Pattern recognition receptors, sensing re(n)al danger
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Discussion
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Renal diseases are regarded as a worldwide public health issue. They have an elevated and increasing incidence in the western population, causing a high degree of mortality and morbidity that affects millions of people worldwide each year, particularly the elderly. Acute kidney injury (AKI) is for instance a common clinical problem that affects approximately 5% of all hospitalized patients. Despite major advancements that were made in last decades, mortality rates of all forms of AKI exceed 50% 1-4. Hence, investigating the underlying pathophysiological mechanisms to dampen the level of kidney injury might provide an immense medical, social and economical benefit. Inflammation is a hallmark of most forms of kidney injury and an important factor in the development of many renal diseases 5-8. Despite the knowledge that inflammation is at the root of many serious complications occurring during renal diseases, the primary mechanism through which the kidney becomes inflamed remains however a major question in understanding renal diseases. Currently, evidence is accumulating that pattern recognition receptors (PRRs) can detect endogenous danger molecules that are released upon tissue and cellular injury or accumulate in non-physiologically sites or amounts 9, 10. The detection of these DAMPs results in the induction of inflammatory responses that are essential for the clearance of cellular debris in order to regenerate injured tissue and for the secretion of many components that are involved in repair mechanisms. On the other hand, prolonged or exaggerated inflammatory responses may result in severe collateral damage due to the secretion of many cytotoxic molecules, and therefore can become detrimental.

In the last decades it has been demonstrated that several PRRs, including TLRs, NLRs and their associated proteins are, next to inflammatory cells, also expressed by parenchymal cells of solid organs, among which kidneys 11-13. Therefore, the classical primary focus on inflammatory cells as (part of) the innate immune system is slightly broadening towards a role for intrinsic parenchymal cells of solid organs, such as the kidney.

The aim of the research described in this thesis was to investigate the role of different PRRs in the pathogenesis of acute and progressive kidney injury and to investigate the contribution of renal cells as sensors of sterile cellular injury and inducers of inflammation that occurs upon kidney injury. In particular, we focused on the individual role that TLR4, NLRP3 and RAGE play during renal damage by using experimental models of ischemia/reperfusion (I/R) injury and unilateral ureter obstruction (UUO) that are indicative for respectively the onset of acute kidney injury (AKI) and chronic kidney diseases (CKD).
PRRs induce an exaggerated inflammatory response upon AKI.

The results we described in chapter 2 and 3 of this thesis underline the current concept that PRRs contribute to the induction of an exaggerated proinflammatory response following AKI that leads to renal dysfunction and injury. The results documented in chapter 2 show that genetic absence of TLR4 diminishes renal inflammation that is associated with less tubular damage and a preserved renal function upon renal I/R injury. Bone-marrow chimeric mice and primary renal TECs subjected to I/R injury indicated that renal cell-associated TLR4 plays an important role in the induction of an inflammatory response. These results agree with studies demonstrating the presence of renal tubular TLR4 expression \(^{14}\) and its important role in inducing exaggerated inflammatory responses upon renal I/R injury \(^{15}\). In addition, the observed function for TLR4 was comparable to the role we previously found for TLR2 upon renal ischemic injury \(^{16}\).

In order to investigate which factors are capable to induce inflammatory responses upon renal injury, in chapter 3 we demonstrate that specific forms of necrotic cell death are capable of inducing IL1\(\beta\) secretion by macrophages in a NLRP3-inflammasome-dependent manner. Moreover, mice with genetic absence of inflammasome components NLRP3 or ASC display less renal inflammation associated with a preserved renal function and morphology and a lower mortality rate upon respectively non-lethal and lethal ischemic injury. The role NLRP3 exerts upon AKI and cellular necrosis was later confirmed by other studies \(^{17,18}\).

Besides the well-characterized PRR families, the multi-ligand receptor RAGE has also been shown to induce inflammatory responses following activation by endogenous danger ligands. Our results in chapter 5 demonstrate however that despite a clear increase in RAGE protein and its potential ligands, RAGE deficiency does not influence renal inflammatory parameters or affect the level of renal dysfunction upon renal I/R injury. Apparently, RAGE does not play a crucial role in the onset of inflammation upon renal I/R injury as it does in other organ (ischemic) injury models \(^{19,20}\), which suggests a tissue-specific role for RAGE in ischemic injury.

In contrast to blocking RAGE, the use of anti-HMGB1 antibodies displays protective effects upon renal I/R injury. This suggests that the effect of HMGB1 on renal I/R injury can not be explained by RAGE signaling, but is probably mediated by its interaction with other PRRs, such as TLR4 and NLRP3 \(^{21,22}\). Indeed, others demonstrated that administration of anti-HMGB1 antibodies or recombinant HMGB1 did not have reno-protective effects in TLR4-deficient mice upon I/R injury \(^{23}\). This study suggests that HMGB1 primarily signals via TLR4 upon renal I/R injury. Altogether, this illustrates a certain level of redundancy between different PRRs upon tissue injury.

In order to dissect the intracellular signaling pathways used after TLR activation, we determined in chapter 2 the relative contribution of the MyD88-dependent and
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TRIF-dependent pathways. The selective use of intracellular adaptor proteins might determine the diversity and specificity of a TLR function. We did neither observe an important role for the TRIF-dependent cascade, nor for the MyD88-dependent pathway during I/R injury, which is downstream of almost all TLRs. Hence, we suggest that additional, yet undefined TLR4-dependent/MyD88-independent signaling pathways might exist, as was also suggested in other research lines. In contrast to our study, others showed a preferential usage of the MyD88-dependent signaling cascade upon renal I/R injury. A possible explanation for the observed discrepancy could be that the activation of a specific downstream TLR pathway partially depends on the type and/or amount of DAMPs that are present as a consequence of the severity of injury induced.

Taken together, our data on primary renal tubular cells and bone marrow chimeric mice indicate that renal parenchymal cells play a crucial role upon sterile injury. This broadens the current concept that renal cells are not just innocent bystanders to a concept that renal cells actively participate in sensing cellular damage and translating it into inflammation. Targeting or interfering within inflammatory signaling cascades upon renal (ischemic) injury might dampen the excessive inflammatory response induced by PRRs in order to prevent tissue injury and collateral damage. Hence, blocking signaling of specific innate immune receptors such as TLR4 or NLRP3 or interfering within ligand-receptor binding with anti-HMGB1 antibodies might have beneficial properties following AKI by preserving morphology and function. In line, it has been shown that TLR expression is elevated in renal transplant biopsies with acute rejection and that targeting TLRs might have potential in order to prevent or treat post-ischemic AKI after transplantation. Moreover, experimental studies suggest that inhibition of TLR signaling with therapeutic agents provide protection following renal I/R injury. Although one needs to keep in mind that blockade of TLR signaling might have detrimental effects with respect to defense against invading pathogens, and as such can cause severe clinical complications, arguing against the use of compounds that would completely block a part of the immune system.

The role of endogenous ligands in inducing inflammation upon renal injury.

Initially, it was thought that PRRs were only activated by specific pathogen-associated molecular patterns. In the past few decades it however became clear that many endogenous ligands released upon cellular injury, collectively referred to as ‘danger-associated molecular patterns’ (DAMPs), are also sensed by PRRs and in turn induce inflammation. In this thesis we look into more detail to the expression profile of DAMPs and the role endogenous ligands exert on inflammation following renal injury.

In chapter 5 we show that expression of endogenous danger ligands HMGB1 and
S100B are highly elevated in ischemic kidneys. The use of anti-HMGB1 antibodies provided protective effects during renal I/R injury and highlight that HMGB1 is an important mediator of inflammatory processes\textsuperscript{23, 31, 32}. In chapter 3 and 6 we show elevated protein levels of the TLR4 ligands biglycan, hyaluronan and S100A8/A9 upon ischemic renal injury in vivo. In chapter 3 we furthermore demonstrate that biglycan and hyaluronan can serve a priming role for NLRP3-inflammasome activation. This priming step is a prerequisite in order to enhance levels of NLRP3 and induce pro-IL1β and pro-IL18 expression. These results provide evidence that DAMPs accumulate in the kidney following injury and are essential for NLRP3-inflammasome activation. Ongoing projects will further investigate the relative contribution of epithelial-associated NLRP3 herein. A direct protective effect of NLRP3 on renal epithelial cells upon ischemic injury was also suggested by another group\textsuperscript{17}, although they proposed that the effect of NLRP3 on renal cells is inflammasome-independent, since ASC-deficient mice did not display a comparable phenotype.

Another key observation of chapter 3 is that in particular the mitochondrial subfraction of necrotic cells is responsible for the observed NLRP3-inflammasome-dependent proinflammatory effects. Currently, mitochondria are thought to form a direct link with innate immune signalling\textsuperscript{33-35}. In our experiments signaling via the ATP-receptor P2X7R was required for inflammasome-dependent cytokine production, indicating that ATP released from disrupted cells or generated by actively respiring mitochondria is capable of activating the NLRP3-inflammasome. Mitochondria are thought to be descendants of ancient bacteria. As a consequence, mitochondrial molecules such as formylated peptides and mitochondrial DNA closely resemble bacterial molecular patterns that are normally kept hidden for the innate immune system. Once released and exposed to the immune system, these mitochondrial components have immunopotency\textsuperscript{34, 36}. Moreover, disturbing the intracellular mitochondrial integrity may already result in NLRP3-mediated inflammatory processes in macrophages\textsuperscript{35, 37}.

In chapter 4 we demonstrate that the kidney has the capacity to detect mitochondrial components as signs of cellular injury. We found that renal TECs express several PRRs that can sense mitochondrial components and induce a proinflammatory response that is partially dependent on the mitochondrial subfraction. By performing in vivo studies and analysis of clinical patient material we moreover provide evidence that mitochondrial DNA may form a link between tissue injury and inflammation, as was suggested by different groups\textsuperscript{33, 34, 36, 38, 39}. Collectively, our data underline the current concept that tissue damage leads to accumulation and increased expression of multiple ligands that are described to function as DAMPs for the immune system. In addition, our data indicate that the innate immune system can be activated by
a wide list of endogenous danger molecules that are normally tightly controlled or kept hidden for the immune system, but whose expression patterns and presence are altered during tissue injury.

**Renal repair or sustained renal injury.**
Inflammation is a prerequisite for the kidney to recover from injury as long as its degree is not too severe and does not exceed the kidney’s intrinsic regeneration capacity. An exaggerated or prolonged inflammatory response can induce collateral damage and can progress into end-stage renal disease with loss of renal function. Inflammation can therefore be regarded as a double-edged sword. In chapter 6 we investigated the role of endogenous S100A8/A9 proteins upon ischemic injury with the primary thought that their absence would be beneficial in the acute phase since these proteins may function as DAMPs, in particular for TLR4. As such, their absence might dampen the acute inflammatory response, as was shown in cerebral ischemic models. Our data however demonstrate that S100A8/A9 proteins are responsible for repair and protection against tubular damage. Their absence results in sustained renal dysfunction and a fibrotic and chronic inflammatory response after I/R injury. In particular, we provide evidence that S100A8/A9 controls macrophage polarization and impairs the polarization towards the M2 macrophage subtype that is associated with fibrotic and anti-inflammatory characteristics. These data were in line with the results of Fujiu et al. demonstrating that S100A8/A9 proteins recruit monocytes to the kidney and promote their activation into M1-macrophages. Whether our observed effects of S100A8/A9 on the ischemic kidney appear to be organ-specific or are indicative for a specific renal pathologic mechanism remains unclear and needs to be further investigated. Although, these data indicate that deleting or blocking endogenous molecules might have beneficial effects in the early phase of ischemic injury by dampening acute inflammatory processes, such as shown for HMGB1. On the contrary, these results also indicate that targeting or blocking endogenous molecules might affect repair mechanisms that are a prerequisite to in turn regenerate the injured tissue.

**The role of PRRs following progressive renal injury.**
Irrespective of the primary insult, the final common pathway of many progressing kidney diseases is the development of renal fibrosis. The underlying pathology of progressive renal injury can be characterized by multiple processes, including inflammation, loss of epithelial integrity and fibrosis. Mechanistic links between inflammation and fibrosis have been proposed. We therefore aimed to study whether the PRRs, TLR4 and NLRP3, play a role in renal pathology following progressive renal disease by using a model of unilateral ureteral obstruction (UUO).
In chapter 7 we demonstrate that TLR4 attenuates the development of tubular damage following UUO but promotes renal fibrosis by modulating the susceptibility of renal cells to TGFβ stimulation. Surprisingly, TLR4 deficiency did not affect renal inflammatory parameters, which is in sharp contrast with the findings we observed for TLR4 deficiency upon AKI (see chapter 2). Apparently, TLR4 activation leads to a profoundly different outcome of injury and inflammation during acute or chronic kidney injury. Even though TLR4-deficient mice do not display an effect with respect to renal inflammation during UUO as compared to wild type mice, they display dramatically lower amounts of renal fibrosis. This indicates that the magnitude of inflammation does not correlate with the extent of fibrosis. The lower amount of renal fibrosis observed in TLR4-deficient mice might be explained by a decreased susceptibility towards TGFβ-mediated fibrosis as a result of higher expression of the negative regulator of TGFβ signaling, Bambi. In accordance, another group demonstrated that TLR4 is a significant mediator of fibrotic renal injury. Moreover, TLR4, but not TLR2 was shown to function as the molecular link between proinflammatory and profibrotic signals in the liver after LPS administration, via modulation of Bambi-mediated TGFβ susceptibility. In contrast to TLR4, we and others previously demonstrated that TLR2 does not affect renal fibrosis or renal interstitial damage upon UUO. The endogenous danger ligands Gp96, biglycan and HMGB1 that can signal via both TLR2 and TLR4 are shown to be upregulated in the obstructed kidney. The difference in phenotype observed between TLR2- and TLR4-deficient mice suggests that signaling of these endogenous ligands might be primarily TLR4-dependent upon UUO-induced injury. In addition, a molecular link as described between TLR4 and fibrosis is lacking for TLR2 that may explain the differences observed in fibrotic responses upon UUO. Furthermore, this would argue against the possibility that the function of individual PRRs can be compensated or taken over by others.

Comparable to the results observed for TLR4, we demonstrate in chapter 8 that the NLRP3-inflammasome attenuates tubular damage upon UUO. In particular, we observe elevated levels of interstitial edema in NLRP3-deficient mice and protective effects of NLRP3 on epithelial and endothelial integrity in the early phase upon UUO. This suggests that NLRP3 exerts additional roles besides its traditional function in caspase-1-dependent activation of precursor cytokines, as was also suggested by other studies. It remains however unresolved whether these effects appear to be mediated directly via NLRP3 or indirectly. In contrast to what has been shown by others, we do not observe a role for NLRP3 in renal fibrosis and found only a minor role on renal inflammation upon UUO. These opposing results could merely reflect differences in the models and sensitivity of experimental techniques used between studies.
The protective effect of NLRP3 on renal injury following UUO was in sharp contrast to the results we (chapter 3) and others have shown for NLRP3 upon AKI. Collectively, we demonstrate opposite functions for TLR4 (chapter 2 and 7) and NLRP3 (chapter 3 and 8) between acute and progressive kidney injury. Obviously, signalling via PRRs 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, and highlights the importance of unraveling individual PRR signaling pathways in different pathological models.

Overall, the research described in this thesis indicates that several pattern recognition receptors that are part of the innate immune system are also expressed in the kidney. Renal TECs actively sense cellular danger through these PRRs and in turn are able to translate this into an effective inflammatory response. Inflammation is at the root of many kidney diseases, but its magnitude determines whether the outcome is beneficial or will contribute to the progression of kidney injury.

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