THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 290, NO. 29, pp. 18018–18028, July 17, 2015
Published in the U.S.A.

Megalin/Cubulin-Lysosome-mediated Albumin Reabsorption Is Involved in the Tubular Cell Activation of NLRP3 Inflammasome and Tubulointerstitial Inflammation*

Received for publication, May 6, 2015, and in revised form, May 25, 2015 Published, JBC Papers in Press, May 29, 2015, DOI 10.1074/jbc.M115.662064

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Background: NLRP3 inflammasome activation is involved in albuminuria-induced renal injury.
Results: The inhibition of megalin/cubilin or lysosomal cathepsin B reduced albuminuria-induced NLRP3 inflammasome activation.
Conclusion: Megalin/cubilin and lysosome rupture is involved in albumin-triggered tubular injury and TI.
Significance: This study provides novel insights into albuminuria-induced TI and implicates the active control of albuminuria as a critical strategy to halt the progression of CKD.

Albuminuria contributes to the development and progression of chronic kidney disease by inducing tubulointerstitial inflammation (TI) and fibrosis. However, the exact mechanisms of TI in response to albuminuria are unresolved. We previously demonstrated that NLRP3 and inflammasomes mediate albumin-induced lesions in tubular cells. Here, we further investigated the role of endocytic receptors and lysosome rupture in NLRP3 inflammasome activation. A murine proteinuric nephropathy model was induced by albumin overload as described previously. The priming and activation signals for inflammasome complex formation were evoked simultaneously by albumin excess in tubular epithelial cells. The former signal was dependent on a albumin-triggered NF-κB pathway activation. This process is mediated by the endocytic receptor, megalin and cubilin. However, the silencing of megalin or cubilin inhibited the albumin-induced NLRP3 signal. Notably, subsequent lysosome rupture and the corresponding release of lysosomal hydrolases, especially cathepsin B, were observed in tubular epithelial cells exposed to albumin. Cathepsin B release and distribution are essential for NLRP3 signal activation, and inhibitors of cathepsin B suppressed the NLRP3 signal in tubular epithelial cells. Taken together, our findings suggest that megalin/cubilin and lysosome rupture are involved in albumin-triggered tubular injury and TI. This study provides novel insights into albuminuria-induced TI and implicates the active control of albuminuria as a critical strategy to halt the progression of chronic kidney disease.

Proteinuria is a common feature of chronic kidney diseases that is a result of renal injury and a causative or aggravating factor for progressive renal damage (1). Accumulating evidence suggests that proteinuria is an important driver for the development of TI[2] and fibrosis (2). This occurs through multiple intracellular signaling pathways, including the induction of tubular chemokine expression, tubular epithelial cells (TECs) atrophy/apoptosis triggered by endoplasmic reticulum stress, oxidative stress, inflammatory cell infiltration in the interstitium, and sustained fibrogenesis (2–4). However, the major mechanisms behind proteinuria-induced pathological changes are largely unclear.

NLRP3 (NACHT, LRR, and PYD domain-containing protein 3) inflammasome, is a cytoplasmic macromolecular complex that orchestrates early inflammatory responses of the innate immune system by mediating IL-1β and IL-18 production (5). NLRP3 senses a large variety of stimuli, ranging from bacterial toxins, extracellular ATP (6), and extracellular matrix components to crystalline structures, such as monosodium urate, silica, and alum (7, 8). Emerging evidence indicates that NLRP3 and inflammasomes are associated with the progression of kidney diseases (9–11). We recently demonstrated that proteinuria causes inflammasome activation in the proximal tubules (12). This study found that TECs expressed significant levels of NLRP3 and the maturation of IL-1β and IL-8 in a time- and dose-dependent manner that contributed to tubular injury and interstitial inflammation (12). These observations suggest a role for NLRP3 inflammasome activation in renal injury, but the molecular signaling of NLRP3 inflammasome activation in TECs is not known.

The activator of inflammasomes are a diverse group, but NLRP3 may not interact directly with its stimuli. NLRP3 may sense a set of common cellular changes that are downstream of

* This work was supported by National Natural Scientific Foundation Grant 81130010, Major State Basic Research Development Program ("973") Grant 2012CB517706, and Program for Clinical Medical Science of Jiangsu Province Grant BL2014080. The authors declare that they have no conflicts of interest with the contents of this article.

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[2] The abbreviations used are: TI, tubulointerstitial inflammation; TECs, tubular epithelial cells; NGAL, neutrophil gelatinase-associated lipocalin; Cath B, cathepsin B.

the initial triggering events. Reactive oxygen species (13), mitochondrial translocation (14), potassium efflux (15), and the cytosolic release of lysosomal cathepsins (16) were identified as possible intermediate cellular signals (17). Several recent reports further demonstrated that the overloading of urinary proteins caused albumin accumulation in lysosomes, lysosomal membrane permeabilization and decreased lysosomal degradation in TECs (18, 19). However, whether lysomoal dysfunction affected inflammasome activation in proteinuria-induced renal injury is the subject of intensive research.

Megalin and cubilin are TEC receptors that exist in tandem and form a complex that mediates albumin uptake by proximal tubular epithelial cells (PTECs). Megalin is a major endocytic receptor that is involved in proximal tubular uptake of glomerular-filtered proteins, including albumin, for intracellular processing and degradation. A renal-specific megalin knock-out mouse displays low molecular weight proteinuria and albuminuria (20, 21). Cubilin, also known as the intrinsic factor cobalamin receptor, is a peripheral membrane protein (22). A previous study demonstrated that cubilin is essential for albumin reabsorption by proximal tubule cells, and megalin drives the internalization of cubilin-albumin complexes (23). However, the mechanisms of albumin reabsorption via megalin/cubilin receptors are largely unknown. The present study investigated whether proteinuria via megalin/cubilin receptors mediated lysosomal dysfunction directly to induce the activation of NLRP3 inflammasome in tubular cell injury and TI.

Experimental Procedures

Animal Model—Protein-overload nephropathy (12, 24) was induced via intraperitoneal injection of bovine serum albumin (BSA) in male Wistar rats 1 week after right nephrectomy (initial weight 120 to 130 g, Academy of Military Medical Science, Animal Experiment Centre). Rats were fed standard rat chow *ad libitum*, given free access to water, and randomly divided into two groups. Rats in the albumin-overload group (AO, $n = 10$) rats received a daily intraperitoneal injection of BSA (5.0 g/kg/day, fatty acid-free, low endotoxin, Roche Diagnostics). Rats in the control group ($n = 8$) received an intraperitoneal injection of an equivalent volume of saline, pH 7.4. BSA was dissolved in normal saline at a concentration of 33%, pH 7.4. BSA injections were administered for 9 weeks. Animals were anesthetized with chloral hydrate and sacrificed at the end of week 10. The Ethics Review Committees for Animal Experimentation of Southeast University approved the animal care and experimental protocols used in this study.

Urine and Blood Measurements—Body weights were measured weekly. Samples were collected every 24 h from rats housed in metabolic cages with access to drinking water only for 0, 2, 5, 7, 9, and 10 weeks. Urinary protein and albumin excretion were measured using the Coomassie Blue method (Jiancheng, Nanjing) or an ELISA kit according to the manufacturer's instructions. Urinary neutrophil gelatinase-associated lipocalin (NGAL) levels were measured using an ELISA kit (Jiancheng, Nanjing). Blood samples were collected on weeks 0, 2, 5, 7, 9, and 10 (at death) from the inner canthus or heart after sacrifice in the 10th week to assess changes in biochemical parameters (Hitachi, Tokyo, Japan).

Renal Histological Preparation—Left kidneys were collected after perfusion with 50 ml of ice-cold normal saline. A portion of coronal tissue was fixed with 10% buffered formalin and embedded in paraffin for staining with hematoxylin/eosin (H&E), periodic acid-Schiff reagent and immunohistochemistry. Another slice was snap-frozen in optimum cutting temperature compound and stored at −80 °C for immunofluorescence staining of frozen sections. The remaining tissue was frozen in liquid nitrogen and stored at −80 °C until analysis using Western blotting and real-time RT-PCR.

Immunohistochemistry Staining—Paraffin-embedded sections from the kidney cortex were cut at a thickness of 3 μm (Cryostat 2800 Frigocut-E, Leica Instruments), and a standard protocol was employed using xylene and a graded ethanol series to deparaffinize and rehydrate the tissue. Sections were washed with PBS and treated with blocking buffer containing 50 mM NH_4Cl, 2% BSA, and 0.05% saponin in PBS for 20 min at room temperature. The sections were incubated overnight at 4 °C with a primary antibody for megalin, cubilin, CD20, CD3, or CD68 (Santa Cruz Biotechnology, Santa Cruz, CA). Sections were washed with PBS, and the secondary antibody was applied. Signals were visualized using an ABC kit (Santa Cruz Biotechnology). The sections were analyzed using an appropriate immunohistochemical processing kit (Maxim, China) according to the manufacturer's instructions. Semiquantitative analysis was conducted using the Image Pro Plus image analysis system.

Transmission Electron Microscopy—Ultrastructural changes in kidney proximal tubular epithelial cells were observed using transmission electron microscopy. Kidneys were immersed in a fixative containing 2.5% glutaraldehyde and 4% paraformaldehyde in 0.1 M phosphate buffer. After fixation and dehydration with ethanol, the samples were embedded in Durcupan resin for ultra-thin sectioning and transmission electron microscopy examination in the VCU electron microscopy core facility.

Cell Culture and siRNA Treatment—A human tubular epithelial cell line (HK-2) was purchased from the China Centre for Type Culture Collection (CCTCC) and cultured in specialized tubular epithelial cell growth media (DMEM-Ham's/F-12 (GIBCO); insulin (5 μg/ml); transferrin (5 μg/ml); hydrocortisone (0.4 μg/ml), Sigma); penicillin G/streptomycin (100 units/ml of penicillin G and 100 μg/ml of streptomycin; Hyclone); and 10% fetal bovine serum (GIBCO, Uruguay)). Cells were primed using 25 μg/ml of LPS (Sigma, *Escherichia coli* 055:B5) for 1, 3, and 6 h with or without 10 mM ATP (Sigma) treatment. The transfection regents and siRNA for NLRP3 were obtained from Invitrogen (RNAiMax reagent and Stealth RNAi^{TM}). The non-targeting scramble-sequence siRNA (Stealth RNAi^{TM}) was used as a negative control. Cultured HK-2 cells were transfected according to the manufacturer's protocol (Invitrogen).

Supernatant ELISA Detection—IL-1β and IL-18 contents in cell-free supernatants were measured using a Valukine^{TM} ELISA kit (R&D Systems) according to the manufacturer's instructions.

Flow Cytometric Analyses—TUNEL staining of HK-2 cell was performed using the One Step TUNEL Apoptosis Assay Kit per the manufacturer's protocol (Beyotime, China). Cells were washed with a PBS solution and re-suspended in a cold PBS solution containing 1% FBS for FACS analysis.

Quantitative Real-time RT-PCR Analyses—Total RNA from HK-2 cells or tubular epithelial cells isolated from the kidney cortex was extracted using the RNAiso plus reagent, and cDNA was synthesized using a reverse transcription (RT) system kit (Takara, Japan) according to the manufacturer's instructions. Real-time RT-PCR was performed using an ABI PRISM 7300 Real-time PCR System (Applied Biosystems). This assay was used to determine NGAL gene expression. GAPDH served as a control for the reaction efficiency of the target genes. The results were analyzed using the comparative cycle threshold ($\Delta\Delta C_t$) method.

Western Blotting Assays—Western blot analysis using whole cell lysates and cell culture supernatants was performed as described previously (16, 25). The cell culture supernatants (400 μl) were lysed, precipitated by the addition of an equal volume of methanol, and 0.25 volumes of chloroform, vortexed, and centrifuged for 10 min at 20,000 × *g*. The upper phase was discarded, and 500 μl of methanol was added to the interphase. This mixture was centrifuged for 10 min at 20,000 × *g*, and the protein pellet was dried at 55 °C, resuspended in Laemmli buffer and boiled for 5 min at 99 °C. Protein lysates from HK-2 cells and kidney cortex extracts were lysed using a total protein extraction kit (KeyGEN, China) according to the manufacturer's instructions, separated using SDS-PAGE, and transferred onto PVDF membranes (Millipore) blocked with 5% milk proteins. The membranes were incubated overnight at 4 °C with the following primary antibodies: anti-NLRP3, cathepsin B, cathepsin D (Abcam), IL-18, ASC, caspase-1, MCP-1, IL-6, Megalin, and Cubilin (Santa Cruz Biotechnology), and IL-1β (Cell Signaling Technology). The blots were washed and incubated with secondary horseradish peroxidase-conjugated antibodies as appropriate, and the signals were detected using an ECL advanced system (GE Healthcare, UK).

Confocal Microscopy—HK-2 cells were plated on confocal dishes for 2 days and subjected to albumin stimulation. Cells were washed twice with PBST, fixed with 4% PFA in PBS for 15 min at 37 °C, and washed three times with PBST. Cells were permeabilized with Triton X-100, blocked with 10% BSA in PBS, and incubated with primary antibodies (in 5% BSA) overnight at 4 °C. After washing with PBST, the cells were incubated with fluorescent secondary antibodies (Invitrogen) in 5% BSA/PBS for 60 min and rinsed with PBST. Cell nuclei were stained with DAPI (Invitrogen). For the LysoTracker uptake test, HK-2 cells were incubated with 50 nM LysoTracker Red (Invitrogen) for 30 min at 37 °C before fixation, followed by washing with

TABLE 1

Biochemical data in the experimental rats

Values are mean ± S.D. and the abbreviations used are: KW, left kidney weight; SCr, serum creatinine; BUN, blood urea nitrogen; TP, total protein; Alb, albumin. NGAL, neutrophil gelatinase-associated lipocalin.

Group	Saline	Albumin-overload
N	8	10
KW (g)	2.64 ± 0.10	4.05 ± 0.30[a]
Serum		
SCr (μmol/liter)	49.83 ± 3.61	50.33 ± 4.45
BUN (mmol/liter)	8.63 ± 0.25	8.94 ± 0.24
TP (g/liter)	58.43 ± 1.13	62.86 ± 0.96[a]
Alb (g/liter)	16.57 ± 0.97	15.11 ± 2.20
Urine		
Protein	6.53 ± 0.23	137.63 ± 10.98[a]
Albumin	4.91 ± 1.42	54.25 ± 33.91[a]
NGAL	87.38 ± 6.81	156.37 ± 6.27[a]

[a] $p < 0.05$ *vs* saline.

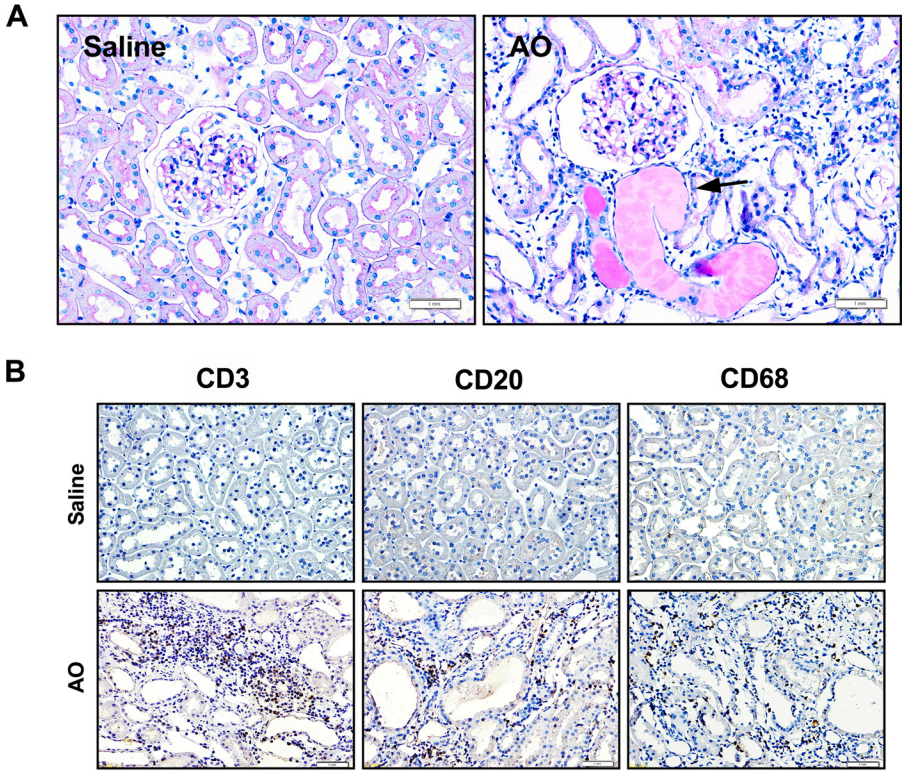

FIGURE 1. **Morphological changes in the kidney of experimental rats.** *A,* periodic acid-Schiff staining of the kidney (magnification ×200). The *arrow* indicates the protein cast in the tubular lumen. *B*, inflammatory cells were analyzed using immunohistochemistry staining in renal interstitium (magnification ×200). CD3 is the marker of T cells; B cells were stained by CD20; CD68-positive staining indicates macrophages. *AO*, albumin-overload.

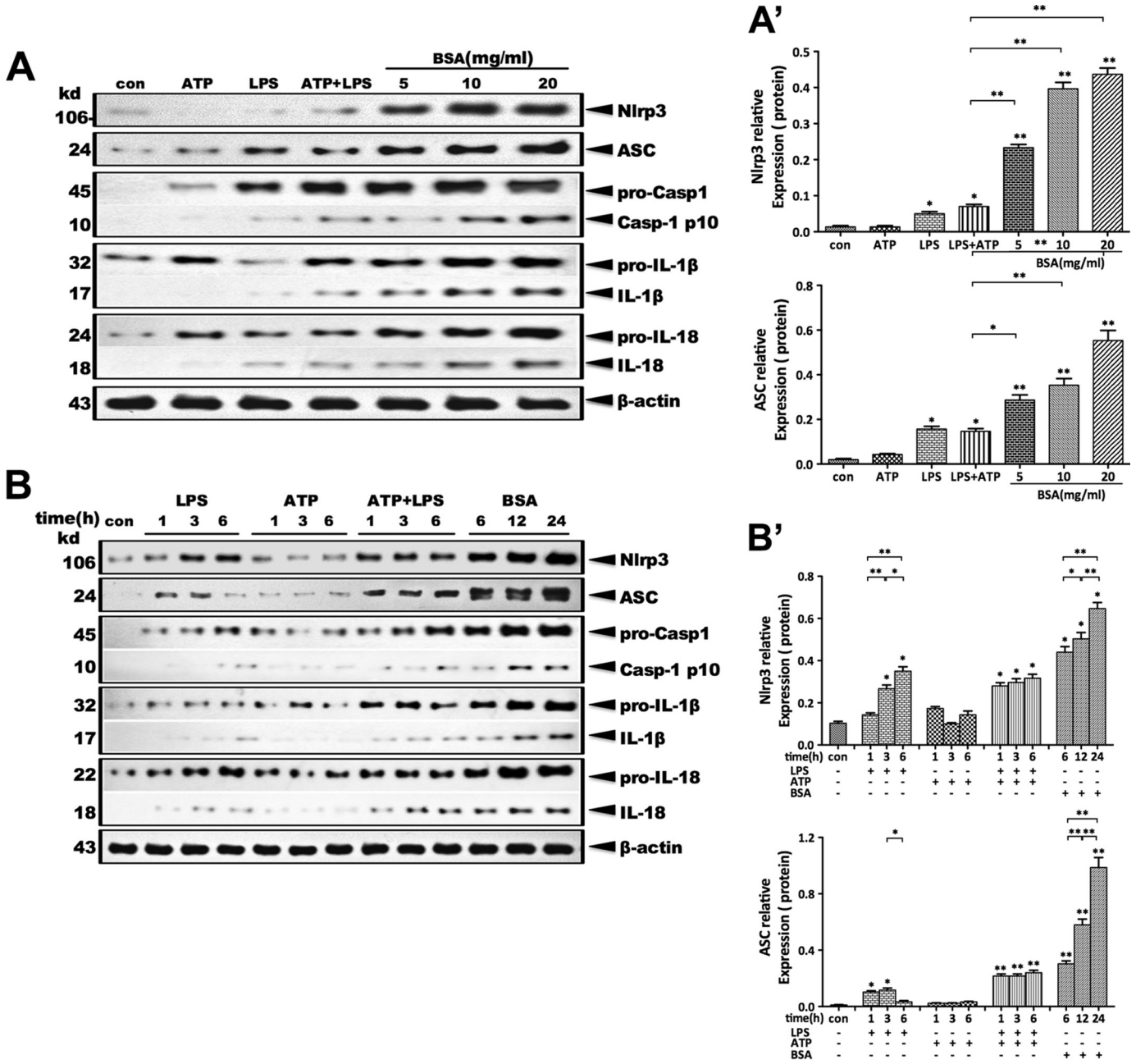

FIGURE 2. **Albumin-overload triggers Nlrp3 activation and proinflammatory cytokine secretion in cultured HK-2 cells.** *A*, Western blotting for Nlrp3, ASC, pro-caspase-1, pro-IL-1β, and pro-IL-18 protein expression in cell lysates for different concentrations of BSA (5, 10, and 20 mg/ml) stimulation. Cleaved caspase-1 p10, IL-1β, and IL-18 were measured from collected supernatants. LPS (25 μg/ml) primed HK-2 cells for 1 h, and ATP (10 mM) stimulation was provided with or without LPS priming for 1 h. *B,* Nlrp3, ASC, pro-caspase-1, pro-IL-1β, and pro-IL-18 (cell lysates) and processed caspase-1, IL-1β, and IL-18 (supernatants) were examined using Western blotting after different time courses of treatment (6, 12, and 24 h). LPS (25 μg/ml) was primed for 1, 3, and 6 h and ATP (10 mM) treatment alone or after LPS priming for an additional 1, 3, and 6 h. *A'* and *B'*, quantification of the protein expression levels. The data are presented as the mean ± S.D. from three independent experiments. *, $p < 0.05$ *versus* the control group; **, $p < 0.001$ *versus* control group. All data shown in these studies are representative of at least three separate experiments.

PBS. TUNEL staining on HK-2 cell was performed using the One Step TUNEL Apoptosis Assay Kit per the manufacturer's protocol (Beyotime, China), and cells were observed using confocal microscopy. Confocal microscopy analyses were performed using an Olympus FV 1000 Viewer.

Statistical Analysis—All data are expressed as the mean ± S.D., and results were analyzed using one-way analysis of variance in SPSS 20.0 statistical software. Nonparametric data were analyzed using the Mann-Whitney *U* test. p values less than 0.05 were considered statistically significant. Correlation analyses were performed using the Spearman rank-order correlation. $p < 0.05$ was considered statistically significant.

Results

Albumin Overload Induces Proteinuria and Tubular Injury—We first evaluated urinary protein and renal function in albumin-overload rats to explore the action of albumin overload *in vivo*. Albumin overload caused tubular injury in rats, which led

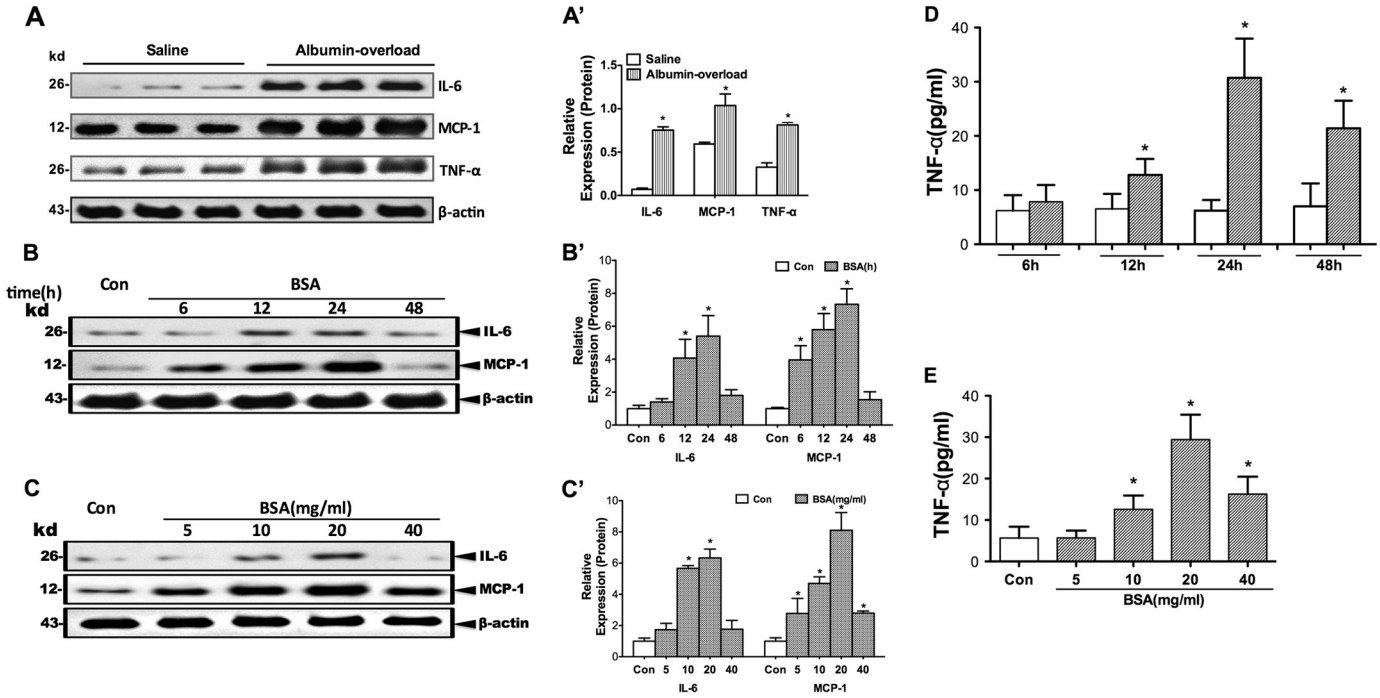

FIGURE 3. **Expression of inflammatory cytokines in experimental rats and cultured HK-2 cells.** *A,* Western blot analysis of IL-6, MCP-1, and TNF-α in renal lysates; β-actin was used as a control. *B* and *C*, expression of MCP-1 and IL-6 in dose and time course stimulations were analyzed using Western blots. *A′–C′*, quantification of the protein expression levels. *D* and *E*, ELISA detection of TNF-α expression in supernatants after stimulation with different doses (5, 10, 20, and 40 mg/ml) of BSA for different times (6, 12, 24, and 48 h). The data are presented as the mean ± S.D. from three independent experiments. *, $p < 0.05$ *versus* the saline group or control group.

to increased urinary protein excretion, albuminuria, and hyperproteinemia at week 10 compared with the saline control group (Table 1). However, there were no statistically significant differences in serum creatinine, blood urea nitrogen, or serum albumin between the two groups. The tubular injury marker urinary NGAL was significantly increased in experimental rats compared with control rats (Table 1). Morphologically, some TECs exhibited atrophy, flattening, and deletion from the basement membrane, accompanied with marked protein cast formation, tubule dilatation, and inflammatory cell infiltration in albumin-overload rats (Fig. 1*A*). Immunostaining of kidney sections revealed interstitial infiltration of inflammatory cells, including T cells (CD3 positive), B cells (CD20 positive), and macrophages (CD68 positive) in albumin overload but not control animals (Fig. 1*B*).

Albumin Triggers Two Signals That Activate NLRP3 Inflammasome Assembly in TECs—Our previous study demonstrated that albumin overload induces NLRP3 inflammasome activation in TECs that are involved in tubulointertial inflammation (12). Other studies demonstrated that NLRP3 inflammasome activation requires a priming signal, like LPS via TLR, which stimulates NF-κB signal to regulate pro-IL-1β gene expression (26). We used LPS and ATP as classic stimuli for NLRP3 activation in HK-2 cells to ascertain whether albumin stimulation activated NLRP3 inflammasome in TECs. Our data indicated that the protein expression of inflammasome components (NLRP3, ASC, and caspase-1) and inflammasome activation markers (IL-18 and IL-1β) in HK-2 cells were significantly enhanced under ATP stimulation with 25 μg/ml of LPS priming for 6 h, but not 10 mM ATP alone (Fig. 2). However, albumin alone triggered the same protein up-regulation without LPS or ATP assistance.

We detected IL-6, TNF-α, and MCP-1 protein expression, as markers of NF-κB signaling, using Western blotting to determine the molecular mechanism of albumin-induced NLRP3 inflammasome activation. First, increased whole kidney cytokine protein (Fig. 3, *A* and *A′*) expression was noted 10 weeks post-albumin injection, which was coincident with the onset and progression to severe albuminuria. We also found that exposure of HK-2 cells to albumin enhanced IL-6 and MCP-1 secretion in a dose- and time-dependent manner, peaking at 20 mg/ml and 24 h, respectively, which is consistent with the *in vivo* experiments (Fig. 3, *B* and *C′*). Exposure to 20 mg/ml of albumin also significantly elevated TNF-α levels in culture supernatants at 24 h (Fig. 3, *D* and *E*).

Albumin Activates the NLRP3 Inflammasome and Triggers IL-18 and IL-1β Secretion by Interacting with Megalin/Cubilin Receptors—Megalin and cubilin are multiligand receptors that form a complex that is responsible for protein reabsorption from the proximal tubule (19). We measured megalin and cubilin protein expression in kidney tissues of experimental animals using immunostaining and Western blotting to examine the role of these proteins in albumin-induced TI (Fig. 4, *A* and *B′*). The results demonstrated that megalin and cubilin were significantly up-regulated in albumin-overload rats compared with saline controls. We evaluated the expression of these endocytic receptors in HK-2 cells over different time and dose courses of albumin exposure (Fig. 4, *C* and *D′*). Consistent with our *in vivo* study, albumin stimulation enhanced megalin and cubilin expression in HK-2 cells, and expression peaked at 24 h using 20

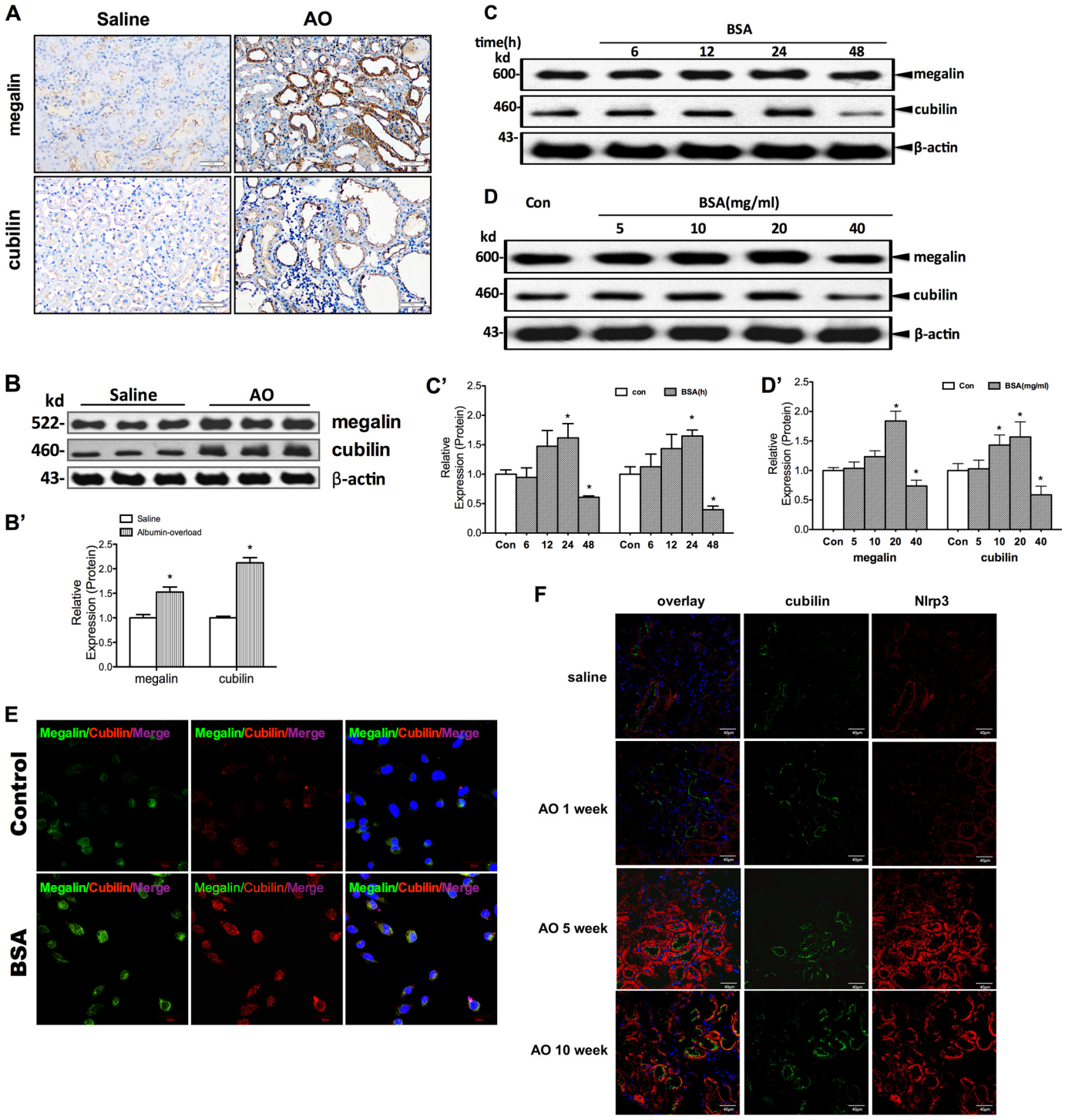

FIGURE 4. **Effect of BSA on megalin and cubilin expression in albumin-overload rats and HK-2 cell lines.** *A*, immunohistochemistry for endocytic receptors of megalin and cubilin expression in kidney tissue of an animal model of albumin overload (magnification ×200). The data are the mean ± S.D. ($n = 5$, *, $p < 0.05$ *versus* the saline-treated group). *B*, the protein expression of megalin and cubilin in renal lysates were examined using Western blotting. *C* and *D*, Western blotting analyses display megalin and cubilin expression in HK-2 cells at different times (6, 12, 24, and 48 h) and different concentrations (5, 10, 20, and 40 mg/ml) of BSA treatment. *B'–D'*, quantification of the protein expression levels. The data are presented as the mean ± S.D. from three independent experiments. *, $p < 0.05$ *versus* the saline group or control group. *E*, the co-localization of megalin and cubilin following 20 mg/ml of BSA stimulation for 24 h was detected using confocal microscopy (magnification ×400). *F*, cubilin (*green*) and Nlrp3 (*red*) protein expression in tubular cells at different time points (1st, 5th, and 10th week) in albumin-overload rats observed by confocal microscopy (magnification, ×400). All data shown in these studies are representative of at least three separate experiments. *AO*, albumin-overload.

mg/ml of BSA. Moreover, immunofluorescence staining confirmed that megalin and cubilin were up-regulated in HK-2 cells following 20 mg/ml of BSA treatment for 24 h (Fig. 4*E*). Cubilin plays an essential role in albumin reabsorption by proximal tubule cells (23), and turnover of the receptors changes during the progression of proteinuria. We investigated whether receptor turnover changed during the progression of proteinuria. Cubilin was presented increasingly on the brush border of

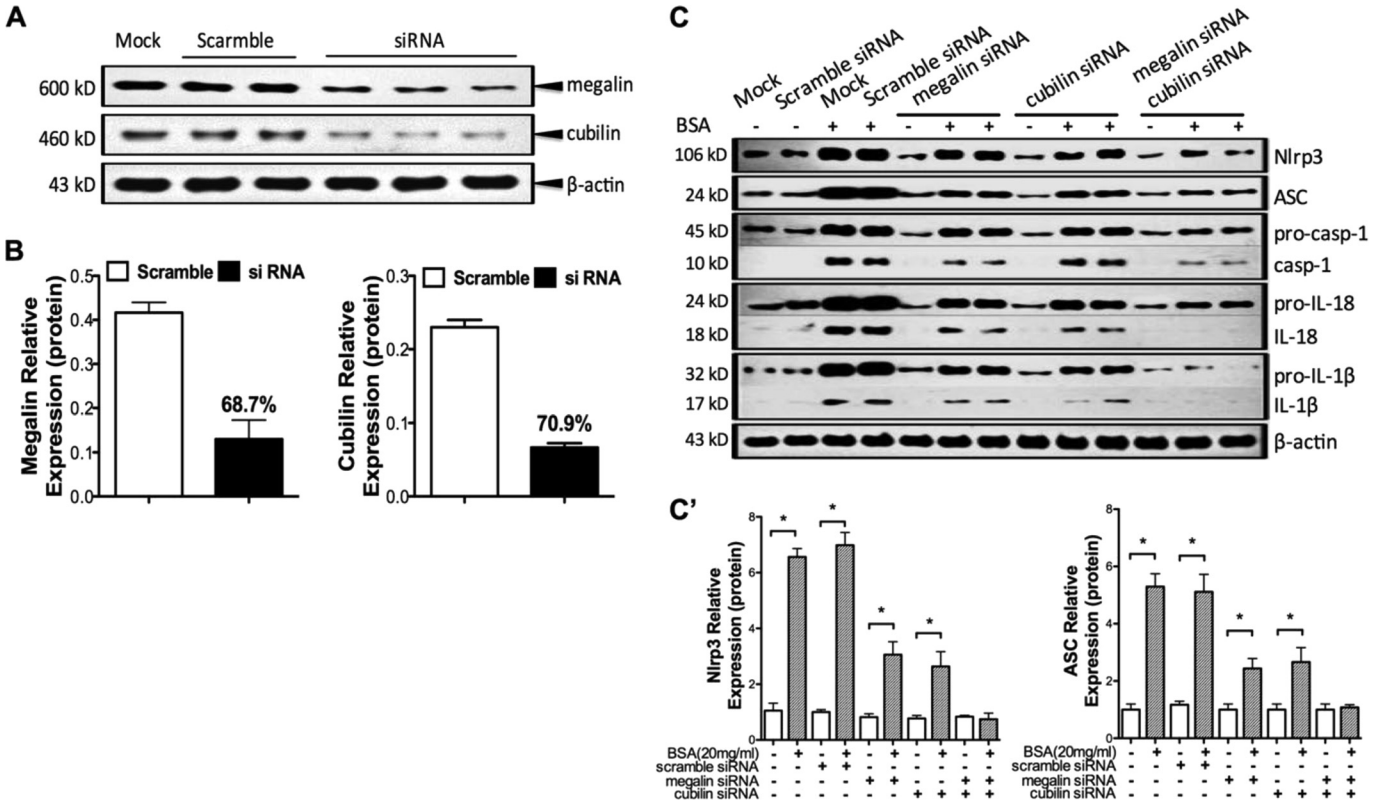

FIGURE 5. **Nlrp3 inflammasome activation and cytokine maturation are reduced in megalin siRNA and/or cubilin siRNA affected HK-2 cells.** *A,* megalin and cubilin protein expression were analyzed in HK-2 cells transfected with small interfering RNA of megalin and cubilin using Western blotting. A nontargeting scramble-sequence siRNA (scramble) was used as the negative control. Quantitation of megalin and cubilin siRNA-transfected knockdown efficiency. Data are presented as mean ± S.D. of three independent experiments. *, $p < 0.05$ *versus* scramble group. *B,* silence efficiency of siRNA transfected in HK-2 cell. *C,* Nlrp3, ASC, pro-caspase-1, pro-IL-18, pro-IL-1β (cell lysates), and caspase-1, IL-18, and IL-1β (supernatant) expression in siRNA-transfected HK-2 cells with or without BSA (20 mg/ml) stimulation compared with mock or scramble controls. *C′*, quantification of the protein expression levels. The data are presented as the mean ± S.D. *, $p < 0.05$ *versus* the control group. The expressed data are from 3 separate experiments.

tubular cells, whereas NLRP3 was localized in the cytoplasm post-albumin injection from 1 to 5 weeks. It reached a new equilibrium with a relative positive location of NLRP3 at week 10 (Fig. 4*F*).

We transfected megalin and/or cubilin siRNA into HK-2 cells to determine whether megalin/cubilin receptors mediated the albumin-induced activation of the NLRP3 inflammasome. siRNA transfection reduced the expressions of megalin and cubilin by 68.7 and 70.9%, respectively (Fig. 5, *A* and *B*). Expression of NLRP3 and cleaved caspase-1, IL-18, and IL-1β in HK-2 cells were markedly inhibited by depletion of megalin and cubilin (Fig. 5, *C* and *C′*). These observations indicate that the endocytic receptor complex of megalin and cubilin mediates albumin-induced NLRP3 inflammasome activation.

Albumin Induced TECs Lesions and Cell Death with Lysosomal Disruption—It is generally accepted that filtered albumin binds to megalin/cubilin receptors of proximal tubular cells, and albumin is transferred to lysosomes by lysosomal proteases for degradation (27). We first investigated the ultrastructure alterations of tubular cells *in vivo* to address the subsequent impact of incoming albumin. The number of lysosomes was significantly elevated in renal proximal TECs of albumin-overload rats compared with the saline group. Lysosomes were also enlarged in these rats compared with controls (Fig. 6*A*).

Enzymatic activity is closely related to the acid milieu of lysosomes (28). Therefore, we next investigated whether albumin attenuated the acidification of lysosomes in renal TECs. We used a specific fluorescent probe, LysoTracker Red, to label acidic intracellular compartments (lysosomes) in HK-2 cells. Punctuated red fluorescence (lysosomes) was clearly revealed under control conditions. However, exposure to albumin abolished LysoTracker Red labeling (Fig. 6*B*). Moreover, NGAL mRNA levels increased in HK-2 cells with albumin stimulation (Fig. 6*C*). HK-2 cells also displayed a general increase in cell death as determined by terminal deoxynucleotidyl transferase-mediated digoxigenin-deoxyuridine nick-end labeling (TUNEL) staining at 24 h of exposure to 40 mg/ml of albumin (Fig. 6, *D* and *E*). Notably, the cathepsin (Cath) B inhibitor Ca-074Me demonstrated a potential role to block cell injury caused by albumin stimulation (Fig. 6, *C–E*).

Lysosome Destabilization and Cathepsin B Are Essential in Albumin-induced NLRP3 Activation—We examined total lysosomal Cath B and D contents using Western blotting to elucidate whether albumin-induced lysosome destabilization results in the release of cathepsin B, which is a lysosomal hydrolase that induces NLRP3 inflammasome in albumin-treated cells. We found that the amount of Cath B increased significantly, but Cath D levels were slightly elevated following albumin treatment compared with controls (Fig. 7, *A* and *A′*). We further examined the levels of NLRP3 inflammasome components (NLRP3, ASC, and caspase-1) and downstream cytokines

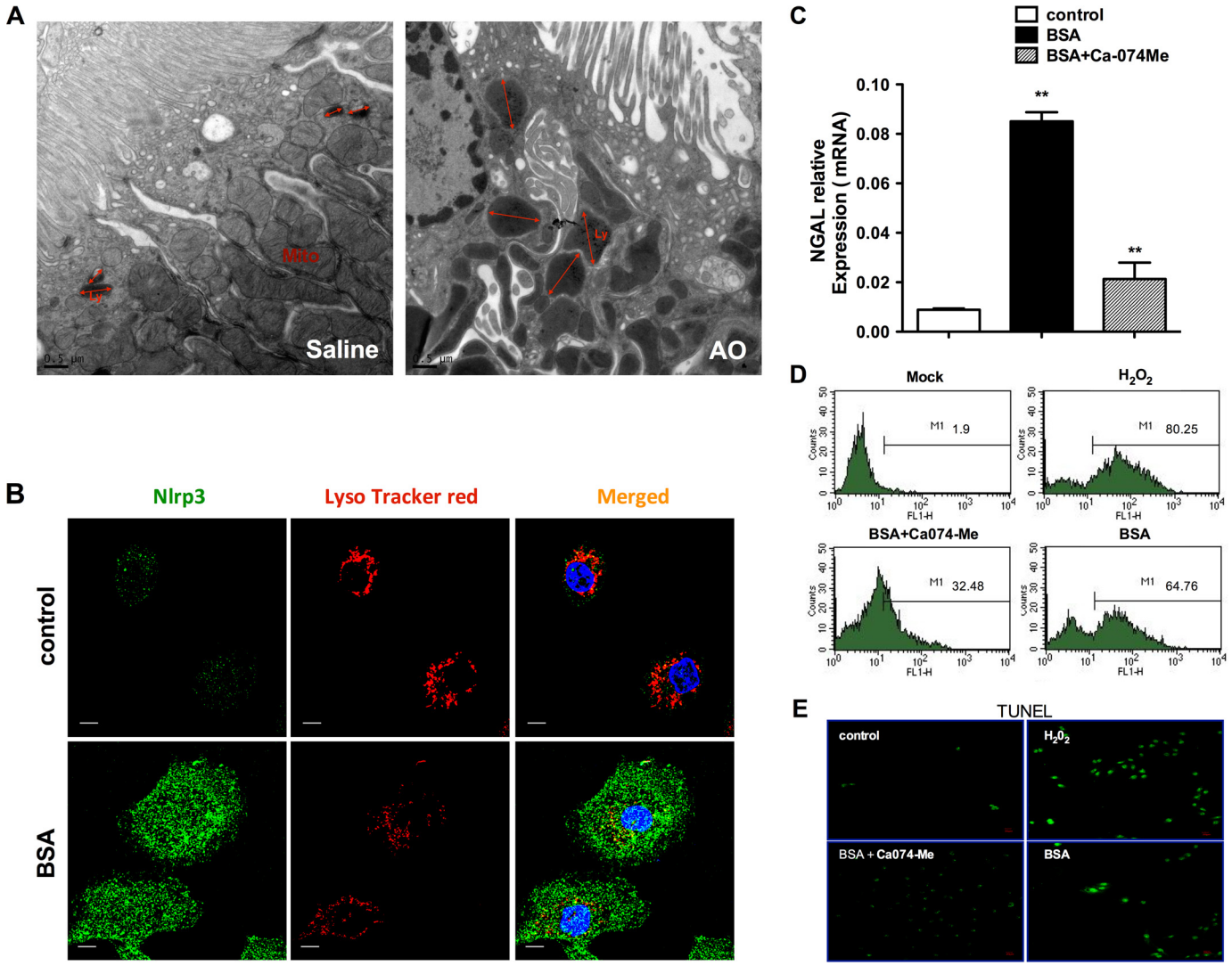

FIGURE 6. **Effects of BSA on TEC injury and death by lysosome rupture.** *A,* lysosomal numbers and diameters with transmission electron microscopy in TECs from saline control and albumin-overload rats. *Scale bars* = 0.5 μm. *Mito*, mitochondrion; *Ly*, lysosome. *B,* HK-2 cells were treated with 20 mg/ml of BSA for 24 h. Incubation with LysoTracker Red for 1 h before Nlrp3 (*green*) staining, and the intensity of cell fluorescence was observed. *Scale bars*, 10 μm. *C*, NGAL mRNA levels in HK-2 cells with 20 mg/ml of BSA treatment for 24 h with or without the cathepsin B inhibitor Ca-074Me (25 μM) were determined using quantitative real-time RT-PCR. GAPDH was used as an mRNA loading control. *D* and *E,* TUNEL staining for HK-2 cells *in vitro* incubated with BSA (40 mg/ml) for 24 h with or without the cathepsin B inhibitor Ca-074Me (25 μM), analyzed using flow cytometry or observed using confocal microscopy (magnification $\times$200). H_2O_2 (500 μM) used as a positive control for cell death by addition 30 min before TUNEL staining. Data are mean $\pm$ S.D., *, $p < 0.05$ *versus* control; **, $p < 0.001$ *versus* control. All data shown in these studies are representative of at least three separate experiments.

(IL-1β and IL-18) and detected an initial significant increase in response to albumin in HK-2 cells. Ca-074Me repressed the expression of these proteins, except pro-cytokines. However, the Cath D inhibitor pepstatin A did not exhibit the same inhibitory effect. Similarly, we observed a diffuse immunostaining pattern of Cath B and only a partial association with NLRP3 in control cells. Albumin treatment significantly altered the relative positions or locations of these proteins (Fig. 7*B*). Albumin treatment did not alter Cath D distribution or enhance colocalization with NLRP3. Furthermore, Ca-074Me, but not pepstatin A, was as an inhibitor of NLRP3 expression. As stated above, albumin treatment reduced lysosomal acidification and LysoTracker Red labeling, and it had an opposite association with the amount of NLRP3 expression in HK-2 cells exposed to albumin (Fig. 6*B*).

Discussion

It is well accepted that proteinuria is a leading cause of tubulointerstitial lesions and subsequent renal function deterioration (1, 29). However, the potential mechanisms of the deleterious effect of proteinuria have not been definitively established. The present study investigated the effect of excess albumin on renal tubules with respect to TEC injury and megalin/cubilin lysosome-mediated NLRP3 inflammasome activation.

NLRP3 and the inflammasome are linked to many human diseases, including gout, autoimmune disease, diabetes, and inflammatory bowel disease (30). Emerging evidence suggests an important role for the NLRP3 inflammasome in the pathogenesis of acute and chronic inflammation and tissue remodeling in the kidney (31). Notably, up-regulation of the NLRP3 inflammasome was demonstrated in both classical immune

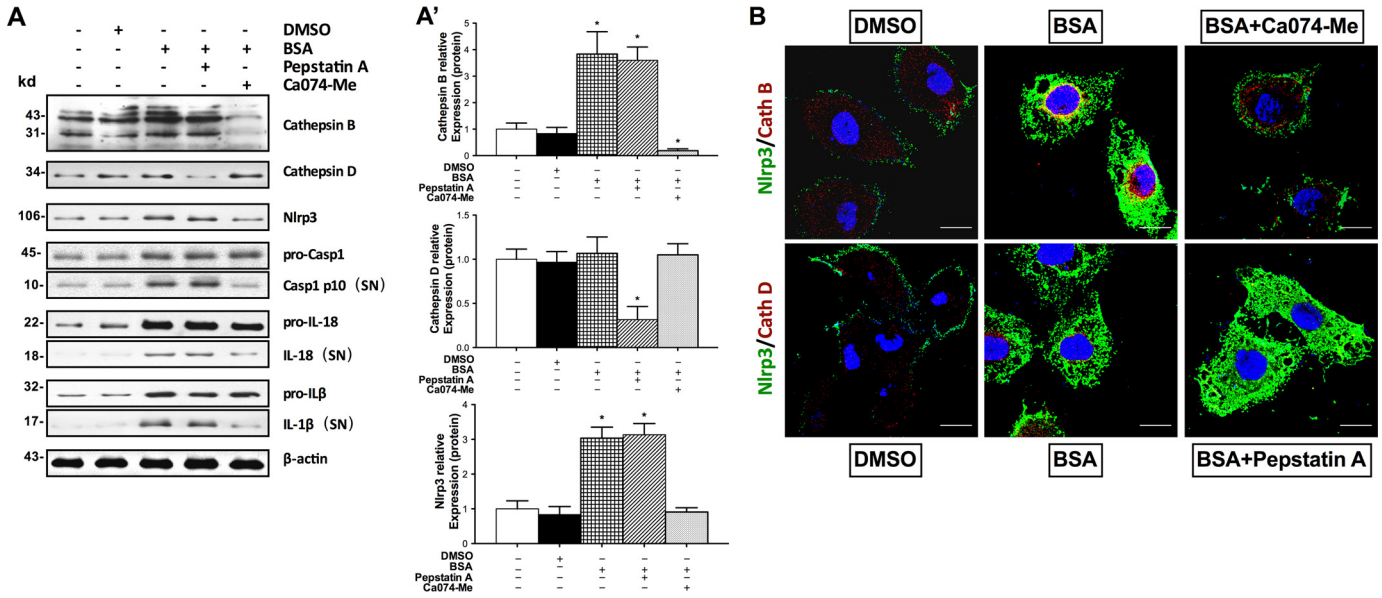

FIGURE 7. **Lysosome destabilization and cathepsin B are essential in albumin-induced NLRP3 activation.** *A,* Western blot analysis of Cath B, Cath D, and Nlrp3 signal activation in HK-2 cells stimulated by 20 mg/ml of BSA for 24 h with or without the cathepsin B inhibitor Ca-074Me (25 μM) or the cathepsin D inhibitor pepstatin A (25 μM). Dimethyl sulfoxide (*DMSO*) served as the vehicle control group. β-Actin was used as a control. *A'*, quantification of the protein expression levels. The data are presented as the mean $\pm$ S.D. *, $p < 0.05$ *versus* the control group. *B,* immunofluorescent staining of Nlrp3 and Cath B/Cath D in HK-2 cells after exposure to 20 mg/ml of albumin for 24 h. *Scale bar* = 10 μm. All data shown in these studies are representative of at least three separate experiments.

cells, such as resident dendritic cells and infiltrating macrophages, and podocytes and tubule epithelial cells in a wide range of glomerular and tubulointerstitial diseases (10). Previously, we demonstrated that proteinuria caused NLRP3 inflammasome activation in the proximal tubules in an albumin-overload model (12). This result is consistent with a report from another group that ER stress induced by albumin involved the activation of inflammasomes (32). Our data in murine and human tubular cells in an albumin-overload model further suggest that megalin/cubilin endocytic receptors and lysosomal rupture are essential for albumin-triggered NLRP3 activation.

The main function of the NLRP3 inflammasome is to cleave and activate certain critical proinflammatory cytokines, particularly IL-1β and IL-18. However, the inflammasome-dependent generation of cleaved cytokines requires a priming step that generates procytokines by the activation of the transcription factor NF-κB (33). Previous studies suggest that the up-regulation of the chemokines MCP-1, IL-6, IL-8, and TNF-α in proximal tubular cells are involved in the transcriptional activation of NF-κB (9, 34–36). This evidence supports our finding that albumin induced IL-6 and TNF-α up-regulation, which are involved in the NF-κB priming signal activation. Briefly, albumin directly triggers NLRP3 inflammasome activation and inflammasome-dependent IL-1β and IL-18 secretion without requiring the preactivation of other activators in the priming process.

Lipopolysaccharide (LPS) and ATP are potent inducers of inflammation and known NLRP3 inflammasome activators in macrophage (37). This study proposed to use these agents as positive controls for inflammasome activation. However, neither LPS alone nor LPS in conjunction with the NLRP3 inflammasome activator ATP overtly enhanced NLRP3 inflammasome levels as expected. These findings exemplify the distinction between inflammatory cells and resident renal cells (38).

A variety of stimuli activate NLRP3 inflammasomes, but none of these stimuli bind to NLRP3 inflammasomes as direct agonists (7, 39). Renal handling of plasma proteins, of which albumin constitutes the majority, are reabsorbed by megalin/cubilin-mediated endocytosis in tubular proximal epithelial cells (40). Caruso-Neves *et al.* (41) showed that LLC-PK1 cells incubated with high concentrations of BSA exhibited a significant reduction in megalin mRNA and protein levels, but peroxisome proliferator-activated receptor α and peroxisome proliferator-activated receptor γ agonists increased mRNA and/or protein levels of megalin (42). In contrast, a recent investigation indicated that megalin/cubilin receptor expression exhibited no significant difference in normal mice following a short-term albumin-overload or a model of focal segmental glomerulosis (FSGS) (43, 44). Notably, megalin and cubilin receptors were significantly elevated in kidney cortex in our 10-week albumin-overload rat model with mild TIF but without tubulointerstitial fibrosis. Our *in vitro* study similarly found that megalin and cubilin were slightly up-regulated in 20 mg/ml of BSA-exposed HK-2 cells at 24 h compared with control cells. These data implicate megalin and cubilin as the crucial receptors for the promotion of albumin reabsorption in proximal tubular epithelial cells with mild/moderate and early stages of albuminuria. However, our findings showed that increased BSA concentrations up to 40 mg/ml or prolonged incubation times (48 h) led to a decline of megalin and cubilin expression in HK-2 cells, which was associated with albumin-induced apoptosis. These results are consistent with a previous study (41). Moreover, interference of megalin and cubilin expression affected NLRP3 inflammasome activation in albumin-overloaded HK-2 cells. Therefore, these could account for the megalin/cubilin endo-

cytic receptor-mediated excessive albumin reabsorption that induced NLRP3 inflammasome activation in proximal tubular cells.

Filtered albumins via megalin/cubilin receptor-mediated internalization are degraded by lysosomal proteases, and this process also requires endosomal acidification to dissociate proteins from the receptors (45). Lysosome destabilization results in the release of Cath B, which is a lysosomal hydrolase that induces NLRP3 inflammasomes in several immune cells (46). The results of the present study revealed that Cath B accumulated in parallel with increased lysosome numbers and in volume *in vivo* and *in vitro* following albumin overload. These data indicate that lysosomal rupture occurred following albumin exposure. Notably, a previous study reported that an increased protein content accumulated per tubule in a proteinuric environment, which subsequently amplified proteolytic activity (44). In addition, lysosomal proteases, such as Cath B, prefer a low pH for proteolysis. However, we found that Cath B levels increased, but the acidification of lysosomes was suppressed by albumin load, which suggests that albumin induced protease inactivation. Notably, an association between Cath B (not Cath D)/lysosomal rupture and inflammasome activation by albumin treatment was observed in our study, and this activation was inhibited by the Cath B inhibitor Ca-074Me. These results suggest that Cath B/lysosome mediates albumin-induced NLRP3 inflammasome activation. We recently reported that mitochondrial dysfunction and mitochondrial reactive oxygen species are also involved in albumin-induced inflammasomes, but whether lysosomal dysfunction is related with oxidative stress is not known. Another recent study suggested that oxidative stress was involved in the lysosomal dysfunction induced by urinary proteins (18).

In summary, this study demonstrated that albumin excess resulted in TEC injury, accompanied by Cath B accumulation, decreased lysosomal acidification, and a reduction of Cath B activity. We further demonstrated that megalin/cubilin-mediated albumin retention and lysosomal rupture are involved in albumin-induced tubulointerstitial inflammation and injury. Our findings provide a novel insight for renal inflammation and fibrosis induced by proteinuria, which might induce new thinking for the treatment of proteinuric nephropathy.

Author Contributions—D. L. and B. C. L. designed the study and wrote the paper, D. L., Y. W., and T. T. T. carried out the experiments and data analyses and prepared the manuscript; L. L. L., H. L., and R. N. T. helped with results interpretation; S. D. C. revised the draft of the article for important intellectual content; and K. L. M. designed the study and provided critical revision of the manuscript. All authors were involved in writing the paper and had final approval of the submitted and published versions.

References

1. Abbate, M., Zoja, C., and Remuzzi, G. (2006) How does proteinuria cause progressive renal damage? *J. Am. Soc. Nephrol.* **17,** 2974–2984
2. Li, X., Pabla, N., Wei, Q., Dong, G., Messing, R. O., Wang, C. Y., and Dong, Z. (2010) PKC-δ promotes renal tubular cell apoptosis associated with proteinuria. *J. Am. Soc. Nephrol.* **21,** 1115–1124
3. Erkan, E., Devarajan, P., and Schwartz, G. J. (2007) Mitochondria are the major targets in albumin-induced apoptosis in proximal tubule cells. *J. Am. Soc. Nephrol.* **18,** 1199–1208
4. Eddy, A. A. (2004) Proteinuria and interstitial injury. *Nephrol. Dial Transplant.* **19,** 277–281
5. Schroder, K., and Tschopp, J. (2010) The inflammasomes. *Cell* **140,** 821–832
6. Mariathasan, S., Weiss, D. S., Newton, K., McBride, J., O'Rourke, K., Roose-Girma, M., Lee, W. P., Weinrauch, Y., Monack, D. M., and Dixit, V. M. (2006) Cryopyrin activates the inflammasome in response to toxins and ATP. *Nature* **440,** 228–232
7. Martinon, F. (2012) Dangerous liaisons: mitochondrial DNA meets the NLRP3 inflammasome. *Immunity* **36,** 313–315
8. Dostert, C., Pétrilli, V., Van Bruggen, R., Steele, C., Mossman, B. T., and Tschopp, J. (2008) Innate immune activation through Nalp3 inflammasome sensing of asbestos and silica. *Science* **320,** 674–677
9. Anders, H. J., and Muruve, D. A. (2011) The inflammasomes in kidney disease. *J. Am. Soc. Nephrol.* **22,** 1007–1018
10. Chang, A., Ko, K., and Clark, M. R. (2014) The emerging role of the inflammasome in kidney diseases. *Curr. Opin. Nephrol. Hypertens.* **23,** 204–210
11. Vilaysane, A., Chun, J., Seamone, M. E., Wang, W., Chin, R., Hirota, S., Li, Y., Clark, S. A., Tschopp, J., Trpkov, K., Hemmelgarn, B. R., Beck, P. L., and Muruve, D. A. (2010) The NLRP3 inflammasome promotes renal inflammation and contributes to CKD. *J. Am. Soc. Nephrol.* **21,** 1732–1744
12. Liu, D., Xu, M., Ding, L. H., Lv, L. L., Liu, H., Ma, K. L., Zhang, A. H., Crowley, S. D., and Liu, B. C. (2014) Activation of the Nlrp3 inflammasome by mitochondrial reactive oxygen species: a novel mechanism of albumin-induced tubulointerstitial inflammation. *Int. J. Biochem. Cell Biol.* **57,** 7–19
13. Zhou, R., Tardivel, A., Thorens, B., Choi, I., and Tschopp, J. (2010) Thioredoxin-interacting protein links oxidative stress to inflammasome activation. *Nat. Immunol.* **11,** 136–140
14. Zhou, R., Yazdi, A. S., Menu, P., and Tschopp, J. (2011) A role for mitochondria in NLRP3 inflammasome activation. *Nature* **469,** 221–225
15. Muñoz-Planillo, R., Kuffa, P., Martínez-Colón, G., Smith, B. L., Rajendiran, T. M., and Núñez, G. (2013) K^+ efflux is the common trigger of NLRP3 inflammasome activation by bacterial toxins and particulate matter. *Immunity* **38,** 1142–1153
16. Hornung, V., Bauernfeind, F., Halle, A., Samstad, E. O., Kono, H., Rock, K. L., Fitzgerald, K. A., and Latz, E. (2008) Silica crystals and aluminum salts activate the NALP3 inflammasome through phagosomal destabilization. *Nat. Immunol.* **9,** 847–856
17. Franchi, L., Muñoz-Planillo, R., and Núñez, G. (2012) Sensing and reacting to microbes through the inflammasomes. *Nat. Immunol.* **13,** 325–332
18. 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. *Am. J. Physiol. Renal Physiol.* **308,** F639–649
19. Nielsen, R., and Christensen, E. I. (2010) Proteinuria and events beyond the slit. *Pediatr. Nephrol.* **25,** 813–822
20. Leheste, J. R., Melsen, F., Wellner, M., Jansen, P., Schlichting, U., Renner-Müller, I., Andreassen, T. T., Wolf, E., Bachmann, S., Nykjaer, A., and Willnow, T. E. (2003) Hypocalcemia and osteopathy in mice with kidney-specific megalin gene defect. *FASEB J.* **17,** 247–249
21. Nykjaer, A., Dragun, D., Walther, D., Vorum, H., Jacobsen, C., Herz, J., Melsen, F., Christensen, E. I., and Willnow, T. E. (1999) An endocytic pathway essential for renal uptake and activation of the steroid 25-(OH) vitamin D3. *Cell* **96,** 507–515
22. Birn, H., Verroust, P. J., Nexo, E., Hager, H., Jacobsen, C., Christensen, E. I., and Moestrup, S. K. (1997) Characterization of an epithelial approximately 460-kDa protein that facilitates endocytosis of intrinsic factor-vitamin B12 and binds receptor-associated protein. *J. Biol. Chem.* **272,** 26497–26504
23. Amsellem, S., Gburek, J., Hamard, G., Nielsen, R., Willnow, T. E., Devuyst, O., Nexo, E., Verroust, P. J., Christensen, E. I., and Kozyraki, R. (2010) Cubilin is essential for albumin reabsorption in the renal proximal tubule. *J. Am. Soc. Nephrol.* **21,** 1859–1867
24. Eddy, A. A. (1989) Interstitial nephritis induced by protein-overload proteinuria. *Am. J. Pathol.* **135,** 719–733

25. Liu, B. C., Gao, J., Li, Q., and Xu, L. M. (2009) Albumin caused the increasing production of angiotensin II due to the dysregulation of ACE/ACE2 expression in HK2 cells. *Clin. Chim. Acta* **403,** 23–30
26. Latz, E., Xiao, T. S., and Stutz, A. (2013) Activation and regulation of the inflammasomes. *Nat. Rev. Immunol.* **13,** 397–411
27. Christensen, E. I., Verroust, P. J., and Nielsen, R. (2009) Receptor-mediated endocytosis in renal proximal tubule. *Pflugers Arch.* **458,** 1039–1048
28. Boya, P. (2012) Lysosomal function and dysfunction: mechanism and disease. *Antioxid. Redox Signal.* **17,** 766–774
29. Yamahara, K., Kume, S., Koya, D., Tanaka, Y., Morita, Y., Chin-Kanasaki, M., Araki, H., Isshiki, K., Araki, S., Haneda, M., Matsusaka, T., Kashiwagi, A., Maegawa, H., and Uzu, T. (2013) Obesity-mediated autophagy insufficiency exacerbates proteinuria-induced tubulointerstitial lesions. *J. Am. Soc. Nephrol.* **24,** 1769–1781
30. Mason, D. R., Beck, P. L., and Muruve, D. A. (2012) Nucleotide-binding oligomerization domain-like receptors and inflammasomes in the pathogenesis of non-microbial inflammation and diseases. *J. Innate. Immun.* **4,** 16–30
31. Zoja, C., Abbate, M., and Remuzzi, G. (2015) Progression of renal injury toward interstitial inflammation and glomerular sclerosis is dependent on abnormal protein filtration. *Nephrol. Dial. Transplant.* **30,** 706–712
32. Fang, L., Xie, D., Wu, X., Cao, H., Su, W., and Yang, J. (2013) Involvement of endoplasmic reticulum stress in albuminuria induced inflammasome activation in renal proximal tubular cells. *PLoS One* **8,** e72344
33. Martinon, F., Mayor, A., and Tschopp, J. (2009) The inflammasomes: guardians of the body. *Annu. Rev. Immunol.* **27,** 229–265
34. Zoja, C., Donadelli, R., Colleoni, S., Figliuzzi, M., Bonazzola, S., Morigi, M., and Remuzzi, G. (1998) Protein overload stimulates RANTES production by proximal tubular cells depending on NF-κB activation. *Kidney Int.* **53,** 1608–1615
35. Wang, Y., Rangan, G. K., Tay, Y. C., Wang, Y., and Harris, D. C. (1999) Induction of monocyte chemoattractant protein-1 by albumin is mediated by nuclear factor κB in proximal tubule cells. *J. Am. Soc. Nephrol.* **10,** 1204–1213
36. Morigi, M., Macconi, D., Zoja, C., Donadelli, R., Buelli, S., Zanchi, C., Ghilardi, M., and Remuzzi, G. (2002) Protein overload-induced NF-κB activation in proximal tubular cells requires H_2O_2 through a PKC-dependent pathway. *J. Am. Soc. Nephrol.* **13,** 1179–1189
37. Rathinam, V. A., Vanaja, S. K., and Fitzgerald, K. A. (2012) Regulation of inflammasome signaling. *Nat. Immunol.* **13,** 333–342
38. Lichtnekert, J., Kulkarni, O. P., Mulay, S. R., Rupanagudi, K. V., Ryu, M., Allam, R., Vielhauer, V., Muruve, D., Lindenmeyer, M. T., Cohen, C. D., and Anders, H. J. (2011) Anti-GBM glomerulonephritis involves IL-1 but is independent of NLRP3/ASC inflammasome-mediated activation of caspase-1. *PLoS One* **6,** e26778
39. Menu, P., and Vince, J. E. (2011) The NLRP3 inflammasome in health and disease: the good, the bad and the ugly. *Clin. Exp. Immunol.* **166,** 1–15
40. Zhai, X. Y., Nielsen, R., Birn, H., Drumm, K., Mildenberger, S., Freudinger, R., Moestrup, S. K., Verroust, P. J., Christensen, E. I., and Gekle, M. (2000) Cubilin- and megalin-mediated uptake of albumin in cultured proximal tubule cells of opossum kidney. *Kidney Int.* **58,** 1523–1533
41. Caruso-Neves, C., Pinheiro, A. A., Cai, H., Souza-Menezes, J., and Guggino, W. B. (2006) PKB and megalin determine the survival or death of renal proximal tubule cells. *Proc. Natl. Acad. Sci. U.S.A.* **103,** 18810–18815
42. Cabezas, F., Lagos, J., Céspedes, C., Vio, C. P., Bronfman, M., and Marzolo, M. P. (2011) Megalin/LRP2 expression is induced by peroxisome proliferator-activated receptor-alpha and -γ: implications for PPARs' roles in renal function. *PLoS One* **6,** e16794
43. Lee, D., Gleich, K., Fraser, S. A., Katerelos, M., Mount, P. F., and Power, D. A. (2013) Limited capacity of proximal tubular proteolysis in mice with proteinuria. *Am. J. Physiol. Renal Physiol.* **304,** F1009–1019
44. Nielsen, R., Mollet, G., Esquivel, E. L., Weyer, K., Nielsen, P. K., Antignac, C., and Christensen, E. I. (2013) Increased lysosomal proteolysis counteracts protein accumulation in the proximal tubule during focal segmental glomerulosclerosis. *Kidney Int.* **84,** 902–910
45. Dickson, L. E., Wagner, M. C., Sandoval, R. M., and Molitoris, B. A. (2014) The proximal tubule and albuminuria: really!. *J. Am. Soc. Nephrol.* **25,** 443–453
46. Jin, C., and Flavell, R. A. (2010) Molecular mechanism of NLRP3 inflammasome activation. *J. Clin. Immunol.* **30,** 628–631