Lipopolysaccharide induces filtrate leakage from renal tubular lumina into the interstitial space via a proximal tubular Toll-like receptor 4–dependent pathway and limits sensitivity to fluid therapy in mice

see commentary on page 847

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Sustained oliguria during fluid resuscitation represents a perplexing problem in patients undergoing therapy for septic acute kidney injury. Here, we tested whether lipopolysaccharide induces filtrate leakage from the proximal tubular lumen into the interstitium, thus disturbing the recovery of urine output during therapy, such as fluid resuscitation, aiming to restore the glomerular filtration rate. Intravital imaging of the tubular flow rate in the proximal tubules in mice showed that lipopolysaccharide did not change the inflow rate of proximal tubule filtrate, reflecting an unchanged glomerular filtration rate, but significantly reduced the outflow rate, resulting in oliguria. Lipopolysaccharide disrupted tight junctions in proximal tubules and induced both paracellular leakage of filtered molecules and interstitial accumulation of extracellular fluid. These changes were diminished by conditional knockout of Toll-like receptor 4 in the proximal tubules. Importantly, these conditional knockout mice showed increased sensitivity to fluid resuscitation and attenuated acute kidney injury. Thus, lipopolysaccharide induced paracellular leakage of filtrate into the interstitium via a Toll-like receptor 4-dependent mechanism in the proximal tubules of endotoxemic mice. Hence, this leakage might diminish the efficacy of fluid resuscitation aiming to maintain renal hemodynamics and glomerular filtration rate.

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Translational Statement

Using a mouse model of acute kidney injury, our results suggest that leakage of filtrate at the proximal tubules could be a cause of sustained oliguria during fluid resuscitation. The proximal tubular flow rate and urine volume at a normal glomerular filtration rate were reduced in lipopolysaccharide-injected endotoxemic mice, indicating that the filtrate diffused into the interstitium at the proximal tubules. Additional volume load did not alleviate oliguria under these conditions because the increased filtrate simply leaked at the tubules. Targeting this leakage may improve the efficacy of fluid therapy during endotoxemic acute kidney injury.

Oliguria in septic acute kidney injury (AKI) is thought to be caused by glomerular filtration rate (GFR) reductions induced by hypoperfusion or vascular dehydration. However, fluid resuscitation and treatment with additional vasoactive agents to maintain renal hemodynamics frequently fail to restore urine output. The mechanism by which urine output starts declining or becomes unresponsive to the therapy maintaining renal circulation during AKI has not been elucidated.

In 1929, the medical literature first reported that phenolsulfonphthalein, a freely filtered dye, disappeared from the kidney tubular lumen of frogs in mercury-induced renal failure.[1] More than 43 years later, Bank *et al.*[2] elegantly demonstrated (using a micropuncture method in combination with intravital imaging) that lissamine green, another freely filtered dye injected i.v., also disappeared from the proximal tubular lumen, whereas GFR (measured by inulin clearance in each segment) was maintained within the normal range in rats with mercury-induced renal injury. In 2015, we reported that lipopolysaccharide (LPS) accumulated in the proximal tubular wall in mice and induced a reduced tubular flow rate, but not GFR; this was visualized using freely filtered

dyes such as Lucifer yellow and fluorescein isothiocyanate-inulin using multiphoton microscopy analysis.[3] All these findings indicate a fluid "disappearance" from the proximal tubules that could cause oliguria independent of GFR reduction during AKI.

Because in previous studies[4,5] it was found that mercury and LPS downregulated electrolyte reabsorption through intracellular transporter pathways, we hypothesized that both a slowed tubular flow rate and oliguria occurred from leakage of tubular flow through a paracellular pathway in the proximal tubules. Furthermore, we hypothesized that this leakage at the proximal tubules created the poor efficacy of fluid therapy, even if the GFR was adjusted. Finally, because we previously observed that tubular flow decreased specifically in the proximal tubules that accumulated fluorophore-conjugated LPS, we further examined whether Toll-like receptor 4 (TLR4), an LPS receptor expressed in proximal tubules, was responsible for the reduced tubular flow rate.

RESULTS

GFR-dependent and -independent oliguria after LPS

There was no statistically significant difference in the urine outputs at 0 and 6 hours after LPS injection (5 or 15 mg/kg) (Figure 1a). LPS injection at both doses led to reduced urine output between 6 and 12 hours after injection compared with saline injection. In mice injected with LPS at 5 mg/kg, the difference in oliguria between 12 and 24 hours after injection was less than that between 6 and 12 hours after injection, whereas mice that received LPS at 15 mg/kg showed sustained oliguria, regardless of the time elapsed.

Intravital imaging by 2-photon microscopy showed that tubules maintained their lumina (central black areas in blue tubules, Figure 1b) at 6 hours after injection of LPS at 5 mg/kg. In contrast, tubules showed shrunken tubular lumina at 6 hours after LPS injection at 15 mg/kg (Figure 1b). These results suggest that injection of LPS at 15 mg/kg reduced the GFR and the tubular flow rate, whereas LPS injection at 5 mg/kg allowed the GFR to remain at a level sufficient to maintain tubular lumen volume 6 hours after injection.

We then evaluated the inflow and outflow rates of tubular flow in the early segments of the proximal tubules via i.v. bolus injections of Lucifer yellow, a dye freely filtered from glomeruli, to estimate the GFR and washout of tubular flow from the proximal tubules to the downstream nephron, respectively (Figure 1c).[3,6] Injection of LPS at 5 mg/kg did not significantly change the inflow time, suggesting that the GFR was maintained at a normal level. In contrast, the outflow time was significantly extended compared with that of the saline-injected mice, indicating that the tubular flow rate was reduced at the proximal tubules, as we previously reported.[3] Injection of LPS at 15 mg/kg significantly extended both inflow and outflow times, indicating reduced GFR.

Tubular flow leakage and fluid retention

In all subsequent experiments, LPS was injected at 5 mg/kg unless otherwise indicated. Factors that reduce the proximal tubular flow rate are increases in sodium and water reabsorption or paracellular water transport or leakage. Because previous studies have shown that LPS reduced, rather than increased, the expression levels of Na^+/H^+ exchanger 3, a major sodium transporter in proximal tubules,[5] we focused on paracellular leakage as a potential cause of the slowed tubular flow rate. We first attempted to find direct evidence of paracellular leakage using intravital imaging. We assessed the fluorescein isothiocyanate-inulin–derived fluorescence between the proximal tubular cells (Figure 2a), while visible detection was very occasional because of the physical spatial limitations in cell–cell junctions compared with the spatial resolution of microscopy, in addition to quick washout in the interstitium. Indeed, either fluorescein isothiocyanate-inulin or Lucifer yellow infused in the subcapsular space (interstitium) was not visibly detectable in the interstitium/capillary area, and appeared in the tubular lumen >1 minute later (Supplementary Movie S1); this suggests that the injected Lucifer yellow was cleared from the interstitium into the venous blood, returned to the systemic circulation, filtered from the glomeruli, and then condensed in the tubular lumen, making the fluorescent level visible.

To support the paracellular leakage hypothesis, we analyzed the expression of the renal tight junction components. LPS reduced occludin expression at the mRNA and protein levels in the kidney 6 hours after injection (Figure 2b and Supplementary Figure S1) and also reduced claudin 2 mRNA level but not protein level (Figure 2c and Supplementary Figure S1). Immunohistochemical staining of claudin 2 revealed that its localization was disrupted by LPS within 6 hours (Figure 2d). In cultured proximal tubular cells, LPS increased the permeability of Lucifer yellow through the tubular cell layer (Figure 2e) and reduced the continuity of Zonula occludens-1 (ZO-1) expression at cell–cell junctions and claudin 2 mRNA level after 6 hours of LPS incubation (Figure 2f and Supplementary Figure S2).

The leakage of filtrate through proximal tubular cells during oliguria was predicted to cause fluid accumulation in the kidney. Indeed, LPS increased the wet kidney weight/body weight ratio, water content/dry kidney weight ratio, Na^+ content/dry kidney weight ratio, and Na^+/K^+ ratio (Figure 3a). The increased Na^+/K^+ ratio indicated that there was a greater increase in extracellular fluid than in intracellular fluid after LPS injection, although LPS is supposed to increase the total number of cells in the kidney because of inflammatory cell infiltration. Furthermore, renal interstitial hydrostatic pressure was increased by LPS (Figure 3b). These results demonstrated that LPS increased extracellular fluid accumulation in the renal interstitium (Figure 3c).

TLR4 in the proximal tubules induces tubular flow leakage

Tubular flow rate analysis using 2-photon microscopy provided information on the time needed for the tubular fluid to flow into the distal superficial nephron.[3,6] We noted that 2-photon microscopy was unable to visualize changes deeper

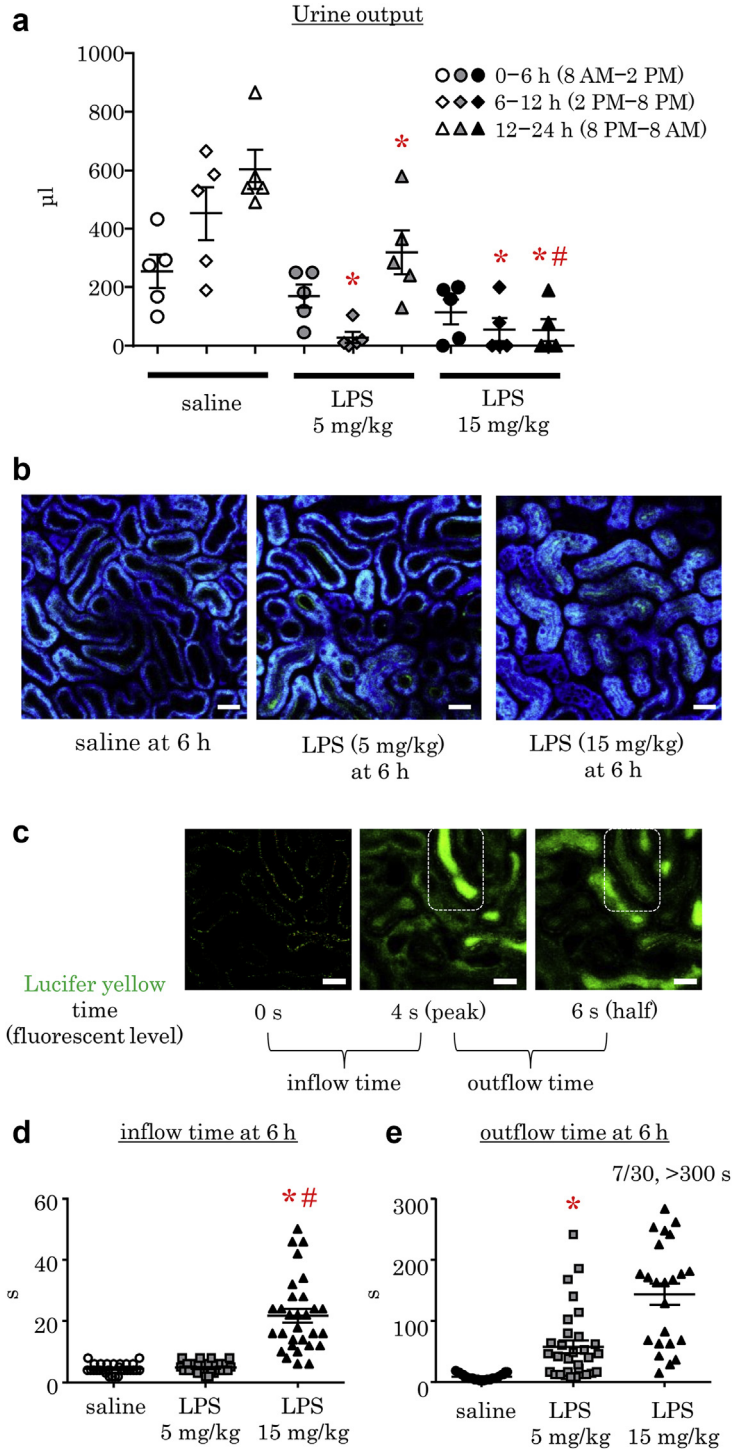

Figure 1 | Lipopolysaccharide (LPS) induces oliguria by both glomerular filtration rate (GFR)–dependent and GFR-independent mechanisms. (**a**) Time-dependent changes in urine output after the administration of low (5 mg/kg, i.p.) and high (15 mg/kg, i.p.) doses of LPS ($n = 5$). (**b**) Multiphoton imaging of the kidney after injection of saline or LPS in live mice. Blue: cytosolic reduced nicotinamide adenine dinucleotide autofluorescence; green: tubular autofluorescence derived from multiple metabolites. (**c–e**) Indirect measurement of GFR and tubular flow washout time by visualization of the i.v.-injected freely filtered dye, Lucifer yellow ($n = 10$). Bar = 50 µm. (**c**) Multiphoton time-lapse imaging of tubules showing filtered Lucifer yellow (green-yellow) first among all tubules in the imaging window. The time to achieve peak fluorescence intensity was used as inflow time, and the time to halve the fluorescence intensity was used as outflow time. Bar = 50 µm. (**d**) The inflow time and (**e**) the outflow time in each tubule were plotted. Observation was stopped at 300 seconds to avoid excessive phototoxicity. Lucifer yellow fluorescence did not halve within 300 seconds in 7 of the 30 tubules in the high-dosage LPS group, and no statistical tests were performed versus this group. *$P < 0.05$ versus control group; #$P < 0.05$ versus LPS (5 mg/kg) group. To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.

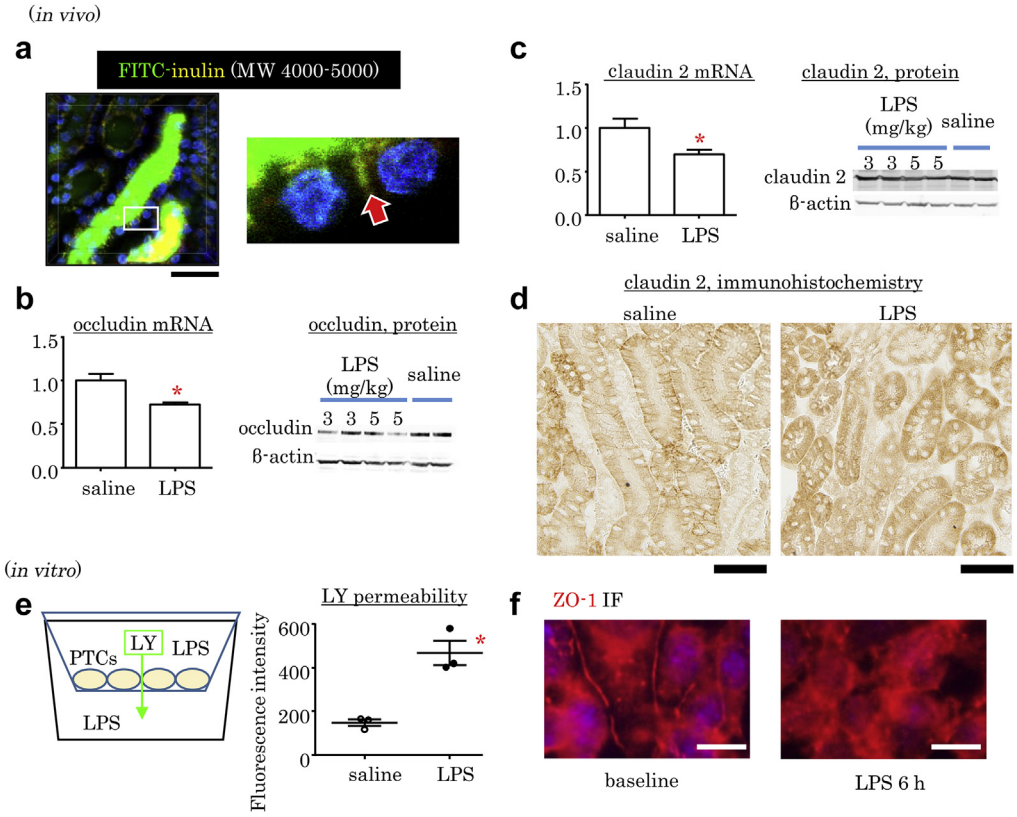

Figure 2 | Lipopolysaccharide (LPS) increased proximal tubular permeability. (**a**) I.v.-injected Lucifer yellow (LY) was condensed in the tubular lumen. The right image is an enlarged image of the area selected by the rectangle in the left image. Bar = 50 μm. (**b**) Expression levels of occludin mRNA (left, $n = 6$–7) and protein (right, $n = 4$). (**c**) Expression levels of claudin 2 (Cldn2) mRNA (left, $n = 6$–7) and protein (right, $n = 4$). (**d**) Immunohistochemistry for Cldn2. Bar = 50 μm. (**e**) Permeability of LY through the proximal tubular cell (PTC) layer. LY was added only to the upper layer and LPS was added to both layers (left). The fluorescence intensity of LY in the medium from the bottom layer was measured (right) ($n = 3$). (**f**) Immunofluorescent images of the tight junction protein ZO-1 in cultured PTCs ($n = 3$). Bar = 16 μm. FITC, fluorescein isothiocyanate; MW, molecular weight. *$P < 0.05$ versus control group. To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.

in the nephron. However, we presumed that leakage occurred mainly in the cortical proximal tubules because Na^+ and water levels increased at similar rates, resulting in isotonic fluid accumulation (Supplementary Figure S3). Moreover, using the 2-photon microscopy technique, we previously reported that fluorophore-conjugated LPS accumulated in the proximal tubules of mice, and that only the tubular flow rate slowed in the LPS-accumulated tubules. We therefore conditionally knocked out (KO) TLR4 in the proximal tubules in *Ndrg1*^{CreERT2} mice (Supplementary Figure S4).[7] LPS slowed the tubular flow in control TLR4-floxed mice at 6 hours, and proximal tubular TLR4 deletion remarkably suppressed this tubular flow rate reduction (Figure 4a). These results were accompanied by changes in urine volume for 6 hours (Figure 4b) and water accumulation in the kidney (Figure 4c). LPS reduced occludin protein levels in control TLR4-floxed mice but not in proximal tubular TLR4-KO mice at 6 hours (Figure 4d and Supplementary Figure S5). LPS had no effect on claudin 2 protein levels at 6 hours, but did disrupt its localization in control TLR4-floxed mice (Figure 4e and Supplementary Figure S5), whereas proximal tubular TLR4-KO mice maintained their claudin 2 expression pattern. The TLR4 inhibitor C34 prevented the LPS-induced tubular flow rate reduction by pre-LPS (at −1 hour) but not post-LPS (at 4 hours) treatment (Supplementary Figure S6) in normal C57Bl6 mice. C34 also prevented the increased permeability of Lucifer yellow and the disruption of ZO-1 continuity in the cultured proximal tubular cells (Figure 4f and g). Pharmacological inhibitors of nuclear factor κB and p38 MAP kinase attenuated the LPS-induced disruption of ZO-1 at 6 hours, but only the nuclear factor κB inhibitor suppressed it at 12 hours (Supplementary Figure S7).

Leakage reduces fluid resuscitation efficacy

These results suggest that LPS induced filtrate leakage at the proximal tubules through a proximal tubular TLR4-dependent mechanism during early endotoxemia. Next, we examined whether proximal tubular TLR4 deletion rescued mice from LPS-induced AKI in a later phase of endotoxemia. When mice did not receive fluid resuscitation, there were no significant differences in urine output and blood urea nitrogen 24 hours after LPS injection between the control floxed and KO mice (Figure 5a). This may be because of the GFR reduction at this time point (Supplementary Figure S8); the

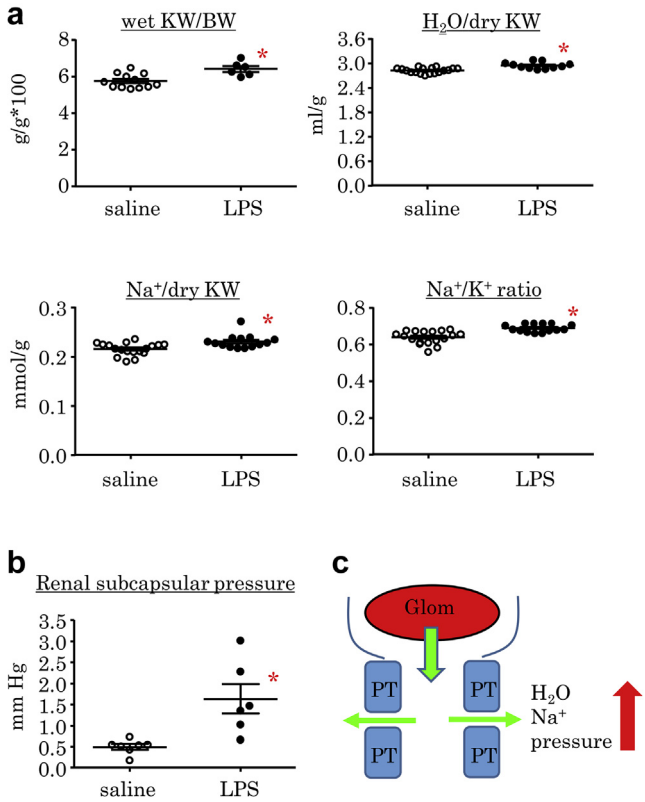

Figure 3 | Fluid congestion in the kidney. (**a**) Wet kidney weight (KW)/body weight (BW), water content (wet KW − dry KW)/dry KW, Na^+ content/dry KW, and Na^+/K^+ ratio in the kidney ($n = 6$–18). (**b**) Renal subcapsular interstitial hydrostatic pressure ($n = 6$–7). (**c**) Proposed working scheme for tubular leakage. Glom, glomerulus; LPS, lipopolysaccharide; PT, proximal tubular cell. *$P < 0.05$ versus control group.

presence of leakage at the proximal tubules does not impact urine output when the GFR is reduced (little fluid volume to be leaked). However, the KO mice, which did not show proximal tubular filtrate leakage, excreted more urine than the control mice in response to fluid resuscitation performed intermittently at 4 and 18 hours (50 ml/kg, subcutaneously) (Figure 5b). KO mice also showed significantly lower blood urea nitrogen, renal kidney injury molecule 1, and neutrophil gelatinase–associated lipocalin mRNA expression, but there was no significant difference in neutrophil gelatinase–associated lipocalin excretion in the urine, collected from 0 to 24 hours, between the groups (Figure 5c and d). The control floxed mice that excreted <3 mg/dl neutrophil gelatinase–associated lipocalin in the urine showed little response to fluid resuscitation (red circle in Figure 5b–e); this suggests that the urine samples used for the neutrophil gelatinase–associated lipocalin assay were mainly excreted in the first 4 hours after LPS injection, before fluid resuscitation.

Proximal tubular TLR4 deletion tended to reduce renal inflammatory markers, including tumor necrosis factor α and CC chemokine ligand 3 expression levels in the kidney (Figure 5e). However, this difference did not achieve statistical significance, potentially because of TLR4 stimulation in the renal inflammatory cells by LPS. Indeed, $CD11b^+$ cells isolated from the kidney of floxed control and KO mice expressed equivalent mRNA levels of macrophage polarity markers (Supplementary Figure S9). Finally, we analyzed inflammatory cytokine levels in the lung to determine whether the alleviation of AKI by proximal tubular TLR4 deletion plus fluid resuscitation protected remote organs. Proximal tubular TLR4 deletion was not sufficient to alleviate inflammatory cytokine levels in the lung at 24 hours after LPS (Supplementary Figure S10).

DISCUSSION

Multiple hypotheses exist regarding the mechanism of oliguria during sepsis, such as GFR reduction induced by afferent-efferent arteriole tone changes, vascular dehydration, and shock-induced hypoperfusion of the kidney. Our findings also indicate that GFR reduction, with or without filtrate leakage from proximal tubules, causes oliguria. In addition to understanding the importance of GFR maintenance, this study explored whether prevention of tubular flow leakage was necessary for standard fluid resuscitation to be most effective. We found that if leakage was left untreated, the increased filtrate resulting from fluid therapy was continually lost in the tubular lumen, resulting in sustained oliguria. Importantly, under fluid resuscitation, oliguria was attenuated and blood urea nitrogen and tubular injury markers were improved in proximal tubular TLR4-KO mice, which showed reduced leakage. These data indicate that prevention of filtrate leakage may protect the kidneys against both oliguria and AKI.

Current therapies against AKI, including fluid resuscitation and vasoconstrictor injection, aim to recover the reduced GFR, but do not address changes in the tubules. AKI research in recent decades has increasingly focused on tubular injury and the necessity of new therapies and reliable markers.[8] However, no new therapies have been added to date, and there are a limited number of candidate drugs. Human recombinant alkaline phosphatase was expected to be effective against septic AKI,[9] but a recent phase IIa/IIb randomized clinical trial reported that it did not significantly improve short-term kidney function among patients with septic AKI.[10] TLR4 antagonists, which were partially effective in the present study, may be candidates for treating both systemic inflammation and filtrate leakage, but it is not clear whether they would be most effective as a treatment or for prophylactic use; post-LPS treatment with C34 did not attenuate the reduction in the tubular flow rate in the present study. We used a bolus injection of LPS, and it is likely that TLR4 antagonism at 4 hours did not work because TLR4-induced signals were already triggered. In the clinical setting, LPS-TLR4 binding lasts for the duration of antibacterial and anti-endotoxin treatment, but further studies, both basic and clinical, are needed to confirm the effective time window. We recently showed that a subpressor dosage of human recombinant atrial natriuretic peptide (hANP) acted on endothelial and proximal tubular cells through its receptor, guanylyl cyclase-A, to

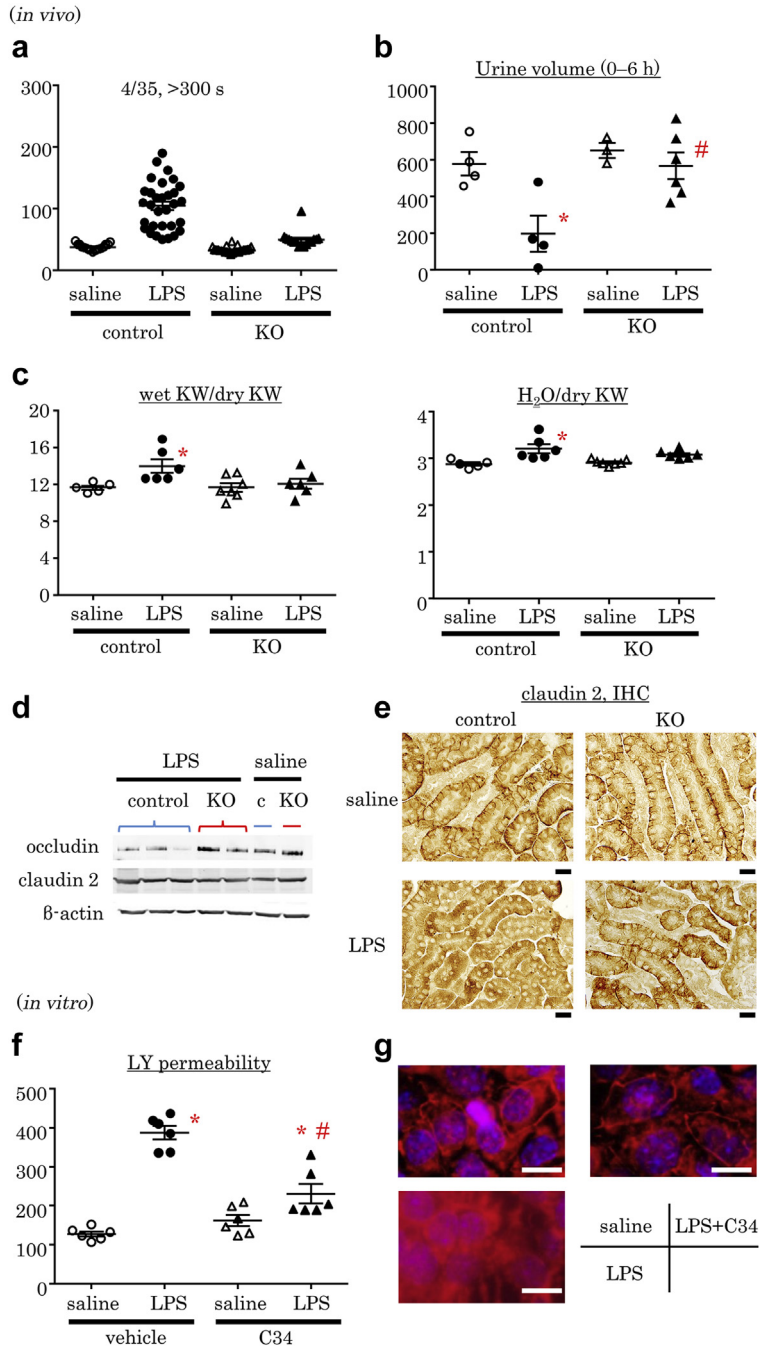

Figure 4 | Oliguria resistance in proximal tubular Toll-like receptor 4 (TLR4)–knockout mice. (**a**) The duration of Lucifer yellow (LY) flowing into the distal nephron lumen after i.v. injection ($n = 3$–5). Observations stopped at 300 seconds to avoid phototoxicity. Four of 35 tubules in lipopolysaccharide (LPS)-injected control mice showed no LY in the distal nephron lumen within 300 seconds. (**b**) Urine output during the first 6 hours after LPS injection ($n = 4$–6). (**c**) Wet kidney weight (KW)/dry KW, and water content/dry KW ($n = 5$–7). (**d**) Western blotting for occludin and claudin 2. c, control. (**e**) Immunohistochemistry for claudin 2. Bar = 20 μm. (**f**) LY permeability through the cultured proximal tubular cell layer, with and without the TLR4 antagonist C34 ($n = 6$). (**g**) ZO-1 immunofluorescence with and without C34 ($n = 3$). Bar = 16 μm. Control, control floxed mice; IHC, immunohistochemistry; KO, proximal tubular TLR4-KO mice. *$P < 0.05$ versus saline-injected mice; #$P < 0.05$ versus LPS-injected control or vehicle-treated mice. To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.

improve the GFR and the tubular flow rate, respectively.[6] We also showed that mortality was decreased if hANP was administered immediately after LPS-induced oliguria.[6] Because hANP recovered the tubular flow rate in a GFR-independent manner, it may also prevent filtrate leakage from tubules, increasing sensitivity to fluid resuscitation, and increasing urine output. Limitations to hANP treatment were potential hypotension from a high dosage[11] and a narrow

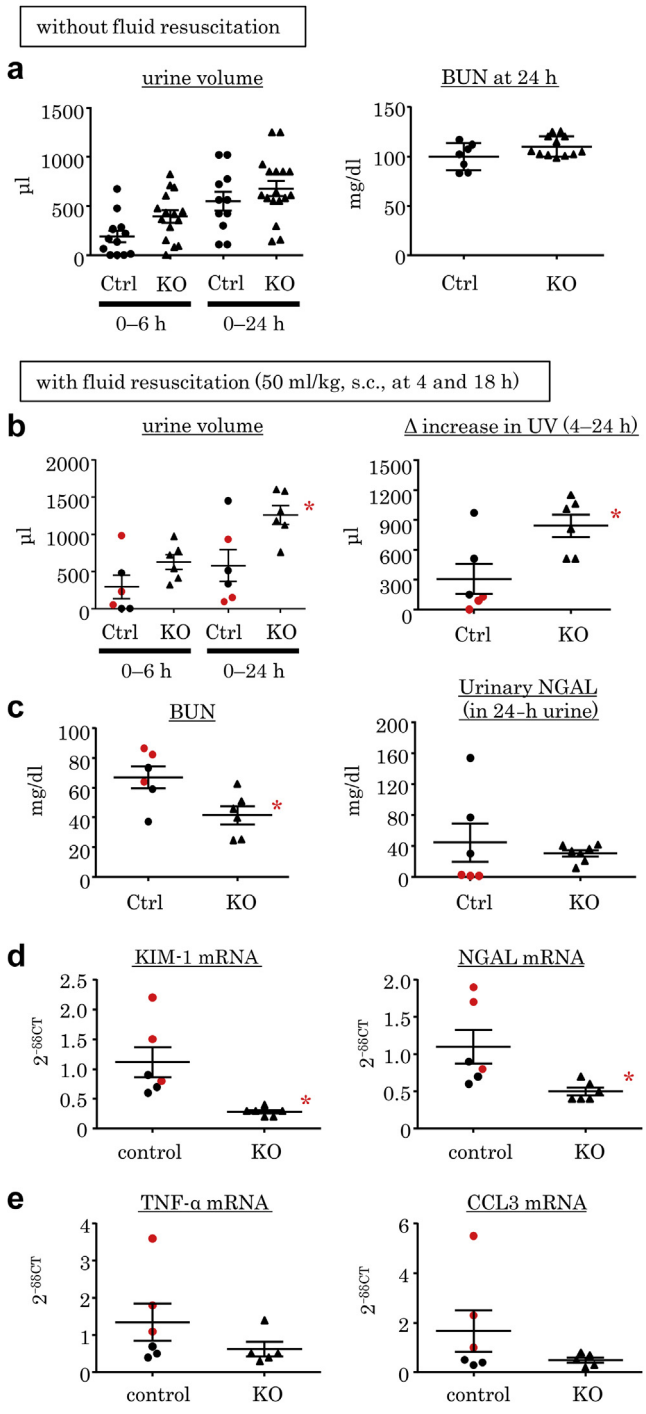

Figure 5 | Acute kidney injury in proximal tubular TLR4-knockout mice at 24 hours after lipopolysaccharide (LPS) injection. (**a**) Twenty-four-hour urine output ($n = 11$–17) and blood urea nitrogen (BUN) ($n = 7$–12) in mice without fluid resuscitation. (**b**) Twenty-four-hour urine output (left) and increases in urine output after fluid resuscitation performed at 4 and 18 hours (right) after LPS injection ($n = 6$). Data for 3 control floxed mice (Ctrl) that responded poorly to fluid resuscitation are colored red. (**c**) BUN and urinary neutrophil gelatinase–associated lipocalin (NGAL) excretion ($n = 6$). (**d**) Renal expression of kidney injury molecule 1 (KIM-1) and NGAL mRNA ($n = 6$). (**e**) Renal expression of tumor necrosis factor α (TNF-α) and CC chemokine ligand 3 (CCL3) mRNA ($n = 5$–6). KO, proximal tubular TLR4-KO mice; s.c., subcutaneously. *$P < 0.05$ versus control mice.

effective time window; hANP was ineffective when applied in a relatively later phase (>18 hours after LPS).[6]

The changes in renal inflammatory cytokine levels between the normal control and proximal tubular TLR4-KO mice did not achieve statistical significance, even though the KO mice experienced attenuated AKI. This may be due to the expression levels of TLR4 in the infiltrated inflammatory cells. Indeed, macrophages isolated from the kidneys of LPS-injected KO mice showed similar cytokine expression levels compared with control mice in the present study. These results indicate that renal inflammation involving nonproximal tubular cells occurred in both mouse strains with and without tubular flow leakage.

AKI often causes coexisting damage in the respiratory system.[12,13] The release of damage-associated proteins and inflammatory cells has been reported to ignite renal-pulmonary syndrome after bilateral nephrectomy[13] and ischemia/reperfusion injury[14] as models of kidney injury. The same study also showed that global TLR4-KO mice had attenuated renal-pulmonary syndrome.[13] Because deletion of TLR4 in the proximal tubules attenuated AKI in our study, we examined whether this proximal tubule–dependent protection prevented renal-pulmonary syndrome in LPS-injected mice with systemic inflammation. However, lung pro- and anti-inflammatory cytokine levels were not significantly affected by the proximal tubular TLR4 deletion. Inhibition of inflammatory cell TLR4, rather than tubular TLR4 or AKI, might be essential for preventing renal-pulmonary syndrome coexisting with sepsis.

TLR4-dependent tight junction disruption has been reported in nonrenal cell types, including intestinal epithelial cells[15,16] and cholangiocytes,[17] and has been implicated in barrier dysfunction and leakage in these organs. In kidney tubules, leakage of filtrate results in stagnant tubular flow downstream and decreased shear stress. Apical shear stress has been shown to maintain proximal tubular morphology, including tight junction formation.[18,19] In addition, secreted substances including inflammatory cytokines and exosomes can stagnate, concentrating in the tubular lumen and inducing a tubule–tubule network.[20] Thus, TLR4 and reduced tubular flow might create a vicious cycle leading to further tight junction loss in downstream proximal tubules.

El-Achkar *et al.*[21] reported that TLR4 was expressed in other parts of the kidney, such as the glomeruli and distal tubules. Neither their study nor our previous study observed labeled LPS binding or accumulation in the distal tubules during endotoxemia.[3] LPS in the glomeruli diminished the glomerular filtration barrier[22] and may affect the fluid filtration rate. However, the reduction in the tubular flow rate in the proximal tubules occurred immediately after LPS exposure (<2 hours) while the GFR was still preserved,[3] and urine volume was almost normalized in proximal tubular TLR4-KO mice. We therefore presumed that the contribution of TLR4 to tubular flow in the other segments of the nephron was scarce, at least, in an early phase (<6 hours).

Tubule-dependent oliguria with normal GFR has been reported in models of mercury-induced AKI in frogs[1] and rats,[2] as well as LPS-induced AKI in rats[6] and mice.[3] Although it has not yet been elucidated in human AKI, urine output frequently decreases or halts during AKI treatment, despite maintenance of the renal circulation by fluid resuscitation. The results of the present study improve our understanding of the reasons for fluid-resistant oliguria during endotoxemia and emphasize the need to prevent proximal tubule leakage during AKI therapy.

METHODS

A detailed description of the materials and methods can be found in the Supplementary Methods.

Animals

All experiments were approved by the Institutional Animal Care and Use Committee of Kagawa University and followed standard guidelines for the humane care and use of animals in scientific research. Male C57/BL6J mice were purchased from CLEA (Tokyo, Japan) and housed in our colony until they were of proper age (12 weeks). TLR4-floxed mice were kindly provided by Dr. Joel Elmquist (University of Texas Southwestern Medical Center, Dallas, TX). Both TLR4-floxed and *Ndrg1*^{CreERT2} mice had a C57Bl/6 background and were from our breeding colony.[7,23]

Surgical procedure and *in vivo* multiphoton imaging settings

LPS (O-55:B5, Sigma, St Louis, MO) was injected i.p. (5 mg/kg) and *in vivo* imaging was performed either 6 or 24 hours later. One group of animals received the TLR4 antagonist C34 (3 mg/kg, i.p.; Tocris Bioscience, Bristol, UK) either 1 hour before or 4 hours after LPS injection. Another group of animals received fluid resuscitation (50 ml/kg saline, subcutaneously) at both 4 and 18 hours after LPS injection and were evaluated by *in vivo* imaging after 24 hours. Imaging was performed as previously described.[6,8,24,25] Mice were anesthetized with 2% isoflurane (Mylan, Osaka, Japan) and placed on a servo-controlled heating table to maintain a constant rectal temperature of 37 °C. The left kidney of each mouse was exteriorized through a small flank incision and attached to a coverslip.

Intravital multiphoton microscopy was performed using an Olympus FV1000MPE multiphoton confocal fluorescence imaging system (Tokyo, Japan), powered by a Chameleon Ultra-II MP laser at 720 and 860 nm (Coherent Inc., Santa Clara, CA). The imaging settings for the microscope (gain and offset for all 3 channels: blue, green, and red) were fixed throughout the experiment. Lucifer yellow (100 μg/kg, i.v. bolus) or fluorescein isothiocyanate-inulin (16 mg/kg, i.v. bolus) was injected to visualize tubular fluid flow. Time-lapse images were obtained at a resolution of 512 × 512 pixels. The inflow time of Lucifer yellow was defined as the time elapsed from a >1000-fold increase in fluorescence intensity (12-bit images) per second to the time of peak fluorescence intensity; outflow time was defined as the time elapsed from peak fluorescence intensity to half peak intensity. Inflow and outflow times were measured in 3 to 4 spatially separated tubular lumens in each imaging window that showed Lucifer yellow at a similar time point.

Tissue sodium and water measurement

Separate groups of mice were injected with LPS and euthanized 6 hours later. Their kidneys were removed, weighed, desiccated at 90 °C for 72 hours, and then reweighed (dry kidney weight). The kidneys were then ashed at 600 °C for 48 hours and the Na^+ and K^+ concentrations were measured by flame photometry (EFOX5053, Eppendorf, Germany).

Statistical analysis

All values in the normal control group were normally distributed, whereas skewing was observed in the oliguric groups. The results were expressed as mean ± SEM. Statistical significance was assessed using 1-way analysis of variance followed by Tukey multiple comparison test. Means were compared between the 2 groups using Student *t* test. All statistical analyses were performed using GraphPad Prism 6 (GraphPad Software Inc., La Jolla, CA), with values of $P < 0.05$ considered statistically significant.

DISCLOSURE

All the authors declared no competing interests.

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AUTHOR CONTRIBUTIONS

DN contributed to the conception and design of the research, prepared figures, drafted the manuscript, and prepared funding; DN, KK, NW, KW, and YZ performed experiments and analyzed data; DN, KK, HW, KW, and JMT interpreted results of experiments; DN, KK, and HW edited and revised the manuscript; MY, SL, and LJ developed the transgenic mouse line; and DN and AN approved the final version of the manuscript.

SUPPLEMENTARY MATERIAL

Supplementary File (PDF)

Figure S1. Expression levels of occludin and claudin 2 protein in the kidney ($n = 4$). LPS reduced occludin but not claudin 2 protein level.

Figure S2. mRNA expression levels of tight junction components in the cultured mProx24 cells ($n = 4$). LPS reduced claudin 2 mRNA expression level.

Figure S3. Sodium per water content in the kidney ($n = 14$–18). LPS increased water and sodium to similar degrees.

Figure S4. Genotyping of the floxed TLR4. (**A**) PCR analysis of genomic DNA isolated from the tail. Tamoxifen induced Cre recombinase activity and cleaved the loxP-flanked TLR4 (delta Tlr4). (**B**) TLR4 mRNA levels in the kidneys of TLR4-floxed control and TLR4-floxed *Ndrg1*^{CreERT2} KO mice 6 weeks after tamoxifen treatment.

Figure S5. Expression levels of occludin and claudin 2 protein in the kidney ($n = 2$ for saline-injected mice and $n = 5$ for LPS-injected mice). LPS reduced occludin protein level in the control but not in the proximal tubular TLR4-KO mice. Claudin 2 protein expression level was similar between the groups.

Figure S6. Effects of the TLR4 antagonist C34 on tubular flow rate at 6 h after LPS injection. Pretreatment with C34 at −1 h of LPS injection significantly shortened the duration required for Lucifer yellow to

reach the distal nephron (left) ($n = 4$). Posttreatment with C34 at 4 h after LPS injection had no significant influence on the tubular flow rate (right) ($n = 3$–4).

Figure S7. ZO-1 (green) immunofluorescence in cultured proximal tubular cells. LPS treatment for 6 h disrupted the continuity of ZO-1. The NF-κB inhibitor BAY11-7082 and p38 MAP kinase inhibitor SB203580 attenuated the disruption, whereas the TBK inhibitor BX795 did not. The tight junction disruption by LPS for 18 h was suppressed by BAY11-7082, but not by SB203580 or BX795. Scale bar = 32 μm.

Figure S8. GFR indirectly measured by inflow time of Lucifer yellow into the earliest segment of proximal tubule in the imaging window ($n = 6$). There was no significant difference in inflow time between the groups at either 6 or 24 h after LPS injection without fluid resuscitation. In contrast to the values at 6 h, the inflow time at 24 h was remarkably prolonged, indicating reduced GFR in both strains.

Figure S9. mRNA expression levels of TNF-α, CCL3, IL-10, and Mrc1 in CD11b-positive cells isolated from the kidney of mice receiving LPS and fluid resuscitation (at 24 h) ($n = 3$–4). There was no significant difference between the control floxed and proximal tubular TLR4-KO mice.

Figure S10. mRNA expression levels of TNF-α, CCL3, and IL-10 in the lung of mice treated with LPS and fluid resuscitation (at 24 h) ($n = 5$–6). There was no significant difference between the control floxed mice and the proximal tubular TLR4-KO mice. Data from 3 control floxed mice in which the urine flow responded poorly to fluid resuscitation are colored red.

Supplementary File (Movie)

Movie S1. Lucifer yellow was injected into the subcapsular space, outside the imaging window, in mice subjected to LPS injection 6 h before imaging. Images were taken every 60 s. The mice had been subjected to tubular flow measurement, and mild Lucifer yellow–derived fluorescence thus remained in the distal nephron at time 0. After the injection of Lucifer yellow, a few proximal tubules showed Lucifer yellow in the tubular lumen at 60 s, which was much longer than after i.v. injection of Lucifer yellow (<10 s in most cases). No fluorescence was detected in the interstitium or capillary. Blue: second harmonic generation of renal capsule; green: tubular autofluorescence and Lucifer yellow; red: tubular autofluorescence.

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