavage site showed unchanged levels of unspliced *Xbp1* mRNA (Primer set 1, Supplementary Figure 3 and Figure 1A). In order to determine whether the increase in renal *Xbp1s* expression is mediated by TLR4 activation during bacterial inflammation, we injected $Tlr4^{Lps\text{-}d}$ mice, which have an inactivating mutation in *Tlr4* and are resistant to LPS, with Ultrapure LPS that only activates the TLR4, but not the TLR2, pathway. *Xbp1s* response to Ultrapure LPS was blunted in kidneys from $Tlr4^{Lps\text{-}d}$ mice, but not in wild-type mice, indicating that the induction of renal *Xbp1s* mRNA splicing is downstream of TLR4 activation in LPS sepsis (Supplementary Figure 4).

Since ER-stress response is activated in chronic conditions such as diabetes, obesity and cancer,[30,31] we tested *Xbp1* splice variants in three models of genetically-induced chronic kidney disease (CKD; Figure 1B). We did not detect any changes in *Xbp1s* levels in *ob/ob* (obesity and the metabolic syndrome), Ksp/*Cre*;$Pkd1^{F/F}$, and Pkhd1/*Cre*;$Pkd2^{F/F}$ (polycystic kidney disease) mouse kidney samples compared to their corresponding controls (Figure 1B). These results indicate that increased renal *Xbp1s* mRNA expression is unique to LPS-induced AKI. The next and logical question is whether the increased Xbp1s is a paraphenomenon, protective, or pathogenic for AKI.

Tubular overexpression of *Xbp1s* induces tubular injury in kidney-inducible *Xbp1s* (KIXs) mice

The systemic and multifactorial nature of LPS injection renders it difficult to dissect the role of renal *Xbp1s* expression in LPS-induced AKI, and disables one from concluding whether the high *Xbp1s* induced by LPS is a mere biomarker, a protective cellular adaptation, or pathogenic. We made a mouse where the *Xbp1s* transgene downstream from a tetracycline-responsive element (TRE) allows inducible renal tubule-specific expression of *Xbp1s* by the tetracycline reverse transcriptional activator (rtTA) driven by a Ksp promoter with doxycycline (Dox), without the need to expose the mice to endotoxin. Crossing TRE/Xbp1s with Ksp/*rtTA* yielded a mouse with kidney-specific inducible expression of Xbp1s (KIXs; Figure 2A). After Dox, *Xbp1s* mRNA and protein was readily detected within 24 hours (Figure 2B and Supplementary Figure 5A–C) and persisted 2 and 5 days after induction

(Supplementary Figure 5A–C). The functionality of the transgenic *Xbp1s* was validated by increased expression of known Xbp1s target genes in KIXs mice such as *GalE* and *Dnajb9*,[32,33] and of genes involved in the unfolded protein response (UPR), namely *Bip* and CCAAT-enhancer-binding protein homologous protein (CHOP) gene *Chop* (Figure 3A–B).[17] As expected, the expression of *Bip,* encoding the major chaperone in the ER stress response, rapidly and steadily increased over time, while the expression of *Chop,* a pro-apoptotic transcription factor involved in the late response to stress, continued to increase up to 5 days. Co-staining of KIXs kidney sections with Xbp1s and nephron segment-specific markers showed that Xbp1s overexpression occurred in both proximal and distal tubules (Supplementary Figure 5D).

KIXs mice showed reduced weight gain over time, 65% increase in kidney-weight-to-body-weight ratio and 50% mortality 5 days after induction (Figure 2C–E). Kidney function was severely impaired in KIXs compared to controls as demonstrated by markedly elevated blood urea nitrogen (BUN) and serum Cr levels at 24 hours after induction, which continued to rise up to 5 days (Figure 2F–G). Kidney histopathology revealed tubular necrosis, tubule dilation, and intracellular vacuoles (Figure 2H). Periodic acid-Schiff (PAS) staining showed loss of brush border in all KIXs mice (Supplementary Figure 6) compatible with proximal tubule injury. Due to the short observational period, fibrosis based on trichrome staining was not observed in KIXs kidneys (Supplementary Figure 6). The expression of the kidney injury marker neutrophil gelatinase-associated lipocalin *(Ngal),* and inflammatory molecules *Il6* and *Tlr4* was increased in KIXs compared to control mice at 2 and 5 days after induction (Figure 3C–D). The increase in the injury marker kidney injury molecule-1 *(Kim1)* was more variable and did not reach statistical significance at 5 days (P=0.14). Recovery of injured tubular cells is achieved by biphasic expression of genes normally expressed during kidney development, and transient activation of cytokine-induced signaling.[34,35] We found downregulation of hepatocyte nuclear factor-1beta *(Hnf1b),* a transcription factor involved in kidney development, and dysregulation of its target genes, suppressor of cytokine signaling 3 (*Socs3)*, and polycystic kidney and hepatic disease 1 (*Pkhd1)* (Figure 3E). Therefore, overexpression of Xpb1s alone is sufficient to induce renal damage, suggesting that the elevation of Xpb1s in sepsis-induced AKI is not just a biomarker but likely pathogenic for the kidney injury.

Xbp1s deletion is protective against LPS-induced kidney injury

The above studies established that Xbp1s is sufficient to cause kidney injury. We next proceeded to test the necessity of Xbp1s in septic renal injury. In order to determine the role of renal tubular Xbp1s in sepsis-induced AKI, we generated renal tubule-specific *Xbp1* deletion using the kidney-specific Six2/*Cre* (Figure 4A). During kidney development, Six2/*Cre* is expressed in the nephron progenitors that will form the epithelial tubule cells. *Xbp1* expression was decreased 50% in total kidney lysate of Six2/Cre^{+};$Xbp1^{F/F}$ mice compared to Six2/Cre^{-}; $Xbp1^{F/F}$ mice (Figure 4B). At baseline, Six2/Cre^{+};$Xbp1^{F/F}$ animals did not exhibit abnormalities in body weight or kidney morphology and function (Figure 4C–D). A similar finding was previously described in Ksp/Cre^{+};$Xbp1^{F/F}$ mice in which Cre activity resulted in deletion of *Xbp1* in distal nephron segments.[28]

Next, we challenged both the Six2/*Cre*$^{+}$;*Xbp1*$^{F/F}$ and Six2/*Cre*$^{-}$;*Xbp1*$^{F/F}$ control mice with an injection of LPS, and compared them to animals injected with vehicle (Figure 5 and Supplementary Figure 7A). Histology did not show any significant morphologic changes after LPS injection in either the Six2/*Cre*$^{+}$;*Xbp1*$^{F/F}$ or Six2/*Cre*$^{-}$;*Xbp1*$^{F/F}$ control mice (Supplementary Figure 7A), similar to other reports of minimal histological tubular injury in septic AKI.[36–38] LPS treatment induced *Xbp1s* to approximately 20 times in the kidney of Six2/*Cre*$^{-}$;*Xbp1*$^{F/F}$ mice, but failed to increase *Xbp1s* expression in the kidney of Six2/*Cre*$^{+}$;*Xbp1*$^{F/F}$ mice (Figure 5C). *Tlr4* and *Kim1* mRNA expression were upregulated in Six2/*Cre*$^{-}$;*Xbp1*$^{F/F}$ LPS-injected mice, but not in Six2/*Cre*$^{+}$;*Xbp1*$^{F/F}$ LPS-injected mice (Figure 5C). The difference in *Kim1* and *Tlr4* expression between the two LPS-treated genotypes reached statistical significance for *Kim1* ($P<0.01$), but not for *Tlr4* ($P=0.07$). *Il6* and *Tnfa* expression levels were not different across the experimental groups. While *Ngal* mRNA was equally increased in both genotypes upon LPS treatment (Figure 5C), the increase in kidney protein levels of NGAL was significantly less in kidneys from Six2/*Cre*$^{+}$;*Xbp1*$^{F/F}$ LPS-injected mice compared to those from *Six2/Cre*$^{-}$;Xbp1$^{F/F}$ LPS-injected mice (Figure 5B). Within 24 hours after LPS treatment, kidney dysfunction developed as shown by elevated serum Cr in both Six2/*Cre*$^{-}$;*Xbp1*$^{F/F}$ and Six2/*Cre*$^{+}$;*Xbp1*$^{F/F}$ mice (Figure 5A). However, the increase in serum Cr in Six2/*Cre*$^{+}$;*Xbp1*$^{F/F}$ was lower than in controls (Figure 5A). Although IREα-Xbp1s signaling is important in promoting pro-survival UPR gene expression, prolonged or excessive ER stress will lead Xbp1s mediated cell death via pro-apoptotic factor CHOP.[39] After LPS challenge, kidneys from Six2/*Cre*$^{+}$;*Xbp1*$^{F/F}$ mice had lower levels of CHOP compared to those from Six2/*Cre*$^{-}$;*Xbp1*$^{F/F}$ mice (Figure 6A–B). In order to examine the association between changes in *Xbp1s* and renal function, we further analyzed the data obtained from Six2/*Cre*$^{-}$;*Xbp1*$^{F/F}$ mice injected with vehicle or LPS. We found a direct relationship between *Xbp1s* expression level and serum Cr, *Kim1, Ngal, Tlr4* and *Chop* expression in kidney (Supplementary Figure 8). These data further support the model that Xpb1s has a pathogenic role in LPS-induced AKI.

Xbp1s overexpression exacerbates LPS-induced kidney injury

To interrogate whether Xbp1s is the sole or major mediator of LPS-induced AKI, we examined whether *Xpb1s* overexpression and LPS are additive in imparting renal injury. Histology did not show any significant morphologic changes after LPS injection in either the control or KIXs kidneys (Supplementary Figure 7B).[36–38] Within the vehicle-treated group, KIXs mice had significantly higher *Tlr4* and *Chop* expression compared to controls (Figure 6C–D and Figure 7C). After LPS injection, control animals showed an increase in NGAL/*Ngal* levels compared to vehicle-treated controls (Figure 7B–C). Similarly, after LPS injection, KIXs animals showed an increase in NGAL/*Ngal* levels comparable to vehicle-treated KIXs (Figure 7B–C). The increase in NGAL protein levels were not significantly higher in LPS-treated KIXs than in LPS-treated controls (Figure 7B). Serum Cr levels, *Kim1, Tlr4* and *Chop*/CHOP expression did not differ between KIXs treated with vehicle or LPS (Figure 7A and 7C, Figure 6C–D). *Il6* and *Tnfa* expression levels were not different across the experimental groups. These studies suggest that in the presence of already elevated *Xbp1s* levels, LPS only modestly increases injury.

DISCUSSION

This study investigated the role of renal tubular UPR transcription factor Xbp1s affects AKI during sepsis. We found that the response of renal tubules to systemic endotoxin involves Xbp1s signaling, and that activation of this pathway contributes to tubular injury, inflammation and renal failure based on the following findings. First, two *in vivo* models of sepsis increased native *Xbp1s* in kidney. The increase of *Xbp1s* in kidney after LPS injection was recently observed also by Hato et al. between 4 hours to 28 hours.[40] Second, inducible *Xbp1s* overexpression in renal tubules *per se* was sufficient to produce pathology that resembles sepsis-induced AKI including increased serum Cr, injury markers and pro-inflammatory molecules *Il6* and *Tlr4*. Moreover, a frequent observation after *Xbp1s* overexpression was vacuolation of tubule epithelium which is the most consistent pathology in septic AKI.[8] Third, *Xbp1s* mRNA reduction in renal tubules is associated with lower NGAL and CHOP/*Chop* levels in the kidney after LPS treatment compared to LPS-treated controls with intact *Xbp1s* expression. These last two findings support the conclusion that the increased *Xpb1s* in LPS- induced AKI is pathogenic, necessary, and sufficient. Fourth, concomitant *Xbp1s* overexpression and LPS injection exhibits additive effects only to a certain extent indicating that LPS utilizes pathways in addition to Xpb1s to induce AKI.

The level of nuclear Xbp1s in renal cortex of wild-type mice concurs with previous studies demonstrating that Xbp1s is required in a tissue-specific manner for constitutive homeostatic UPR.[26] However, Xbp1s hyperactivity during infection is detrimental to the kidney. We observed no changes in kidney *Xbp1s* mRNA expression in AKI models other than LPS, such as FA and IRI experiments, suggesting no significant Xbp1s activation at the whole kidney level. However, it is possible that Xbp1s could be increased in small subsets of cells. Further studies will be needed to determine whether Xbp1s is activated in specific limited cell populations within the kidney in AKI models other than LPS, and are clinically relevant in those disease processes.

Importantly, *Xbp1s* overexpression *per se* leads to kidney injury, inflammation, and mortality in the absence of systemic infection and inflammation. KIXs mice had higher renal *Chop* levels within a day after induction, and higher *Ngal, Kim1, Il6* and *Tlr4* levels starting at 2 and 5 days after induction. In our LPS-induced sepsis experiments, vehicle-treated KIXs mice showed a significant increase only in *Tlr4* and *Chop* expression compared to vehicle-treated controls, possibly due to the shorter induction time used in this protocol (i.e. 16 hours versus 1 day). The combination of LPS and *Xbp1s* overexpression did not have additive effect on *Tlr4* and *Chop* expression in KIXs, but it increased renal *Chop* expression compared to LPS-alone. It is important to consider that the transgenic induction of *Xbp1s* in KIXs was much higher than the *Xbp1s* induction by LPS alone (approximately 2,000–4,000 times in KIXs versus 6–20 times after LPS injection, respectively). It is possible that in LPS-treated KIXs the *Xbp1s* overexpression overwhelmed the cellular UPR response making it difficult to unmask any contribution by other parallel pathways activated by LPS.

Overall, we found that *Xbp1s* overexpression activates pathways that are common to LPS-induced injury, which contrasts with previous findings showing that pretreatment with ER stress inducers reduces the degree of renal injury.[41–44] Nevertheless, Xbp1s in macrophages

was shown to be required downstream of TLR4 after LPS recognition for sustained production of innate immune mediators like IL-6 and TNF-α.[23] Our data suggest that such a cascade occurs in renal tubular cells. *Xbp1s* overexpression increased *Il6* and *Tlr4,* and increased *Socs3, a* major regulator that inhibits the anti-inflammatory response in infection.[45,46] The increased *Socs3* is compatible with the decrease of its transcriptional repressor *Hnf1b.* Downregulation of *Hnf1b* also decreased its target gene *Pkhd1* in KIXs.[47] The *Hnf1b-Socs3-Pkhd1* axis is essential for tubulogenesis, a process that is reactivated during the regeneration of injured tubular cells.[47] In KIXs mice, this pathway was silenced meaning that the repair potential of renal tubules is impaired, which in turn may contribute to the burden of kidney failure in these mice.

On the other hand, Six2/*Cre*$^{+}$;*Xbp1*$^{F/F}$ mice had no detectable phenotype at baseline but showed a tendency towards less kidney injury and inflammation upon LPS challenge. Using Six2/*Cre*, we achieved a 50% decrease in *Xbp1* mRNA in total kidney of 21 day old mice (Figure 4). Using *Xbp1s*-specific primers, we found that *Xbp1s* levels were similar between Six2/*Cre*$^{+}$;*Xbp1*$^{F/F}$ and Six2/*Cre*$^{-}$;*Xbp1*$^{F/F}$ mice that were 8–12 weeks old and injected with vehicle (Figure 5C). This suggests that in the absence of stress, *Xbp1s* levels are maintained constant in the kidney of Six2/*Cre*$^{+}$;*Xbp1*$^{F/F}$ mice via increased splicing of newly transcribed or already available *Xbp1* mRNA in tubular cells that did not undergo recombination. However, when treated with LPS, Six2/*Cre*$^{+}$;*Xbp1*$^{F/F}$ mice failed to further increase kidney *Xbp1s* expression compared to LPS-injected Six2/*Cre*$^{-}$;Xbp1$^{F/F}$ controls. After LPS injection, Six2/*Cre*$^{+}$;*Xbp1*$^{F/F}$ mice showed lower *Kim1* and *Chop* expression, lower NGAL protein levels, and lower serum Cr values compared to LPS-treated controls. NGAL is also a marker of systemic inflammation as it is produced by both hematopoietic and non-hematopoietic cells as part of the inflammatory response.[48] The differences observed in NGAL expression in the KIXs and *Xbp1s* knockdown kidneys during sepsis may be explained by both changes in the number of infiltrating immune cells and in NGAL production by renal tubular cells.

ER stress signaling controls inflammation in an organ-specific manner.[19] While the knockdown of pathways that activate *Xbp1s* in other systems is associated with increased inflammation,[24,49,50] we showed that Xbp1s actually promotes inflammation and injury in LPS-induced AKI, while a decrease in Xbp1s in renal tubular cells attenuates the inflammatory response and injury (Figure 8). Using *Tlr4*$^{Lps\text{-}d}$ mice, which have a whole body expression of mutant *Tlr4* and are resistant to LPS, we showed that TLR4 activation by LPS is required for the increase in Xbp1s in kidney. However, due to the global nature of the deletion, this experiment *per se* is not sufficient to distinguish between the role of tubular TLR4 signaling versus TLR4 activation in infiltrating immune cells; two pathways that can both contribute to the increase in Xbp1s in kidney. Recently, administration of STF-083010, an inhibitor of the endonuclease activity of IRE1α, to rats with ischemic AKI, suppressed Xbp1s, inflammation and apoptosis, and improve renal function and structure.[51] In this study, we did not observe any improvement in the kidney histology of Six2/*Cre*$^{+}$;*Xbp1*$^{F/F}$ mice ih the short duration of 24 hours after LPS injection (Supplementary Figure 7), but we cannot exclude that the lower NGAL (and serum creatinine) levels may lead to a better renal outcome in longer follow-up. As sepsis causes multi-organ dysfunction, systemic therapy to suppress Xbp1s activation could have implications for organs other than the kidney. Future

studies are needed to determine whether and how LPS sepsis activates Xbp1s in non-renal organs as well as whether renal Xbp1s-mediated AKI affects distant organs.[52]

In conclusion, our findings have significant translational potential. Further investigation in septic patients will be required to confirm or refute whether renal Xbp1s signaling drives AKI in sepsis, whether Xbp1s could serve as an early sentinel biomarker to signal impending sepsis- associated AKI and eventually, pharmacologic blockade of Xbp1s signaling can prevent, forestall, or alleviate AKI in sepsis and improve overall survival.

METHODS

Animal studies

Mice were maintained on a 12-hour light/dark cycle (6 am to 6 pm) with unrestricted access to food and water and no more than 4 mice per cage. All protocols were approved by the Institutional Animal Care and Use Committee of University of Texas Southwestern Medical Center. Male and female mice were randomly assigned to experimental groups in all studies.

To study kidney expression of *Xbp1s* in AKI, several disease models were used. C57BL/6 mice (8–12 weeks old) were injected with intraperitoneal (IP) folic acid (FA, 240 μg/g body weight; F7876, Sigma-Aldrich, St.Louis, MO) or vehicle ($NaHCO_3$, 0.2 ml, 0.3 M; S6014, Sigma-Aldrich, St.Louis, MO), and killed 48 hours later.[53] Another set of C57BL/6 mice (8–12 weeks old) received an IP injection of 16 mg/kg lipopolysaccharide (LPS) from *Escherichia coli* 0111:B4 (LPS-EB; tlrl-eblps, InvivoGen, San Diego, CA) or vehicle, and a single dose of IP injection of 1 mg/kg buprenorphine to control pain. 500 μl of saline was administered subcutaneously as fluid replacement. Animals were sacrificed at 24-hour following LPS injection. Cecal ligation puncture (CLP) was performed in C57BL/6 mice as a second sepsis model *in vivo.* Briefly, the cecum was ligated 1 cm from the distal end with a silk suture and punctured once with a 23-gauge needle. Sham operated animals underwent laparotomy without ligation or puncture of the cecum. 1 mg/kg sustained release buprenorphine was administered IP at the time of surgery to all animals. Mice were sacrificed 24 hours following surgery. Finally, another set of C57BL/6 mice (8–12 weeks old) underwent right nephrectomy and contralateral ischemia-reperfusion injury (IRI; 20' ischemia by renal artery cross-clamp, 4-hour reperfusion), and sacrificed 4-hour after surgery. To study the expression of *Xbp1s* in chronic kidney disease (CKD), three models were tested. First, the *ob/ob* mouse strain was used as model timed for phases I and II of type II diabetes and obesity. These mice exhibit obesity, hyperphagia, transient hyperglycemia, glucose intolerance, and elevated plasma insulin due to a homozygous mutation in leptin. Next, two previously described mouse models of polycystic kidney disease (PKD) were tested, Ksp/*Cre*;*Pkd1*$^{F/F}$ and Pkhd1/*Cre*;*Pkd2*$^{F/F}$.[54] Ksp/*Cre*; *Pkd1*$^{F/F}$ mice exhibit an aggressive phenotype and die from kidney failure by postnatal day 16 (P16). Pkhd1/*Cre*;*Pkd2*$^{F/F}$ mice suffer from a milder form of PKD and die between P7–125. To prove that the Xbp1s increase in kidney after LPS treatment is mediated by TLR4 activation, C3H/HeJ mice, which have a spontaneous mutation in *Tlr4* at the LPS response locus (*Tlr4*$^{Lps\text{-}d}$) that makes C3H/HeJ mice more resistant to endotoxin, were used. C3H/HeJ or wild-type C57BL/6J mice received an IP injection of 16 mg/kg Ultrapure LPS from

Escherichia coli 0111 :B4 (Ultrapure LPS-EB: tlrl- 3pelps, InvivoGen, San Diego, CA) that only activates the TLR4, but not TLR2, pathway, or vehicle, and sacrificed after 24 hours.

To generate the kidney-inducible *Xbp1s* (KIXs) transgenic mice, we crossed the TRE/*Xbp1s* transgenic mouse[32] in C57BL/6 background with the Ksp/*rtTA*[55] mouse model in CD-1 background. Doxycycline (Dox)-containing water (0.1 or 0.5 mg/mL; D9891, Sigma-Aldrich) was used to induce *Xbp1s* expression at weaning (21 days after birth). Ksp/*rtTA* transgenic mice given Dox were used as controls to exclude confounding effects of rtTA expression and activation, and Dox exposure. *Xbp1s* expression was tested at 1, 2 and 5 days after induction. Fresh Dox-containing water was provided 3 days after the initial induction.

Conditional *Xbp1* knockdown mice ($Xbp1^{F/F}$)[24] in C57BL/6 background were bred with Six2/*Cre*[56] mice with CD-1 background to achieve renal tubule-specific deletion of *Xbp1.* KO mice at weaning were compared to age-matched Six2/*Cre* control animals. KIXs or Six2/Cre^{+};$Xbp1^{F/F}$ mice (8–12 weeks old) were used for LPS injections. KIXs underwent preconditioning with doxycycline-containing water (0.1 mg/mL) for 16 hours (6 pm to 10 am). At sacrifice, tissues were frozen in liquid nitrogen for subsequent processing.

Tissue harvesting and analysis

Mice were anesthetized and blood was obtained by cardiac puncture, and the right kidney was flash-frozen for molecular assays. Left kidneys were perfused with cold PBS and 4% (w/v) paraformaldehyde (PFA) and then removed. For immunofluorescence, isolated kidneys were fixed in 4% (w/v) PFA overnight, dehydrated in sucrose/PBS 30% (w/v) overnight, embedded in optimal cutting temperature (OCT) compound, and sectioned by cryostat. For immunohistochemistry, kidneys were fixed in 4% (w/v) PFA overnight and embedded in paraffin. Sagittal sections of kidneys were stained with hematoxylin and eosin (H&E), trichrome or Periodic acid-Schiff stain (PAS), and examined by light microscopy using an Axioplan 2 Imaging system (Carl Zeiss Micro-Imaging, Inc. Thornwood, NY).

For histology, kidney sections stained with H&E were examined and photographed by an operator blinded to the experimental protocol. A semi-quantitative pathological scoring system was used as described with minor modifications.[57] The severity of renal tubular damage was scored in a blinded fashion with a grading system: 0 = no visible lesions, normal or near normal morphology; 1 = loss of brush border in less than 25% of tubular cells, integrity of basal membrane; 2 = loss of brush border in more than 25% of tubular cells, thickened basal membrane; 3 = as in 2 plus inflammation cast formation, necrosis up to 60% of tubular cells; 4 = as in 3 plus necrosis in more than 60% of tubular cells. Ten fields of cortex and medulla were scored in each kidney sections using ImageJ. The total score for each kidney was calculated by addition of all scores with a numeric maximum of 40.

Blood urea nitrogen (BUN) and creatinine measurements

Blood was collected, kept at room temperature (RT) for 30 minutes, and serum was isolated (3,600 × g for 10 min). Serum creatinine (Cr) was measured using capillary electrophoresis, and BUN using the Vitros 250 Analyzer.

Immunofluorescence

Frozen 8-mm sections were washed with PBS, blocked with 5% (w/v) BSA/PBS (1 h at RT), incubated with a primary antibody anti-Xbp1s (1:50; 619502, BioLegend, San Diego, CA), anti- NKCC2 (1:200; NKCC21-A, Alpha Diagnostic International, San Antonio, TX), or anti-NCC (1:200; SPC-402D, StressMarq, Biosciences Inc., Victoria, Canada) at 4°C overnight, and detected by secondary antibodies conjugated with Alexa Fluor 594 or Alexa Fluor 488 (1:500; Molecular Probes, Eugene, OR). Sections stained with Lotus Tetragonolobus Lectin (LTA) were incubated with fluorescein-labeled LTA (1:500; FL-1321, Vector Laboratories, Burlingame, CA) at RT for 30 min before washing. Sections stained with Dolichos Biflorus Agglutinin (DBA) were incubated with biotinylated DBA (1:400; B-1035, Vector Laboratories, Burlingame, CA) at 4°C overnight, and detected by fluorescein Avidin D (1:500; A-2001, Vector Laboratories, Burlingame, CA).

Immunoblotting

Total protein was extracted from kidneys using T-PER buffer (78510, Thermo Fisher Scientific, Waltham, MA) with proteinase/phosphatase inhibitors (88669, Thermo Fisher Scientific, Waltham, MA). Protein was quantified by the BCA Protein Assay Kit (23227, Thermo Fisher Scientific, Waltham, MA). 20 μg of protein was loaded on a 4–15% SDS-polyacrylamide gel, and the proteins were transferred nitrocellulose membranes. The membrane was blocked in either 5% bovine serum albumin (BSA) or 1% powdered milk, and then incubated with a primary rabbit anti-Xbp1s (1:500; 619502, BioLegend, San Diego, CA), goat anti-Ngal (1:1000; AF1757, R&D Systems, Minneapolis, MN), mouse anti-GADD153 (clone B-3; 1:200; sc-7351, Santa Cruz Biotechnology Inc., Dallas, TX), or mouse anti-β-actin (1:50,000; A3854; Sigma-Aldrich, St.Louis, MO) overnight at 4°C. Goat anti-rabbit, donkey anti-goat or sheep anti-mouse HRP- conjugated IgG were used as secondary antibodies, and blots were developed using the SuperSignal West Dura Extended Duration substrate (Pierce, Thermo Fisher Scientific, Waltham, MA). The protein bands were quantified using Quantity One imaging software from Bio-Rad.

Reverse transcription polymerase chain reaction (RT-PCR) and quantitative RT-PCR (RT-qPCR)

Total RNA from mouse kidney was isolated using Trizol (Gibco, Thermo Fisher Scientific, Waltham, MA). Total RNA (2 μg) was treated with DNase (18068–015, Invitrogen, Thermo Fisher Scientific, Waltham, MA) and reverse transcribed using an iScript cDNA Synthesis Kit (170–8891, Bio-Rad, Hercules, CA). RT-PCR was performed using a Platinum Taq DNA Polymerase High Fidelity (11304–011, Invitrogen, Thermo Fisher Scientific, Waltham, MA). Transcript levels were quantified by RT-qPCR using SybrGreen (172–5121, Bio-Rad, Hercules, CA) on a CFX Connect real-time PCR detection system. Expression amount was normalized to 18S mRNA and was represented as fold change. Data were analyzed using the Bio-Rad CFX software. PCR primers (Supplementary Table 2) were designed with the online NCBI/Primer- BLAST software.

Statistical analyses

All results are depicted as mean ± standard error of the mean (SEM). Unpaired student's t-test was used for pairwise comparisons, and two-way analysis of variance (ANOVA) with Sidak's test for multiple comparisons. $P<0.05$ was considered statistically significant. The correlation between Xbp1s gene expression and serum Cr values, *Kim-1, Ngal, Tlr4* and *Chop* gene expression was assessed with linear regression using Prism 7 (v.7.0a, GraphPad Software Inc, La Jolla, CA).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGEMENTS

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TRANSLATIONAL STATEMENT

Increased Xbp1s signaling in renal tubules is unique to sepsis-associated AKI and contributes to inflammation and injury. Inhibition of Xbp1s-mediated pathways is a potential portal to alleviate AKI onset, severity, and progression in sepsis. Xbp1 knockdown mice exposed to endotoxin (LPS) had lower NGAL and serum creatinine levels which may translate to better renal outcomes in longer follow-up. Future studies should determine if inhibition of Xbp1s activity improves renal outcome in septic AKI. Finally, urine Xbp1s may be a potential biomarker of impending AKI onset during sepsis.

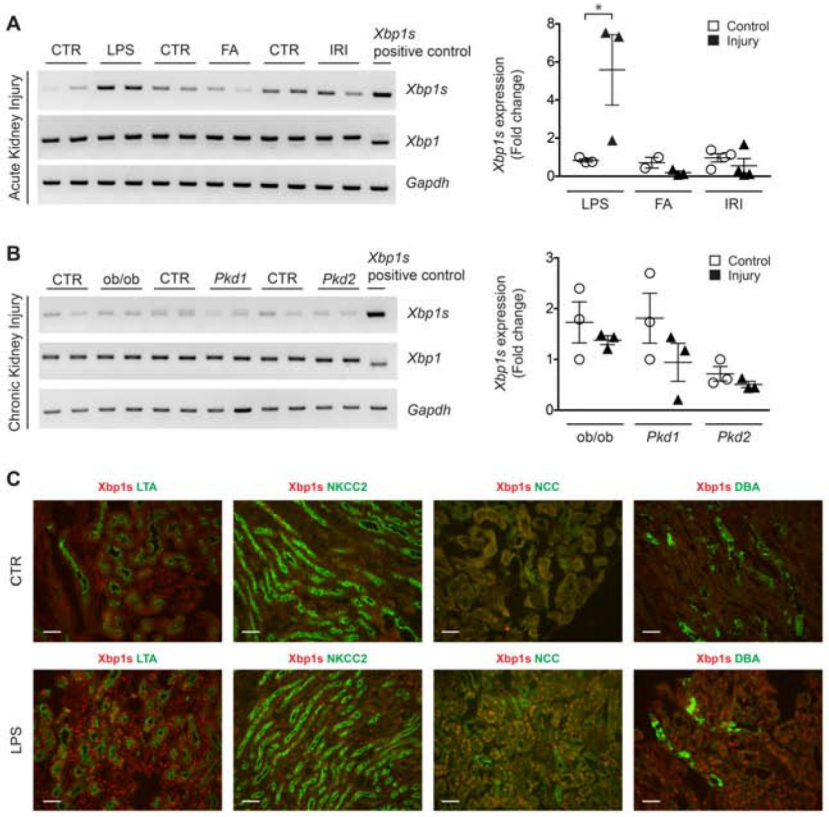

Figure 1. *Xbp1s* expression in mouse kidney is increased by LPS-induced sepsis.

(**A**) RT-PCR (left panel) and qPCR (right panel) analysis showing *Xbp1s* transcript levels in mouse kidney is increased by lipopolysaccharide (LPS; CTR and LPS-injected, n=3 each), but not by other acute kidney injury (AKI) insults such as folic acid (FA; CTR and FA, n=3 each) or ischemia-reperfusion injury (IRI; CTR and IRI, n=4) compared to respective control. (**B**) RT-PCR (left panel) and qPCR (right panel) showing that *Xbp1s* transcript levels are unchanged in the kidney of genetic mouse models of chronic kidney disease, i.e. type II diabetes (CTR and ob/ob mice, n=3) and polycystic kidney disease [PKD; Ksp/*Cre*;*Pkd1*$^{F/F}$ (*Pkd1*, n=3) mice at postnatal day 10, P10; Pkhd1/Cre;Pkd2$^{F/F}$ (*Pkd2*, n=3) mice at postnatal day 21, P21] compared to respective control (n=3 for each condition). Mouse kidney with known Xbp1 splicing was used as positive control. (**C**) Xbp1s antibody co-staining with nephron segment-specific markers revealed that Xbp1s expression was induced in epithelia cells from all nephron segments in kidneys of mice treated with LPS compared to vehicle-treated controls. LTA: lotus tetragonolobus agglutinin; NKCC2: Na^+-K^+-Cl^- cotransporter; NCC: Na^+-Cl^- cotransporter; DBA: dolichos biflorus agglutinin. Scale bar, 20 μm. Bars and error bars indicate mean and SEM. Student's unpaired t-test of injury vs. control within each model. * indicates P<0.05, compared to respective control. CTR: control.

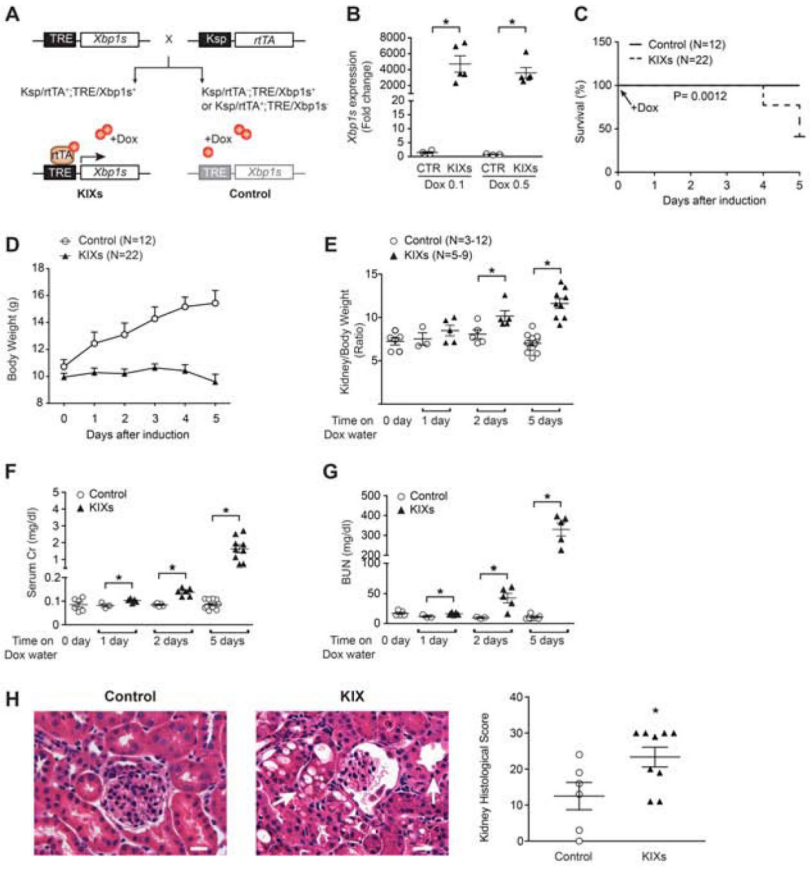

Figure 2. Overexpression of Xbp1s in the kidney produces tubular injury.

(**A**) To generate a kidney-inducible Xbp1s (KIX) mouse model, TRE/*Xbp1s* mice were crossed with Ksp/*rtTA* mice. KIXs were Ksp/*rtTA*;TRE-*Xbp1s* positive, whereas control animals only expressed either TRE-*Xbp1s* or Ksp/*rtTA* (lighter grey color). Doxycycline (Dox) was used to induce *Xbp1s* expression in renal tubular cells at postnatal day 21 (P21). Control animals also received Dox. (**B**) qPCR showed that *Xbp1s* expression increased equally after one day treatment with Dox- containing water at concentrations of either 0.1 mg/ml or 0.5 mg/ml. (**C-G**) 0.5 mg/ml Dox- containing water was provided daily for the duration of the experiment. (**C**) Kaplan-Meier survival curves of KIXs and control mice upon treatment with Dox. The experiment was stopped after 5 days. (**D**) Body weight of KIXs and control mice upon *Xbp1s* induction. (**E**) Kidney to body weight ratios for KIXs and control mice at baseline and after *Xbp1s* induction. (**F-G**) Kidney function of KIXs and control mice was assessed by serum Cr (**F**) and blood urea nitrogen (BUN, **G**) levels. (**H**) H&E staining revealed histological changes consistent with acute tubular necrosis (e.g. tubule dilation and intracellular vacuoles; arrows) in KIXs mice at 5 days after induction. Kidney histological score is shown (control, n=6; KIXs, n=9). Scale bar, 20 μm. Bars and error bars are mean and SEM. Student's unpaired t-test of KIXs vs. control at each time-point. * indicates P<0.05, compared to respective control. TRE: tetracycline responsive element; Ksp: Ksp-cadherin promoter; rtTA: reverse tetracycline-controlled transactivator; KIXs: kidney- inducible Xbp1s mice; Cr: creatinine.

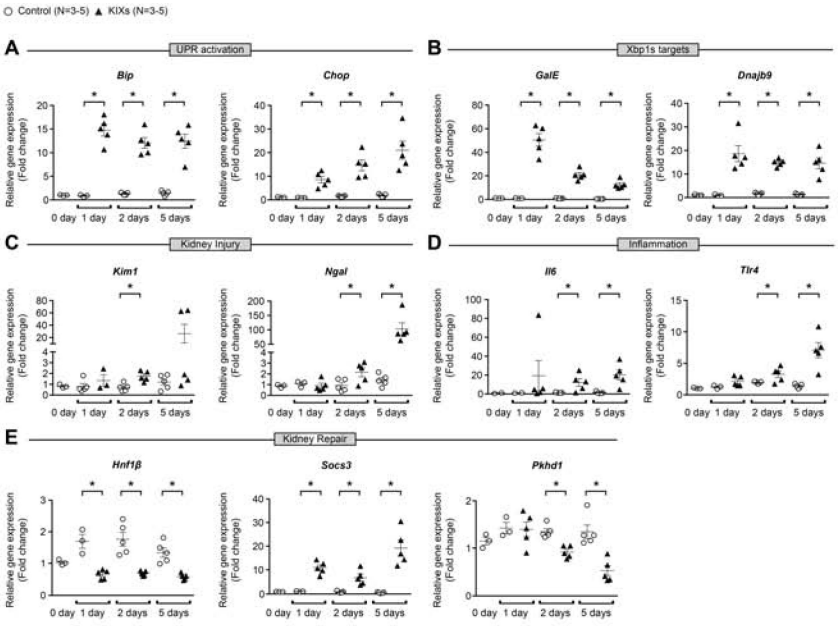

Figure 3. Overexpression of *Xbp1s* in the kidney increases the expression of UPR effectors, kidney injury markers and inflammatory molecules.

qPCR showed that *Xbp1s* overexpression in KIXs mice induced significant increase of the UPR effectors *Bip* and *Chop* (**A**), and of the Xbp1s target genes *GalE* and *Dnajb9* (**B**) from one to five days after treatment with Dox. Expression of the kidney injury markers *Kim1* and *Ngal* (**C**), and of the inflammatory molecules *Il6* and *Tlr4* (**D**) were increased starting at two days after treatment. The transcription factor HNF-1β regulates genes important for the homeostatic control of kidney repair during stress-induced proliferation. KIXs mice have significantly reduced *Hnf1b* expression, increased *Socs3,* and decreased *Pkhd1* expression that are negatively and positively regulated by HNF- 1β, respectively (**E**). 0.5 mg/ml Dox-containing water was provided daily for the duration of the experiment. Fold change is relative to 0 day on Dox-containing water. Bars and error bars are mean and SEM. Student's unpaired t-test of control vs. KIXs at each time-point. * indicates $P<0.05$, compared to respective control at each time-point. KIX: kidney-inducible Xbp1s mice; UPR: unfolded protein response.

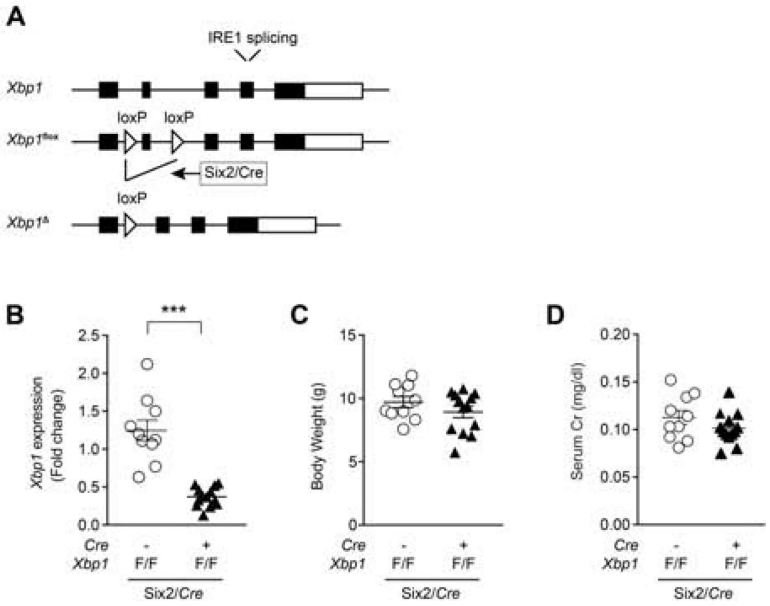

Figure 4. Kidney-specific Xbp1s knockdown mice have normal kidney function.

(**A**) Floxed *Xbp1* mice were bred with Six2/*Cre* mice to generate kidney-specific deletion of *Xbp1.* Open triangles indicate loxP sites. (**B**) *Xbp1* expression in kidneys of Six2/*Cre*$^{+}$;*Xbp1*$^{F/F}$ (n=13) mice at P21 was approximately 50% decreased compared to Six2/*Cre*$^{-}$;*Xbp1*$^{F/F}$ (n=10) control mice. Six2/*Cre*$^{+}$;*Xbp1*$^{F/F}$ mice showed similar body weight (**C**) and kidney function (**D**) at P21 compared to control animals. Bars and error bars are mean and SEM. Student's unpaired t-test of Six2/*Cre*$^{-}$;*Xbp1*$^{F/F}$ vs. Six2/*Cre*$^{+}$;*Xbp1*$^{F/F}$. *** indicates P<0.001, compared to control.

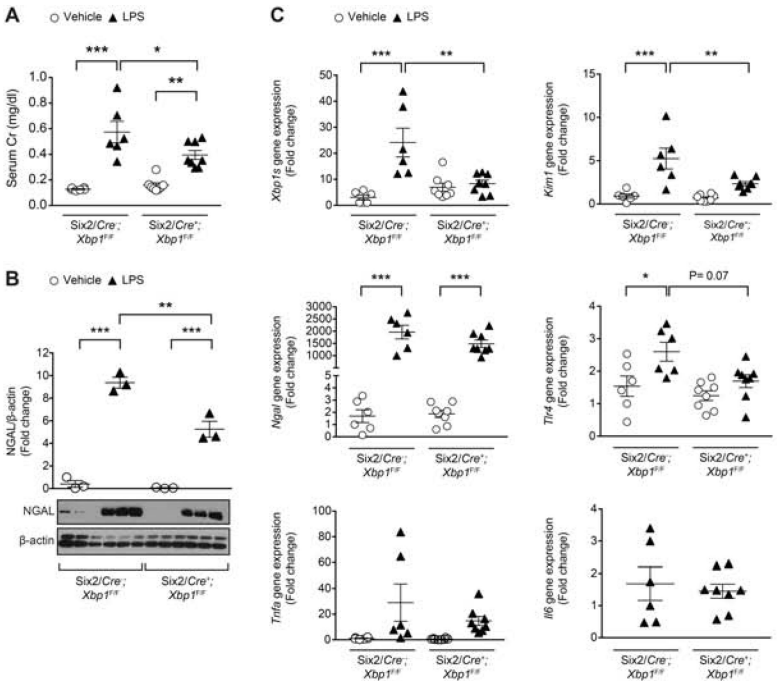

Figure 5. Kidney-specific *Xbp1s* knockdown affects kidney function in response to acute LPS-induced AKI.

Mice received an intraperitoneal (IP) injection of LPS (16 mg/kg) or vehicle, and were sacrificed 24 hours later. (**A**) Both Six2/*Cre*$^{-}$;Xbp1$^{F/F}$ and Six2/*Cre*$^{+}$;Xbp1$^{F/F}$ animals showed significant increase in serum Cr levels after LPS injection (Six2/*Cre*$^{-}$;*Xbp1*$^{F/F}$: n=8; Six2/*Cre*$^{+}$;*Xbp1*$^{F/F}$: n=10) compared to vehicle (Six2/*Cre*$^{-}$;*Xbp1*$^{F/F}$: n=6; Six2/*Cre*$^{+}$;*Xbp1*$^{F/F}$: n=10), but the increase was lower in Six2/*Cre*$^{-}$;*Xbp1*$^{F/F}$ mice. (**B**) After LPS injection, NGAL protein expression levels normalized to β-actin were lower in the kidney of Six2/*Cre*$^{+}$;*Xbp1*$^{F/F}$ mice compared to controls. Fold change is relative to vehicle-treated Six2/*Cre*$^{-}$;Xbp1$^{F/F}$ mice. (**C**) qPCR on mouse kidney samples from mice injected with vehicle (Six2/*Cre*$^{-}$;*Xbp1*$^{F/F}$: n=6; Six2/*Cre*$^{+}$;*Xbp1*$^{F/F}$: n=8) or LPS (Six2/*Cre*$^{-}$;*Xbp1*$^{F/F}$: n=6; Six2/*Cre*$^{+}$;*Xbp1*$^{F/F}$: n=8). Fold change is relative to vehicle-treated Six2/*Cre*$^{-}$;Xbp1$^{F/F}$ mice. *Il6* mRNA expression was undetectable in the kidney of mice treated with vehicle. Bars and error bars are mean and SEM. 2-way ANOVA, Sidak's multiple comparisons test. * indicates P<0.05, ** indicates P<0.01, *** indicates P<0.001. Cr: creatinine; LPS: lipopolysaccharide.

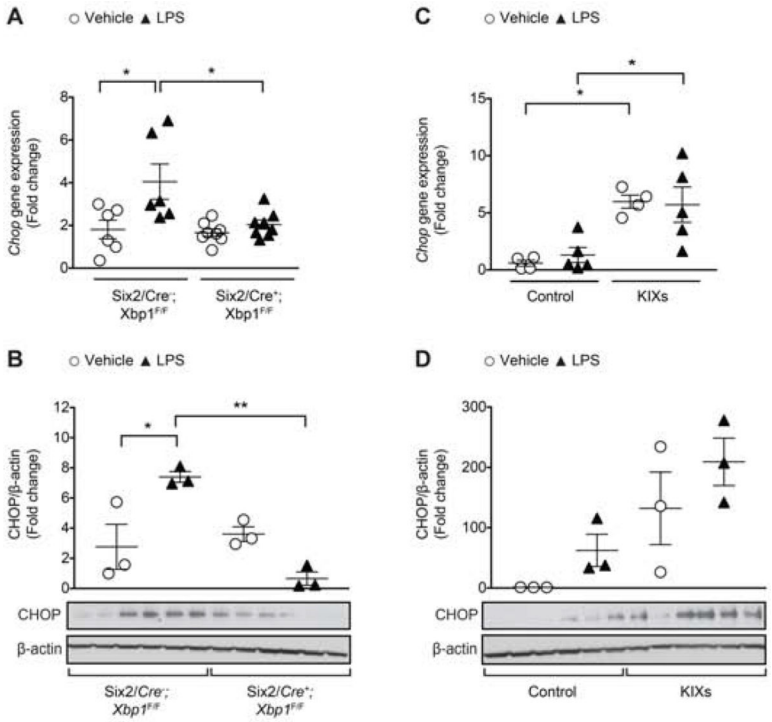

Figure 6. Kidney-specific *Xbp1s* knockdown associates with decreased expression of the pro-apoptotic transcription factor CHOP.

qPCR and immunoblot of the pro-apoptotic marker *Chop*/CHOP in the kidney of tissue-specific *Xbp1s* knockdown (**A-B**) and KIXs (**C-D**) mice at 24 hours after an intraperitoneal (IP) injection of LPS (16 mg/kg) or vehicle. Fold change is relative to vehicle-treated control mice. KIXs and control mice received 0.1 mg/ml Dox- containing water for 16 hours prior LPS injection. Bars and error bars are mean and SEM. 2-way ANOVA, Sidak's multiple comparisons test. * indicates P<0.05, ** indicates P<0.01, *** indicates P<0.001. LPS: lipopolysaccharide.

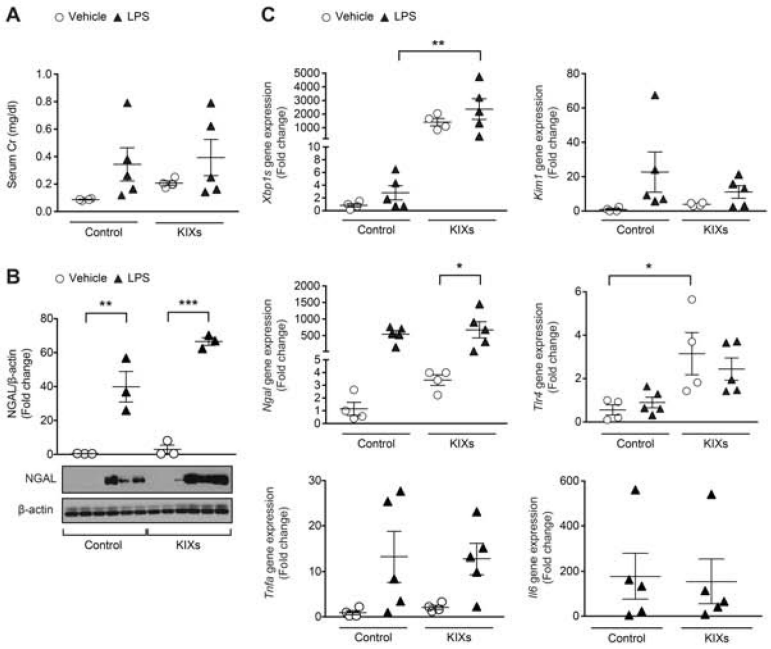

Figure 7. Kidney-specific *Xbp1s* overexpression affects kidney function in response to acute LPS-induced injury.

KIXs and control mice received 0.1 mg/ml Dox-containing water for 16 hours, then an intraperitoneal (IP) injection of LPS 16 mg/kg (control: n=5; KIXs: n=5) or vehicle (control: n=4; KIXs: n=4), and sacrificed 24 hours later. (**A**) Serum Cr levels from control or KIXs mice injected with vehicle or LPS. (**B**) NGAL protein expression normalized by β-actin in the kidney of KIXs mice compared to controls. (**C**) qPCR on kidney samples from mice injected with vehicle or LPS. Fold change is relative to vehicle-treated control mice. *Il6* mRNA expression was undetectable in the kidney of mice treated with vehicle. Bars and error bars are mean and SEM. 2-way ANOVA, Sidak's multiple comparisons test. * indicates P<0.05, ** indicates P<0.01, *** indicates P<0.001. Cr: creatinine; LPS: lipopolysaccharide.

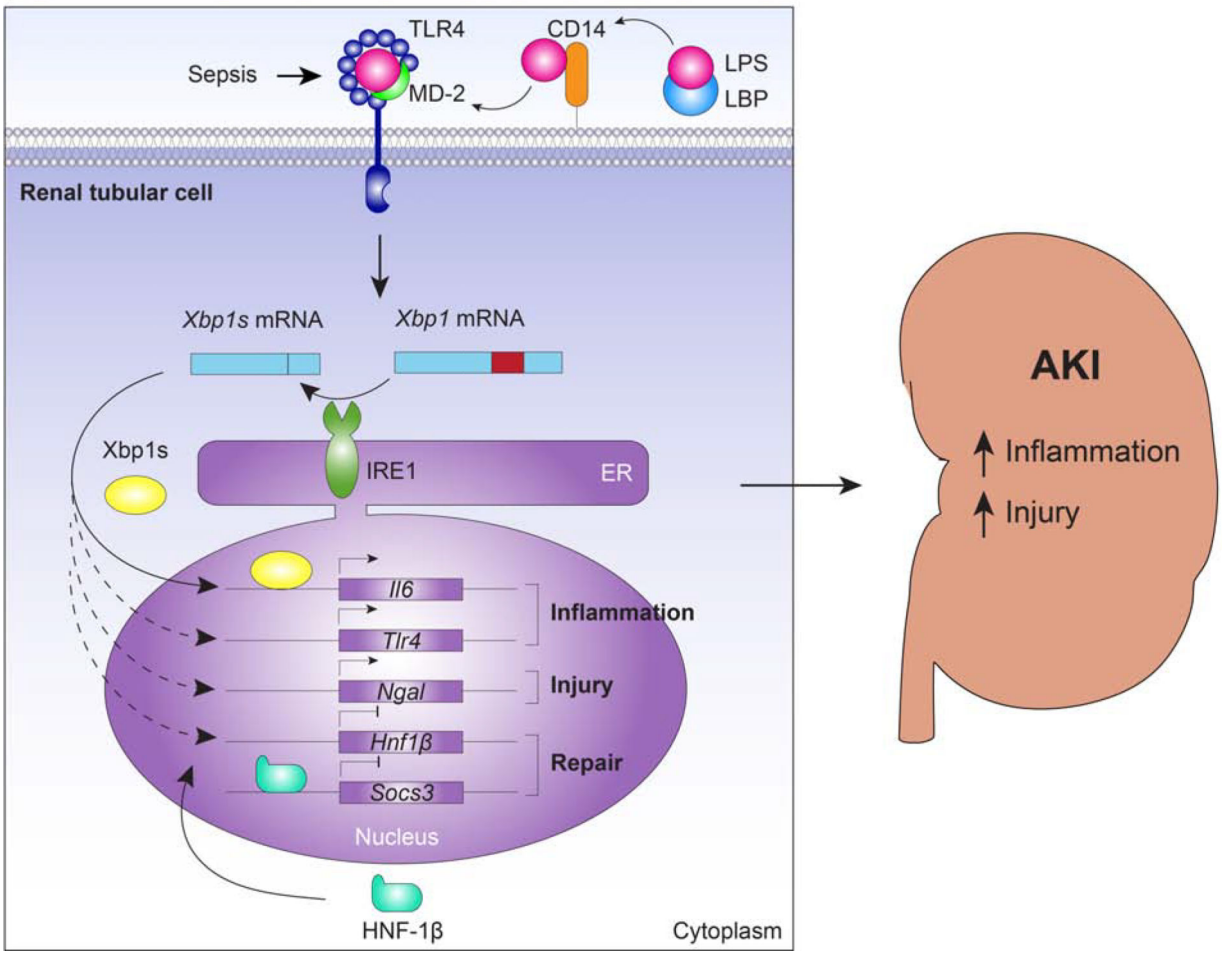

Figure 8. The proposed mechanism by which Xbp1s promotes inflammation and injury in renal tubular cells during AKI in sepsis.

LPS is bound to LBP which transfers it to CD14; CD14 then brings LPS to the TLR4-MD-2 complex. The binding of the endotoxin LPS to the TLR4-MD-2 complex activates IRE1 that cleaves the mRNA encoding for Xbp1 by excision of 26 nucleotides (red) from the *Xbp1* mRNA, generating the spliced *Xbp1* form, *Xbp1s,* which is translated into a nuclear transcription factor. Xbp1s increases the transcription of its known target gene *Il6.* Transcriptional pathways not fully characterized lead to an increase in *Ngal* and a decrease in *Hnf1b* transcription. HNF-1β is a nuclear transcription factor that inhibits the expression of *Socs-3.* Altogether these transcriptional networks augment inflammation and injury, while decreasing kidney repair. Other pathways downstream the activation of TLR4, such as the NF-κB pathway, may augment the sepsis-induced AKI. Importantly, both tubular TLR4 signaling and TLR4 activation in infiltrating immune cells can possibly contribute to the increase in Xbp1s in kidney. LPS: lipopolysaccharide; LBP: LPS binding protein; CD14: cluster of differentiation 14; MD-2: myeloid differentiation factor 2; TLR4: toll-like receptor-4; Xbp1s: spliced X-box binding protein-1; ER: endoplasmic reticulum; IRE1: inositol-requiring enzyme 1; *Il6:* interleukin-6; *Ngal:* neutrophil gelatinase-associated lipocalin; *Hnf1b:* hepatocyte nuclear factor 1β; *Socs3:* suppressor of cytokine signaling 3.