DMM Advance Online Articles. Posted 6 August 2015 as doi: 10.1242/dmm.019398
Access the most recent version at http://dmm.biologists.org/lookup/doi/10.1242/dmm.019398

Albumin Stimulates Renal Tubular Inflammation through a HSP70-TLR4 Axis in Early Diabetic Nephropathy

Huei-Fen Jheng[1,2], Pei-Jane Tsai[1,3], Yi-Lun Chuang[4], Yi-Ting Sheng[5], Ting-An Tai[4], Wen-Chung Chen[6], Chuan-Kai Chou[7], Li-Chun Ho[8], Ming-Jer Tang[4], Kuei-Tai A. Lai[9], Junne-Ming Sung[5*], and Yau-Sheng Tsai[1,2,10*]

[1]Institute of Basic Medical Sciences, [2]Institute of Clinical Medicine, [3]Department of Medical Laboratory Science and Biotechnology, and [4]Department of Physiology, National Cheng Kung University, Tainan; [5]Division of Nephrology, Department of Internal Medicine, [6]Department of Pathology, and [10]Research Center of Clinical Medicine, National Cheng Kung University Hospital, Tainan; [7]National Laboratory Animal Center, National Applied Research Laboratories, Taipei; [8]Division of Nephrology, Department of Internal Medicine, E-DA Hospital/I-Shou University, Kaohsiung; [9]NovoTaiwan Biotech, Taipei, Taiwan, ROC

Address correspondence to

Junne-Ming Sung, 138 Sheng Li Road, Tainan 704, Taiwan, ROC. Phone: 886-6-2353535 ext. 2591; Fax:886-6-2766175; Email: jmsung@mail.ncku.edu.tw

Yau-Sheng Tsai, 1 University Rd, Tainan 701, Taiwan, ROC. Phone: 886-6-2353535 ext. 4242; Fax: 886-6-2758781; E-mail: yaustsai@mail.ncku.edu.tw

Keywords: diabetic nephropathy, toll-like receptor, tubular injury, albuminuria, DAMP

Summary Statement

Activation of the HSP70-TLR4 axis by albumin in the tubular cell induces tubular inflammation and injury.

Abstract

Increased urinary albumin excretion is not simply an aftermath of glomerular injury, and also involves in the progression of diabetic nephropathy (DN). While toll-like receptors (TLRs) are incriminated in renal inflammation of DN, whether and how albumin is involved in TLR-related renal inflammatory response remains to be clarified. Here we showed that both TLR2 and TLR4, one of their putative endogenous ligands HSP70, and NF-κB promoter activity were markedly elevated in the kidney of diabetic mice. A deficiency of TLR4, but not TLR2, alleviated albuminuria, tubulointerstitial fibrosis, and inflammation induced by diabetes. The protection against renal injury in diabetic *Tlr4*$^{-/-}$ mice was associated with reduced tubular injuries and preserved cubilin levels, rather than amelioration of glomerular lesions. *In vitro* studies revealed that albumin, a stronger inducer than high-glucose, induced the release of HSP70 from proximal tubular cells. HSP70 blockade ameliorated albumin-induced inflammatory mediators. HSP70 triggered the production of inflammatory mediators in a TLR4-dependent manner. Moreover, HSP70 inhibition *in vivo* ameliorates diabetes-induced albuminuria, inflammatory response, and tubular injury. Finally, we found that DN patients had higher levels of TLR4 and HSP70 in the dilated tubules than non-diabetic controls. Thus, activation of the HSP70-TLR4 axis, stimulated at least in part by albumin, in the tubular cell is a novel mechanism associated with inducing tubulointerstitial inflammation and aggravating pre-existing microalbuminuria in the progression of DN.

Introduction

The clinical-pathologic signs of early diabetic nephropathy (DN) are renal hypertrophy, mesangial expansion and glomerular basement membrane (GBM) thickening, and the presence of albuminuria (Breyer et al., 2005; Brosius et al., 2009; Gross et al., 2005). Although the glomerulus has been the focus of investigations into DN, tubulointerstitial inflammation and tubular injury are also major features. Thus, pathological changes in tubulointerstitium closely correlate with the magnitude of renal dysfunction and albuminuria (Gilbert and Cooper, 1999). It has been shown that tubular functional and morphological changes precede the onset of microalbuminuria in early DN (Thomas et al., 2005). Thus, the role of tubulointersititial injury in the progression of DN cannot be neglected.

Although the effect of hyperglycemia on tubular damage is documented, increased albumin leakage may cause tubulointersititial injury and progression of renal diseases (Burton and Harris, 1996; Remuzzi et al., 1997). For example, excess albumin has been shown to induce tubular phenotypic changes, apoptosis, and production of inflammatory mediators (Ohse et al., 2006; Zoja et al., 2003). However, the detailed mechanism by which albumin induces tubular injury is not yet clear. Within the tubulointerstitium, renal tubules abundantly express innate immune receptors, including toll-like receptors (TLRs), suggesting they are ready to sense environmental changes and transduce the inflammatory signals upon

renal injury (Batsford et al., 2011). In addition to components from pathogens, cell-surface TLRs, such as TLR2 and TLR4, monitor tissue homeostasis by sensing endogenous ligands, which are known as damage-associated molecular patterns (DAMPs) (Miyake, 2007; Rubartelli and Lotze, 2007).

TLR2 and TLR4 contribute to the pathogenesis of inflammatory-associated renal injury and kidney disease (Brown et al., 2007; Cunningham et al., 2004; Leemans et al., 2005; Pulskens et al., 2010). The levels of TLR2 and TLR4 increase in the monocytes of type 2 diabetic patients and in the kidneys of diabetic rats (Dasu et al., 2010; Li et al., 2010). Although recent studies have demonstrated that a deficiency of TLR2 or TLR4 attenuates the inflammatory response and the development of DN (Devaraj et al., 2011; Kuwabara et al., 2012; Lin et al., 2012a), what is not clear is which stimuli, in addition to hyperglycemia, predominantly activate the TLR-related inflammatory response. Whether TLR2 or TLR4 plays a more important role in diabetic renal injury remains unclear. In this study, we hypothesized that endogenous ligands released by the stimulation of albuminuria activate tubular cell inflammation *via* a TLR signaling, which in turn accelerates the development and increases the severity of renal injury in DN.

Results

Activation of NF-κB and inflammatory response in the diabetic kidney

To directly assess the inflammatory status *in vivo*, we induced diabetes by intraperitoneal injection of streptozotocin (STZ) into NF-κB-luciferase reporter mice. Two weeks after STZ induction, an increase of luminescence was detected in the abdominal region of diabetic mice and the signal had lasted for 5 months after the induction of diabetes (Fig. 1a). Expression of luciferase was increased in the kidney, but not in the liver and lung (Fig. S1). Consistently, increased expression of cytokines, chemokines, and macrophage markers, associated with elevated urinary albumin excretion (UAE) and downregulated nephrin and podocin levels, was found in the kidney of 1-month diabetic C57BL/6 mice (Fig. 1b~c and Fig. S1). Furthermore, induction of diabetes in wild-type mice for 3 months resulted in early features of DN, including elevated UAE, tubulointerstitial fibrosis, mesangial matrix expansion, and GBM thickening (Fig. S2).

Upregulation of TLRs and HSP70 in the diabetic kidney

The expression of *Tlr2* and *Tlr4* was 2.5-fold higher in the diabetic kidney than in non-diabetic controls, whereas those were not different in the liver and lung (Fig. 1d and Fig. S3). The diabetic kidney exhibited increased expression of TLR2 and TLR4 predominantly in the tubules, without apparently increased expression in the glomeruli (Fig. 1e). The increased

TLR4 expression was more prominent in the dilated proximal tubules with thinning or loss of the brush border in the kidney of diabetic mice. In addition, diabetes significantly increased the level of HSP70, whereas the levels of HSP60 and biglycan were not affected (Fig. 1f). Although HMGB1 has been shown to be involved in DN (Lin et al., 2013; Lin et al., 2012a), its upregulation was relatively mild. Furthermore, the increased HSP70 and HMGB1 protein levels were predominantly located in the tubules of the diabetic kidney. Consistently, immunoblotting analysis also confirmed a significant upregulation of HSP70, but no change in HMGB1 (Fig. 1g). Gene expression of HSP70, but not others, is significantly upregulated in the diabetic kidney (Fig. 1h). Thus a diabetic milieu predominantly increases HSP70 and marginally upregulated HMGB1, and these molecules may serve as endogenous ligands for TLRs.

Attenuation of albuminuria in *Tlr4*$^{-/-}$ diabetic mice

To directly address the functional significance of TLR2 and TLR4 in the pathogenesis of DN, *Tlr2*$^{-/-}$ and *Tlr4*$^{-/-}$ mice were used. Susceptibility to STZ induction was not influenced by the lack of either TLR2 or TLR4, evidenced by the similar blood glucose levels (Tables S1 and S2). UAE, urinary albumin/creatinine ratio (UACR), and tubulointerstitial fibrosis were significantly attenuated in *Tlr4*$^{-/-}$ diabetic mice (Fig. S4), but they did not differ between *Tlr2*$^{-/-}$ and WT diabetic mice (Fig. S5). However, we did not find significant differences in

mesangial matrix expansion, GBM thickening, and nephrin and podocin levels between *Tlr2*$^{-/-}$, *Tlr4*$^{-/-}$ and WT diabetic mice (Fig. S4 and S5). Thus, a deficiency in TLR4, but not TLR2, attenuates diabetes-induced albuminuria and tubulointerstitial fibrosis, which is not associated with significant improvements in the structural and molecular changes of glomerulus.

Attenuation of inflammatory response in *Tlr4*$^{-/-}$ diabetic mice

The kidney of 1-month diabetic *Tlr4*$^{-/-}$ mice showed significantly lower expression of chemokines, macrophage marker, and profibrotic genes (Fig. S4). Macrophage infiltration was significantly compromised in *Tlr4*$^{-/-}$ diabetic kidney (Fig. S4). However, these parameters were not different between *Tlr2*$^{-/-}$ and WT diabetic kidney (Fig. S5). These results suggest that the improved renal function in *Tlr4*$^{-/-}$ diabetic mice is associated with decreases of macrophage infiltration and expression of key genes for profibrotic and proinflammatory mediators.

Reduction of tubular injury in *Tlr4*$^{-/-}$ diabetic mice

The kidney of 1-month diabetic *Tlr4*$^{-/-}$ mice showed less tubular pathological changes, evidenced by reductions of tubular dilation, brush border loss, and flattened tubular epithelium (Fig. 2a). Quantification revealed a decreased nucleus-to-cytoplasm ratio and an

increased epithelial thickness in *Tlr4*$^{-/-}$ diabetic kidney (Fig. 2b and 2c). Furthermore, we found that diabetes dramatically induced kidney injury molecule 1 (Kim-1), a marker for proximal tubular injury, and downregulated cubilin, a receptor for albumin uptake (Fig. 2d~g). TLR4 deficiency significantly blunted Kim-1 and preserved apical cubilin levels in the diabetic kidney. Caspase-3 activation in the kidney, particularly within the tubules, was attenuated in diabetic *Tlr4*$^{-/-}$ mice (Fig. 2h and 2i). In contrast, diabetic *Tlr2*$^{-/-}$ and WT mice showed similar degrees of tubular damage and apoptosis (Fig. S6). These results suggest that TLR4 plays a dominant role in renal tubular injury under the diabetic condition.

Albumin induces NF-κB activation and HSP70 release in LLC-PK1 cells

Treatment of porcine proximal tubular LLC-PK1 cells with high-glucose (HG) and albumin significantly increased the expression of *Ccl2*, *Tnfα*, and *Mip2* (data not shown) and translocation of NF-κB into the nucleus (Fig. 3a). Co-treatment with a NF-κB inhibitor caffeic acid phenethyl ester (CAPE) attenuated the increased *Ccl2* expression (Fig. 3b). We next examined DAMP production and release. HSP70 and HMGB1 levels were not different in the lysates of LLC-PK1 cells treated with low-glucose (LG), HG and albumin (Fig. 3c). In contrast to the proposed effect of HG on tubular cells (Lin et al., 2012a; Mudaliar et al., 2013), only albumin efficiently induced HSP70 release, accompanied by a slight HMGB1 release, into the medium. The effect of albumin on HSP70 secretion was independent of species

difference and contamination of fatty acid, but was abolished by boiling (Fig. 3d). However, only human albumin was able to stimulate HMGB1 release from LLC-PK1 cells. Finally, depletion of HSP70 by antibodies significantly attenuated albumin-induced expression of *Ccl2* and *Tnfα* in LLC-PK1 cells (Fig. 3e).

HSP70 mediates albumin-induced inflammatory mediators in mPTCs

In a primary culture of mouse proximal tubular cells (mPTCs), HG and albumin treatments significantly induced the production of HSP70, but not HMGB1 (Fig. 4a). Although HG marginally induced HSP70 release, albumin at the concentration as low as 0.2 mg/mL efficiently induced HSP70 release from mPTCs. In the same condition, HMGB1 was not detectable regardless of the stimulation. Moreover, both human and bovine albumins stimulated HSP70 release from mPTCs, but only human albumin was able to stimulate HMGB1 release (Fig. 4b). HSP70 blockade by various inhibitors, including pifithrin-μ (PFTμ), VER-155008 (VER), and KNK437 (KNK), attenuated albumin-induced expression of *Ccl2* and *Tnfα* in mPTCs (Fig. 4c~e). We also examined the effects of HG/albumin on changes of TLRs and endocytic receptors. TLR2 was only upregulated by albumin, whereas TLR4 was dramatically upregulated by albumin and modestly by HG in mPTCs (Fig. 4f). Additionally, cubilin was only downregulated by albumin, whereas megalin was downregulated by both HG and albumin (Fig. 4g). These results suggest that albumin plays a

major role in induction of HSP70 release, upregulation of TLRs, and downregulation of endocytic receptors in the tubule.

TLR4 mediates HSP70-induced production of inflammatory mediators

We next addressed which TLR mediated DAMP-induced inflammatory response in the tubular cell. Treatment with HSP70 at 5 μg/mL increased expression of *Ccl2* and *Tnfα* in the mPTCs from WT and *Tlr2*$^{-/-}$, but not *Tlr4*$^{-/-}$, mice (Fig. 5a). HSP70 treatment also increased TLR2 expression, but had no effect on TLR4 expression. Although HMGB1 has been suggested to activate TLRs (Mudaliar et al., 2013), HMGB1 at this concentration was not able to induce significant increases of *Ccl2*, *Tnfα*, *Tlr2*, and *Tlr4* (Fig. 5b). To confirm whether TLRs are sufficient to mediate the stimulation from HSP70, we reconstituted TLR2 or TLR4 in human embryonic kidney (HEK) 293T cells. HEK293T cells overexpressing TLR2 or TLR4 successfully responded to their specific agonists, Pam3CSK4 and LPS (data not shown). However, treatment with HSP70 increased expression of *CCL2* and *TNFα* in TLR4-expressing cells, but not in TLR2-expressing cells (Fig. 5c). These results suggest that HSP70 triggers the production of inflammatory mediators in a TLR4-dependent manner. The proposed model of the albumin-HSP70-TLR4 axis in the renal tubular inflammation is summarized in Fig. 5d.

HSP70 blockade *in vivo* ameliorates diabetes-induced albuminuria, inflammatory response, and tubular injury

We next applied two cell-permeable inhibitors (PFTμ and VER), one HSP70 transcriptional inhibitor (KNK), and one neutralizing anti-HSP70 antibody in DN mice. Treatment with PFTμ and VER attenuated UAE and expression of *Ccl2*, *Tnfα*, and *Kim1*, without the change in glucose levels (Fig. 6a and 6b). Transcriptional inhibition of HSP70 by KNK also attenuated UAE and expression of *Ccl2*, *Tnfα*, and *Kim1* (Fig. 6c). Functional antagonism of extracellular HSP70 by a neutralizing anti-HSP70 antibody decreased UAE and tended to decrease expression of *Ccl2* and *Tnfα* (Fig. 6d). Therefore, HSP70 inhibition *in vivo* is protective from diabetes-induced albuminuria, inflammatory response, and tubular injury.

Expression of TLRs and DAMPs in the renal biopsies of DN patients

Finally, we assessed the expression of TLR2, TLR4, HSP70, and HMGB1 in the kidney tissues from DN patients and non-diabetic controls. Although TLR2 and TLR4 were modestly expressed in the tubules of non-diabetic controls, they both were robustly increased in the dilated tubules of DN biopsies (Fig. 7 and Table S3). Consistently, HSP70 was significantly upregulated in the tubules, especially in dilated ones, of DN biopsies. Nuclear HMGB1 staining was observed in both DN and control biopsies, whereas cytoplasmic HMGB1 staining was significantly increased in the tubules of DN biopsies.

Discussion

Accumulating evidence indicates that inflammation plays a significant role in the development and progression of DN (Navarro-Gonzalez and Mora-Fernandez, 2008). The increased luminescence signal in our transgenic mice unequivocally demonstrated that NF-κB is activated at the initial stage, and this activation persists through the development of DN. Furthermore, our results showed that HSP70, TLR2, and TLR4 were upregulated in the kidney of diabetic mice and subjects. The *in vitro* study showed that albumin, which is a stronger inducer than HG, induced upregulation of both *Tlr2* and *Tlr4*, and a significant release of HSP70 into the culture medium. TLR4, rather than TLR2, mediated the HSP70-induced upregulation of inflammatory cytokines in the tubular cell. Thus, we propose a model that, in the early stage of diabetes, a leakage of albumin into the renal tubules upregulates TLR2 and TLR4, and induces HSP70 release and TLR4 activation, leading to a more severe injury and a stronger inflammatory response in the tubule and interstitium (summarized in Fig. 5d).

There has been much focus on the glomerular permeability change as the primary factor for the manifestation of albuminuria in kidney (Molitch et al., 2004). Instead, other studies have emphasized the importance of diminished albumin uptake by the tubules in governing albuminuria in diabetes (Russo et al., 2009; Tojo et al., 2001). It is possible that albuminuria

that develops in diabetes may be the consequent impairments of both glomerular filtration barrier and tubular reabsorption. Since we did not find significant improvements of glomerular structure and podocyte functional molecules in *Tlr4*$^{-/-}$ diabetic mice, we speculated that amelioration of albuminuria in *Tlr4*$^{-/-}$ diabetic mice may be the consequent improvement of tubular reabsorption. Because albumin molecules are taken up by the megalin-cubilin complex receptor located on the apical surface of proximal tubules (Gekle, 2005), the tubular injury would impair albumin reabsorption (Christensen et al., 2012). In addition, mice downregulated in megalin or cubilin show proteinuria or albuminuria (Leheste et al., 1999; Liu et al., 2011). The function and quantity of megalin and cubilin are declined in early DN (Kaseda et al., 2011; Tojo et al., 2001). Our results indeed showed that diabetes markedly upregulated Kim-1 and downregulated cubilin. Deficiency of TLR4, but not TLR2, blunted the increased Kim-1 level and tubular injury score, and preserved the apical cubilin level. These results suggest that TLR4 deficiency reduces the proximal tubule injury, which, in turn, contributes to albumin recovery.

Recently, Russo et al. have shown that in even normal conditions nephrotic levels of albumin (~ 1 mg/ml) are filtered through the glomerular basement membrane (Russo et al., 2007). Therefore, the apical side of proximal tubules should be exposed to high concentration of albumin even in healthy conditions without overt inflammatory responses. The process of

albumin-induced tubular inflammation involves the initial receptor-mediated endocytic uptake of albumin (Birn and Christensen, 2006). Endocytosed albumin is transported across the tubular cell (transcytosis), or transferred to lysosomes for degradation and released into cytosol. Excessive binding of extracellular albumin to megalin may initiate intracellular signaling events, including NF-κB activation (Wang et al., 1999). It is still controversial how great the capacity of normal proximal tubule is to handle increasing albumin load before tubular injury or stress could develop. One study showed lysosomal enzyme activity and membrane permeabilization were preserved in HK-2 cells loaded with 2 mg/ml urinary proteins (Liu et al., 2015). Currently, we do not have good explanations for the lack of inflammation if proximal tubules face high apical albumin load. It is reasonable to speculate that healthy tubules may preserve lysosomal function or signaling desensitization machineries to prevent further stress response and signaling. Nevertheless, proximal tubules on their basolateral side are exposed to blood *per se* via peritubular capillaries, where much albumin (~ 30 mg/ml) exists. No overt inflammatory responses in this situation can be explained by the predominantly apical location of the receptors for albumin *(Birn and Christensen, 2006; Tang et al., 2003b).*

Several studies documented the involvement of TLR4 in DN development. For example, Kuwabara et al. showed a predominant activation of S100A8/TLR4 signaling in the glomeruli of diabetic mice (Kuwabara et al., 2012). However, the principal site, renal tubules,

for TLR4 expression in the diabetic condition may mediate through different stimulations. Indeed, the role of TLR4 in tubular inflammation was shown by the genetic and pharmacological approaches (Lin et al., 2013; Lin et al., 2012a). Both studies proposed that HG induces a pro-inflammatory effect using the cell model. Although our results also showed that HG induced NF-κB activation and increased *Tlr4* expression in proximal tubular cells, no apparent releases of HSP70 and HMGB1 were detected. Importantly, while similar blood glucose levels were reached in the STZ-only and STZ+uninephrectomy groups of Lin's study (Lin et al., 2012a), the induction of TLR4 and inflammatory response was exacerbated in their STZ+uninephrectomy group. These results suggest factors other than HG, possibly contributed by enhancing hyperfiltration, may induce the release of DAMPs and more severe inflammatory response. Since uninephrectomy has been shown to hasten the development of DN and albuminuria, we thus speculated albumin is a trigger for tubular inflammation. Our results consistently showed that albumin treatment induced NF-κB nuclear translocation, DAMP production and release, and inflammatory mediator production in the proximal tubular cell. The putative role of urinary albumins in inducing tubulointerstitial changes is also supported by others (Eddy, 1989; Tang et al., 2003a). Although the concentration of albumin in the proximal convoluted tubule in diabetes has been debated (Oken and Flamenbaum, 1971; Tojo and Endou, 1992), we found that treatment of mPTCs with pathophysiologically relevant concentrations of albumin for 24 h induced these changes.

These results suggest that albuminuria is not simply an aftermath of glomerular injury, and can directly cause tubular inflammation and renal injury.

In the search for putative endogenous ligands for TLR activation, HMGB1 has been suggested for activation of TLRs in DN (Mudaliar et al., 2013). Our results also showed that HMGB1 was slightly increased in the kidney of diabetic mice, and cytoplasmic HMGB1 staining was significantly upregulated in DN biopsies. We next tested the causative relationship in a cell model, and found that HMGB1 was minimally detected in the medium of LLC-PK1 and mPTC cells treated with albumin, and cannot be detectable in those treated with HG. Moreover, HMGB1 at 5 μg/mL could not efficiently upregulate inflammatory mediators in mPTCs. Thus, the role of HMGB1 in DN progression remains speculative. In contrast, we found that HSP70 was upregulated in mPTCs, and released by LLC-PK1 cells and mPTCs treated with albumin. HSP70 blockade attenuated albumin-induced production of inflammatory mediators. HSP70 triggered the production of inflammatory mediators in a TLR4-dependent manner. Finally, HSP70 was significantly upregulated in the kidney of diabetic mice and subjects. Although the upregulation of tubular HSP70 in DN patients is controversial (Calabrese et al., 2007; Lin et al., 2012a; Nakhjavani et al., 2010), selection bias, study population, and individual diversity may mediate the discrepancies. Nevertheless, our results establish a causative relationship among albumin, HSP70, and TLR4 in tubular inflammation during DN progression.

Intracellular HSP70 is generally considered to be cytoprotective and anti-inflammatory, whereas extracellular HSP70 functions as DAMP and is pro-inflammatory. Therefore, it is important to discriminate the role of intracellular and extracellular HSP70 in DN. Two cell-permeable inhibitors, VER and PFTμ, are proposed to target different sites in HSP70 (Schlecht et al., 2013; Zhang et al., 2013). VER, which binds to the nucleotide binding site of HSP70, acts as an ATP-competitive inhibitor. PFTμ, in spite of its inhibition of p53, interferes with the substrate binding domain of HSP70 and disrupts its association with client proteins. These two compounds are thought to inhibit intracellular HSP70. KNK437, a specific inhibitor of HSF-1, inhibits the transcription of HSP70 (Cai et al., 2010), thus decreasing production of both intracellular and extracellular HSP70. In contrast, a neutralizing anti-HSP70 antibody acts only on extracellular HSP70 (Cai et al., 2010). Since the delivery efficiency of anti-HSP70 neutralizing antibody to apical side of tubular cells could be lower than other small molecule compounds, the effect of extracellular HSP70 antagonism is relatively weaker. Nevertheless, our results showed that blockade of both intracellular and extracellular HSP70 ameliorated diabetes-induced albuminuria, inflammatory response, and perhaps tubular injury. Recently, HSF-1 deficient mice with an absent stress response are protected against ischemic renal injury, confirming the contribution of intracellular HSP70 to ischemic renal injury (Sreedharan et al., 2014).

The role of TLR2 in the renal injury of DN has been recently investigated. Our finding of modestly increased TLR2 expression in the kidney of diabetic mice and human DN biopsies is consistent with the previous study (Li et al., 2010). Devaraj et al. demonstrated that TLR2 knockout attenuates the renal inflammatory state and incipient DN (Devaraj et al., 2011). Although we found some restoration of the increased inflammatory cytokines in *Tlr2*$^{-/-}$ diabetic mice, most of these changes were modest. Our findings suggest that the effect of TLR2 deficiency on protection from renal injury in DN is relatively modest. The discrepancy between these two studies may stem from the insulin supplementation and STZ dosage/frequency. A smaller number of animals in our TLR2 study may not be sufficient to detect a significant difference. Nevertheless, because the phenotypic characterization of *Tlr2*$^{-/-}$ and *Tlr4*$^{-/-}$ mice was carried out simultaneously in our study, it is reasonable to compare the magnitude of renal injury and inflammatory status between them. Our results showed that a deficiency of TLR4 had more beneficial effects than that of TLR2, suggesting that activation of the TLR4 signaling pathway plays a more important role in diabetic renal injury than that of TLR2.

In conclusion, our findings identify TLR4 as a critical mediator of inflammatory responses that lead to renal injury and dysfunction in DN. Blocking of TLR4-mediated

inflammatory responses attenuated the development of albuminuria, partly through decreases in factors associated with fibrosis, the inflammatory response, and tubular injury in the kidney. *In vitro* studies of tubular cells established a causative effect of albumin on inducing the release of HSP70 and the expression of inflammatory mediators *via* a TLR4-dependent pathway. Thus, leaked albumins result in a cycle of chronic tubular injury, inflammation, and dysfunction through activation of the HSP70-TLR4 axis. Our study highlights the HSP70-TLR4 axis as a key mediator of tubular inflammation and emphasizes the potential contribution of albuminuria on tubular injury in DN.

Materials and Methods

Animals *Tlr2*$^{-/-}$and *Tlr4*$^{-/-}$ mice were kindly provided by Dr. S. Akira, and maintained on a C57BL/6 genetic background. Diabetes was induced in 8-week-old male mice by intraperitoneal administration of STZ (Sigma-Aldrich-Aldrich) at 65 mg/kg body weight for five consecutive days. Mice with a fasting blood glucose >300 mg/dL were considered diabetic. For HSP70 blockade *in vivo*, pifithrin-μ (5 mg/kg; Calbiochem), VER-155008 (16 mg/kg; Sigma), and KNK437 (25 mg/kg; Sigma) were intraperitoneally administrated on 2-week diabetic mice every other day for 7 days. For antibody blocking, 2-week diabetic mice were intraperitoneally received either anti-HSP70 or isotype-matched IgM antibodies (25 μg/kg, Novus) every other day for 7 days. Mice were fed *ad libitum* with regular chow (Purina Laboratory Rodent Diet 5001, PMI Nutrition International, Richmond, IN). Animals were housed in a specific-pathogen-free barrier facility and were handled in accordance with procedures approved by the Institutional Animal Care and Use Committee of National Cheng Kung University.

***In vivo* imaging system** The generation of NF-κB-luciferase reporter mice was carried out by injection of a linearized plasmid with four NF-κB-responsive elements upstream of firefly luciferase cDNA (4x-κB-*luc*) into FVB fertilized oocytes, and maintained on a FVB genetic background. Mice were anesthetized and imaged with an ultrasensitive camera (IVIS Spectrum, Xenogen). Signal intensity was measured as the sum of all detected photon counts

per second within the region of interest after subtracting the background luminescence and is presented as photons/sec/cm^2/steradian.

Renal function For the assessment of renal function, mice were housed in metabolic cages (Solo Mouse Metabolic Cage, Tecniplast) for 24 h, and water intake and urine output were recorded. Urinary albumin concentration was measured by immunoassay (Bethyl Laboratories), and urine creatinine was determined by the Jaffe enzymatic method.

RNA analysis Tissues were stored in RNAlater (Ambion), and total RNA was extracted with REzol (Protech Technology). Samples of mRNA were analyzed by SYBR Green-based real-time quantitative RT-PCR, with *β-actin* as the reference gene in each reaction.

Immunostaining and immunobloting analyses For the immunohistochemical staining, paraffin-embedded sections (5 μm) were incubated with primary antibodies against mouse TLR2 (Abcam), TLR4 and cubilin (Santa Cruz), Kim-1 (R&D), and cleaved caspase 3 (Cell Signaling); or human TLR2 and TLR4 (Santa Cruz), HSP70 (Enzo Life Science), and HMGB1 (Abcam). Slides were developed using 3,3′-diaminobenzidine substrate-chromogen solution (Dako). For the immunofluorescence staining, frozen sections (12 μm) were incubated with antibodies against HSP60 and HSP70 (Enzo Life Science), and biglycan and

HMGB1 (Abcam) followed by secondary antibodies conjugated with Alexa Fluor dyes (Invitrogen). For the immunobloting, proteins were probed with antibodies against Kim-1, podocin and HSP70 (Santa Cruz), HMGB1 (Abcam), nephrin (PROGEN), caspase 3 (Cell Signaling), and β-actin and α-tubulin (Sigma-Aldrich).

Image quantification The ratio of nucleus to cytoplasm in tubules was calculated as the area of hematoxylin (nucleus staining) divided by the area of background staining (cytoplasmic staining) in five to ten fields of renal cortex per mouse. The thickness of tubular epithelium was measured on a line across the apical/basolateral tubular interface, and the average was based on >500 tubules examined per mouse. The quantification was performed using image analysis software (AxioVision, Zeiss). NF-κB activation was examined with p65 immunofluorescence staining using a confocal microscope (C1-Si, Nikon). The percentage of p65-positive cells was calculated as the number of cells showing nuclear red staining divided by total cell number from three images per group. The expression of TLR2, TLR4, HSP70 and HMGB1 in the cortex of renal biopsies was graded on a scale from 0 to 5: 0 (negative), 1 (1~20% positive), 2 (21~40% positive), 3 (41~60% positive), 4 (61~80% positive), and 5 (>80% positive) (Lin et al., 2012b).

***In vitro* study** The mPTCs were isolated according to a method described previously (Chen et al., 2014), and maintained in a 1:1 DMEM:Ham's F12 medium containing 10% FBS and 17.5 mM glucose. LLC-PK1 and HEK293T cells were maintained in low-glucose DMEM supplemented with 10% FBS. For the treatments of glucose and albumin, LLC-PK1 cells and mPTCs were pre-conditioned with low-glucose DMEM containing 0.1% FBS for 24 h, and then treated with low-glucose (5.6 mM), high-glucose (30 mM) or albumin (0.02~20 mg/mL in 5.6 mM glucose) for 24 h. Various sources of albumin, including human (Alb1, Sigma #A3782) and bovine albumin (Alb2, Sigma #A8806 and Alb3, #A6003), were used. Unless otherwise defined herein, bovine albumin (Sigma #A6003) was used. The endotoxin levels in all albumin preparations were less than 0.03 EU/mL, determined by an endotoxin detection kit (Pyrotell). For HSP70 depletion, the culture medium from LLC-PK1 cells were incubated with anti-HSP70 antibody (Santa Cruz) or control IgG overnight at 4°C, followed by incubation with Protein G beads (GenScript) for 2 h. The supernatant was used for the subsequent experiments. For HSP70 inhibition, mPTCs were pretreated with pifithrin-μ, VER-155008, and KNK437 for 30 min followed by albumin treatment for 24 h. For DAMP stimulation, mPTCs from TLR2- or TLR4-deficent mice were stimulated with 5 μg/mL human HSP70 (Enzo Life Science) or HMGB1 (Sigma) for 8 h. For TLR overexpression, confluent HEK293T cells were transfected with TLR2 (90 ng) or TLR4/CD14/MD2 (30 ng of

each) plasmids for 24 h, and then changed to medium with 40 μg/mL human HSP70 or HMGB1 for 8 h.

Human renal tissues Renal biopsies diagnosed as DN without evidence of other pathological changes were obtained from 11 diabetic patients. Ten normal kidney tissues obtained from non-diabetic patients who received nephrectomy for renal tumor were used as the controls. Their clinical data are shown in Supplemental Table 3. Renal biopsies from patients who had diabetes history and a final histologic diagnosis of DN without other types of kidney disease, confirmed by microscopic, immunofluorescent and electron microscopic examinations, were used as the DN group. The control renal tissues were obtained through searching the tumor registry at Human Biobank of National Cheng Kung University Hospital for patient who underwent nephrectomy for renal tumor. These 10 subjects had no medical history of diabetes and all had fasting blood glucose lower than 126 mg/dL. The control tissues from normal renal tissues adjacent to the tumor without other renal diseases except for the solitary renal tumor were confirmed by microscopic examination. All subjects provided written informed consent for this study, which was approved by the Institutional Review Board of National Cheng Kung University Hospital.

Data analysis Values are reported as mean ± SEM. Statistical analyses were conducted by Student's *t*-test or one-way ANOVA followed by Dunnett's multiple comparison test. Differences were considered to be statistically significant at $P < 0.05$.

Acknowledgments

We thank for Human Biobank and Department of Pathology of National Cheng Kung University Hospital for technical assistances, and Dr. Iain. C. Bruce of Department of Physiology at Zhejiang University for discussion.

Competing interests

The authors declare that there is no duality of interest associated with this manuscript.

Author contributions

The author contribution is listed below: H.F.J. researched data, contributed to discussion, wrote manuscript. P.J.T. contributed to discussion, reviewed/edited manuscript. Y.L.C. researched data, wrote manuscript. T.A.T. researched data, wrote manuscript. W.C.C. researched data. C.K.C. researched data. L.C.H. contributed to discussion. M.J.T. contributed to discussion. K.T.L. contributed to discussion. J.M.S. collected biopsy, contributed to discussion, reviewed/edited manuscript. Y.S.T. contributed to discussion, wrote manuscript, reviewed/edited manuscript.

Funding

This work was supported by grants from National Science Council (NSC-99-2320-B-006-014 and NSC-101-2320-B-006-036), National Health Research Institute (NHRI-EX104-10231SI), and National Cheng Kung University Top-Notch Project.

References

Batsford, S., Duermueller, U., Seemayer, C., Mueller, C., Hopfer, H. and Mihatsch, M. (2011). Protein level expression of Toll-like receptors 2, 4 and 9 in renal disease. *Nephrology, dialysis, transplantation : official publication of the European Dialysis and Transplant Association - European Renal Association* **26**, 1413-6.

Birn, H. and Christensen, E. I. (2006). Renal albumin absorption in physiology and pathology. *Kidney international* **69**, 440-9.

Breyer, M. D., Bottinger, E., Brosius, F. C., 3rd, Coffman, T. M., Harris, R. C., Heilig, C. W. and Sharma, K. (2005). Mouse models of diabetic nephropathy. *Journal of the American Society of Nephrology : JASN* **16**, 27-45.

Brosius, F. C., 3rd, Alpers, C. E., Bottinger, E. P., Breyer, M. D., Coffman, T. M., Gurley, S. B., Harris, R. C., Kakoki, M., Kretzler, M., Leiter, E. H. et al. (2009). Mouse models of diabetic nephropathy. *Journal of the American Society of Nephrology : JASN* **20**, 2503-12.

Brown, H. J., Lock, H. R., Wolfs, T. G., Buurman, W. A., Sacks, S. H. and Robson, M. G. (2007). Toll-like receptor 4 ligation on intrinsic renal cells contributes to the induction of antibody-mediated glomerulonephritis via CXCL1 and CXCL2. *Journal of the American Society of Nephrology : JASN* **18**, 1732-9.

Burton, C. and Harris, K. P. (1996). The role of proteinuria in the progression of chronic renal failure. *American journal of kidney diseases : the official journal of the National Kidney Foundation* **27**, 765-75.

Cai, W. F., Zhang, X. W., Yan, H. M., Ma, Y. G., Wang, X. X., Yan, J., Xin, B. M., Lv, X. X., Wang, Q. Q., Wang, Z. Y. et al. (2010). Intracellular or extracellular heat shock protein 70 differentially regulates cardiac remodelling in pressure overload mice. *Cardiovascular research* **88**, 140-9.

Calabrese, V., Mancuso, C., Sapienza, M., Puleo, E., Calafato, S., Cornelius, C., Finocchiaro, M., Mangiameli, A., Di Mauro, M., Stella, A. M. et al. (2007). Oxidative stress and cellular stress response in diabetic nephropathy. *Cell Stress Chaperones* **12**, 299-306.

Chen, W. C., Lin, H. H. and Tang, M. J. (2014). Regulation of proximal tubular cell differentiation and proliferation in primary culture by matrix stiffness and ECM components. *American journal of physiology. Renal physiology* **307**, F695-707.

Christensen, E. I., Birn, H., Storm, T., Weyer, K. and Nielsen, R. (2012). Endocytic receptors in the renal proximal tubule. *Physiology* **27**, 223-36.

Cunningham, P. N., Wang, Y., Guo, R., He, G. and Quigg, R. J. (2004). Role of Toll-like receptor 4 in endotoxin-induced acute renal failure. *Journal of immunology* **172**, 2629-35.

Dasu, M. R., Devaraj, S., Park, S. and Jialal, I. (2010). Increased toll-like receptor (TLR) activation and TLR ligands in recently diagnosed type 2 diabetic subjects. *Diabetes Care* **33**, 861-8.

Devaraj, S., Tobias, P., Kasinath, B. S., Ramsamooj, R., Afify, A. and Jialal, I. (2011). Knockout of toll-like receptor-2 attenuates both the proinflammatory state of diabetes and incipient diabetic nephropathy. *Arterioscler Thromb Vasc Biol* **31**, 1796-804.

Eddy, A. A. (1989). Interstitial nephritis induced by protein-overload proteinuria. *The American journal of pathology* **135**, 719-33.

Gekle, M. (2005). Renal tubule albumin transport. *Annual review of physiology* **67**, 573-94.

Gilbert, R. E. and Cooper, M. E. (1999). The tubulointerstitium in progressive diabetic kidney disease: more than an aftermath of glomerular injury? *Kidney Int* **56**, 1627-37.

Gross, J. L., de Azevedo, M. J., Silveiro, S. P., Canani, L. H., Caramori, M. L. and Zelmanovitz, T. (2005). Diabetic nephropathy: diagnosis, prevention, and treatment. *Diabetes Care* **28**, 164-76.

Kaseda, R., Hosojima, M., Sato, H. and Saito, A. (2011). Role of megalin and cubilin in the metabolism of vitamin D(3). *Therapeutic apheresis and dialysis : official peer-reviewed journal of the International Society for Apheresis, the Japanese Society for Apheresis, the Japanese Society for Dialysis Therapy* **15 Suppl 1**, 14-7.

Kuwabara, T., Mori, K., Mukoyama, M., Kasahara, M., Yokoi, H., Saito, Y., Ogawa, Y., Imamaki, H., Kawanishi, T., Ishii, A. et al. (2012). Exacerbation of diabetic nephropathy by hyperlipidaemia is mediated by Toll-like receptor 4 in mice. *Diabetologia* **55**, 2256-66.

Leemans, J. C., Stokman, G., Claessen, N., Rouschop, K. M., Teske, G. J., Kirschning, C. J., Akira, S., van der Poll, T., Weening, J. J. and Florquin, S. (2005). Renal-associated TLR2 mediates ischemia/reperfusion injury in the kidney. *J Clin Invest* **115**, 2894-903.

Leheste, J. R., Rolinski, B., Vorum, H., Hilpert, J., Nykjaer, A., Jacobsen, C., Aucouturier, P., Moskaug, J. O., Otto, A., Christensen, E. I. et al. (1999). Megalin knockout mice as an animal model of low molecular weight proteinuria. *The American journal of pathology* **155**, 1361-70.

Li, F., Yang, N., Zhang, L., Tan, H., Huang, B., Liang, Y., Chen, M. and Yu, X. (2010). Increased expression of toll-like receptor 2 in rat diabetic nephropathy. *Am J Nephrol* **32**, 179-86.

Lin, M., Yiu, W. H., Li, R. X., Wu, H. J., Wong, D. W., Chan, L. Y., Leung, J. C., Lai, K. N. and Tang, S. C. (2013). The TLR4 antagonist CRX-526 protects against advanced diabetic nephropathy. *Kidney Int* **83**, 887-900.

Lin, M., Yiu, W. H., Wu, H. J., Chan, L. Y., Leung, J. C., Au, W. S., Chan, K. W.,

Lai, K. N. and Tang, S. C. (2012a). Toll-like receptor 4 promotes tubular inflammation in diabetic nephropathy. *J Am Soc Nephrol* **23**, 86-102.

Lin, M., Yiu, W. H., Wu, H. J., Chan, L. Y., Leung, J. C., Au, W. S., Chan, K. W., Lai, K. N. and Tang, S. C. (2012b). Toll-like receptor 4 promotes tubular inflammation in diabetic nephropathy. *Journal of the American Society of Nephrology : JASN* **23**, 86-102.

Liu, J., Li, K., He, Y., Zhang, J., Wang, H., Yang, J., Zhan, J. and Liang, H. (2011). Anticubilin antisense RNA ameliorates adriamycin-induced tubulointerstitial injury in experimental rats. *The American journal of the medical sciences* **342**, 494-502.

Liu, W. J., Xu, B. H., Ye, L., Liang, D., Wu, H. L., Zheng, Y. Y., Deng, J. K., Li, B. and Liu, H. F. (2015). Urinary proteins induce lysosomal membrane permeabilization and lysosomal dysfunction in renal tubular epithelial cells. *American journal of physiology. Renal physiology* **308**, F639-49.

Miyake, K. (2007). Innate immune sensing of pathogens and danger signals by cell surface Toll-like receptors. *Semin Immunol* **19**, 3-10.

Molitch, M. E., DeFronzo, R. A., Franz, M. J., Keane, W. F., Mogensen, C. E., Parving, H. H. and Steffes, M. W. (2004). Nephropathy in diabetes. *Diabetes Care* **27 Suppl 1**, S79-83.

Mudaliar, H., Pollock, C. A., Komala, M. G., Chadban, S. J., Wu, H. and Panchapakesan, U. (2013). The Role of Toll-Like Receptor Proteins (Tlr) 2 and 4 in Mediating Inflammation in Proximal Tubules. *Am J Physiol Renal Physiol*.

Nakhjavani, M., Morteza, A., Khajeali, L., Esteghamati, A., Khalilzadeh, O., Asgarani, F. and Outeiro, T. F. (2010). Increased serum HSP70 levels are associated with the duration of diabetes. *Cell Stress Chaperones* **15**, 959-64.

Navarro-Gonzalez, J. F. and Mora-Fernandez, C. (2008). The role of inflammatory cytokines in diabetic nephropathy. *J Am Soc Nephrol* **19**, 433-42.

Ohse, T., Inagi, R., Tanaka, T., Ota, T., Miyata, T., Kojima, I., Ingelfinger, J. R., Ogawa, S., Fujita, T. and Nangaku, M. (2006). Albumin induces endoplasmic reticulum stress and apoptosis in renal proximal tubular cells. *Kidney Int* **70**, 1447-55.

Oken, D. E. and Flamenbaum, W. (1971). Micropuncture studies of proximal tubule albumin concentrations in normal and nephrotic rats. *J Clin Invest* **50**, 1498-505.

Pulskens, W. P., Rampanelli, E., Teske, G. J., Butter, L. M., Claessen, N., Luirink, I. K., van der Poll, T., Florquin, S. and Leemans, J. C. (2010). TLR4 promotes fibrosis but attenuates tubular damage in progressive renal injury. *J Am Soc Nephrol* **21**, 1299-308.

Remuzzi, G., Ruggenenti, P. and Benigni, A. (1997). Understanding the nature of renal disease progression. *Kidney international* **51**, 2-15.

Rubartelli, A. and Lotze, M. T. (2007). Inside, outside, upside down: damage-associated molecular-pattern molecules (DAMPs) and redox. *Trends Immunol* **28**, 429-36.

Russo, L. M., Sandoval, R. M., Campos, S. B., Molitoris, B. A., Comper, W. D. and Brown, D. (2009). Impaired tubular uptake explains albuminuria in early diabetic nephropathy. *Journal of the American Society of Nephrology : JASN* **20**, 489-94.

Russo, L. M., Sandoval, R. M., McKee, M., Osicka, T. M., Collins, A. B., Brown, D., Molitoris, B. A. and Comper, W. D. (2007). The normal kidney filters nephrotic levels of albumin retrieved by proximal tubule cells: retrieval is disrupted in nephrotic states. *Kidney international* **71**, 504-13.

Schlecht, R., Scholz, S. R., Dahmen, H., Wegener, A., Sirrenberg, C., Musil, D., Bomke, J., Eggenweiler, H. M., Mayer, M. P. and Bukau, B. (2013). Functional analysis of Hsp70 inhibitors. *PloS one* **8**, e78443.

Sreedharan, R., Chen, S., Miller, M., Haribhai, D., Williams, C. B. and Van Why, S. K. (2014). Mice with an absent stress response are protected against ischemic renal injury. *Kidney international* **86**, 515-24.

Tang, S., Leung, J. C., Abe, K., Chan, K. W., Chan, L. Y., Chan, T. M. and Lai, K. N. (2003a). Albumin stimulates interleukin-8 expression in proximal tubular epithelial cells in vitro and in vivo. *J Clin Invest* **111**, 515-27.

Tang, S., Leung, J. C., Abe, K., Chan, K. W., Chan, L. Y., Chan, T. M. and Lai, K. N. (2003b). Albumin stimulates interleukin-8 expression in proximal tubular epithelial cells in vitro and in vivo. *The Journal of clinical investigation* **111**, 515-27.

Thomas, M. C., Burns, W. C. and Cooper, M. E. (2005). Tubular changes in early diabetic nephropathy. *Advances in chronic kidney disease* **12**, 177-86.

Tojo, A. and Endou, H. (1992). Intrarenal handling of proteins in rats using fractional micropuncture technique. *The American journal of physiology* **263**, F601-6.

Tojo, A., Onozato, M. L., Ha, H., Kurihara, H., Sakai, T., Goto, A., Fujita, T. and Endou, H. (2001). Reduced albumin reabsorption in the proximal tubule of early-stage diabetic rats. *Histochemistry and cell biology* **116**, 269-76.

Wang, Y., Rangan, G. K., Tay, Y. C. and Harris, D. C. (1999). Induction of monocyte chemoattractant protein-1 by albumin is mediated by nuclear factor kappaB in proximal tubule cells. *Journal of the American Society of Nephrology : JASN* **10**, 1204-13.

Zhang, L., Fok, J. J., Mirabella, F., Aronson, L. I., Fryer, R. A., Workman, P., Morgan, G. J. and Davies, F. E. (2013). Hsp70 inhibition induces myeloma cell death via the intracellular accumulation of immunoglobulin and the generation of proteotoxic stress. *Cancer letters* **339**, 49-59.

Zoja, C., Morigi, M. and Remuzzi, G. (2003). Proteinuria and phenotypic change of proximal tubular cells. *J Am Soc Nephrol* **14 Suppl 1**, S36-41.

Figures

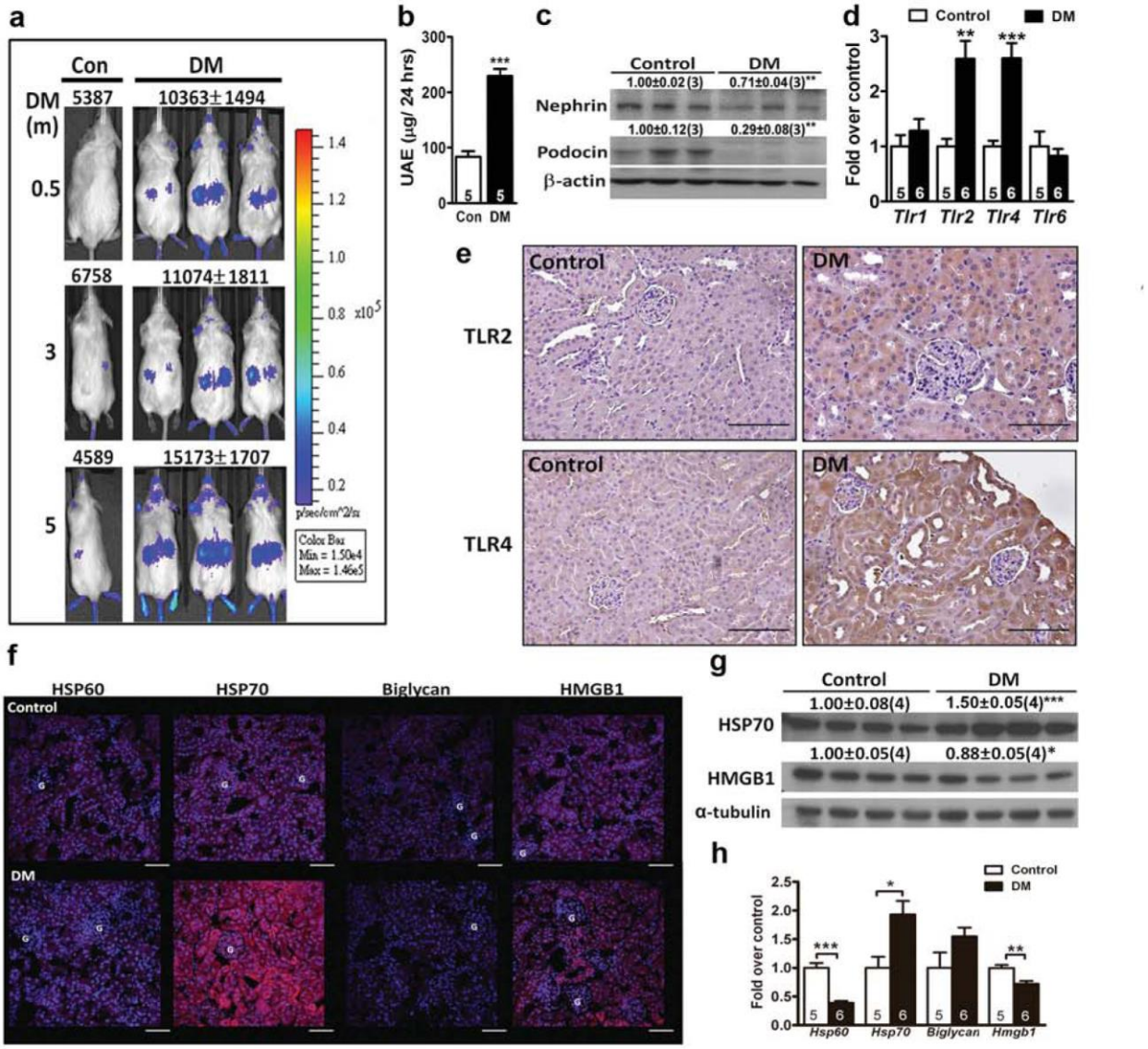

Fig. 1. Expression and localization of inflammatory mediators in the diabetic kidney. (**a**) Imaging and photon counting in diabetic and control male transgenic (NF-κB-RE-luciferase) mice. The color overlays on the images represent the photons/sec emitted, and the quantified

photon signals are shown. (**b**) Daily urinary albumin excretion (UAE) and (**c**) immunoblot analyses on nephrin and podocin from the kidney of 1-month diabetic C57BL/6 mice. The relative intensities of the bands by densitometric quantification to control mice with the number of mice in parentheses are indicated. (**d**) Expression of TLRs in the kidney of 1-month diabetic relative to control mice. (**e**) Immunohistochemical staining for TLR2 and TLR4 and (**f**) immunofluorescence staining for HSP60, HSP70, biglycan, and HMGB1 (*red*) in the kidney of 1-month diabetic and control mice. The DAPI nuclear counterstain appears *blue*. Scale bars, 50 μm. G, glomerulus. (**g**) Immunoblot analyses on HSP70 and HMGB1 from the kidney of 1-month diabetic C57BL/6 mice. (**h**) Expression of DAMPs in the kidney of 1-month diabetic relative to control mice. **P*<0.05, ***P*<0.01 and ****P*<0.001.

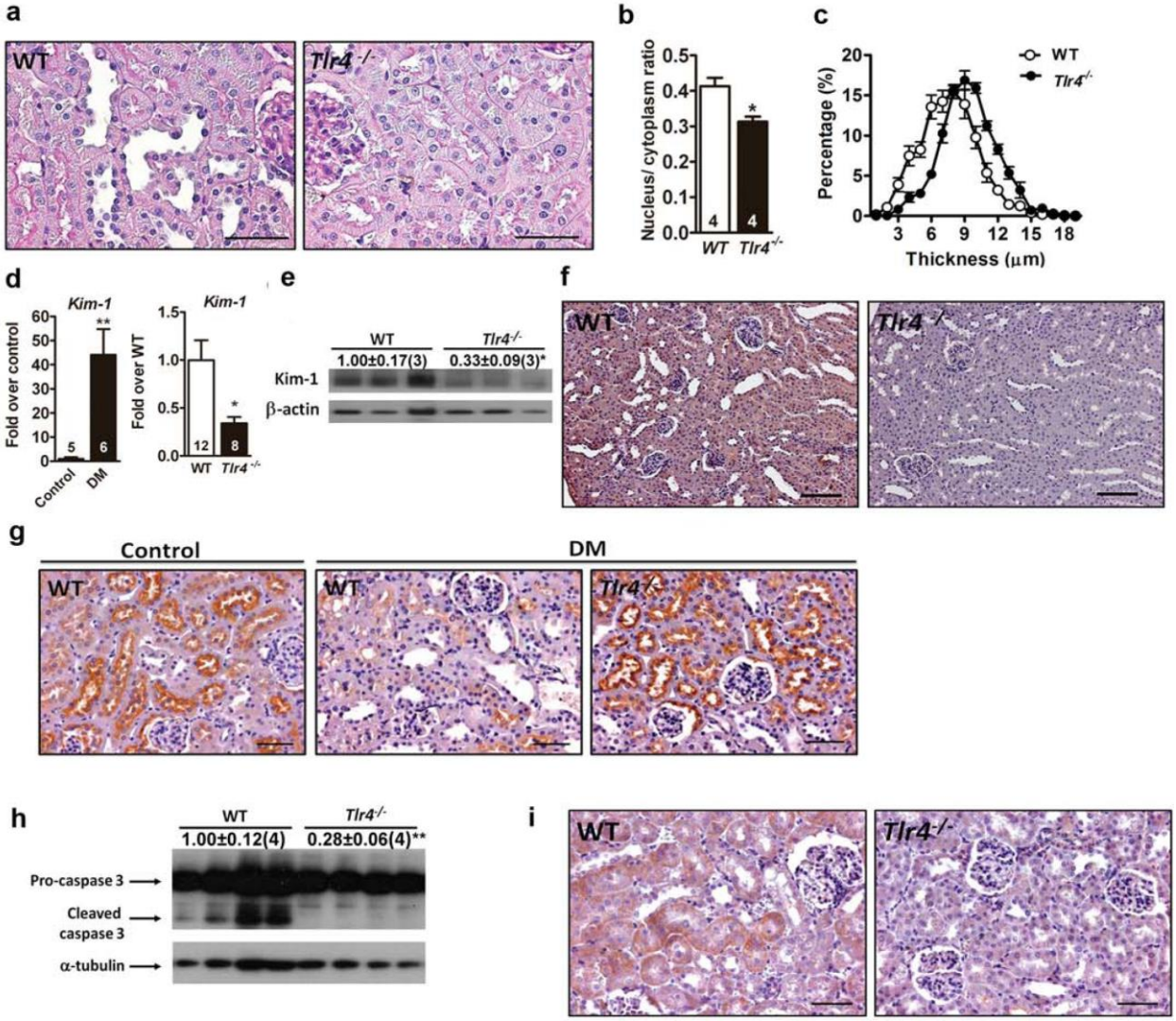

Fig. 2. Renal tubular injury and apoptosis in *Tlr4*$^{-/-}$ diabetic mice. (**a**) Representative tubular morphology, (**b**) quantification of the tubular nucleus-to-cytoplasm ratio, and (**c**) distribution of tubular epithelial thickness in *Tlr4*$^{-/-}$ and WT 1-month diabetic mice. Scale bar, 50 μm. (**d**) Expression of *Kim-1* in WT control, and WT and *Tlr4*$^{-/-}$ 1-month diabetic mice. Numbers inside bars indicate the mouse number for each group. (**e**) Immunoblot analyses and (**f**) immunohistochemical staining of Kim-1 in the kidney of *Tlr4*$^{-/-}$ and WT 1-month diabetic mice. Scale bar, 100 μm. (**g**) Immunohistochemical staining of cubilin in the kidney of WT control, and WT and *Tlr4*$^{-/-}$ 1-month diabetic mice. Scale bar, 50 μm. (**h**) Immunoblot

analyses and (**i**) immunohistochemical staining of caspase 3 in the kidney of 1-month diabetic mice. The relative intensities of the bands by densitometric quantification to WT with the number of mice in parentheses are indicated. Scale bar, 50 μm. *P<0.05 and **P<0.01.

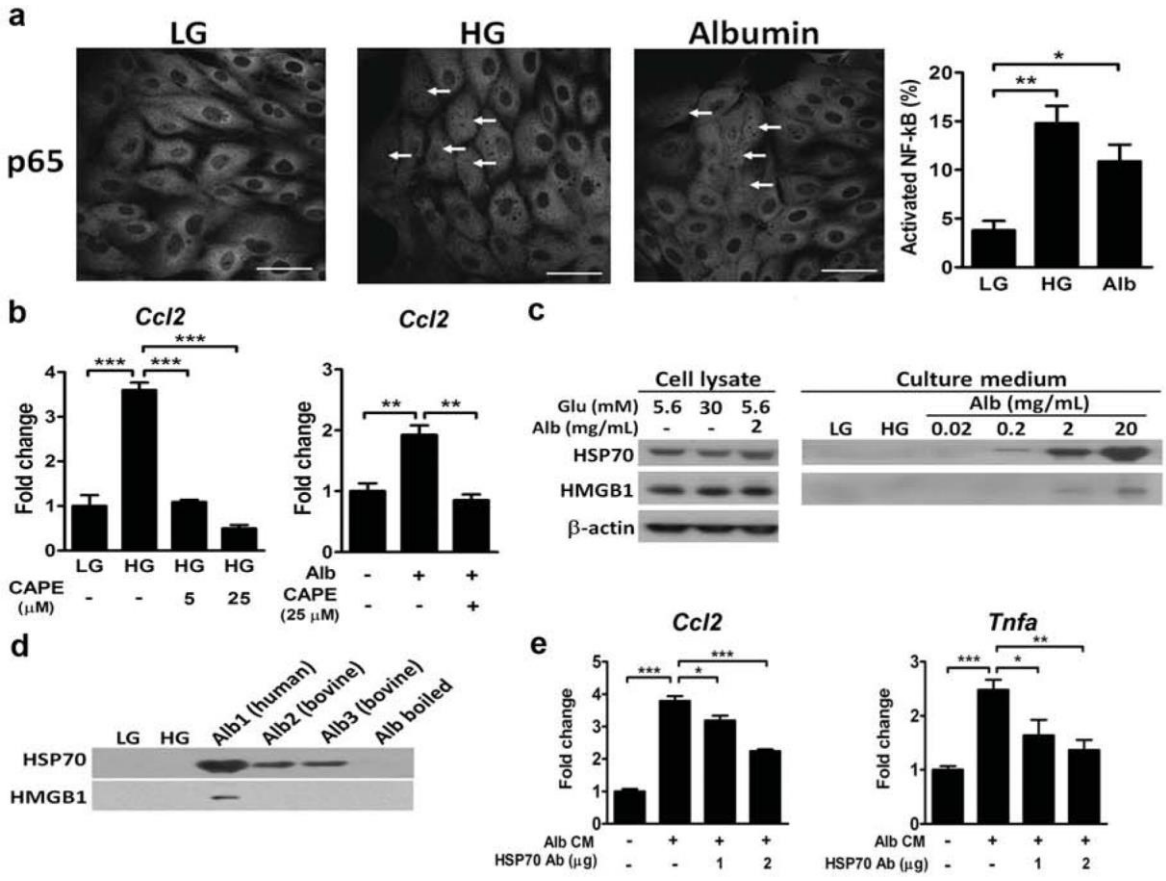

Fig. 3. Effects of HG and albumin on NF-κB activation and DAMP release in LLC-PK1 cells. (**a**) Immunofluorescence staining of NF-κB in LLC-PK1 cells after 24 h-incubation in the medium containing 5.6 mM glucose (LG), 30 mM glucose (HG), or 5.6 mM glucose with 2 mg/mL albumin. The percentage of NF-κB translocation into the nucleus is presented. *n*=3 in each group. (**b**) Expression of *Ccl2* in LLC-PK1 cells treated with LG, HG, and albumin with or without CAPE for 24 h. *n*=3 in each group. (**c**) Immunoblot analyses of HSP70 and HMGB1 from the cell lysate and culture medium of LLC-PK1 cells treated with LG, HG, and different concentrations of albumin for 24 h. (**d**) Immunoblot analyses of HSP70 and HMGB1 from the culture medium of LLC-PK1 cells treated with various sources of albumin (2 mg/mL). Alb1, fatty acid free human albumin; Alb2, fatty acid free bovine albumin; Alb3, essential fatty acid free bovine albumin; Alb boiled, Alb3 boiled for 10 min. (**e**) Expression

of *Ccl2* and *Tnfα* in LLC-PK1 cells treated with the conditioned medium (CM) with or without depletion of HSP70 for 8 h. **P*<0.05, ***P*<0.01 and ****P*<0.001 by one-way ANOVA followed by Dunnett's test.

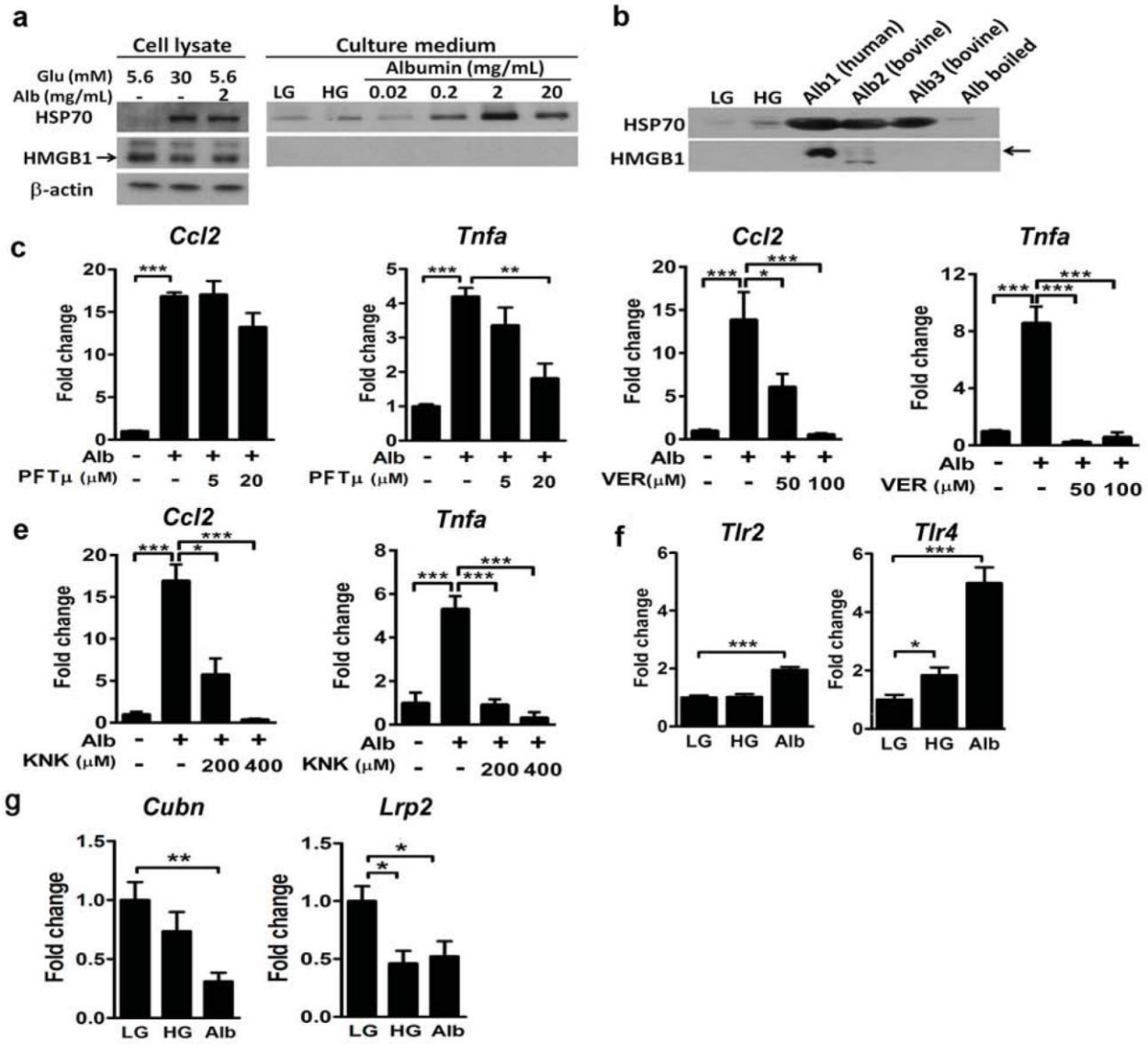

Fig. 4. Effects of HG and albumin on DAMP release and gene expression in mPTCs. (**a**) Immunoblot analyses of HSP70 and HMGB1 from the cell lysate and culture medium of mPTCs treated with LG, HG, and different concentrations of albumin for 24 h. (**b**) Immunoblot analyses of HSP70 and HMGB1 from the culture medium of mPTCs treated with various sources of albumin (2 mg/mL) as described in Fig. 3d. Expression of *Ccl2* and *Tnfα* in WT mPTCs pretreated with (**c**) pifithrin-μ, (**d**) VER-155008, and (**e**) KNK437 for 30 min before albumin stimulation. *n*=3~4 in each group. Expression of (**f**) *Tlr2* and *Tlr4* and (**g**)

Cubn and *Lrp2* in WT mPTCs treated with LG, HG, and albumin (0.2 mg/mL) for 24 h. **P*<0.05, ***P*<0.01 and ****P*<0.001 by one-way ANOVA followed by Dunnett's test.

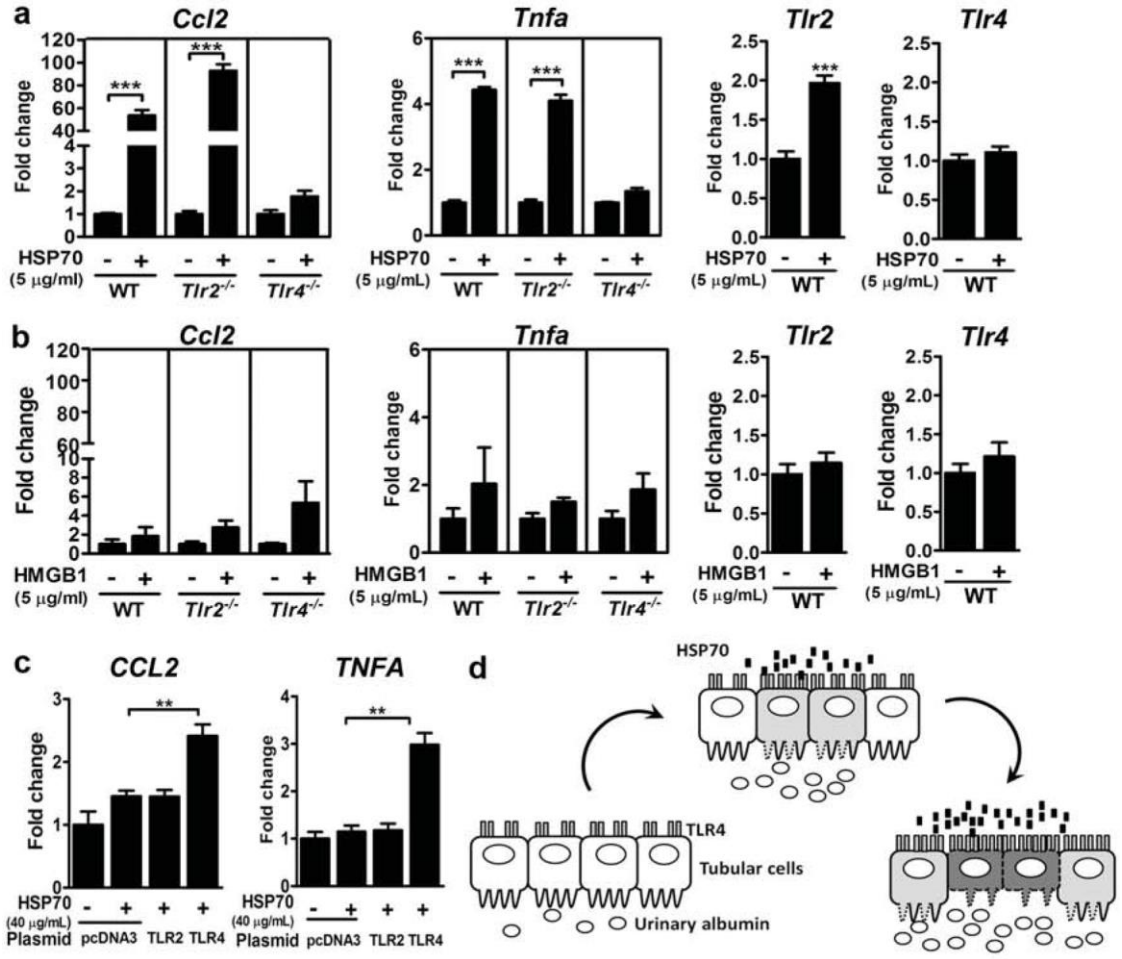

Fig. 5. TLRs in DAMP-induced inflammatory response. Expression of *Ccl2*, *Tnfα*, *Tlr2*, and *Tlr4* in mPTCs from *Tlr2*$^{-/-}$, *Tlr4*$^{-/-}$ and WT mice treated with (**a**) 5 μg/mL human HSP70 and (**b**) 5 μg/mL human HMGB1 for 8 h. ***P<0.001 by Student's *t*-test. (**c**) Expression of *CCL2* and *TNFA* in TLR2- and TLR4/CD14/MD2-overexpressing HEK293T cells treated with 40 μg/mL human HSP70 for 8 h. **P<0.01 by one-way ANOVA followed by Dunnett's test. (**d**) The proposed model of the albumin-HSP70-TLR4 axis in the renal tubular inflammation.

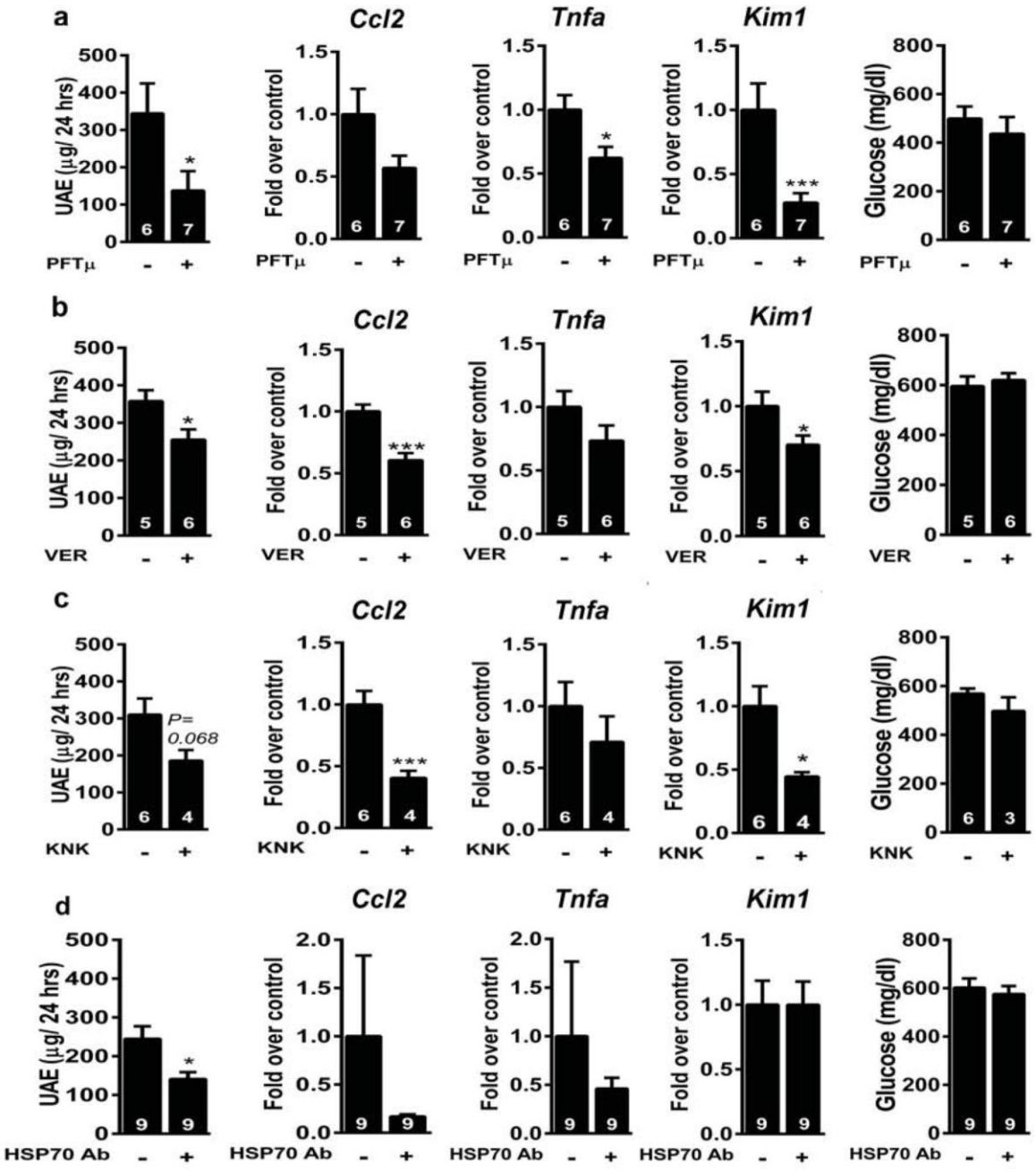

Fig. 6. HSP70 blockade in DN mice. UAE, expression of *Ccl2*, *Tnfa*, and *Kim1*, and glucose levels of 2-week diabetic mice received (**a**) pifithrin-μ (5 mg/kg), (**b**) VER-155008 (16 mg/kg), (**c**) KNK437 (25 mg/kg), and (**d**) anti-HSP70 or isotype-matched IgM antibodies intraperitoneally for one week. **P*<0.05 and ****P*<0.001 by Student's *t*-test.

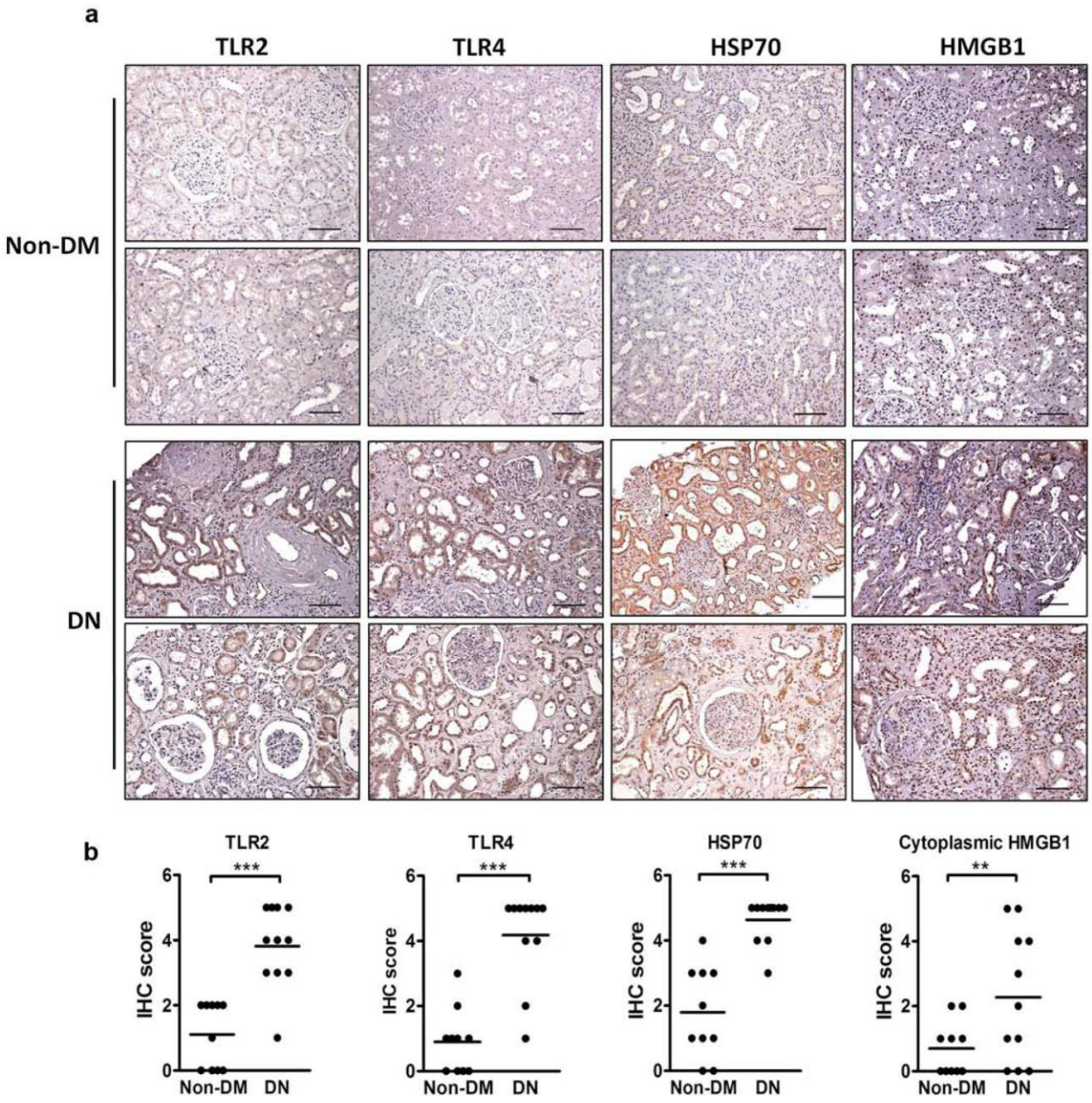

Fig. 7. Renal expression of TLR2, TLR4, HSP70, and HMGB1 in human biopsies.

(**a**) Representative photomicrographs and (**b**) scoring of TLR2, TLR4, HSP70, and HMGB1 immunohistochemical staining in the renal tissue from DN patients (*n*=11) and non-diabetic controls (Non-DM, *n*=10). Scale bars, 200 μm. ***P*<0.01 and ****P*<0.001.

Translational Impact

Clinical issue

Diabetic nephropathy (DN), one of diabetes complications, is the most common cause of end-stage renal disease. Increased urinary albumin excretion, a hallmark of DN, has been suggested to be involved in the progression *of DN*. While toll-like receptors (TLRs) are incriminated in renal inflammation of DN, whether and how albumin is involved in TLR-related renal inflammatory response remains to be clarified. The authors hypothesized that endogenous ligands released by the stimulation of albuminuria activate tubular cell inflammation *via* a TLR signaling, which in turn accelerates the development and increases the severity of renal injury in DN. To test this hypothesis, the authors used TLR2 and TLR4 deficient mice as modeling organisms.

Results

They found that a deficiency of TLR4, but not TLR2, alleviated albuminuria, tubulointerstitial fibrosis, inflammation, and apoptosis induced by diabetes. The protection against renal injury in diabetic *Tlr4*$^{-/-}$ mice was associated with reduced tubular injuries, rather than amelioration of glomerular lesions. In the search for putative endogenous ligands for TLR activation, they found that HSP70 was markedly elevated in the damaged tubules of diabetic mice. The cell culture studies revealed that albumin, a stronger inducer than high-glucose, stimulated the release of HSP70. Blockade of HSP70 ameliorated albumin-induced inflammatory mediators. Moreover, HSP70 induced the production of inflammatory mediators in a TLR4-dependent manner. To examine the clinical significance, they found that both TLR4 and HSP70 were dramatically up-regulated in damaged tubules of kidneys from DN patients. Thus, this study suggests an important role of the albumin-HSP70-TLR4 axis in the tubular inflammatory response and development of DN.

Implication and future directions

This study highlights the HSP70-TLR4 axis as a key mediator of tubular inflammation and emphasizes the potential contribution of albuminuria on tubular injury in DN. Thus, this work exemplifies how clinical observations can be mechanistically dissected by a basic investigation in the murine and cell model. Whether this mechanism universally exists in other types of renal disease requires further studies. Nevertheless, inhibition of tubular inflammation, with the focus on albumin-HSP70-TLR4 axis, to ease the DN progression may provide a new therapeutic insight for DN.