Pattern recognition receptors, sensing re(n)al danger
Pulskens, W.P.C.
NLRP3 prevents from renal interstitial edema early after unilateral ureteral obstruction in mice

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Abstract

Progressive renal disease is characterized by tubulo-interstitial injury with ongoing inflammation and fibrosis, eventually resulting in loss of tubular architecture, renal morphology and function. One of the mechanisms that could orchestrate these pathophysiological processes is the NLRP3-inflammasome that induces maturation of effector cytokines upon activation by endogenous ligands. In the current study we investigated the role of NLRP3 following progressive renal injury by subjecting WT and NLRP3-deficient (-/-) mice to unilateral ureter obstruction (UUO).

Our results revealed a progressive increase of renal NLRP3 mRNA in WT mice following UUO. NLRP3-/- mice displayed enhanced tubular injury and dilatation and an elevated expression of injury biomarker NGAL compared to WT mice after UUO. Moreover, NLRP3-/- mice showed more severe interstitial edema, which could be explained by increased intratubular pressure and an enhanced tubular and vascular permeability due to reduced mRNA expression of intercellular junction components. The decreased epithelial barrier function in NLRP3-/- mice was not associated with increased apoptosis and/or proliferation of renal epithelial cells. NLRP3 deficiency did not affect renal fibrosis or inflammation.

Together, our data reveal an important role for the NLRP3-inflammasome in preserving renal integrity and protection against tubular injury in the early phase upon progressive renal injury.
Introduction

Fibroproliferative diseases, including progressive renal disease are a leading cause of morbidity and mortality worldwide. In the kidney, interstitial inflammation and fibrosis are main predictors for the risk of progression towards end-stage renal disease. Progressive renal disease can be characterized by a cascade of pathophysiological processes, including disruption of tubular integrity, infiltration of inflammatory cells, accumulation of (myo)fibroblasts, tubular atrophy and an increased deposition of extracellular matrix (ECM) molecules. Eventually, this altogether results in renal fibrogenesis.

The innate immune system may contribute to renal fibrotic processes through the activation of toll-like receptor 4 (TLR4) and TLR9, but not TLR2. An alternative mechanism that may orchestrate renal inflammatory and fibrotic processes is the formation and activation of the intracellular inflammasome that consists of NLRP3, ASC and caspase-1. Activation of the NLRP3-inflammasome induces maturation of effector cytokines, including IL-1β and IL-18, although the exact pathways leading to activation are still a matter of debate.

NLRP3 is expressed in human and murine tubular epithelial cells, and displays an enhanced renal expression following experimental models mimicking acute and chronic kidney disease (CKD). Interestingly, the absence of NLRP3 diminished renal inflammation and injury in the early phase of acute renal ischemia reperfusion injury and in the late phase of chronic kidney disease. We previously showed contrasting functions for both TLR2 and TLR4 with respect to renal injury and inflammation between experimental models of either acute or progressive kidney injury. Apparently, signaling via innate immune receptors can lead to a profoundly different outcome in local injury and inflammation during acute (ischemic) or progressing renal injury.

To extend the findings of the study of Vilaysane et al. and to investigate the role of NLRP3 in the early phase of progressive renal injury, in this study we subjected wild type and NLRP3-/- mice to unilateral ureter obstruction (UUO). This study revealed a novel and important function of NLRP3 in preserving renal integrity during the acute phase of progressive renal disease.
Material & Methods

Ethics Statement
The Animal Care and Use Committee of the University of Amsterdam approved this study and all included animal experiments (study# DPA101016). Experiments have been conducted according to national guidelines.

Mice
Pathogen-free 8-12 week old female wild type C57Bl/6 mice were purchased from Charles River Laboratories. NLRP3-/- mice were generated as described previously\textsuperscript{17}, backcrossed nine times to a C57Bl/6 background and bred in the animal facility of the Academic Medical Center in Amsterdam, the Netherlands. Only age and sex-matched mice were used in experiments. Genetic NLRP3 deficiency in KO mice was confirmed by routinely screening by RTPCR.

Unilateral Ureter Obstruction
Unilateral ureter obstruction was induced as described previously\textsuperscript{18}. Briefly, all mice received preoperative analgesia (subcutaneous injection of 50μg/kg buprenorphin (Temgesic, Shering-Plough)) and the right ureter was subsequently ligated with 6.0 silk through a small abdominal incision under 2.0% isoflurane-induced anesthesia. The abdomen was closed in two layers and mice were allowed to recover from surgery for 12 hours at 28°C in a ventilated stove. The contralateral non-obstructed kidney (t=14) served as control. Mice (n=7/group) were sacrificed 1, 3, 7 and 14 days after surgery.

Measurement of mRNA expression
Total RNA was extracted from thirty 20μm frozen total renal tissue sections of each animal with Trizol reagent (Invitrogen) according to the manufactures protocol. All RNA samples were quantified by spectrophotometry and stored at -80°C until processed for reverse transcription. RNA was converted to cDNA by using oligo-dT as primer. NLRP3, ASC, NGAL, Claudin-1, -2, -5 and VE-Cadherin mRNA expression was analyzed by real-time quantitative reverse transcription-PCR (RT-PCR) performed on a Roche light cycler with SYBR green PCR master mix. Specific gene expression was normalized to mouse hypoxanthine-guanine-phosphoribosyltransferase (HPRT) house keeping gene expression. SYBR green dye intensity was analyzed with linear regression analysis. Six animals per group were analyzed by real-time quantitative RT-PCR.
**Scoring of tubular injury and interstitial edema**
Renal tissues were fixed in 10% formalin and embedded in paraffin. All histopathological scorings were made in the cortex using PAS-D stained renal sections and performed on coded slides. The percentage of tubular injury (criteria: epithelial flattening, tubular dilatation and brush border loss) and the degree of interstitial edema was estimated by a pathologist in a blinded fashion using a 4-point scale in ten randomly chosen, non-overlapping fields (200x magnification). Degree of injury was graded onto a scale from 0 to 4: 0=normal; 1=mild, involvement of less than 25% of the cortex; 2=moderate, involvement of 25 to 50% of the cortex; 3=severe, involvement of 50 to 75% of the cortex; 4=extensive damage involving >75% of the cortex. Degree of edema was graded according: 0=normal; 1=mild edema; 2=moderate; 3=severe; 4=extensive edema formation.

**Detection of apoptotic and proliferating tubular epithelial cells**
Antigen retrieval was performed by boiling paraffin-embedded tissue sections for 10min in either 10mM Tris/1mM EDTA (apoptosis) or sodium citrate buffer (pH 6.0) (proliferation), followed by blocking endogenous peroxidase activity. Slides were incubated with rabbit anti-human active caspase-3 polyclonal antibody (Cell Signaling Technology) or rabbit anti-Ki67 antibody (Sp6; Neomarkers). After incubation with the appropriate secondary antibodies, slides were developed using 1% H₂O₂ and DAB (Sigma-Aldrich) in 0.05M Tris-HCl (pH 7.9), and counterstained with methyl green (Sigma). To evaluate the degree of apoptosis and proliferation, caspase-3- and Ki67-positive tubular epithelial cells were counted in at least 10 non-overlapping high power fields (magnification 400x). Since tubular atrophy was very severe 14 days post-UUO in all groups, identification of epithelial cells was difficult. Therefore, the total amount of positive stained cells was quantified for this time point. Staining specificity was confirmed by incubating renal slides without the primary antibody and that remained completely negative.

**Detection of macrophage accumulation**
Paraffin-embedded renal sections were deparaffinized and boiled for 10 minutes in 10mM sodium citrate buffer (pH 6.0) for antigen retrieval. Subsequently, endogenous peroxidase activity and non-specific binding were blocked and slides were exposed to rat anti-mouse F4/80 IgG2b mAb (Serotec). Slides were subsequently incubated with rabbit-anti-biotin (Dako), followed by streptavidin-ABC solution (Dako). Finally, slides were developed as described above and counterstained with methyl green. The percentage of positive F4/80 staining was quantified digitally using Image Pro Plus software version 5.0.
Detection of fibrosis
For staining of collagen, slides were incubated with 0.2% Picro Sirius Red solution (pH 2.0) for 1 hour followed by incubation within 0.01M HCl. For myofibroblast staining antigen retrieval was achieved by boiling for 10 minutes in 10mM sodium citrate buffer (pH 6.0). Subsequently, slides were exposed to mouse anti-human αSMA-IgG2a (DAKO), followed by incubation with goat anti-mouse IgG2a-HRP (Southern Biotech). Slides were developed as described above and counterstained with methyl green. The percentage of positive αSMA and Picro Sirius Red was quantified digitally using Image Pro Plus software version 5.0.

Preparing kidney homogenate
For cytokine measurements, snap-frozen kidneys were homogenized in Greenberger Lysis buffer (150mM NaCl, 15mM Tris, 1mM MgCl₂·H₂O, 1mM CaCl₂ and 1% Triton-X), and incubated for 30 minutes at 4°C. Homogenates were subsequently centrifuged at 14,000rpm for 10 minutes after which supernatants were stored at -80°C until ELISAs were performed. To determine protein content, Bradford Protein Assay (Bio Rad) was used with IgG as standard.

ELISA
Cytokines and chemokines (Keratinocyte Chemoattractant (KC), Monocyte Chemoattractant-1 (MCP-1), Interleukin 1-β (IL1-β), Tumor Necrosis Factor (TNF-α) and total transforming growth factor-β (TGF-β)) were measured in the kidney homogenates using specific ELISA’s (R&D systems) according the manufactures protocol. Activation of latent TGF-β was done according to the protocol supplied by the manufacturer. The detection limits were 12 pg/ml (KC), 6 pg/ml (MCP-1), 31pg/ml (IL1-β), 31 pg/ml (TNF-α) and 31 pg/ml (TGF-β).

Statistics
Differences between groups were analyzed using Mann-Whitney U test. Values are expressed as mean ± standard error of the mean (SEM). A value of p<0.05 was considered as statistically significant.
Results

*Increased NLRP3 mRNA and constitutive ASC mRNA expression in the kidney following UUO*

To determine whether progressive renal injury influences expression kinetics of NLRP3 and ASC, we quantified mRNA levels in total kidney specimens at several time points post-UUO. In line with the results of Vilaysane et al.\textsuperscript{11}, renal NLRP3 mRNA levels were significantly enhanced 1, 3, 7 and 14 days post-UUO, when compared with contralateral kidneys (figure 1A). In contrast, ASC mRNA levels remained constitutively present (figure 1B). These data suggest a potential role for the NLRP3 inflammasome in the underlying pathophysiology of UUO.

![Figure 1: Progressive renal injury enhances renal NLRP3, but not ASC mRNA expression.](image)

Relative expression levels of NLRP3 (A) and ASC (B) mRNA in total kidney specimen following progressive renal injury. NLRP3 mRNA expression was significantly enhanced 1, 3, 7 and 14 days post-UUO compared with contralateral kidneys, whereas ASC mRNA remained constitutively expressed. Data are mean±SEM of 6 mice per group. *:p<0.05.

*NLRP3 attenuates renal injury in UUO*

We next defined the role of the NLRP3 inflammasome in progressive renal injury by subjecting wild type and NLRP3-/- mice to UUO. Wild type mice demonstrated a progressive increase in the level of tubular injury, including epithelial flattening, brush border loss and tubular dilatation which reached a maximum at day 14 (figure 2A). Interestingly, NLRP3-/- mice developed significantly more severe tubular injury 1, 3 and 7 days post-UUO, while after 14 days both genotypes demonstrated maximal score of injury. As shown in representative microphotographs interstitial edema was more pronounced in NLRP3-/- mice 1 day post-UUO compared to wild type mice. Indeed, semi-quantitative score of the degree of interstitial edema revealed higher levels in obstructed kidneys of NLRP3-/- mice compared to wild type mice (1.78±0.19 vs. 2.25±0.18 arbitrary units at t=1; p=0.008). Additionally, NGAL mRNA was measured as an early and sensitive biomarker of renal injury. NLRP3-/- mice
demonstrated enhanced NGAL expression in their obstructed kidneys 1 day post-UUO compared to wild type mice (figure 2B). No differences were observed at later time points when tubules become severely atrophic.

**Figure 2: NLRP3 deficiency increases renal injury following UUO.**
Semi-quantitative tubular injury score in wild type (white bars) and NLRP3-/- (black bars) mice following UUO (A). NLRP3-/- mice developed significantly more tubular injury when compared to their respective wild type mice 1, 3, and 7 days post-UUO. The panels show representative microphotographs of renal tissue subjected to 1 day of UUO, with apparent interstitial edema in the NLRP3-/- kidneys (magnification x200). Additionally, NLRP3-/- mice displayed enhanced mRNA expression of the renal injury biomarker NGAL compared to their wild type mice following UUO (B). Data are mean±SEM of 7 mice per group. Contral. = contralateral kidneys of mice subjected to 14 days of UUO. *:p<0.05.

**NLRP3 deficiency reduces renal expression of epithelial and endothelial intercellular junction molecules following UUO**
In order to investigate whether the enhanced interstitial edema observed in NLRP3-/- mice might be a consequence of a disturbed epithelial integrity or a leaky microvascular endothelium, we determined the expression of renal Claudin-1, and -2 and Claudin-5 and VE-Cadherin as major constituents composing tight junctions between epithelial and endothelial sheets, respectively. Interestingly, 1 day post-UUO when interstitial edema was most prominent, NLRP3-/- mice displayed a reduced expression of Claudin-1, -2, -5 and VE-Cadherin in their obstructed kidneys when compared to wild type mice (figure 3A-D). VE-Cadherin expression remained lower in kidneys of NLRP3-/- mice. In contrast 14 days post-UUO a subtle increase was observed for Claudin-1 and -2. These data suggest that NLRP3-/- mice might have less (effective) tight junctions in order to maintain renal epithelial...
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and endothelial integrity upon UUO as a consequence of reduced Claudin-1, -2, -5 and VE-Cadherin expression, respectively. NLRP3 deficiency did not affect the basal levels of these parameters in renal tissue of sham-mice (data not shown). This observation in combination with increased intratubular pressure as a result of increased dilatation in the NLRP3-/- mice may contribute to the more severe interstitial edema formation observed in these mice compared to wild type mice.

**NLRP3 deficiency reduces apoptosis, but not proliferation of TECs**

We additionally evaluated whether the observed differences in tubular injury and integrity between wild type and NLRP3-/- mice could be ascribed to an effect of NLRP3 deficiency on tubular apoptosis and proliferation. Despite increased tubular damage NLRP3-/- mice had slightly less apoptotic TECs 3 days post-UUO (figure 4), whereas at other time points no differences were observed. The number of proliferating TECs was comparable at all time points between wild type and NLRP3-/- mice (data not shown).
NLRP3 deficiency does not affect macrophage accumulation but impairs early renal IL1-β levels following UUO

To determine whether NLRP3 deficiency modulates tubulo-interstitial inflammation following UUO, macrophage accumulation and the levels of proinflammatory cytokines/chemokines IL1-β, KC, MCP-1 and TNF-α were analyzed in kidneys. Wild type mice displayed a progressive accumulation of macrophages in obstructed kidneys following UUO (figure 5A). NLRP3 deficiency however did not influence macrophage accumulation. Renal IL1-β levels were significantly reduced in contralateral and obstructed kidneys (t=1) of NLRP3-/- mice compared to wild type mice, whereas levels at later time points did not differ (figure 5B). NLRP3 deficiency did not influence renal concentrations of MCP-1, TNF-α or KC following UUO (data not shown).
NLRP3 deficiency does not affect renal fibrosis following UUO

Finally, we evaluated whether NLRP3 deficiency affects renal fibrosis following UUO by analyzing the accumulation of myofibroblasts, total collagen deposition and renal concentrations of the profibrotic molecule Transforming Growth Factor-β (TGF-β). As expected, wild type mice displayed a progressive myofibroblast accumulation (figure 6A) and collagen deposition (figure 6B) in their obstructed kidneys following UUO. NLRP3 deficiency did however not affect myofibroblast accumulation (figure 6A) or collagen deposition, except for a reduction observed at 3 days post-UUO (figure 6B). Moreover, NLRP3 deficiency did not affect the renal concentrations of total TGF-β following UUO (data not shown).
Discussion

The intracellular multi-protein scaffold, the inflammasome contributes to the establishment of an effective inflammatory response by exerting catalytic cleavage and subsequent release of effector cytokines. Endogenous danger ligands that are released following tissue injury can prime or directly activate the inflammasome, thereby initiating an inflammatory response. It has been shown that NLRP3 is expressed by renal tubular epithelial cells and that total renal NLRP3 expression is clearly enhanced following acute\(^{10}\) and chronic kidney disease\(^{11}\). Hence, in the current study we aimed to elucidate the role of the NLRP3 inflammasome at several time points in progressive tubulo-interstitial injury, renal inflammation and fibrosis by subjecting wild type and NLRP3\(^{-/-}\) mice to UUO.

We observed that NLRP3, but not ASC mRNA progressively increased following UUO that may reflect an enhanced expression by renal TECs and/or the accumulation of NLRP3 expressing inflammatory cells or (myo)fibroblasts. These data are in agreement with the results of Vilaysane et al. and other studies showing that NLRP3 expression is induced in response to proinflammatory agents\(^{11, 19}\). Interestingly, we observed that NLRP3 attenuates renal damage following UUO, as reflected by
significant enhanced levels of tubular injury in obstructed kidneys of NLRP3-/- mice 1, 3 and 7 days post-UUO compared to wild type mice. In accordance, NLRP3-/- mice displayed enhanced mRNA expression of the renal injury biomarker NGAL 1 day post-UUO. After 14 days no differences in renal injury could be found anymore as tubular injury was at maximum in both groups. In contrast to these results, we and others previously found that the NLRP3 inflammasome enhanced acute renal injury and dysfunction following acute ischemia reperfusion injury. These opposite effects of NLRP3 deficiency in acute and progressive renal injury are also seen in mice deficient for TLR4. Obviously, signaling via these pattern recognition receptors can lead to a profoundly different outcome of local injury and inflammation during acute (ischemic) or chronic (UUO) renal injury. This implicates that targeting these receptors for the treatment of renal diseases requires careful consideration.

Additionally, a major characteristic observed in obstructed kidneys of NLRP3-/- mice was significant elevated interstitial edema, possibly as a result of increased intratubular pressure-induced mechanical stretch and enhanced vascular and tubular permeability. Indeed, NLRP3-/- mice demonstrated more tubular dilatation and a reduced mRNA expression of claudin-1, -2, -5 and VE-Cadherin 1 day post-UUO compared to wild type mice. Claudin-1 and -2 exhibit a specific expression pattern in the kidney and are thought to be essential in tight junction formation to establish close connections between epithelial cells, thereby maintaining cell polarity and tubular integrity. On the contrary, Claudin-5 and VE-Cadherin are specifically expressed in endothelial cells to maintain vascular barrier functions and regulate endothelial permeability. Together, this suggests that NLRP3 protects against progressive renal injury during UUO by mediating renal expression of epithelial and endothelial intercellular junction molecules leading to a better preservation of vascular permeability and epithelial integrity in response to mechanical stretch. In line, it has been shown that stretch adversely affects tight junction structures on epithelial cells, thereby modulating epithelial permeability. Moreover, a recent study of Zaki et al. showed that the NLRP3 inflammasome protects against the loss of epithelial integrity during experimental colitis. The decreased epithelial barrier function in NLRP3-/- mice was not associated with increased apoptosis of renal epithelial cells and/or decreased cell proliferation in our study. Whether the reduced early expression of intercellular junction molecules is a direct or indirect effect of NLRP3 deficiency and the relevance of the subtle increases in Claudin-1 and -2 expression observed 14 days post-UUO remain unclear and need to be investigated in more detail in the future.

The common acknowledged function of the NLRP3 inflammasome is release of mature cytokines via caspase-1-mediated cleavage, thereby initiating an inflammatory response. Hence, we investigated whether absence of NLRP3...
affected renal inflammation following UUO. Indeed, we observed that NLRP3 deficiency resulted in a mild but significant reduction of early renal IL1-β levels. These differences disappear however while injury progresses, proposing a certain level of redundancy through other members of the NLRP protein family that may form additional IL1-β activating multi-protein scaffolds. The concentrations of renal KC, MCP-1 and TNF-α were not affected by absence of NLRP3. In contrast to the pivotal role we demonstrated for NLRP3 in renal inflammation following acute ischemic injury 10, macrophage accumulation was not altered by NLRP3 deficiency in the present study. In agreement with this result, a study of Jones et al. demonstrated that deficiency of either IL1-β or IL1-R1, both functioning downstream of inflammasome activation, does not affect accumulation of macrophages following UUO 30.

Next, we investigated whether the absence of NLRP3 influenced the progression of renal fibrosis by determining the accumulation of myofibroblasts and collagen deposition following UUO. However, no differences were observed in the accumulation of myofibroblasts or collagen deposition in obstructed kidneys of NLRP3-/- mice following UUO, except a slight but significant decrease 3 days post-UUO. Moreover, NLRP3 deficiency did not affect the concentrations of the renal profibrotic molecule TGF-β following UUO. In line, the study of Jones et al. demonstrated that IL1-β deficiency did not affect the accumulation of myofibroblasts or collagens in kidneys following obstruction-induced renal fibrosis 30. In contrast, transgenic mice with neutralized IL-18 by IL-18 binding protein exhibited reduced collagen deposition and myofibrolast accumulation during UUO, without altering the concentrations of TGFβ 31. Together, it can thus be proposed that the development of progressive renal fibrosis is primarily mediated via IL-18, while the role of the upstream NLRP3-inflammasome is only minor. However, one must keep in mind that neutralizing IL-18 activity via IL-18BP may have pleiotropic effects that could interfere with disease outcome.

A recent study of Vilaysane et al. also investigated the role of NLRP3 in chronic kidney disease. In accordance to our results, they showed that NLRP3-/- obstructed kidneys displayed reduced numbers of apoptotic cells and reduced IL1-β levels 11. In this study however, NLRP3-/- mice exhibited less renal injury after 2 weeks of obstruction as determined by scoring the percentage of cortical tubular necrosis as injury parameter. Apparently, NLRP3 plays a beneficial role at the early phase of progressive renal injury while this role is not extended (this study) or even reversed 11 in the late phase of disease. This observation may reflect the different renal responses that take place in the early and late phase of progressive renal injury 32 and/or suggests that early and late damage is mediated by different processes in this disease 33, 34. The reason that we could not confirm the difference between wild type and NLRP3-/- animals that Vilaysane et al. 11 found in fibrosis, inflammation
and injury at day 14 is currently unclear but could merely reflect differences in the model used. While we \textsuperscript{6,8} and others \textsuperscript{3,35-40} observed that a major pathological change associated with urinary tract obstruction is the loss of renal tubular cells via apoptosis, Vilaysane \textit{et al}. found that renal injury is particularly reflected by high levels of cortical tubular necrosis (up to 80\%) \textsuperscript{11}.

In conclusion, our results imply a central role for the NLRP3-inflammasome in the maintenance of renal integrity and protection against the early effects of UUO-induced progressive renal injury, whereas the NLRP3-inflammasome only plays a minor role in renal inflammation and fibrosis.

**Disclosure**

The authors declare no financial or commercial conflicts of interest.

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