Toll-like Receptor 2 Is Required for LPS-induced Toll-like Receptor 4 Signaling and Inhibition of Ion Transport in Renal Thick Ascending Limb

Received for publication, December 20, 2011, and in revised form, March 26, 2012. Published, JBC Papers in Press, April 20, 2012, DOI 10.1074/jbc.M111.336255

David W. Good,‡§ Thampi George,§ and Bruns A. Watts III‡

From the Departments of ‡ Internal Medicine and § Neuroscience and Cell Biology, University of Texas Medical Branch, Galveston, Texas 77555

Background: Bacterial molecules act through Toll-like receptors to impair renal tubule function. Activation of ERK and inhibition of bicarbonate absorption by LPS requires both TLR4 and TLR2 in thick ascending limb. LPS also did not increase ERK phosphorylation in MTALs from TLR2−/− mice. TLR2 deficiency had no effect on expression of TLR4, MD-2, or MyD88. Preceding our study, we demonstrated that basolateral LPS inhibits 

Previously we demonstrated that basolateral LPS inhibits HCO3− absorption in the renal medullary thick ascending limb (MTAL) through TLR4-dependent ERK activation. Here we report that the response of the MTAL to basolateral LPS requires TLR2 in addition to TLR4. The basolateral addition of LPS (ultrapure Escherichia coli K12) decreased HCO3− absorption in isolated, perfused MTALs from wild-type mice but had no effect in MTALs from TLR2−/− mice. In contrast, inhibition of HCO3− absorption by lumen LPS was preserved in TLR2−/− MTALs, indicating that TLR2 is involved specifically in mediating the basolateral LPS response. Sepsis is a major cause of morbidity and mortality in critically ill patients, accounting for more than 200,000 deaths per year in the United States alone and consuming considerable health resources (1–4). The sepsis syndrome results from a dysregulated inflammatory response to infection that leads to multiple organ failure (2). A common and severe complication of sepsis is renal insufficiency. Impaired renal function accelerates the pathogenesis of sepsis through the loss of metabolic, fluid, and electrolyte homeostasis (3, 5, 6), and the development of kidney dysfunction doubles the risk for mortality in septic patients (3, 4, 7, 8). Bacterial sepsis and acute endotoxemia induce a number of defects in renal tubule function (9–13), but the underlying mechanisms remain poorly understood. Recently, we demonstrated that bacterial molecules can act directly through Toll-like receptors (TLRs) to impair the transport function of renal tubules, identifying a new pathophysiological mechanism that can contribute to renal tubule dysfunction during sepsis. In particular, absorption of HCO3− by the medullary thick ascending limb (MTAL) is inhibited by Gram-negative bacterial LPS through activation of TLR4 and by Gram-positive bacterial structures through activation of TLR2 (14, 15). Direct action of bacterial molecules to inhibit renal tubule HCO3− absorption would impair the ability of the kidneys to correct systemic metabolic acidosis that contributes to multiple organ dysfunction during sepsis (2, 3, 16–18) and is an independent risk factor for mortality in septic patients (19, 20). Understanding the molecular events involved in TLR4- and TLR2-induced transport inhibition in the MTAL may provide insights into how bacterial molecules can adversely affect the function of epithelial cells and aid in identifying potential therapeutic targets to treat or prevent renal tubule dysfunction during sepsis. The TLRs are a family of transmembrane receptors that recognize specific molecular patterns associated with a variety of

This work was supported, in whole or in part, by National Institutes of Health, NIDDK, Grant DK-038217.

1 To whom correspondence should be addressed: Division of Nephrology, 4.200 John Sealy Annex, University of Texas Medical Branch, 301 University Blvd., Galveston, TX 77555-0562. Tel.: 409-772-2472; Fax: 409-772-5451; E-mail: dgood@utmb.edu.

2 The abbreviations used are: TLR, Toll-like receptor; MTAL, medullary thick ascending limb; Pam3CSK4, N-palmitoyl-S-[2,3-bis(palmitoyloxy)-(2RS)-propyl]-[r]-cysteinyl-[S]-seryl-[S]-lysyl-[S]-lysine × 3 HCl; LTA, lipoteichoic acid; JHCO3−, absolute rate of HCO3− absorption; p-ERK, phospho-ERK1/2-Thr202/Tyr204.
microbial pathogens (21, 22). TLR4 functions as the primary signaling receptor for Gram-negative bacterial LPS (21, 23–30). Following LPS recognition, TLR4 activates intracellular signaling pathways that result in the induction of innate immune responses, including the activation of transcription factors that regulate the induction of genes encoding proinflammatory cytokines (22, 31). Our studies of the effects of LPS on MTAL function uncovered a novel sidedness to TLR4 signaling in epithelial cells. LPS inhibits HCO$_3^-$ absorption in the MTAL from either the basolateral or luminal cell surface through the activation of TLR4 (14). However, the underlying signaling mechanisms are different. Basolateral LPS decreases HCO$_3^-$ absorption through the activation of an ERK-dependent pathway (14, 32). In contrast, inhibition by lumen LPS is unaffected by ERK inhibitors but is eliminated by PI3K inhibitors and rapamycin, consistent with the activation of a PI3K-mTOR signaling pathway (14). Thus, LPS inhibits HCO$_3^-$ absorption in the MTAL through the activation of distinct TLR4-mediated signaling pathways in the basolateral and apical membranes. The molecular mechanisms that underlie this membrane-specific TLR4 signal specificity are undefined and have important implications for the selective targeting of TLR4-induced inflammatory responses in epithelial cells.

In addition to inhibition by Gram-negative LPS through TLR4, absorption of HCO$_3^-$ by the MTAL is inhibited by Gram-positive bacterial molecules through TLR2. TLR2 is expressed selectively in the basolateral membrane of MTAL cells, in contrast to TLR4, which is expressed in both basolateral and apical membrane domains (14, 15). MTAL HCO$_3^-$ absorption is inhibited by bacterial lipopeptides [N-palmitoyl-S-[2,3-bis(palmitoyloxy)-(2RS)-propyl]-[R]-cysteiny1-[S]-lysyl-[S]-lysine × 3 HCl (Pam3CSK$_4$)] and Gram-positive bacterial cell wall structures (lipoteichoic acid and peptidoglycan) recognized by TLR2 (15). Moreover, the inhibition by Gram-positive bacterial components is additive to inhibition by basolateral LPS, due to the TLR2 and TLR4 agonists activating different cell signaling pathways (15). These findings have important implications for the pathogenesis of kidney dysfunction during polymicrobial sepsis because they show that Gram-negative and Gram-positive bacterial molecules can act independently and additively to impair renal tubule function by activating different intracellular signals through different TLRs (15). The separate effects of TLR2 and TLR4 ligands to alter renal tubule function are consistent with the distinct roles of these receptors in bacterial recognition, in which TLR4 is critical for defense against Gram-negative infection through recognition of LPS, and TLR2 plays a predominant role in detection and protection against Gram-positive infection (23–30, 33–38). Evidence in support of this separation of function includes studies of LPS responses in mutant mouse models and transfected cell lines; TLR2-deficient cells that express TLR4 exhibit normal responses to enterobacterial LPS, indicating that TLR2 is not required for LPS signaling (25, 27–29, 33).

A unique feature of TLR2 is its ability to interact with other TLRs (TLR1 and TLR6) to form heterodimers that discriminate between different bacterial lipoproteins. The TLR2-TLR1 heterodimer recognizes triacylated lipopeptides, including Pam$_3$CSK$_4$, whereas the TLR2-TLR6 heterodimer recognizes diacylated lipoproteins and Gram-positive bacterial molecules, including lipoteichoic acid (21, 36, 39–41). These cooperative interactions expand the structural array of pathogen-derived components recognized by TLR2 and enhance ligand-induced TLR2 cell activation (22, 36, 39, 40). Studies using an enzyme complementation assay and co-immunoprecipitation to examine TLR dimerization in transfected cells found that TLR2 also is capable of interacting with TLR4 (42). However, to our knowledge, a physical association between TLR4 and TLR2 has not been reported in native cells, and no ligand has been identified that requires the co-presence of TLR2 and TLR4 for cell activation (42). Thus, the possible biological and functional significance of a TLR2-TLR4 association is unknown.

In the present study, we report that TLR2 is required along with TLR4 for the response of the MTAL to Gram-negative bacterial LPS. Our results show that the effects of basolateral LPS to activate ERK and inhibit HCO$_3^-$ absorption are dependent on both TLR4 and TLR2 and provide evidence of an association between TLR4 and TLR2. In contrast, the TLR4-mediated inhibition of HCO$_3^-$ absorption by lumen LPS does not depend on TLR2, indicating that TLR2 is required specifically for the basolateral LPS response. These findings reveal a novel requirement for TLR2 in LPS-induced TLR4 signaling and suggest that interaction between TLR4 and TLR2 may play a role in mediating renal tubule dysfunction during sepsis.

**EXPERIMENTAL PROCEDURES**

**Animals**—Mice deficient in TLR2 (B6.129-Tlr2<sup>tmKir</sup>/J; TLR2<sup>−/−</sup>), CD14 (B6.129S-Cd14<sup>tm1Frm/J</sup>; CD14<sup>−/−</sup>), and TLR4 (C57BL/10ScN; TLR4<sup>−/−</sup>), and wild-type control mice (C57BL/6) for TLR2<sup>−/−</sup> and CD14<sup>−/−</sup> were purchased from the Jackson Laboratory (Bar Harbor, ME). Mice were 6–8 weeks old. Male Sprague-Dawley rats (50–90 g, body weight) were purchased from Taconic (Germantown, NY). The animals were maintained under pathogen-free conditions in microisolator cages and received standard rodent chow (NIH 31 diet, Ziegler) and water up to the time of experiments. All protocols in this study were approved by the Institutional Animal Care and Use Committee of The University of Texas Medical Branch.

**Tubule Perfusion and Measurement of Net HCO$_3^-$ Absorption**—MTALs were isolated and perfused in vitro as described previously (14, 43). Tubules were dissected from the inner stripe of the outer medulla at 10 °C in control bath solution (see below), transferred to a bath chamber on the stage of an inverted microscope, and mounted on concentric glass pipettes for perfusion at 37 °C. The tubules were perfused and bathed in control solution that contained 146 mM Na$^+$, 4 mM K$^+$, 122 mM Cl$^-$, 25 mM HCO$_3^-$, 2.0 mM Ca$^{2+}$, 1.5 mM Mg$^{2+}$, 2.0 mM phosphate, 1.2 mM SO$_4^{2−}$, 1.0 mM citrate, 2.0 mM lactate, and 5.5 mM glucose (equilibrated with 95% O$_2$, 5% CO$_2$, pH 7.45, at 37 °C). Solutions containing experimental agents were prepared as described previously (14, 15). Ultrapure *Escherichia coli* K12 LPS, Pam$_3$CSK$_4$, and purified lipoteichoic acid (LTA) from *Staphylococcus aureus* were purchased from InvivoGen. Experimental agents were added to the bath or lumen solution as described under “Results.”
The protocol for study of transepithelial $\text{HCO}_3^-$ absorption was as described (14, 43, 44). Tubules were equilibrated for 20–30 min at 37 °C in the initial perfusion and bath solutions, and the luminal flow rate (normalized per unit of tubule length) was adjusted to 1.5–1.9 nl/min/mm. One to three 10-min tubule fluid samples were then collected for each period (initial, experimental, and recovery). The tubules were allowed to re-equilibrate for 5–10 min after an experimental agent was added to or removed from the bath or lumen solution. The absolute rate of $\text{HCO}_3^-$ absorption ($\text{HCO}_3^-_{\text{pmol/min/mm}}$) was calculated from the luminal flow rate and the difference between total CO$_2$ concentrations measured in perfused and collected fluids (43). An average $\text{HCO}_3^-$ absorption rate was calculated for each period studied in a given tubule. When repeat measurements were made at the beginning and end of an experiment (initial and recovery periods), the values were averaged. Single tubule values are presented in Figs. 1, 7, and 8. Mean values ± S.E. ($n = \text{number of tubules}$) are presented under “Results.”

**Immunoprecipitation and Immunoblotting**—Immunoprecipitation and immunoblot analyses were carried out on the inner stripe of the outer medulla dissected from mouse and rat kidneys, as described previously (32, 44–47). This tissue preparation is highly enriched in MTALs and exhibits regulatory changes in signaling and transport proteins that accurately reflect changes observed in the MTAL (45, 46, 48–54). The tissue samples were homogenized in ice-cold PBS and lysed for 2 h at 4 °C in radioimmune precipitation assay buffer with protease inhibitors. Separately, protein G magnetic beads (Pure-Proteome, Millipore) were washed and resuspended according to the manufacturer’s instructions and incubated overnight at 4 °C with 10 µg of anti-TLR2 (mouse mAb TL2.1, Invivogen) or 2 µg of anti-TLR4 (goat polyclonal L-14, Santa Cruz Biotechnology, Inc.) antibody. The antibody-bound beads were washed three times, and samples containing 500 µg of cell lysate were added and incubated for 2 h at 4 °C in a volume of 1 ml. After washing, the immunoprecipitated proteins were eluted by heating at 60 °C for 15 min in Laemmli sample buffer. Samples of equal protein content were separated by SDS-PAGE and transferred to PVDF membranes. Membranes were blocked with 5% BSA in TBS/Tween and incubated overnight at 4 °C with anti-TLR4 (1:1000; rabbit polyclonal, Abcam) or anti-TLR2 (1:1000; goat polyclonal D-17, Santa Cruz Biotechnology, Inc.) antibody. After washing in TBS, horseradish peroxidase-conjugated anti-rabbit (for TLR4) or anti-goat (for TLR2) secondary antibody was applied, and immunoreactive bands were detected by chemiluminescence (Luminol Reagent, Santa Cruz Biotechnology, Inc.). In some experiments (Figs. 3C and 5 (B and D)), cell lysates were immunoblotted directly for TLR4. Inner stripe lysates from wild-type and TLR2/−/− mice also were immunoblotted using anti-MyD88 (1:1000; rabbit polyclonal HFL-296, Santa Cruz Biotechnology, Inc.) and anti-MD-2 (1:1000; rabbit polyclonal, Abcam) antibodies. Parallel gels stained with Coomassie Blue were analyzed to confirm equal loading among lanes. Protein bands were quantified by densitometry. Whole cell lysates prepared from rat lung and heart were immunoprecipitated with anti-TLR2 antibody and immunoblotted with anti-TLR4 and anti-TLR2 antibodies using the same protocols as for the inner stripe.

**Confocal Immunofluorescence Microscopy**—MTALs were studied by confocal microscopy as described previously (14, 32, 46, 51). Mouse MTALs were dissected and mounted on Cell-Tak-coated coverslips at 10 °C. The tubules were then incubated for 15 min at 37 °C in a flowing bath using the same control solution as in $\text{HCO}_3^-$ transport experiments. In some experiments, tubules were incubated in the absence and presence of LPS (Figs. 2 and 6). Following incubation, the tubules were washed with PBS and fixed and permeabilized in acetone at −20 °C for 10 min. The tubules were incubated in Image-iT FX signal enhancer (Invitrogen) for 30 min at room temperature, washed, and blocked in 10% goat or donkey serum in PBS for 1 h at room temperature. The tubules were then incubated overnight at 4 °C with antiphospho-ERK1/2-Thr202/Tyr204 (1:200; Cell Signaling Technology), anti-TLR4 (1:100; Abcam), or anti-MyD88 (1:100; Santa Cruz Biotechnology) antibody. They were washed and then incubated for 1 h at room temperature in Alexa 488-conjugated goat or donkey anti-rabbit IgG antibody in blocking buffer. Fluorescence staining was examined using a Zeiss laser-scanning confocal microscope (LSM510 UV META), as described (14, 32, 46, 51). Tubules were imaged longitudinally, and z axis optical sections (0.4 µm) were obtained through a plane at the center of the tubule, which provides a cross-sectional view of cells in the lateral tubule walls. For individual experiments, 2–6 tubules from the same kidney for each experimental condition or from wild-type and TLR2/−/− mice were fixed and stained identically and imaged in a single session at identical settings of illumination, gain, and exposure time. Fluorescence intensity of phospho-ERK1/2 (p-ERK) (Fig. 2), TLR4 (Fig. 4), and MyD88 (Fig. 6) staining was quantified as described previously (32, 46). Two-dimensional image analysis was performed using MetaMorph software, in which boxes were positioned on the cytoplasm in the midregion of the cell (1.4 × 4.2 µm; p-ERK) or on linear regions of basolateral and apical membrane domains (0.7 × 2.1 µm; TLR4 and MyD88), and pixel intensity per unit area was determined for each region. Three different cells were analyzed per optical section, and three optical sections were analyzed per tubule, one section at the center of the tubule and two sections positioned 0.12 µm above and below the center section (32). The measurements were averaged to obtain a value for each tubule. The fluorescence intensity for experimental groups was expressed as a percentage of the control value measured in the same experiment. Mean values (n = number of tubules) were used for statistical analysis. Morphometric analysis showed no difference in total cell volume or cell surface area in MTALs from wild-type and TLR2/−/− mice.

**Analysis**—Results are presented as mean ± S.E. Differences between means were evaluated using Student’s t test for paired or unpaired data or analysis of variance with Newman-Keuls multiple range test, as appropriate. p < 0.05 was considered statistically significant.

**RESULTS**

**Inhibition of $\text{HCO}_3^-$ Absorption by Bath but Not Lumen LPS Is Eliminated in MTALs from TLR2/−/− Mice**—Previously we demonstrated that LPS inhibits $\text{HCO}_3^-$ absorption in the...
LPS-induced TLR4 Signaling Requires TLR2 in Thick Ascending Limb

MTALs from either the basolateral or luminal cell surface and that these effects depend on activation of TLR4 (14, 32). To test whether the response of the MTAL to LPS is influenced by TLR2, we examined the effects of LPS on HCO$_3^-$ absorption in isolated, perfused MTALs from wild-type and TLR2$^{−/−}$ mice. Adding LPS to the bath decreased HCO$_3^-$ absorption by 28% (from 17.2 ± 0.4 pmol/min/mm) in MTALs from wild-type mice but had no effect on HCO$_3^-$ absorption in MTALs from TLR2$^{−/−}$ mice (17.9 ± 0.7 pmol/min/mm for control versus 17.9 ± 0.6 pmol/min/mm for bath LPS) (Fig. 1, A and B). In contrast, adding LPS to the lumen decreased HCO$_3^-$ absorption by 28% (from 17.2 ± 0.4 to 12.4 ± 0.5 pmol/min/mm) in MTALs from wild-type mice and by 25% (from 17.9 ± 0.2 to 13.4 ± 0.2 pmol/min/mm) in MTALs from TLR2$^{−/−}$ mice (Fig. 1, C and D). Thus, inhibition of HCO$_3^-$ absorption by lumen LPS is preserved in TLR2$^{−/−}$ MTALs. The effect of bath addition of E. coli O111:B4 LPS to inhibit HCO$_3^-$ absorption (14) also is eliminated in TLR2$^{−/−}$ MTALs (data not shown), indicating that the absence of basolateral LPS response is not specific for ultrapure E. coli K12 LPS. These results indicate that inhibition of HCO$_3^-$ absorption by basolateral LPS in the MTAL requires TLR2 in addition to TLR4 and that TLR2 is involved specifically in mediating the basolateral LPS response.

**LPS-induced ERK Activation Is Eliminated in MTALs from TLR2$^{−/−}$ Mice**—Previously we demonstrated that basolateral LPS inhibits HCO$_3^-$ absorption in the MTAL through TLR4-mediated activation of ERK (14, 32). Based on the inability of basolateral LPS to inhibit HCO$_3^-$ absorption in TLR2$^{−/−}$ MTALs, we tested whether TLR2 is required for LPS-induced ERK activation. MTALs dissected from wild-type and TLR2$^{−/−}$ mice were incubated in the absence and presence of LPS for 15 min, stained with anti-p-ERK antibody, and then analyzed by confocal immunofluorescence (Fig. 2, A and B). Consistent with previous results (32), stimulation with LPS increased p-ERK staining 2.4 ± 0.1-fold in MTALs from wild-type mice. In contrast, LPS had no effect on phosphorylation of ERK in MTALs from TLR2$^{−/−}$ mice. These results demonstrate that activation of ERK by LPS in the MTAL depends on TLR2. Thus, basolateral LPS fails to inhibit HCO$_3^-$ absorption in TLR2$^{−/−}$ MTALs because TLR2 is required along with TLR4 for LPS-induced ERK activation.

Further studies were carried out to test whether ERK can be activated in TLR2$^{−/−}$ MTALs by a stimulus that functions independently of TLR2 and TLR4. MTALs from TLR2$^{−/−}$ mice were incubated for 15 min in the absence and presence of 1 nm aldosterone, which inhibits HCO$_3^-$ absorption in the MTAL, aldosterone, which inhibits HCO$_3^-$ absorption in the MTAL and activates the ERK pathway in vitro (52). These results demonstrate that the ERK pathway is functional in MTALs from TLR2$^{−/−}$ mice and that the inability of LPS to stimulate ERK is not due to the induction of a nonspecific negative regulator of ERK in the TLR2$^{−/−}$ tubules.

**TLR4 Is Expressed at Normal Levels in MTALs from TLR2$^{−/−}$ Mice**—One possible explanation for the inability of basolateral LPS to activate ERK and inhibit HCO$_3^-$ absorption in MTALs from TLR2$^{−/−}$ mice is that loss of TLR2 may reduce or eliminate TLR4 expression in the MTAL. To test this, expression of TLR4 was examined by confocal immunofluorescence in MTALs dissected from wild-type and TLR2$^{−/−}$ mice. As demonstrated previously (14), TLR4 staining is present in the basolateral and apical membrane domains as well as in the cytoplasm (Fig. 3A). The intensity of TLR4 staining in basolateral and apical membrane domains and cytoplasm did not differ in MTALs from wild-type and TLR2$^{−/−}$ mice (Fig. 3B). To confirm further the expression of TLR4 in TLR2$^{−/−}$ mice, TLR4 protein level was analyzed by immunoblotting of the inner stripe of the outer medullia. This region of the kidney is highly enriched in MTALs and has been studied extensively to identify regulated changes in the expression of MTAL proteins (47–54). As shown in Fig. 3C, no difference in TLR4 protein abundance was observed in inner stripe tissue from wild-type and TLR2$^{−/−}$ mice. Taken together, these results indicate that TLR4 is expressed at normal levels in MTALs from TLR2$^{−/−}$ mice and support the view that the inability of TLR2$^{−/−}$ MTALs to respond to basolateral LPS is not due to a loss of TLR4 protein expression.
LPS-induced TLR4 Signaling Requires TLR2 in Thick Ascending Limb

Expression of MyD88 and MD-2 in TLR2\(^{-/-}\) Mice—The effects of basolateral LPS to activate ERK and inhibit HCO\(_3\)\(^{-}\) absorption in the MTAL are dependent on the intracellular adaptor molecule MyD88 (32). We therefore examined whether reduced expression of MyD88 could explain the lack of responsiveness of TLR2\(^{-/-}\) MTALs to basolateral LPS. As shown in Fig. 4A, expression of MyD88 did not differ in the inner stripe of the outer medulla of wild-type and TLR2\(^{-/-}\) mice. Further studies examined whether the unresponsiveness of TLR2\(^{-/-}\) MTALs to basolateral LPS was due to reduced expression of MD-2, a cell surface glycoprotein that co-associates with TLR4 and is required for LPS-induced TLR4 signaling (55–58). As shown in Fig. 4B, no difference in MD-2 protein expression was observed in inner stripe tissue from wild-type and TLR2\(^{-/-}\) mice. These results support the view that the inability of MTALs from TLR2\(^{-/-}\) mice to respond to basolateral LPS is not the result of reduced expression of MyD88 or MD-2.

TLR2 Associates with TLR4 in Inner Stripe of Outer Medulla—Further experiments were carried out to test for an interaction between TLR2 and TLR4 in the inner stripe of the outer medulla. Initial experiments were carried out using inner stripe tissue dissected from rats to take advantage of the higher protein yield compared with mice. Inner stripe tissue was incubated in vitro in the absence and presence of LPS for 15 min, and cell lysates were immunoprecipitated with anti-TLR2 antibody. Immunoprecipitates were then immunoblotted with anti-TLR4 antibody to detect TLR2-associated TLR4. As shown in Fig. 5A, immunoprecipitation of TLR2 resulted in coprecipitation of TLR4, and the amount of TLR4 in TLR2 immunoprecipitates was increased in the presence of LPS. The presence of TLR4 in TLR2 immunoprecipitates was confirmed by immunoblotting with a second TLR4 antibody (M-16, Santa Cruz Biotechnology, Inc.) (not shown). This same experiment was then carried out on inner stripe tissue from wild-type and TLR2\(^{-/-}\) mice. Similar to results obtained in rats, immunoprecipitation of inner stripe lysates from wild-type mice with anti-TLR2 antibody resulted in coprecipitation of TLR4, and the amount of TLR4 in TLR2 immunoprecipitates was increased in the presence of LPS (Fig. 5B). In contrast, TLR4 was not detected in TLR2 immunoprecipitates from the inner stripe of TLR2\(^{-/-}\) mice, despite the presence of normal amounts of TLR4 in the TLR2\(^{-/-}\) lysates (Figs. 3C and 5B). These results show that the ability of the anti-TLR2 antibody to immunoprecipitate TLR4 depends on the presence of TLR2 and is not the result of a nonspecific interaction of the Protein G bead-bound TLR2 antibody with TLR4 in the inner stripe lysates. The association of TLR2 with TLR4 was con-

**FIGURE 2. LPS-induced ERK activation requires TLR2.** A, MTALs dissected from wild-type and TLR2\(^{-/-}\) mice were incubated in vitro at 37 °C in the absence (control) and presence of LPS (500 ng/ml) for 15 min. The tubules were then fixed and stained with anti-p-ERK antibody and analyzed by confocal immunofluorescence as detailed under “Experimental Procedures.” Images are z axis sections (0.4 μm) taken through a plane at the center of the tubule showing a cross-sectional view of cells in the lateral tubule walls (14, 32, 46, 51). LPS increased p-ERK labeling in MTALs from wild-type mice but had no effect in MTALs from TLR2\(^{-/-}\) mice. Images are representative of at least nine tubules of each type. Scale bar, 5 μm. B, the intensity of p-ERK staining was quantified for experiments in A as described under “Experimental Procedures” and is presented as a percentage of control level measured in the same experiment. Bars, means ± S.E., *, p < 0.05 versus wild-type control. C, MTALs from TLR2\(^{-/-}\) mice were incubated at 37 °C in the absence (control) and presence of aldosterone (1 nm) for 15 min and then analyzed for p-ERK staining by confocal immunofluorescence as in A. Images are representative of at least six tubules of each type. D, the intensity of p-ERK staining was quantified for experiments in C as described under “Experimental Procedures” and is expressed as a percentage of control level measured in the same experiment. *, p < 0.05 versus control.
LPS-induced TLR4 Signaling Requires TLR2 in Thick Ascending Limb

FIGURE 3. Expression of TLR4 in MTALs from TLR2−/− mice. A, MTALs dissected from wild-type and TLR2−/− mice were stained with anti-TLR4 antibody (Abcam) (14) and analyzed by confocal immunofluorescence as described in the legend to Fig. 2A. TLR4 staining is present in the basolateral (arrowheads) and apical (arrows) membrane domains. Images are representative of at least nine tubules of each type. B, the intensity of TLR4 staining in basolateral and apical membrane domains and in the cytoplasm was quantified for experiments in A as described under “Experimental Procedures.” Fluorescence intensity for TLR2−/− tubules is presented as a percentage of the wild-type value measured in the same experiment. Bars, means ± S.E. TLR2−/− values do not differ from wild-type for all three regions. C, lysates of the inner stripe of outer medulla from wild-type and TLR2−/− mice were immunoblotted using polyclonal anti-TLR4 antibody (Abcam). The blot was loaded with 50 μg of protein/lane. Each lane contains a sample from a different mouse. Apparent molecular mass is shown on the right. Parallel gels were subjected to Coomassie Blue staining to verify equal protein loading.

Inhibition of HCO$_3^-$ Absorption by Basolateral LPS—CD14 is a membrane-anchored LPS binding protein that enhances LPS signaling by facilitating transfer of LPS to the TLR4 signaling complex (59–63). CD14 also can facilitate TLR2 signaling (34, 38, 64–66), and it has been suggested that CD14 may be a component of cell surface signaling complexes involving TLR4 and TLR2 (67–70). To determine whether CD14 is involved in mediating inhibition of HCO$_3^-$ absorption by basolateral LPS in the MTAL, tubules from wild-type and CD14−/− mice were perfused in vitro. Adding LPS to the bath decreased HCO$_3^-$ absorption by 28% (from 14.2 ± 0.5 to 10.3 ± 0.6 pmol/min/mm) in MTALs from wild-type mice and by 26% (from 14.1 ± 0.4 to 10.4 ± 0.2 pmol/min/mm) in MTALs from CD14−/− mice (Fig. 7, A and B). The response of the CD14−/− MTALs to LPS cannot be explained by the presence of soluble CD14 because the tubules are perfused in vitro using serum-free artificial solutions. Thus, the TLR2- and TLR4-dependent inhibition of HCO$_3^-$ absorption by basolateral LPS does not require CD14.

Inhibition of HCO$_3^-$ Absorption by TLR2 Agonists Does Not Require TLR4—Previously we demonstrated that the TLR2 agonists Pam$_3$CSK$_4$ and LTA inhibit HCO$_3^-$ absorption in the MTAL through activation of basolateral TLR2 (15). In the present study, we show that the response of the MTAL to the TLR4 agonist LPS depends on TLR2. Therefore, we asked whether the response of the MTAL to TLR2 agonists is reciprocally dependent on TLR4. MTALs from TLR4−/− mice were perfused in vitro. Adding Pam$_3$CSK$_4$ to the bath decreased HCO$_3^-$ absorption by 25% (from 16.6 ± 0.7 to 12.4 ± 0.8 pmol/min/mm), and adding LTA to the bath decreased HCO$_3^-$ absorption by 28% (from 16.1 ± 0.5 to 11.6 ± 0.8 pmol/min/mm) in TLR4−/− MTALs (Fig. 8, A and B). These decreases are virtually identical to those induced by bath Pam$_3$CSK$_4$ and LTA in MTALs from wild-type mice and rats (15). Thus, basolateral TLR2 is able to transmit signals in response to TLR2 agonists independently of TLR4 in the MTAL.

DISCUSSION

Toll-like receptors are responsible for recognizing conserved microbial structures, resulting in the induction of inflammatory responses that defend the host against invading pathogens. When the inflammatory response to bacterial infection is dysregulated, as occurs in sepsis, it can lead to cell and tissue dam-
LPS-induced TLR4 Signaling Requires TLR2 in Thick Ascending Limb

A.

Wild-type  |  TLR2\(^{-/-}\)
---|---
MyD88 |  [Image 180x531 to 377x552]

B.

Wild-type  |  TLR2\(^{-/-}\)
---|---
MD-2 |  [Image 180x650 to 378x679]

FIGURE 4. Expression of MyD88 and MD-2 in TLR2\(^{-/-}\) mice. Lysates of the inner stripe of outer medulla from wild-type and TLR2\(^{-/-}\) mice were immunoblotted using anti-MyD88 (A) or anti-MD-2 (B) antibody. Blots were loaded with 50 µg of protein/lane. Each lane contains a sample from a different mouse. Apparent molecular mass is shown on the right. MyD88 and MD-2 protein abundance was quantified by densitometry, and band densities for TLR2\(^{-/-}\) mice are presented as a percentage of wild-type values. Bars, means ± S.E. TLR2\(^{-/-}\) values do not differ from those of wild-type.

The ability of TLR2 to mediate responses to a broad range of structurally diverse molecules is attributable to its cooperative interactions with TLR1 and TLR6. TLR2 forms heterodimers with TLR1 to recognize triacylated lipopeptides, such as Pam\(_3\)CSK\(_4\) (40, 41), mycobacterial lipoarabinomannan and 19-kDa lipoprotein (40, 41), and Borrelia burgdorferi lipoprotein OsPA (37). TLR2-TLR6 heterodimers recognize diacylated lipopeptides (39), lipoteichoic acid and peptidoglycan from Gram-positive bacteria (36, 39, 73), and yeast zymosan (36). In experiments using enzyme complementation and co-immunoprecipitation approaches in transfected cells, Lee et al. (42) reported that TLR2 also can associate specifically with TLR4 and that intracellular domain interactions appeared to play an important role in this association. The biological relevance of this interaction was unclear, however, because no ligand had been identified that required the co-presence of TLR4 and TLR2 for cell activation (42), and a physical association of TLR2 with TLR4 had not been observed in other co-expression studies (39, 41, 74). In the present study, we show that TLR2 is required along with TLR4 (14, 32) for the response of the MTAL to basolateral E. coli LPS. We also demonstrate an association between TLR2 and TLR4, which are co-expressed in the basolateral membrane of MTAL cells (14, 15). TLR4 and TLR2
can be co-immunoprecipitated in the absence of LPS, indicating that this ligand is not necessary for TLR2 and TLR4 to associate. Importantly, the TLR4-mediated effects of LPS to activate ERK and inhibit HCO$_3^-$ absorption are eliminated completely in MTALs from TLR2$^{-/-}$ mice, indicating that TLR2 plays an essential role in the LPS-induced responses and is not merely an accessory molecule that enhances an already functional TLR4 response. It remains to be determined whether the requirement for TLR2 in LPS-induced signaling in the MTAL is selective for ERK activation or if interaction between TLR4 and TLR2 is involved in the induction of other LPS-induced signaling pathways, such as the recruitment of interleukin-1 receptor-associated kinases that leads to NfκB activation and the production of proinflammatory cytokines (31). Of note, previous studies using cultured renal proximal tubule cells showed that the effects of purified *E. coli* LPS to stimulate production of TNF-α and KC through TLR4 were markedly reduced in cells grown from TLR2$^{-/-}$ mice, suggesting that TLR2 was required to achieve a maximal LPS response (75). These findings conceivably could be explained by a functional interaction between TLR4 and TLR2 similar to that identified in the current study and suggest that cooperative interaction between TLR4 and TLR2 may regulate LPS-induced signaling events in addition to ERK activation and play a role in mediating functional responses to LPS in nephron segments other than the MTAL. Basolateral LPS inhibits HCO$_3^-$ absorption in the MTAL through ERK-dependent inhibition of apical membrane NHE3, the Na$^+$/H$^+$ exchanger isoform responsible for absorption of NaCl and/or NaHCO$_3$ by epithelial cells of the kidney and intestinal tract (32). Results of the current study indicate that cell signals generated through the interaction of TLR4 and TLR2 can modify the transport activity of this exchanger. Although the molecular basis for the requirement of TLR2 in LPS-induced TLR4 signaling remains to be determined, it is possible to comment on some potential contributing factors. First, the involvement of TLR2 in the LPS response is not the result of contaminants in the LPS solutions because 1) our experiments were carried out using ultrapure LPS prepared by the modified phenol extraction protocol shown to eliminate TLR2-dependent ligands from *E. coli* LPS preparations (27), and 2) basolateral LPS has no effect on HCO$_3^-$ absorption in MTALs from TLR4$^{-/-}$ mice despite the presence of functional TLR2 that mediates transport inhibition in response to TLR2 ligands (Fig. 8) (14, 15). Second, our results show that both the level and the subcellular distribution of TLR4 expression are normal in MTALs from
TLR2−/− mice, including labeling along the basolateral membrane. Thus, the inability of TLR2−/− MTALs to respond to basolateral LPS cannot be explained by loss of TLR4 expression. This conclusion is supported further by the finding that TLR4-mediated inhibition of HCO3− absorption by lumen LPS is normal in TLR2−/− MTALs. Third, we have shown previously that the effects of basolateral LPS to activate ERK and inhibit HCO3− absorption in the MTAL are mediated through the adaptor protein MyD88 (32), raising the possibility that loss of TLR2 could diminish MyD88 expression and render it unable to support LPS-induced TLR4 signaling. This explanation also is unlikely based on the observations that 1) MyD88 is expressed at normal levels in the inner stripe of the outer medulla of TLR2−/− mice, and 2) TLR4-mediated inhibition of HCO3− absorption by lumen LPS, which depends on MyD88,3 is normal in TLR2−/− MTALs. We also demonstrate that deletion of

3 D. Good, T. George, and B. A. Watts III, unpublished observations.
TLR2 has no effect on the level of expression of MD-2, an LPS-binding glycoprotein that is required for activation of TLR4 signaling by LPS (55–58), arguing against reduced MD-2 levels as an explanation for the lack of response of TLR2−/− MTALs to LPS. Finally, we show that ERK is activated in TLR2−/− MTALs by aldosterone, a physiological stimulus that inhibits HCO₃⁻ absorption in the MTAL through an ERK-dependent pathway independent of that activated by LPS through TLR4 and TLR2 (32, 52). Thus, the impairment of ERK activation is selective for the TLR4-TLR2 pathway and is not due to nonspecific induction of a negative regulator of the ERK pathway in MTALs from TLR2−/− mice. Our data do not rule out the possibility that loss of TLR2 may induce a negative regulator that selectively suppresses TLR4-dependent ERK signaling in MTAL cells (22, 76); however, we are not aware of any evidence for such a mechanism in cells from TLR2−/− mice.

The results of our study are consistent with a mechanism involving cooperative interaction between TLR4 and TLR2. Our data indicate that the association between TLR4 and TLR2 may be enhanced by stimulation with LPS, suggesting that LPS may initiate intracellular signals in the MTAL by altering a physical interaction between TLR4 and TLR2 in the basolateral membrane. In particular, this receptor interaction appears to be important for LPS signaling via MyD88. MyD88 functions as an adaptor protein in signaling pathways for TLR4 and TLR2 (22). TLR4 activation by LPS induces recruitment and binding of MyD88 to the intracellular signaling domain, triggering the activation of downstream signaling pathways, including MAPK (22). We have shown that MyD88 mediates the TLR4-dependent effects of basal LPS to activate ERK and inhibit HCO₃⁻ absorption in the MTAL (32).

In the present study, we found that the ability of LPS to induce recruitment of MyD88 to the basolateral membrane domain is impaired in MTALs from TLR2−/− mice. These results suggest that interaction of TLR2 with TLR4 is important for LPS-induced recruitment of MyD88 to the basolateral TLR4 signaling complex and that reduced signaling through MyD88 is responsible for the inability of LPS to induce downstream activation of ERK in TLR2−/− MTALs.

The structural basis underlying the TLR4-TLR2 interaction in our experiments is undefined. TLR4 was reported to form heterodimers with TLR2 in cells transiently transfected with TLR4 and TLR2 constructs (42), but it is unknown whether this interaction can occur physiologically or represents a manifestation of receptor overexpression in cells in vitro. An alternative possibility is that TLR4 and TLR2 may interact, directly or indirectly, as components of a receptor complex recruited to microdomains in the MTAL basolateral membrane. Recent studies show that LPS stimulation triggers the recruitment and clustering of TLR4 into lipid rafts in the plasma membrane, along with additional receptor proteins, such as MD-2, heat-shock proteins, and chemokine receptors (58, 69, 77). The formation of these activation clusters on the cell surface is important for LPS-induced cell activation (58, 69, 77). Recruitment of TLR2 into membrane microdomains along with TLR4 in response to LPS has not been demonstrated; however, lipoteichoic acid binding has been shown to induce the recruitment of both TLR2 (70) and TLR4 (68) into lipid rafts. These findings raise the possibility that LPS could concentrate TLR4 and TLR2 in activation clusters in the basolateral membrane of the MTAL, leading to direct or indirect interaction between the receptors that is required for LPS-induced ERK activation. Consistent with this hypothesis, LPS-induced TLR4 clustering in lipid rafts leads to MyD88 recruitment and MAPK activation (69, 77), key components of the basolateral LPS-induced signaling pathway in the MTAL (Fig. 6) (32). An ability of LPS to induce the formation of different receptor clusters within different membranes also may aid in explaining why TLR2 is involved in LPS-induced TLR4 signaling in the basolateral membrane but not in the apical membrane of MTAL cells; TLR2 is not expressed in the apical membrane (15) and thus would not be a component of the TLR4 receptor complex recruited into apical microdomains. An important area for future investigation will be to assess the role of receptor clustering within lipid rafts as a possible factor contributing to the association between TLR4 and TLR2 in the MTAL basolateral membrane and to the ability of LPS to activate different TLR4-mediated signaling pathways at the basolateral and apical membranes. An additional unanswered question is whether TLR4 interacts with TLR2 to recognize LPS or if TLR4 functions by itself (in association with MD-2) as the LPS-binding receptor and then interacts functionally with TLR2 to initiate intracellular signals after LPS binding.

In addition to its interaction with TLR4 to induce signaling by LPS, TLR2 functions independently of TLR4 in the MTAL to recognize other bacterial molecules. We have shown previously that the triacylated lipopeptide Pam₃CSK₄ and the Gram-positive cell wall components lipoteichoic acid and peptidoglycan inhibit HCO₃⁻ absorption in the MTAL through activation of basolateral TLR2 and that the inhibition by these TLR2 ligands is additive to inhibition by LPS due to the activation of different intracellular signaling pathways (15). In the present study, we demonstrate further that the inhibition of HCO₃⁻ absorption by Pam₃CSK₄ and lipoteichoic acid occurs normally in MTALs from TLR4−/− mice. These findings indicate that basolateral TLR2 functions as the recognition and signaling receptor for Pam₃CSK₄, lipoteichoic acid, and peptidoglycan independent of TLR4. Thus, TLR2 plays a dual role in the induction of cell signals that inhibit HCO₃⁻ absorption in the MTAL, both through a functional interaction with TLR4 to activate ERK in response to Gram-negative bacterial LPS and through a TLR4-independent signaling pathway activated by Gram-positive bacterial molecules. As noted above, TLR2 interacts with TLR1 to recognize Pam₃CSK₄ and with TLR6 to recognize lipoteichoic acid. It is currently unknown whether TLR1 and/or TLR6 are expressed in the MTAL and may function to enhance basolateral TLR2 signaling in response to these ligands. It is possible that the differential roles of TLR2 in mediating TLR4-dependent responses to LPS and TLR4-independent responses to Pam₃CSK₄ and lipoteichoic acid in the MTAL may involve the selective recruitment of TLR2 with TLR4 or with TLR1 or TLR6 to form different receptor signaling clusters within membrane microdomains in response to different bacterial structures. A role for TLR2 homodimers that function independently of TLR4 in the basolateral membrane also cannot be ruled out.
CD14 is a high affinity LPS receptor that exists as a glycosylphosphatidylinositol-anchored membrane protein and as a soluble protein in plasma (59, 61, 62). CD14 plays a pivotal role in enhancing LPS responsiveness by facilitating transfer of LPS to the MD-2:TLR4 complex that initiates signal transduction (58, 60, 61, 63). CD14 also interacts with bacterial products that activate TLR2 and enhances cellular responses to TLR2 ligands, including lipoteichoic acid, peptidoglycan, and lipoarabinomannan (34, 38, 62, 64–66). Moreover, recent studies show that CD14 is a component of TLR4 and TLR2 receptor clusters recruited to membrane microdomains in response to ligand activation (68–70, 77). This suggests that ligation of CD14 by bacterial components may play a role in the recruitment of receptor molecules into lipid rafts and that CD14, TLR4, and TLR2 may exist in compartmentalized receptor signaling complexes on the cell surface (67–70, 77). These findings raise the possibility that CD14 could play a role in promoting the cooperative interaction between TLR4 and TLR2 in the basolateral membrane of the MTAL. Our results show, however, that the inhibition of HCO3− absorption by basolateral LPS is fully preserved in MTALs from CD14−/− mice. Thus, neither LPS recognition nor LPS-induced TLR4:TLR2 signaling requires CD14. Whether other cell surface LPS receptor molecules that mediate CD14-independent LPS signaling (62, 78) may play a role in the response of the MTAL to basolateral LPS is not known. Also, our results do not rule out the possibility that the response of the MTAL to LPS may be facilitated in vivo by soluble CD14. Consistent with this possibility, we have shown that serum enhances the responsiveness of the MTAL to basolateral LPS in vitro (14). Nevertheless, it is clear that CD14 is not necessary for the functional interaction between TLR4 and TLR2 induced by LPS in the MTAL.

The effect of LPS to inhibit HCO3− absorption in the MTAL is significant for disease pathogenesis because it impairs the ability of the kidneys to correct systemic metabolic acidosis that contributes to multiple organ failure during sepsis (2, 3, 16–18) and is independently associated with increased mortality in septic patients (19, 20). In addition, we found that the ability of basolateral LPS to inhibit HCO3− absorption through ERK activation is up-regulated in MTALs from septic mice (79). This suggests that the interaction between TLR4 and TLR2 may be modified during sepsis to enhance renal tubule responses to bacterial molecules. The identification of LPS-induced cell responses that require TLR4 and TLR2 may have additional implications for immunopathology. For example, it is possible that interaction of TLR4 with TLR2 may expand the range of LPS phenotypes recognized by renal tubules. An ability of epithelial cells to discriminate between LPS from different bacteria or to detect subtle changes in LPS structure may be important to generate different responses to specific bacteria and to protect against naturally occurring LPS heterogeneity that may result in evasion of host responses and sustained bacterial pathogenicity (30). Interaction between TLR4 and TLR2 also may play a role in recognition and signaling responses induced by endogenous “danger” molecules, such as high mobility group box 1 protein, heat-shock protein 60, and biglycan that have been reported to activate cells through both receptors (22, 62, 80, 81). Such a role would have potential therapeutic implications because activation of TLR4 and TLR2 on renal cells by endogenous ligands produced by stressed or damaged cells is thought to play an important role in mediating inflammatory kidney injury in a variety of non-infectious conditions, including ischemia-reperfusion, glomerular diseases, and nephrotoxic injury (82–88). Our results raise the possibility that the protective effects of TLR2 knock-out in these conditions (83, 84, 86, 88) may be due, in part, to loss of TLR4-mediated signals that depend on TLR2. Last, in addition to the kidney, our studies provide evidence of an association between TLR4 and TLR2 in the lung but not in the heart. These findings suggest that cooperation between TLR4 and TLR2 may play a role in determining cellular responses to LPS in other organ systems and raise the question of whether TLR4:TLR2 interaction may be a selective feature of bacterial recognition in epithelial tissues.

In summary, our results demonstrate that the effects of basolateral LPS to activate ERK and inhibit HCO3− absorption in the MTAL (14, 32) require both TLR4 and TLR2. Co-immunoprecipitation studies show that TLR4 associates with TLR2. These results suggest that TLR4 and TLR2 interact functionally in the basolateral membrane of MTAL cells to mediate LPS-induced ERK activation. Our findings provide evidence of a novel requirement for TLR2 in LPS-induced TLR4 signaling and broaden our understanding of TLR4 function to include interaction with another TLR for the induction of cell responses. Basolateral TLR2 plays a complex, dual role in bacterial recognition and the induction of cell signals that impair HCO3− absorption in the MTAL, both through cooperative interaction with TLR4 to mediate ERK activation by Gram-negative LPS and through a TLR4-independent signaling pathway activated by Gram-positive cell wall molecules (15). Regulation of TLR2 expression and its interaction with TLR4 may provide new mechanisms for controlling TLR4-mediated LPS responses. Understanding the molecular basis of the association between TLR4 and TLR2 in the MTAL may enable selective targeting of LPS responses and lead to new therapeutic strategies to treat or prevent renal tubule dysfunction during sepsis.

REFERENCES

1. Levy, E. M., Viscoli, C. M., and Horwitz, R. I. (1996) The effect of acute renal failure on mortality. A cohort analysis. JAMA 275, 1489–1494
2. Cohen, J. (2002) The immunopathogenesis of sepsis. Nature 420, 885–891
3. Schrier, R. W., and Wang, W. (2004) Acute renal failure and sepsis. NEJM 351, 159–169
4. Doi, K., Lee, N., and Star, R. A. (2009) Animal models of sepsis and sepsis-induced kidney injury. J. Clin. Invest. 119, 2868–2878
5. Klenzak, J., and Himmelfarb, J. (2005) Sepsis and the kidney. Crit. Care Clin. 21, 211–222
6. El-Achkar, T. M., Hosein, M., and Dagher, P. C. (2008) Pathways of renal injury in systemic gram-negative sepsis. Eur. J. Clin. Invest. 38, 39–44
7. Russell, J. A., Singer, J., Bernard, G. R., Wheeler, A., Hulsen, M., Schein, R., Summer, W., Wright, P., and Walley, K. R. (2000) Changing pattern of organ dysfunction in early human sepsis is related to mortality. Crit. Care Med. 28, 3405–3411
8. Oppert, M., Engle, C., Brunhorst, F. M., Bogatsch, H., Reinhart, K., Frei, U., Eckardt, K. U., Loefler, M., and John, S. (2008) Acute renal failure in patients with severe sepsis and septic shock. A significant independent risk factor for mortality. Results from the German Prevalence Study. Nephrol. Dial. Transplant. 23, 904–909
9. Austgen, T. R., Chen, M. K., Moore, W., and Souba, W. W. (1991) Endotoxin and renal glucose metabolism. Arch. Surg. 126, 23–27
10. Grinevich, V., Knepper, M. A., Verbalis, J., Reyes, I., and Aguilara, G.
LPS-induced TLR4 Signaling Requires TLR2 in Thick Ascending Limb

(2004) Acute endotoxemia in rats induces down-regulation of V2 vasopressin receptors and aquaporin-2 content in the kidney medulla. *Kidney Int. 65*, 54–62

11. Schmidt, C., Höcherl, K., and Bucher, M. (2007) Regulation of renal glucose transporters during severe inflammation. *Am. J. Physiol. Renal Physiol. 292*, F804–F811

12. Wang, W., Li, C., Summer, S. N., Falk, S., Wang, W., Ljubanovic, D., and Schrier, R. W. (2008) Role of AQP1 in endotoxemia-induced acute kidney injury. *Am. J. Physiol. Renal Physiol. 294*, F1473–F1480

13. Olesen, E. T., de Seigneur, S., Wang, G., Lütken, S. C., Frekaixia, J., Kwon, T. H., and Nielsen, S. (2009) Rapid and segmental specific dysregulation of AQP2, S256-pAQP2, and renal sodium transporters in rats with LPS-induced endotoxemia. *Nephrol. Dial. Transplant. 24*, 2338–2349

14. Good, D. W., George, T., and Watts, B. A., 3rd (2009) Lipopolysaccharide directly alters renal tubule transport through distinct TLR4-dependent pathways in basolateral and apical membranes. *Am. J. Physiol. Renal Physiol. 297*, F866–F874

15. Good, D. W., George, T., and Watts, B. A., 3rd (2010) Toll-like receptor 2 mediates inhibition of HCO$_3^-$ absorption by bacterial lipoprotein in medullary thick ascending limb. *Am. J. Physiol. Renal Physiol. 299*, F536–F544

16. Kraut, J. A., and Kurtz, I. (2001) Use of base in the treatment of severe acidic states. *Am. J. Kidney Dis. 38*, 703–727

17. Kellum, J. A., Song, M., and Venkataraman, R. (2004) Effect of hyperchloremic acidosis on arterial pressure and circulating inflammatory molecules in experimental sepsis. *Chest 125*, 243–248

18. Baumgart, K., Radermacher, P., Calzia, E., and Hauser, B. (2008) Pathology of tissue acidosis in septic shock. Blocked microcirculation or impaired cellular respiration? *Crit. Care Med. 36*, 640–642

19. Goñi, J., García-Vázquez, M., Banzo, R., Canteras, M., Ruiz, J., Banzo, V., Herrero, J. A., and Valdés, M. (2007) Predictors of mortality in patients with methicillin-resistant *Staphylococcus aureus* (MRSA) bacteremia. *J. Crit. Care Med. 36*, 239–245

20. Lee, S. W., Hong, Y. S., Park, D. W., Choi, S. H., Moon, S. W., Park, J. S., Kim, J. Y., and Baek, J. K. (2008) Lactic acidosis not hyperlactatemia as a predictor of hospital mortality in septic emergency patients. *Emerg. Med. J. 25*, 659–665

21. Akira, S., Uematsu, S., and Takeuchi, O. (2006) Pathogen recognition and innate immunity. *Cell 124*, 783–801

22. Kawai, T., and Akira, S. (2010) The role of pattern-recognition receptors in innate immunity. *Cell 140*, 395–407

23. Good, D. W., George, T., and Watts, B. A., 3rd (2009) Hyposmolality inhibits NHE3 and HCO$_3^-$ absorption through TLR4-mediated signaling. *J. Physiol. Cell Physiol. 301*, C1296–C1306

24. Takeuchi, O., Hoshino, K., Kawai, T., Sanjo, H., Takada, H., Ogawa, T., Takeda, K., and Akira, S. (1999) Differential roles of TLR2 and TLR4 in recognition of gram-negative and gram-positive bacterial cell wall components. *Immunity 11*, 443–451

25. Yoshimura, A., Lien, E., Ingalls, R. R., Tuomanen, E., Dziarski, R., and Golenbock, D. (1999) Cutting edge. Recognition of Gram-positive bacterial cell wall components by the innate immune system occurs via Toll-like receptor 2. *J. Immunol. 163*, 1–5

26. Ozinsky, A., Underhill, D. M., Fontenot, J. D., Hajjar, A. M., Smith, K. D., Wilson, C. B., Schroeder, L., and Aderem, A. (2000) The repertoire for pattern recognition of pathogens by the innate immune system is defined by cooperation between Toll-like receptors. *Proc. Natl. Acad. Sci. U.S.A. 97*, 13766–13771

27. Aplexopoulou, L., Thomas, V., Schnare, M., Lobet, Y., Anguita, J., Schoen, R. T., Medzhitov, R., Fikrig, E., and Flavell, R. A. (2002) Hyporesponsiveness to vaccination with Borrelia burgdorferi OspA in humans and in TLR1- and TLR2-deficient mice. *Nat. Med. 8*, 878–884

28. Schröder, N. W., Morath, S., Alexander, C., Hamann, L., Hartung, T., Zähringer, U., Göbel, U. B., Weber, J. R., and Schumann, R. R. (2003) Lipoteichoic acid (LTA) of *Streptococcus pneumoniae* and *Staphylococcus aureus* activates immune cells via Toll-like receptor (TLR)-2, lipopolysaccharide-binding protein (LBP), and CD14, whereas TLR-4 and MD-2 are not involved. *J. Biol. Chem. 278*, 15587–15594

29. Takeuchi, O., Kawai, T., Mühldorf, P. F., Morr, M., Radolf, J. D., Zychlinsky, A., Takeda, K., and Akira, S. (2001) Discrimination of bacterial lipopolysaccharides by Toll-like receptor 6. *Int. Immunol. 13*, 933–940

30. Takeuchi, O., Sato, S., Horinouchi, T., Hoshino, K., Takeda, K., Dong, Z., Modlin, R. L., and Akira, S. (2002) Cutting edge. Role of Toll-like receptor 1 in mediating innate immune response to microbial lipopolysaccharides. *J. Immunol. 169*, 10–14

31. Sandor, F., Matz, E., Mandell, L., Replik, G., Golenbock, D. T., Espevik, T., Kurt-Jones, E. A., and Finberg, R. W. (2003) Importance of extracellular domains of TLR1 and TLR2 in NFκB signaling. *J. Biol. Chem. 267*, 15064–15074

32. Good, D. W. (1990) Inhibition of bicarbonate absorption by peptide hormones and cyclic adenosine monophosphate in rat medullary thick ascending limb. *J. Clin. Invest. 85*, 1006–1013

33. Good, D. W., Watts, B. A., 3rd, George, T., and Shull, G. E. (2004) Transepithelial HCO$_3^-$ absorption is defective in renal thick ascending limbs from Na$^+$/H$^+$ exchanger NHE1 null mutant mice. *Am. J. Physiol. Renal Physiol. 287*, F1244–F1249

34. Good, D. W., 3rd, and Good, D. W. (2002) ERK mediates inhibition of Na$^+$/H$^+$ exchange and HCO$_3^-$ absorption by nerve growth factor in MTAT. *Am. J. Physiol. Renal Physiol. 282*, F1056–F1063

35. Good, D. W., George, T., and Watts, B. A., 3rd (2008) Nerve growth factor inhibits Na$^+$/H$^+$ exchange and HCO$_3^-$ absorption through parallel phosphatidylinositol 3-kinase-mTOR and ERK pathways in thick ascending limb. *J. Biol. Chem. 283*, 26602–26611

36. Good, D. W., George, T., and Watts, B. A., 3rd (2011) High sodium intake increases HCO$_3^-$ absorption in medullary thick ascending limb through adaptations in basolateral and apical Na$^+$/H$^+$ exchangers. *Am. J. Physiol. Renal Physiol. 301*, F334–F343

37. Ecelbarger, C. A., Terris, J., Hoyer, J. R., Nielsen, S., Wade, J. B., and Knepper, M. A. (1996) Localization and regulation of the rat renal Na$^+$/K$^+$-2Cl$^-$ cotransporter, BSC-1. *Am. J. Physiol. 271*, F619–F628

38. Kim, G. H., Ecelbarger, C., Knepper, M. A., and Packer, R. K. (1999) Regulation of thick ascending limb ion transporter abundance in response to altered acid/base intake. *J. Am. Soc. Nephrol. 10*, 935–942

39. Good, D. W., and Watts, B. A., 3rd (2000) Hyposomolality stimulates Na$^+$/H$^+$ exchange and HCO$_3^-$ absorption in thick ascending limb.
LPS-induced TLR4 Signaling Requires TLR2 in Thick Ascending Limb

Watts, B. A., 3rd, George, T., and Good, D. W. (2002) Aldosterone inhibits apical NHE3 and HCO3− absorption via a nongenomic ERK-dependent pathway in medullary thick ascending limb. Am. J. Physiol. Renal Physiol. 291, F1003–F1016

Lee, J. D., Kato, K., Tobias, P. S., Kirkland, T. N., and Ulevitch, R. J. (1992) LPS-induced TLR4 signaling requires TLR2 in thick ascending limb. J. Biol. Chem. 267, 11439–11447

Watts, B. A., 3rd, George, T., and Good, D. W. (2006) Aldosterone inhibits apical NHE3 and HCO3− absorption via a nongenomic ERK-dependent pathway in medullary thick ascending limb. Am. J. Physiol. Renal Physiol. 291, F1003–F1016