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### CD44, TLR4, TREM-1/DAP12 in renal injury, inflammation and fibrosis

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# 3

## **TLR4 amplifies renal fibrosis and decreases renal damage upon progressive renal injury**

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## Abstract

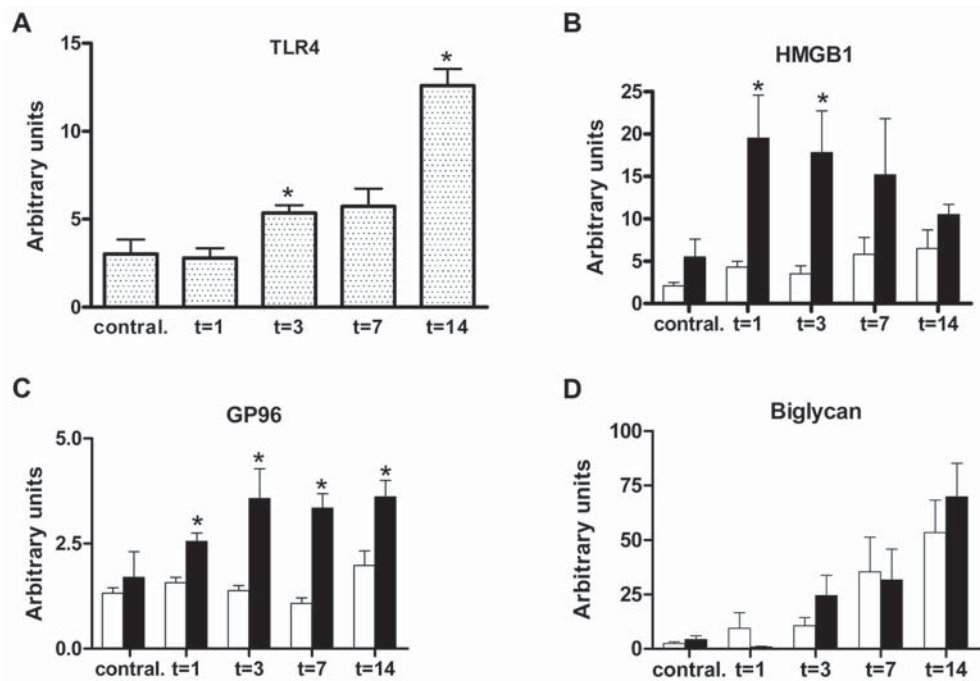
Toll-like receptors (TLRs) can orchestrate an inflammatory response upon activation by pathogen-associated motifs and endogenous stress ligands that are released during tissue injury. Most TLRs, including TLR4, are constitutively expressed in the kidney. Progressive injury of the kidney is characterized by a cascade of events including inflammation, tubular atrophy and interstitial fibrosis. The function of TLR4 herein remains unknown. In the present study, the role of TLR4 in chronic obstructive nephropathy was investigated by subjecting wild type (WT) and TLR4-deficient mice to unilateral ureter obstruction (UUO). We found elevated levels of TLR4 mRNA in the kidney following UUO. TLR4 attenuated tubular damage which was associated with an early enhanced number of proliferating tubular epithelial cells. TLR4-deficient mice developed considerably less renal fibrosis as assessed by collagen deposition than WT mice without differences in myofibroblast accumulation and despite decreased MMP activity. In vitro studies demonstrate that primary tubular epithelial cells and myofibroblasts can mediate TLR4-dependent collagen deposition in response to TGF- $\beta$  stimulation. The decrease in fibrosis in TLR4-deficient mice is associated with an upregulation of Bambi, a negative regulator of TGF- $\beta$  signaling. In conclusion, TLR4 attenuates tubular damage, but mediates renal fibrosis by modifying TGF- $\beta$ -susceptibility of renal cells. Hence, TLR4 signaling seems to be a key event in the induction of fibrosis during progressive renal injury and may be a therapeutic target to prevent renal fibrosis.

## Introduction

Fibroproliferative diseases, including progressive renal disease, are a leading cause of morbidity and mortality worldwide <sup>1</sup>. Renal tubular damage, inflammation and interstitial fibrosis are main predictors for the risk of progression towards end-stage renal failure <sup>2</sup>. Progression of renal fibrosis involves a cascade of pathophysiological processes, including disruption of tubular integrity, a robust inflammatory response, accumulation of (myo)fibroblasts, tubular atrophy and an increased deposition of extracellular matrix (ECM) components resulting in fibrogenesis <sup>3-5</sup>.

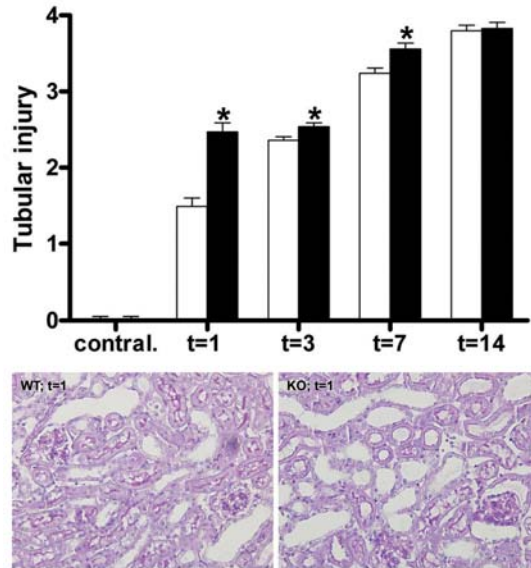
The group of Toll-like receptors (TLRs) may be one of the receptor families that orchestrate this cascade of inflammation, myofibroblast accumulation and fibrosis in the kidney. TLRs can initiate an inflammatory response upon recognition of specific pathogen associated molecular patterns (PAMPs). Currently, it is widely accepted that not only PAMPs can trigger TLR-mediated immune responses, but endogenous danger molecules that are released upon tissue or cell injury as well <sup>6-11</sup>. We already found that several of these endogenous ligands that can potentially activate both TLR2 and TLR4 <sup>9,11-13</sup> are strongly upregulated in murine kidneys after unilateral ureter obstruction <sup>14,15</sup>.

**Figure 1.** Expression levels of TLR4 mRNA.



(A) in wild type obstructed kidneys and its endogenous danger ligands HMGB1 (B), GP96 (C) and Biglycan (D) in wild type (white bars) and TLR4<sup>-/-</sup> (black bars) mice following UUO. TLR4 is markedly upregulated upon UUO when compared to contralateral kidneys. HMGB1 and GP96 mRNA were enhanced in TLR4<sup>-/-</sup> mice compared to wild type mice, whereas Biglycan levels were similar. Data are mean±SEM of six mice per group. \*:p<0.05.

**Figure 2.** Semi-quantitative score of tubular injury in wild type (white bars) and TLR4<sup>-/-</sup> (black bars) mice following UUO.



TLR4<sup>-/-</sup> mice developed more severe tubular injury when compared to wild type mice 1, 3 and 7 days post-UUO. Data are mean±SEM of 7-8 mice per group. Contral. =contralateral kidneys of mice subjected to 14 days of UUO. \*:p<0.05. The lower panels show representative microphotographs of renal tissue of wild type and TLR4<sup>-/-</sup> mice 1 day post-UUO, magnification 200x.

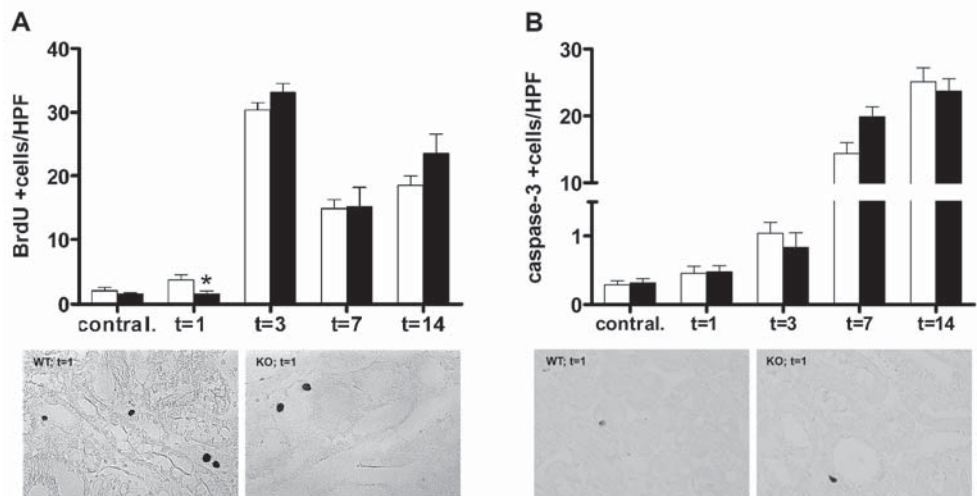
We demonstrated that TLR2 does not play a role in the development of fibrosis or injury after UUO<sup>14</sup>. Until now, the role of TLR4 in progressive renal injury and fibrosis remains unknown. In a model of hepatic fibrogenesis it has been demonstrated that TLR4 can enhance TGF- $\beta$  signaling and myofibroblast activation, suggesting that TLR4 can function as a molecular link between proinflammatory and profibrogenic signals in liver tissue<sup>16</sup>. Interestingly, TLR4 is widely and constitutively expressed in the kidney, e.g. on tubular epithelial cells (TECs)<sup>17,18</sup>. We and others have shown that renal-associated TLR2 and TLR4 can induce an exaggerated inflammatory response in the kidney upon acute ischemic renal injury with subsequent detrimental effects on renal histology and function<sup>19-21</sup>. In order to study the role of TLR4 in progressive renal injury and renal fibrosis, we subjected wild type and TLR4<sup>-/-</sup> mice to unilateral ureter obstruction (UUO).

## Results

### Increased TLR4 mRNA expression upon UUO

To evaluate the expression of TLR4 and some potential endogenous danger ligands upon progressive renal injury, TLR4, HMGB1 and GP96 and Biglycan mRNA levels were quantified at several

**Figure 3.** Proliferating (A) and apoptotic (B) cells in the obstructed and contralateral kidneys from wild type (white bars) or TLR4<sup>-/-</sup> (black bars) mice following UUO.



The number of proliferating (BrdU+) TECs was lower in the obstructed kidneys of TLR4<sup>-/-</sup> mice compared to wild type mice 1 day post-UUO, while there were no differences at later time points. The number of apoptotic (active caspase-3+) cells was similar in obstructed kidneys of wild type and TLR4<sup>-/-</sup> mice at all time points. The number of positive stained TECs was counted in ten non-overlapping high power fields (x400). Since tubular atrophy was very severe 14 days post-UUO in both groups, identification of epithelial cells was difficult. Therefore, the total amount of positive cells was quantified at this time point. Representative pictures of wild type and TLR4<sup>-/-</sup> mice are of 1 day post-UUO. Data are mean±SEM of 7-8 mice per group. Contral. =contralateral kidneys of mice subjected to 14 days of UUO. \*:p<0.05.

time points post-UUO. TLR4 mRNA levels were significantly enhanced 3 and 14 days post-UUO compared with contralateral kidneys (figure 1a).

Interestingly, HMGB1 and GP96 mRNA was higher in obstructed kidneys of TLR4<sup>-/-</sup> mice following UUO when compared to wild type mice, whereas Biglycan mRNA expression was similar (figure 1b-1d). These data suggest a potential role for TLR4 activation and signaling following UUO.

#### TLR4 attenuates tubular injury in obstructed kidneys

Following UUO, wild type mice demonstrated progressive tubular injury, such as widespread dilatation and epithelial flattening. Interestingly, TLR4<sup>-/-</sup> mice developed more severe tubular damage in the obstructed kidneys than wild type mice at 1, 3 and 7 days post-UUO (figure 2). After 14 days end stage renal disease was reached in both groups of mice as demonstrated by maximal score of injury.

#### Impaired proliferation early upon UUO in TLR4<sup>-/-</sup> mice

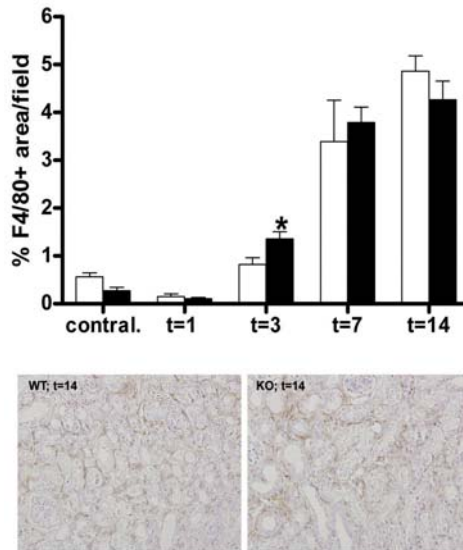
In order to assess whether the increased tubular injury in TLR4<sup>-/-</sup> obstructed kidneys was associated with a disturbed balance between proliferation and apoptosis, the number of proliferating

and apoptotic tubular epithelial cells (TECs) were quantitatively determined by immunohistochemistry, whereas 14 days post-UUO the total number of positive cells was determined. The number of proliferating TECs was significantly lower in the obstructed kidneys of TLR4<sup>-/-</sup> mice 1 day post-UUO, but similar at later time points (figure 3a). The number of apoptotic cells was comparable between wild type and TLR4<sup>-/-</sup> mice at all time points (figure 3b).

Comparable inflammatory response and influx of macrophages in obstructed kidneys of wild type and TLR4<sup>-/-</sup> mice

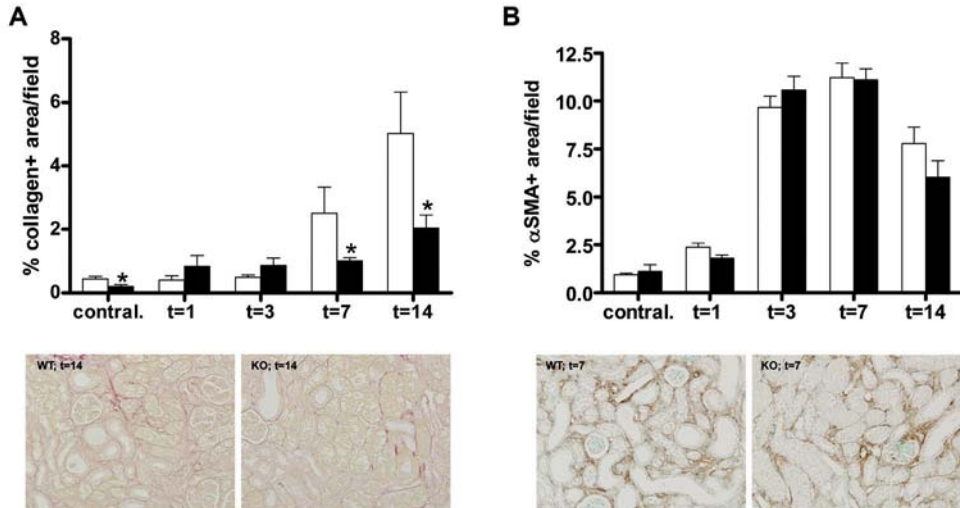
In order to obtain insight into the role of TLR4 in renal inflammation following UUO, the concentration of the proinflammatory chemokines MCP-1 and KC in renal homogenates and the accumulation of macrophages were determined. No differences were observed in the levels of KC and MCP1 in renal homogenates of both groups at all time points (data not shown). Macrophage infiltrate progressively increased in the obstructed kidneys following UUO. In line with the chemokine data, no differences were found in the accumulation of macrophages between obstructed kidneys of wild type and TLR4<sup>-/-</sup> mice 1, 7 and 14 days post-UUO (figure 4). Surprisingly, 3 days post-UUO TLR4<sup>-/-</sup> mice displayed enhanced macrophage influx compared to wild type mice.

**Figure 4.** Quantitative analysis of interstitial macrophages in obstructed and contralateral kidneys of wild type (white bars) and TLR4<sup>-/-</sup> (black bars) mice following UUO.



In wild type and TLR4<sup>-/-</sup> mice an increase of interstitial macrophages was observed in the obstructed kidneys, peaking at 14 days post-UUO. The amount of macrophages was higher in the TLR4<sup>-/-</sup> mice 3 days post-UUO when compared to wild type mice. Below are representative pictures of wild type and TLR4<sup>-/-</sup> mice are of 14 days post-UUO (x200). Data are mean±SEM of 7-8 mice per group. Contral. =contralateral kidneys of mice subjected to 14 days of UUO. \*:p<0.05.

**Figure 5.** Quantitative analysis of total collagen deposition (A) and myofibroblast accumulation (B) in obstructed and contralateral kidneys of wild type (white bars) and TLR4<sup>-/-</sup> (black bars) mice following UUO.



The amount of total collagen deposition progressively increased following UUO and was significantly lower in the TLR4<sup>-/-</sup> mice 7 and 14 days post-UUO (A). No difference was found in the accumulation of  $\alpha$ -SMA positive myofibroblasts between wild type and TLR4<sup>-/-</sup> mice (B). The percentage of Picro Sirius Red and  $\alpha$ -SMA positive staining was quantified by digital analysis. Representative pictures of wild type and TLR4<sup>-/-</sup> mice are of 14 days post-UUO (A) or 7 days post-UUO (B). Data are mean $\pm$ SEM of 7-8 mice per group. Contral. =contralateral kidneys of mice subjected to 14 days of UUO. \*:p<0.05.

#### TLR4 enhances collagen deposition but does not affect myofibroblast accumulation

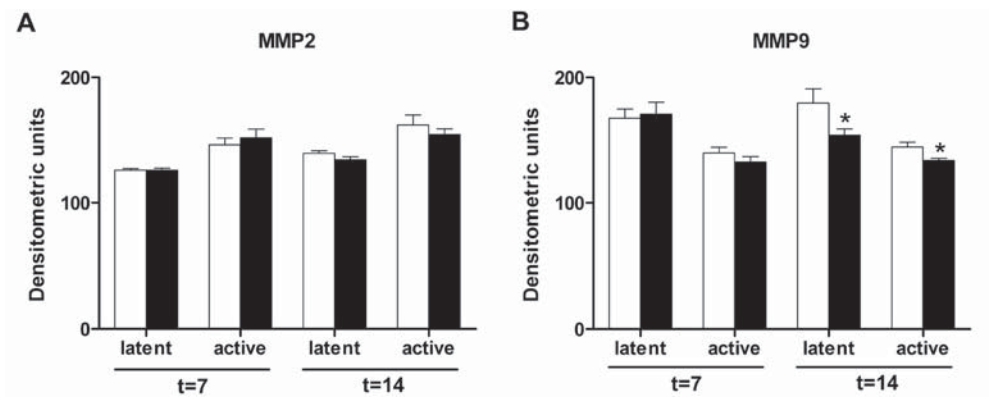
Total collagen deposition was determined by quantitative analysis of Picro Sirius Red staining. This revealed that wild type mice had a progressive increase in the amount of total collagen deposition. Interestingly, total collagen deposition was less in the obstructed kidneys of TLR4<sup>-/-</sup> mice 7 and 14 days post-UUO when compared to wild type mice (figure 5a). Since myofibroblasts are major matrix producing cells in the kidney<sup>22,23</sup>, the presence of myofibroblasts was quantified. A strong accumulation of  $\alpha$ -SMA positive myofibroblasts was observed in obstructed kidneys following UUO. However, no differences were observed between wild type and TLR4<sup>-/-</sup> mice at all time points (figure 5b).

#### TLR4 deficiency lowers MMP9 activity following UUO

By zymography we evaluated whether TLR4 deficiency influenced the activity of MMP2 or MMP9. The levels of both latent and active MMP2 were equal in the obstructed kidneys of wild type and TLR4<sup>-/-</sup> mice 7 and 14 days post-UUO (figure 6a). In contrast, a slight but significant decrease in the levels of latent and active MMP9 was found in the obstructed kidneys of TLR4<sup>-/-</sup> mice when compared to wild type mice 14 days post-UUO (figure 6b).

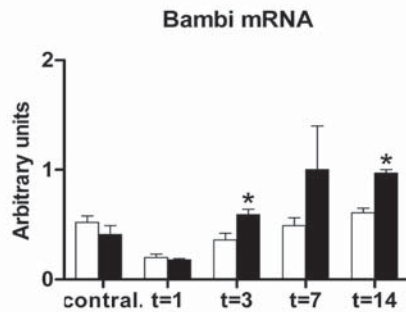


**Figure 6.** Quantification of latent and active MMP2 (A) and MMP9 (B) in obstructed kidneys of wild type (white bars) and TLR4<sup>-/-</sup> (black bars) mice following UUU.



No differences were observed in the renal levels of latent or active MMP2 between wild type and TLR4<sup>-/-</sup> mice. The levels of latent and active MMP9 were significantly lower in the kidneys of TLR4<sup>-/-</sup> mice 14 days post-UUU. Data are mean±SEM of 7 mice per group. \*:p<0.05.

**Figure 7.** Bambi mRNA levels in obstructed kidneys of wild type (white bars) and TLR4<sup>-/-</sup> (black bars) mice following UUU.

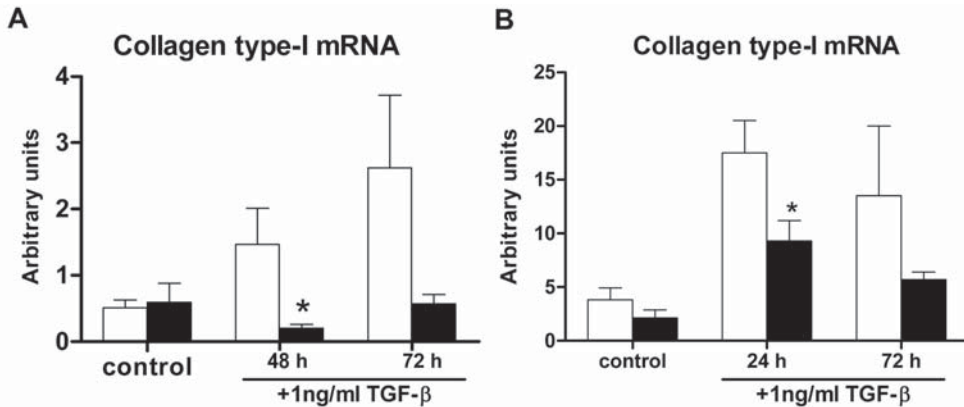


Bambi expression was significantly enhanced in obstructed kidneys of TLR4<sup>-/-</sup> mice compared to wild type mice 3 and 14 days post-UUU. Data are mean±SEM of 6-7 mice per group. Contral. =contralateral kidneys of mice subjected to 14 days of UUU. \*:p<0.05.

### TLR4 deficiency alters TGF-β susceptibility following UUU

In order to investigate whether TLR4 deficiency affected the balance between the profibrotic protein transforming growth factor-β (TGF-β) and the antifibrotic cytokine hepatocyte growth factor (HGF), concentrations of both proteins were determined in kidney homogenates. The concentration of total and active TGF-β protein was similar in homogenates of wild type and TLR4<sup>-/-</sup> mice at all time points (data not shown). In addition, concentrations of HGF were comparable between renal homogenates of wild type and TLR4<sup>-/-</sup> mice, except for 3 days post-UUU (1422.1±71.0 pg/mg protein vs. 891.6±53.3 pg/mg protein of renal homogenate; wild type vs. TLR4<sup>-/-</sup>, p=0.003).

**Figure 8.** Relative collagen type-I mRNA expression by primary TECs (A) and primary myfibroblasts (B) from wild type (white bars) and TLR4<sup>-/-</sup> (black bars) mice, when subjected to stimulation with 1ng/ml TGF- $\beta$ .



Control cells remained 72h in culture medium. TLR4<sup>-/-</sup> primary TECs and TLR4<sup>-/-</sup> primary myfibroblasts produced significantly less collagen type-I mRNA compared to wild type primary TECs and to wild type primary myfibroblasts following TGF- $\beta$  stimulation. \*:p<0.05.

To assess whether TLR4 deficiency altered the susceptibility towards TGF- $\beta$  signaling, the renal expression of BMP and Activin membrane-bound inhibitor (Bambi) was determined. Bambi mRNA was significantly elevated 3 and 14 days post-UUO in the obstructed kidneys of TLR4<sup>-/-</sup> mice when compared to kidneys of wild type mice (figure 7).

#### Renal tubular epithelial cells and myfibroblasts promote fibrosis via TLR4

To explore the mechanism by which TLR4 contributes to renal fibrogenesis primary TECs and myfibroblasts of wild type and TLR4<sup>-/-</sup> mice were stimulated with TGF- $\beta$ , after which the relative levels of collagen type-I mRNA were determined. The level of collagen type-I mRNA expression was enhanced in both wild type TECs and myfibroblasts following TGF- $\beta$  stimulation when compared to respectively unstimulated wild type TECs or myfibroblasts. Interestingly, TLR4<sup>-/-</sup> TECs and myfibroblasts produced significantly less collagen type-I mRNA after TGF- $\beta$  stimulation compared to Wt TECs and myfibroblasts, respectively. A similar trend was still observed after 72h of TGF- $\beta$  stimulation (figure 8a, b).

Bambi mRNA expression was significantly elevated in both unstimulated TLR4<sup>-/-</sup> TECs and TLR4<sup>-/-</sup> TECs that were stimulated for 72h with TGF- $\beta$  when compared to their specific wild type control TECs (unstimulated wild type vs. TLR4<sup>-/-</sup> TECs  $0.47 \pm 0.13$  vs.  $1.42 \pm 0.51$  arbitrary units, p<0.05; stimulated wild type vs. TLR4<sup>-/-</sup> TECs  $0.22 \pm 0.06$  vs.  $1.11 \pm 0.34$  arbitrary units, p<0.05). Moreover, unstimulated TLR4<sup>-/-</sup> myfibroblasts showed significant enhanced Bambi mRNA expression when compared to unstimulated wild type myfibroblasts ( $0.17 \pm 0.04$  vs.  $0.44 \pm 0.16$  arbitrary units, p<0.05). After 24h and 72h of TGF- $\beta$  stimulation TLR4<sup>-/-</sup> myfibro-

blasts showed a tendency towards enhanced Bambi expression when compared to wild type myofibroblasts (24h stimulated wild type vs. TLR4<sup>-/-</sup> myofibroblasts 0.21±0.03 vs. 0.32±0.08 arbitrary units, p=0.083; 72h stimulated wild type vs. TLR4<sup>-/-</sup> myofibroblasts 0.19±0.03 vs. 0.28±0.03 arbitrary units, p=0.083).

## Discussion

Irrespective of the primary insult, the final common pathway of many chronic kidney diseases is the development of renal fibrosis. More insights into the primary mechanisms that cause renal fibrosis may contribute to the development of specific therapeutic strategies aimed to block or slow down progression of renal diseases.

Most TLRs, including TLR4, are expressed in the kidney and have been shown to play a pivotal role in various experimental models of renal injury and in renal transplantation<sup>24,25</sup>. Recently, we reported that the endogenous danger ligands hyaluronan<sup>15</sup>, HMGB1, biglycan and Gp96<sup>14</sup>, which have the potential to activate TLR2 and TLR4<sup>9,11-13</sup> are significantly upregulated during UUO. We also demonstrated that TLR2 deficiency does not affect fibrogenesis and renal injury during chronic obstructive nephropathy<sup>14</sup>. Since these ligands can also activate TLR4, we aimed to elucidate the role of TLR4 in chronic obstructive nephropathy. In the current study, we find that TLR4 mRNA is progressively enhanced following UUO. This may reflect an increased expression of TLR4 by TECs and/or a local accumulation of TLR4-positive macrophages and myofibroblasts. In addition, we show that TLR4 attenuates tubular injury following UUO which may be a consequence of an early increase in tubular proliferation in wild type kidneys compared to TLR4<sup>-/-</sup> kidneys. This finding is consistent with previous studies demonstrating that TLR4 is involved in cell proliferation<sup>26,27</sup>. Another explanation for the protective role of TLR4 on tubular injury could be that TLR4 activation following UUO induces the expression of several adhesion molecules that may be essential for the preservation of tubular architecture and integrity. Indeed, it has already been demonstrated that TLR4 activation can trigger fibroblasts<sup>28</sup>, epithelial cells<sup>29</sup> and endothelial cells<sup>30</sup> to express several adhesion molecules. In addition, an effect of TLR4 deficiency on epithelial-mesenchymal transition (EMT) processes can also be of importance, since EMT processes contribute to disturbance of the epithelium<sup>31,32</sup>. This should be investigated in more detail in future.

One of the characteristics of tubulointerstitial injury is an excessive inflammatory response, mainly characterized by infiltrating macrophages that contribute to renal fibrosis<sup>33</sup>. Since TLRs induce inflammation upon activation, we hypothesized that TLR4 deficiency diminishes inflammation with a subsequent dampening effect on progressive injury in the obstructed kidneys. Surprisingly, inflammatory parameters are comparable in obstructed kidneys of wild type and TLR4<sup>-/-</sup> mice, as reflected by similar levels of renal chemokines KC and MCP1 and comparable numbers of infiltrating macrophages following UUO, whereas three days post-UUO macrophage accumulation is significantly higher in TLR4<sup>-/-</sup> mice. This result is in sharp contrast with previous findings

that showed a severely reduced inflammatory response in kidneys of TLR4<sup>-/-</sup> mice compared to wild type mice after acute renal ischemic injury<sup>20,21</sup>. Apparently, TLR4 activation leads to a profoundly different outcome of injury and inflammation during acute (ischemic) or chronic (UUO) renal injury. One possible explanation for the similar degree of inflammation in both groups of mice after UUO could be that the increased renal injury in TLR4<sup>-/-</sup> mice leads to an increased amount of multiple endogenous stress ligands that can activate several receptors, including members of the TLR/IL1 superfamily. As a consequence, the net effect may be a profound inflammation, thereby neutralizing the negative impact of TLR4 deficiency on inflammation. In support of this hypothesis we observe that TLR4<sup>-/-</sup> mice display enhanced levels of endogenous danger ligands in obstructed kidneys following UUO, including HMGB1 and GP96 that can also signal via TLR4-independent cascades<sup>9,12</sup>. The significance of the higher macrophage accumulation 3 days post-UUO in TLR4<sup>-/-</sup> mice is however not clear and further studies will be necessary to elucidate this. Even though TLR4<sup>-/-</sup> mice show a similar degree of inflammation compared to wild type mice, they display dramatically lower amounts of fibrosis. Apparently, the magnitude of inflammation due to TLR4 activation does not correlate with the extent of fibrosis.

Our data reveal that despite the extensive tubular injury observed in the TLR4<sup>-/-</sup> mice following UUO, renal fibrosis is clearly attenuated as reflected by reduced collagen deposition. This result is in agreement with the report of Seki et al. that demonstrated the importance of TLR4 in hepatic fibrosis following bile duct ligation<sup>16</sup>. Moreover, another study demonstrated that fibroblasts produced profibrotic chemokines (e.g. MCP1) upon activation in a TLR4-dependent manner in a model of systemic sclerosis<sup>34</sup>. The reduced fibrosis in our study can be the consequence of a disturbed balance between ECM component synthesis and degradation. We first investigated whether TLR4 deficiency affects the accumulation of myofibroblasts following UUO, since activated fibroblasts can become matrix-producing myofibroblasts by several stimuli that are associated with tissue injury<sup>22</sup>. Despite a progressive accumulation of myofibroblasts upon UUO, no differences are observed between wild type and TLR4<sup>-/-</sup> mice. Although these data show that TLR4 does not affect the accumulation of myofibroblasts in obstructed kidneys, it does not rule out a role for TLR4 in the activation of (myo)fibroblasts that subsequently can enhance their matrix-synthesis capacity. Indeed, it has been described that (myo)fibroblasts can express TLR4<sup>35,36</sup> and that TLR4 drives myofibroblast activation in the liver<sup>16</sup>. To see whether the reduced renal fibrosis observed in the TLR4<sup>-/-</sup> mice could be ascribed to elevated ECM-degradation, we determined the activity of MMP2 and MMP9. The role of MMPs and their regulators in tubulointerstitial injury has already been investigated extensively<sup>37-39</sup>. Our results show no difference in the activity of MMP2 between both groups. Surprisingly, we observe that MMP9 activity is slightly lower in renal homogenates of TLR4<sup>-/-</sup> mice 14 days post-UUO. These data suggest that MMP-mediated ECM breakdown is not responsible for the markedly decreased fibrogenesis in TLR4<sup>-/-</sup> mice. In progression of renal diseases, TGF $\beta$  and HGF exert reciprocal and essential functions. TGF $\beta$  is known to promote fibrosis, while HGF has been described to act in a reno-protective fashion<sup>40,41</sup>. In order to investigate whether TLR4 deficiency alters the balance between TGF $\beta$  and HGF,

protein levels were determined in the renal homogenates. Despite major differences in renal fibrosis between wild type and TLR4<sup>-/-</sup> mice, similar concentrations are observed for total and active TGF $\beta$  protein. In addition, TLR4 deficiency does not alter concentrations of HGF, except for 3 days post-UUO. To determine whether TLR4 could affect the susceptibility towards TGF $\beta$ -signaling, we analyzed the renal levels of Bambi. Previous studies demonstrated that Bambi lacks an intracellular kinase domain and as a consequence inhibited TGF $\beta$  signaling by forming non-productive complexes with TGF $\beta$  receptors<sup>42</sup>. We observe that TLR4<sup>-/-</sup> mice have elevated levels of Bambi mRNA when compared to wild type mice following UUO. This result is in line with the study of Seki et al. revealing that TLR4 can sensitize hepatic stellate cells to TGF $\beta$ -mediated signals by down regulation of Bambi<sup>16</sup>. Thus, the lower amount of renal fibrosis that we observe in TLR4<sup>-/-</sup> mice might be explained by a decreased susceptibility towards TGF $\beta$ -mediated fibrosis as a result of higher expression of the negative regulator Bambi.

Finally, we explored the mechanism by which TLR4 contributes to renal fibrogenesis. We found that TECs and myofibroblasts induce a profibrogenic response upon TGF $\beta$ -stimulation in a TLR4-dependent manner, as reflected by a reduced collagen type-I mRNA synthesis by TLR4<sup>-/-</sup> TECs and TLR4<sup>-/-</sup> myofibroblasts. Interestingly, TGF $\beta$ -stimulated primary TLR4<sup>-/-</sup> TECs demonstrate elevated Bambi mRNA expression, when compared to stimulated wild type TECs. We even find elevated Bambi mRNA expression in unstimulated primary TLR4<sup>-/-</sup> TECs and myofibroblasts compared to respectively non-stimulated wild type TECs and myofibroblasts. Together these data suggest that Bambi may sensitize TECs and myofibroblasts to TGF $\beta$ -induced signals and as a consequence to reparative or reactive processes. An alternative explanation could be that TECs and myofibroblasts are directly stimulated to produce collagens via TLR4 and stress ligand interaction. Taken together, these data show that TLR4 can increase renal fibrosis most probably by altering the susceptibility of renal cells towards TGF $\beta$ -signaling, which is associated with a down regulation of Bambi mRNA.

In a previous study we did not find a role for TLR2 in the development of renal fibrosis and injury after UUO, although TLR2 deficiency reduced the accumulation of myofibroblasts<sup>14</sup>. Likely, the contrasting function of TLR2 and TLR4 in this experimental setting reflects the individual functions these molecules may have during progressive renal injury, among which the exclusive link between TLR4 signaling and Bambi-mediated TGF $\beta$ -susceptibility. Hence, these data highlight the importance of unraveling individual TLR signaling pathways. Despite similarity in potential endogenous ligands (i.e. biglycan, HMGB1, and Gp96 can activate both TLR2 and TLR4), individual TLRs exert different functions. Indeed, Seki et al. reported that TLR4 and not TLR2 was required for hepatic fibrosis<sup>16</sup>.

In conclusion, our results demonstrate that TLR4 exerts important functions during chronic obstructive nephropathy. This study is the first to show that TLR4 attenuates early tubular damage and exerts profibrogenic properties following UUO via increased TGF $\beta$ -susceptibility. Thus, TLR4 inhibition may be a new therapeutic target to prevent the progression of chronic renal diseases.

## Materials & Methods

### Mice

Pathogen-free 8 week old female wild type C57Bl/6 mice were purchased from Charles River Laboratories. TLR4<sup>-/-</sup> mice were a generous gift of Dr. S. Akira and generated as described previously<sup>43</sup>, backcrossed six times to a C57Bl/6 background and bred into the animal facility of the Academic Medical Center in Amsterdam, the Netherlands. Only age and sex-matched mice were used in experiments. The Animal Care and Use Committee of the University of Amsterdam approved all experiments.

### Unilateral ureter obstruction

Unilateral ureter obstruction (UUO) was induced as described previously<sup>15</sup>. Briefly, all mice received preoperative analgesia (subcutaneous injection of 50µg/kg buprenorphin (Temgesic, Shering-Plough)) and subsequently the right ureter was 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 nonobstructed kidney served as control. Mice (n=7-8/group) were sacrificed 1, 3, 7 and 14 days post-UUO. To detect proliferating cells, 5-bromo-2'-deoxyuridine (BrdU; Sigma Chemical Co, St Louis, MO) was injected intraperitoneally one hour before sacrifice (40mg/kg body weight).

### Quantitative real-time RT-PCR

Total RNA was extracted from thirty 20µm frozen renal tissue sections of wild type and TLR4<sup>-/-</sup> mice 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. Mouse TLR4, HMGB1, GP96, Biglycan, Bambi,  $\alpha$ -smooth muscle actin ( $\alpha$ SMA), E-cadherin, collagen type-I mRNA expression was analyzed by real-time quantitative reverse transcription-PCR (RT-PCR) performed on a Roche lightcycler with SYBR green PCR master mix. Specific gene expression was normalized to household genes (mouse HPRT, TBP and CycloG gene expression). SYBR green dye intensity was analyzed with linear regression analysis.

### Scoring of histopathology

Renal tissues were fixed for 24hrs in 10% formalin and subsequently 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 was estimated by a pathologist in a blinded fashion using a 4-point scale according to the following criteria: tubular dilatation, cast deposition, brush border loss and necrosis in 10 randomly chosen, non-overlapping high power 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.

#### Detection of apoptosis and proliferation

For detection of apoptosis, antigen retrieval was performed by boiling tissue sections of 5 $\mu$ m for 10min in 10mM Tris/1mM EDTA, followed by blocking endogenous peroxidase activity and incubation with rabbit anti-human active caspase-3 polyclonal antibody (Cell Signaling Technology). To stain for BrdU, DNA was denatured in 2M HCl, and antigen retrieval was performed by 0.4% pepsin in 0.01M HCl. Sections were subsequently exposed to mouse IgG1 anti-BrdU antibodies (Sigma-Aldrich). The slides were developed using 1% H<sub>2</sub>O<sub>2</sub> and DAB (Sigma-Aldrich) in 0.05M Tris-HCl (pH 7.9), and counterstained with methyl green<sup>2</sup> (Sigma-Aldrich). Caspase-3 and BrdU immuno stainings were quantified by counting the numbers of positive tubular epithelial cells in at least 10 non-overlapping high power fields (magnification 400x). Since tubular atrophy was very severe 14 days post-UUO in both groups, identification of epithelial cells was difficult. Therefore, the total amount of positive cells was quantified at this time point.

#### Detection of macrophage infiltrates

Renal sections were deparaffinized and antigen retrieval was performed by 0.1% trypsin digestion (BDH). Slides were subsequently exposed to rat anti-mouse F4/80 IgG2b mAb (Serotec), followed by incubation with rabbit anti-rat biotin (DakoCytomation) and subsequently incubated with streptavidin-ABC solution (DakoCytomation). The slides were developed as described above and counterstained with methyl green. The immunostaining for macrophages was quantified digitally using Image Pro Plus software version 5.0.

#### Detection of fibrosis

For staining of total 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 (myo)fibroblast staining antigen retrieval was performed by boiling for 10 minutes in 10mM sodium citrate buffer (pH 6.0). Subsequently, slides were exposed to mouse anti-human  $\alpha$ SMA-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  $\alpha$ SMA and Picro Sirius Red staining 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 MgCl<sub>2</sub>·H<sub>2</sub>O, 1mM CaCl<sub>2</sub> and 1% Triton-X), and incubated for 30min at 4°C. Homogenates were subsequently centrifuged at 14,000rpm for 10min after which the supernatants were stored at -80°C until ELISA's were performed. To correct for protein content, Bradford Protein Assay (Bio Rad) was used with IgG as standard to determine protein levels.

## ELISA

Cytokines and chemokines (Keratinocyte Chemoattractant (KC), monocyte chemoattractant-1 (MCP1), hepatocyte growth factor (HGF) and active and latent transforming growth factor (TGF- $\beta$ ) were measured in kidney homogenates using specific ELISA (R&D systems) according to the manufacturer's protocol. Activation of latent TGF- $\beta$  was done according to the protocol supplied by the manufacturer. The detection limits were 12 pg/ml (KC), 6 pg/ml (MCP1), 156 pg/ml (HGF) and 30 pg/ml (TGF- $\beta$ ).

## Zymography

Total protein was isolated from frozen renal tissue sections of each animal using RIPA-buffer (without protease inhibitors). Equal amounts of protein (12 $\mu$ g) were loaded onto a 10% SDS-PAGE gel containing 2mg/ml gelatin-B (Bloom 225, Sigma). Gels were washed twice with 2.5% Triton-X (BioRad Laboratories) and subsequently incubated overnight at 37 °C in a buffer containing 50mM Tris-HCl (pH 7.5), 200mM NaCl, 5mM CaCl<sub>2</sub> and 0.02% Brij-35 (Sigma). To visualize MMP activity, gels were stained with Coomassie Brilliant Blue, subsequently de-stained and photographed. Levels of latent and active MMP2 and MMP9 were determined by densitometric analysis.

## Isolation of primary renal tubular epithelial cells and myofibroblasts and in vitro stimulation assays

Primary renal tubular epithelial cells (TECs) from wild type (n=4) and TLR4<sup>-/-</sup> (n=3) mice were harvested and cultured as described previously<sup>21</sup>, and grown to confluence in HK2 culture medium, supplemented with 10% FCS, 100 IU/ml penicillin, 100  $\mu$ g/ml streptomycin, 2 mM L-glutamine (all Invitrogen), 1% ITSe and 1% S1 hormone mixture (Sigma). To obtain primary renal myofibroblasts, wild type and TLR4<sup>-/-</sup> mice (n=4/group) were subjected for 7 days to UUO to enhance interstitial fibrosis. Cells of obstructed kidneys were subsequently isolated as described previously<sup>21</sup>, and cultured for 2 weeks in standard culture medium (RPMI 1640 (Gibco), supplemented with 10% FCS, 100IU/ml penicillin, 100 $\mu$ g/ml streptomycin, 2mM L-Glutamine (all Invitrogen)) to improve selective outgrowth of myofibroblasts. Cultured cells disclosed a clear fibroblast-like morphology by phase-contrast microscopy (elongated spindle-shaped cells). To verify myofibroblast-like origin of cells, quantitative RT-PCR analysis was performed for E-cadherin and  $\alpha$ -smooth muscle actin ( $\alpha$ SMA) as described above. E-cadherin PCR was negative, whereas both genotypes demonstrated equal levels of  $\alpha$ SMA mRNA expression, indicating presence of comparable levels of myofibroblasts between wild type and TLR4<sup>-/-</sup> samples (data not shown). After washing with PBS, cells were serum starved for 2 hours and subsequently stimulated with 1ng/ml recombinant human TGF- $\beta$  (R&D systems). Unstimulated control cells remained in normal medium for 3 days. After stimulation, total RNA was isolated using Trizol reagent (Invitrogen) and converted to cDNA as described above. As marker for fibrogenesis, collagen type-I mRNA levels were measured by RT-PCR in the obtained samples and corrected for house-keeping gene expression.



### Statistics

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

### Disclosure

The authors do not have any financial conflict of interest.

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