Pattern recognition receptors, sensing re(n)al danger
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General Introduction
1. The kidney

The primary function of a kidney is the maintenance of body homeostasis. This role is exerted through the filtration of circulating blood and the subsequent reabsorption of water, salts and other essential small molecules within different renal compartments. Additionally, the kidney excretes waste products such as metabolites, breakdown products and salts by the formation of pro-urine. In order to maintain body homeostasis, kidneys filtrate approximately 180 liters of blood in a daily manner. Other important roles for the kidney are the regulation of blood pressure through the regulation of water and salt reabsorption/secretion and the release of important hormones, including renin, erythropoietin, prostaglandins and vitamin D ¹.

A normal human kidney consists of about 1.2 million nephrons, which are the smallest functional units of a kidney. A nephron can be segmented in different structures beginning with the glomerulus, where filtration of the blood takes place. In the next connected tubular compartment, reabsorption of proteins, water and salts is regulated via either active or passive transport mechanisms. Individual segments of this tubular compartment include the proximal tubule, the loop of Henle, the distal tubule and the collecting duct system, which each exert specific roles with respect to the reabsorption of water and solutes and the resultant formation and concentration of pro-urine. The inner layer of the tubular compartments is formed of a single layer of tubular epithelial cells (TECs) and controls reabsorption via transcellular and paracellular pathways. In the end, the remaining pro-urine will flow off via the urethras to the bladder.

2. Renal diseases relevant for this thesis

Kidney diseases and renal injuries are regarded as a worldwide public health issue. They have a high and increasing incidence in the western population, causing a high degree of mortality and morbidity that affects millions of people worldwide each year, particularly in the elderly ², ³. As a consequence of western life style and the raise in the absolute number of elderly people due to the climbing mean age of humans, the incidence of diabetes, metabolic syndrome and cardiovascular events is becoming a major burden to the society. Since these varying etiologies are commonly associated with or results in the development of renal injury, the incidence of various renal injuries in turn increases. Hence, investigating the underlying pathophysiological mechanisms that contribute to halt or dampen the level of renal injury might provide an immense medical, social and economical benefit.
There exist several pathological renal etiologies that can be roughly characterized either as a form of acute kidney injury (AKI) or once progressing and sustaining for a prolonged period of >3 months may lead to and regarded as chronic kidney disease (CKD). The underlying pathology might however differ completely between various renal injury etiologies. In order to delimitate the scope of this thesis, we focused on two different models of renal diseases, the first is a model of ischemia/reperfusion (I/R) injury which is a major cause of AKI and the second is a model of progressive renal disease that is representative for the development of chronic kidney disease.

2.1 Acute kidney injury

Acute kidney injury (AKI) is defined as a sudden drop of kidney function that results in retention of waste products, including plasma urea and creatinine. Depending on its severity and duration, AKI can be accompanied by metabolic disturbances, changes in body fluid balance and effects on many other organ systems. Causes leading to AKI can be broadly subdivided into three categories, pre-renal, intrinsic renal and post-renal causes. Of these, the major intrinsic trigger of AKI and the main focus of this thesis is renal ischemia, caused by an insufficient blood supply to the kidney followed by reperfusion. Ischemia/reperfusion (I/R) injury occurs in several clinical settings, among which shock, trauma, sepsis, vascular surgery and during renal transplantation procedures. Despite major advancements in knowledge of the underlying pathophysiology, AKI still remains a common clinical problem affecting approximately 5% of all hospitalized patients, while mortality rates of all forms of AKI remain above 50%. Moreover, treatment is still only supportive, emphasizing the need to develop therapeutic interventions to prevent or reduce renal injury associated with AKI.

As a consequence of impaired renal blood flow following ischemia, a dysbalance develops between supply and demand of oxygen and nutrients to the kidney, concomitant with depletion of ATP, the major cellular energy source. The resulting pathophysiological processes can be characterized as a complex interplay between renal hemodynamics, tubular epithelial and endothelial cell injury and inflammatory processes.

Endothelial cells are important for the regulation of vascular tone and perfusion, vasopermeability, prevention of coagulation and the regulation of inflammatory cell migration. Following I/R injury, endothelial cells may become damaged, can detach from vessels and are prone to undergo necrosis and/or apoptosis. As a result, regulation of normal physiological processes is disturbed which can cause vascular
congestion, edema formation and a diminished blood flow. Renal I/R injury is also associated with an upregulation of endothelial adhesion molecules (e.g. ICAM-1 and selectins) which enables the infiltration of subsets of inflammatory cells 18. The majority of renal parenchyma consists of tubular epithelial cells (TECs) that are highly polarized cells carrying out reabsorption of proteins and minerals from the urinary fluid by active and passive transport mechanisms. TECs have a physiologically high demand for oxygen and energy (e.g. ATP) to maintain normal processes, which makes them highly susceptible to injury upon hypoxic conditions. Especially TECs from the proximal tubules located in the S3 segment of the cortico-medullary region are sensitive to I/R injury because of the local microvascular architecture and high oxygen demands of the tubule segments located in this region 19.

During hypoxic conditions tubular integrity is threatened as TECs lose their cellular polarization thereby affecting cell-cell contacts, cell-matrix interactions and the apical microvillar extensions that form the tubular brush border of proximal tubules 20-22. Moreover, a significant part of TECs will eventually die by either necrosis or apoptosis 23, resulting in disruption of tubular integrity and back-leakage of primary urine to the interstitium that in turn aggravates interstitial edema. In addition, the production of reactive oxygen species (ROS) by mitochondrial dysfunction increases upon ischemia and sustains during reperfusion, thereby causing cellular injury by oxidation of proteins, DNA damage and induction of apoptosis 24. Altogether, the early pathological events caused by I/R injury can be observed by morphological changes such as loss of the brush border, TEC simplification, TEC detachment, apoptosis and massive necrosis of TECs, cast deposition in the tubular lumen and tubular dilatation (figure 1). In a cross-sectional renal tissue slide, a typical horseshoe-shaped region in the cortico-medullary region can be identified that is most prone to become damaged following I/R injury.

A major characteristic of renal I/R injury is a local inflammatory response. This can be viewed morphologically by an apparent increase in the number of infiltrating polymorphonuclear cells (PMNs; e.g. granulocytes) early upon damage, whereas in a later extend during the reperfusion phase the number of accumulating macrophages and lymphocytes becomes more evident 25, 26. Moreover, upon renal injury the levels of several proinflammatory cytokines/chemokines are highly elevated compared with non-inflamed tissue. These include for example the early central cytokines Tumor Necrosis Factor-α (TNFα), Interleukin (IL)-1β and -6 and the chemoattractants Keratinocyte Chemoattractant (KC; the murine homologue of IL-8) and Monocyte Chemoattractant Protein-1 (MCP1) 27. Diverse processes that are ongoing upon renal I/R injury altogether contribute to the induction of both innate and adaptive immune responses 25, 28. Damaged TECs that undergo either apoptosis or more commonly necrosis contribute to inflammation
following I/R injury. Apoptosis is generally considered as being immunologically silent since apoptotic cells disintegrate into multiple cellular blebs that are subsequently cleared and taken up by phagocytic cells like macrophages. On the contrary, necrosis results in cellular burst and the (uncontrolled) release of intracellular immunopotent molecules into extracellular space, including several danger-associated molecular patterns (DAMPs; see next paragraph in introduction). Notably, apoptotic cells can also undergo a process of secondary necrosis, in turn amplifying the inflammatory response by release of endogenous molecules. As a consequence of massive TEC necrosis, elevated numbers of leukocytes accumulate in the kidney, enabling the recognition of specific DAMPs released by these necrotic cells. In addition, remaining surviving TECs can also sense and recognize the released DAMPs. The subsequent intracellular signals are then translated into effective inflammatory responses by these leukocytes and surviving TECs, thereby exaggerating the inflammation. In addition, remaining viable TECs are important in the generation and secretion of proinflammatory mediators, like cytokines and chemokines. In addition, remaining TECs enhance the expression of adhesion molecules that in turn favors the attraction of inflammatory cells. Furthermore, local activation of the complement system also contributes to I/R-induced damage in the kidney.

Currently, it is still a matter of debate how the kidney is able to translate (ischemic) cellular damage into an inflammatory response. Evidence is currently accumulating that intrinsic renal cells, in particular TECs, are not just innocent bystanders but in fact actively participate in monitoring renal (cellular) injury. In this respect it is interesting to note that renal parenchyma expresses a diverse array of several pattern recognition receptors (PRRs) that can induce a proinflammatory response upon the recognition of cellular injury-associated molecules, and hence contribute to renal inflammation (described elsewhere in this introduction). It thus seems that a complex interplay exists between intrinsic renal cells with resident and/or infiltrating inflammatory cells enabling the induction of inflammation following AKI. Part of this thesis will further investigate the contribution of specific receptors belonging to the innate immune system in sensing and mediating acute kidney injury in a sterile environment. Providing that the severity of (ischemic) injury does not exceed the kidney’s intrinsic regeneration capacity, it can completely recover from an ischemic insult. While necrotic cells and intratubular casts are removed actively by professional phagocytes, remaining TECs will proliferate and replace the injured epithelium and thereby restore tubular integrity. The primary mechanism behind tubular regeneration is thought to be the dedifferentiation, spreading and migration and subsequent proliferation of remaining viable TECs. Recovery from I/R injury is furthermore accompanied by angiogenesis and the expression of i.e. growth factors, adhesion molecules, and transcription factors. Morphologically, the phenomenon of tissue repair and
regeneration can be observed by an increasing amount of dedifferentiated and proliferating cells in the corticomedullary area a few days after I/R injury (figure 1). In the end, as tubular architecture and morphology are restored completely, this can be associated with a restored renal function.

In this respect, inflammation is often characterized as a process with a double-edged sword. Eventually, inflammation is a prerequisite for the kidney to recover from injury, since infiltrating inflammatory cells, which are attracted by chemokines locally produced by surviving TECs and resident leukocytes, are necessary for removal of damaged cells and locally produce and secrete cytotoxic molecules. As long as the degree of inflammation is not too severe and does not exceed the kidney’s intrinsic regeneration capacity, the onset of inflammation is beneficial. On the other hand, an exaggerated or prolonged inflammatory response may induce collateral damage that is ultimately detrimental for a kidney’s integrity, and this can progress into end-stage renal disease with loss of renal function.

**Figure 1**: This picture shows PAS-D-stained cross-sectional slides of a murine kidney subjected to a model of renal I/R injury. After 1 day of I/R injury, the cortico-medullary region can be characterized by loss of brush border, massive necrosis of TECs, cast deposition and obstruction of tubular lumen and a robust inflammatory infiltrate. After 3 and 7 days of renal I/R injury, cast and necrotic debris is being removed and remaining TECs start to proliferate. After 14 days, renal epithelial histology is completely restored as can be observed by the presence of functional tubuli.
2.2 Progressive renal injury

Renal fibrosis is considered as the common final and irreversible pathway by which kidney diseases with variable etiology progress to end-stage renal failure, irrespective of the nature of the primary underlying renal disease. Fibro-proliferative diseases, including progressive renal disease, are still a leading cause of morbidity and mortality worldwide. In the kidney, the degree of tubular damage, inflammation and fibrosis of the tubulo-interstitial compartment are main predictors for the loss of renal function and the risk for progression towards end-stage renal failure. Management includes chronic dialysis and/or eventually renal transplantation. As many renal diseases with variable etiology commonly progress to end-stage renal disease, it is hard to define one ‘unique’ or predominant pathological process that results in the development of chronic kidney disease. A commonly acknowledged experimental model that is frequently used to mimic the development of progressive tubulo-interstitial injury is the model of unilateral ureter obstruction (UUO). UUO results in chronic obstructive nephropathy that is still one of the most important causes of renal insufficiencies in children. UUO-induced progressive renal injury involves a complex cascade of several pathological processes that are reviewed elsewhere. Briefly, as a result of enhanced intra-tubular pressure, TECs are subjected to enhanced mechanical stretch that has been implicated as one of the primary insults upon UUO. In addition, a marked number of TECs will die by regulated processes of apoptosis. As a consequence, tubular integrity is lost that, in combination with fluid leakage due to vascular congestion, can result in interstitial edema. Furthermore, the tubulo-interstitium can be characterized by a robust infiltration of inflammatory cells, accumulation of (myo)fibroblasts, tubular atrophy, regression of peritubular capillaries and the enhanced deposition of extracellular matrix (ECM) components, among which collagens (figure 2). Fibrosis is thought to be the consequence of a disturbed balance between ECM synthesis by (myo)fibroblasts and ECM degradation by matrix metallo proteases (MMPs) and excessive and persistent inflammation. The majority of inflammatory cells that massively accumulate in the chronically failing kidney due to ureteral obstruction are macrophages, which promote renal fibrosis. Macrophages contribute to renal injury by inducing cell toxicity, damage to the basement membrane and interstitial fibrosis. Moreover, macrophages produce and secrete a panel of proinflammatory cytokines/chemokines, hence accelerating renal inflammation and may produce other profibrotic factors such as transforming growth factor-β (TGFβ). On the other hand, macrophages are also involved in renal repair mechanisms by clearance of apoptotic debris and the secretion of antifibrotic mediators.
Another key event during progressive renal injury is the accumulation of α-smooth muscle actin (αSMA) expressing myofibroblasts. Through different mechanisms, myofibroblasts become activated and contribute to excessive and progressive deposition of ECM proteins. The origin and character of myofibroblasts is still controversial, although matrix-producing myofibroblasts may derive from different cellular origins, including resident fibroblasts, endothelial cells, pericytes or cells from hematopoietic progenitors. Moreover, emerging evidence suggests that TECs undergoing a process referred to as epithelial to mesenchymal transition (EMT) may contribute to the destruction and loss of tubular epithelium and the subsequent accumulation of renal myofibroblasts.

TGFβ is considered as the most important cytokine exerting profibrotic effects that also promotes EMT processes upon progressive renal injury. Once bound to its receptor, many intracellular signaling cascades may be activated such as SMAD protein-dependent downstream pathways. TGFβ signaling induces downstream expression of other profibrotic mediators including connective tissue growth factor.

**Figure 2:** This figure (adapted and modified from: Bascands J.L. et al.) schematically presents the continuing pathological processes that occur following progressive renal injury due to obstructive nephropathy. Following UUO, TECs become damaged due to accumulated intratubular pressure and mechanical stretch and in turn undergo apoptosis. As a consequence, tubular integrity disappears and eventually tubuli withdraw through tubular atrophy. Moreover, inflammatory cells (e.g. macrophages) infiltrate into the renal interstitium, whereas fibroblasts become activated and an accumulation of myofibroblasts can be observed. This leads to a progressive deposition of extracellular matrix molecules (e.g. collagens) and scarring of renal tissue.
(CTGF) that in turn stimulates TGFβ-signaling in a positive feedback manner \(^6^4\). The profibrotic properties of TGFβ include the enhancement of matrix synthesis and the decrease of matrix degradation, and its essential role in renal injury has already been demonstrated by intervention assays and genetic knockdown studies \(^6^5, ^6^6\). As TGFβ signaling has very powerful profibrotic properties, its activity needs to be tightly regulated in order to maintain renal homeostasis. Regulation is exerted via several mechanisms, including negative regulation through the protein Bambi, a member of the family of TGFβ receptors \(^6^7, ^6^8\). TGFβ signaling, in turn, induces Bambi expression by a negative feedback mechanism \(^6^9\). In addition, signaling by bone morphogenetic proteins (BMPs; in particular BMP7) counteract the effects of TGFβ with respect to EMT and tubular damage \(^7^0\).

The role that individual receptors belonging to the (innate) immune system exert in the initiation of inflammation and fibrosis following progressive renal injury still remains unclear. Part of this thesis will focus on the role of individual PRRs in the underlying pathology of UUO.

### 3. The innate immune system

Following evolution almost all species acquired a more or less complex immune system enabling the detection of potential hazardous and foreign pathogens such as bacteria, parasites, viruses and fungi. In most vertebrates the immune system can be roughly divided into a non-specific innate immune system (responsible for the initial and rapid response towards dangerous particles) and an acquired, highly-specific adaptive immune system. Generally, exposure to a foreign structure initially activates an innate immune response to battle against the invading pathogen while concomitantly an adaptive immune response is arranged that eventually results in highly specific protection against a particular pathogen and besides also develops immunological memory. Hence, the immune system essentially protects an organism against exposure towards foreign pathogens to maintain a body’s integrity. The innate immune system exerts the initial response to a foreign pathogen through the recognition of various pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharide (LPS or endotoxin as a component of gram-negative bacteria) or peptidoglycans (a component of gram-positive bacteria). In addition, the innate immune system detects dying or damaged intrinsic cells in a sterile environment. The resulting inflammatory response facilitates the clearance of these necrotic and damaged cells and supports the subsequent healing process of tissue \(^7^1\). At that time, the concept comprising the ability of the innate immune system to detect both foreign particles as well as intrinsic endogenous molecules led to the formation of
the ‘danger-hypothesis’ of Polly Matzinger \(^{72}\). Herein she proposed that the immune system evolved to respond not to infection *per se* but to non-physiological cell death, damage and cellular stress. As a consequence, it was appreciated that the innate immune system could also respond to several endogenous ‘danger’ molecules, to which collectively was referred as the ‘damage-associated molecular patterns’ (DAMPs). This heterogeneous group of molecules includes degradation products of macromolecules, products of proteolytic cascades, intracellular components of ruptured cells and products of genes that are activated by inflammation. Several of these DAMPs accumulate in non-physiological sites or amounts during tissue injury \(^{73}\).

### 3.1 Pattern Recognition Receptors

For the rapid detection of a diversity of PAMPs as well as DAMPs, the innate immune system has evolved a number of families of highly conserved germ line-encoded pattern recognition receptors (PRRs). These include the well-categorized families of membrane-bound Toll-like receptors (TLRs) \(^{74}\), the intracellular nucleotide-binding domain and leucine-rich repeat containing receptors (NLRs) \(^{75, 76}\), RIG-I-like receptors (RLRs), C-type lectin receptors (CLRs) and absence in melanoma-2 (AIM2)-like receptors \(^{77}\). Moreover, other immunoglobulin receptors are also regarded as PRRs, like the multi-ligand receptor Receptor for Advanced Glycation End products (RAGE) \(^{78}\) as well as members of the family of formyl-peptide receptors (FPRs) \(^{79-81}\). All PRRs demonstrate a cell type-specific expression pattern widely throughout the body. PRRs act as cellular sentinels for detection of hazardous pathogens or cellular damage and stress, and upon activation they in turn exert an inflammatory response that can be characterized by the upregulation of proinflammatory gene expression and the secretion of cytokines, chemokines and other proinflammatory mediators. In this thesis, we focused particularly on specific members of the TLR and NLR family in sensing sterile injury and mediating inflammation.

**Toll-like receptors (TLRs)**

Currently, the family of mammalian TLRs consists of 13 different members, whereas in humans 10 different TLRs are identified \(^{82}\). All TLRs are composed of an extracellular recognition domain consisting of leucine-rich repeats (LRR), a transmembrane region and an intracellular Toll/IL1-Receptor (TIR) signaling domain.

Each TLR recognizes different kind of PAMPs and/or DAMPs and after subsequent formation of homo- or heterodimers intracellular downstream signaling cascades are activated. The potential ligands for TLRs include lipids, lipoproteins and nucleic acids derived from a wide range of microbes such as bacteria, viruses and fungi \(^{74, 83}\).
Additionally, TLRs can be activated by a wide array of DAMPs released upon tissue injury. The type of ligands recognized by a specific TLR is partially dependent on its intracellular localization. According their localization, TLRs can be roughly divided into two arbitrary subgroups. The first group can be found on the cellular surface and includes TLR2 and TLR4, on which part of this thesis is focusing, whereas the other group, including TLR3, TLR7, TLR8 and TLR9, is expressed in intracellular vesicles such as endosomes, lysosomes and in the endoplasmatic reticulum (ER).

Upon ligand recognition the intracellular TIR-domain of a TLR functions as a scaffold for the recruitment of specific adaptor proteins via homotypic interactions between their TIR-domains. Currently, there are five different adaptor proteins identified, among which Myeloid Differentiation primary response gene 88 (MyD88), Myeloid differentiation factor-88 adaptor-like protein (Mal), TIR-domain-containing adapter-inducing interferon-β (TRIF), TRIF-related adapter molecule (TRAM) and sterile α- and armadillo motif-containing protein (SARM). Last decade a lot of effort is put in discovering proteins and transcription factors involved in the intracellular signaling cascades downstream of TLRs and their adaptor molecules. All these signaling pathways are extensively described elsewhere, and are schematically presented in figure 3 in a simplified manner (figure 3). Generally, all TLRs, except TLR3, signal through recruitment of the adaptor protein MyD88 that primarily results in nuclear factor-κB (NFκB)-dependent signaling pathways. Additional signaling routes like map-kinase-kinase (MKKs) pathways are also activated upon TLR signaling. Mal specifically contributes to MyD88-dependent signaling via TLR2 and TLR4. TLR3 and TLR4 however, can signal via MyD88-independent pathways through recruitment of the TRIF adaptor protein leading to interferon (IFN)-β production and late NFκB activation. Herein TRAM provides specificity for the MyD88-independent component for TLR4 signaling. The role adaptor molecule SARM plays upon TLR activation is less well described, although it has been identified as a negative regulator of TRIF-dependent TLR signaling. Hence, the differential recruitment of adaptor proteins by TLRs provides a certain level of specificity to TLR-signaling pathways. Furthermore, upon tissue injury or infection, many DAMPs or PAMPs are released or accumulate that have the potential to activate specific TLRs, and hence a combined activation of several TLRs simultaneously could result in complementary, synergistic effects modulating the downstream innate immune responses.
General Introduction

Nucleotide-binding domain and leucine-rich repeat containing proteins (NLR)

The family of NLRs currently represents an intracellular family of approximately 22 PRRs that can respond to various stimuli, including diverse PAMPs and DAMPs. This family of cytosolic proteins, including the Nucleotide-binding domain and leucine-rich repeat containing proteins (NLRP proteins, e.g. NLRP3), can be structurally characterized as multi-domain proteins containing a region identified by a series of LRRs, a central nucleotide domain termed the NACHT domain and an effector domain. The LRR domain has been implicated in ligand sensing such as in the TLRs, although the exact mechanism is still under investigation. The central NACHT domain is crucial for oligomerization, thereby forming active high molecular weight complexes important for activation. Furthermore, the effector domain mediates

Figure 3: This modified picture (adapted from: O’Neill LA et al.) schematically presents the intracellular signaling pathways that are used by TLRs. TLRs exert downstream signaling by recruitment of (combinations of) specific intracellular adaptor molecules, such as MyD88, Mal, TRIF and TRAM. Briefly, MyD88-dependent pathways mainly result in activation and translocation of NFκB to the nucleus where it stimulates transcription of proinflammatory genes, including cytokines/chemokines. Additional cascades including map-kinase-kinase (M KK) pathways are activated upon MyD88-dependent signaling that induce proinflammatory responses. In addition, TLR4 and also TLR3 signal via the TRIF-dependent pathway that primarily leads to activation of transcription factors IRF-3 and IRF-7 and the subsequent induction of IFN-β inducible genes. Moreover, TRIF-dependent signaling pathways can result in a second, delayed activation of the transcription factor NFκB.
signal transduction to downstream targets that varies between different NLRs and hence determines their biological effect. As we focused specifically on the role of the NLRP3 inflammasome in part of this thesis, hereafter the NLRP3 inflammasome will be used for introduction. The effector domain of NLRP3 contains a PYD-domain that subsequently interacts and recruits the PYD-domain of the cytosolic adaptor molecule ASC (apoptosis-associated speck-like protein containing a caspase recruitment domain). The ASC adaptor then redistributes into the cytosol and subsequently binds its CARD to the CARD-domain of procaspase-1. Together, the resultant formation is a multi-protein scaffold referred to as the ‘inflammasome’. The most important biological function of the NLRP3-inflammasome is the autocatalytic activation of procaspase-1 that subsequently processes and matures precursor cytokines, such as pro-IL1β, pro-IL18 and pro-IL33. IL1β is considered as a central and highly potent cytokine that plays an important role in the innate immune response. Uncontrolled IL1 release or signaling might however result in excessive inflammation with subsequent detrimental effects on tissue homeostasis and hence highlights the necessity of a tight regulation. Indeed, spontaneously secreted active IL1β is already described as the molecular basis of different auto-inflammatory disorders.

Inflammasome activation and subsequent IL1β release is regulated at multiple levels and as first requires an essential priming signal that induces NFκB-dependent expression of precursor cytokines, like pro-IL1β. This priming can be exerted by for instance activation of TLR-signaling pathways. Moreover, the priming signal enhances expression of the NLRP3 protein. An additional secondary stimulus is subsequently necessary for formation of the inflammasome that results in maturation and release of effector cytokines by caspase-1-mediated cleavage. A diversity of ligands is already described that triggers the secondary step activating the inflammasome including several PAMPs and DAMPs (see other paragraph). Due to the high diversity in size and heterogeneity in structure between potent ligands, the mechanism by which these ligands trigger NLRP3 oligomerization and subsequent inflammasome formation are still controversial.

Currently, three different activation models are proposed (figure 4). The first one is the ‘channel model’ proposing that ATP released from necrotic cells, binds to the purinergic P2X7-receptor leading to opening of cation channels that induces potassium efflux and in turn the formation of a large pore mediated by the hemichannel protein pannexin 1. Hence, a subset of agonists could enter cells through these pores and bind directly to NLRP3. Since several ligands are too big for cellular entrance through any type of pore, this model cannot account for all stimuli of the inflammasome. The second proposal is the ‘lysosomal rupture model’ hypothesizing that inefficient clearance of large endocytosed particles leads
to phagosomal destabilization and lysosomal rupture. The resultant cytoplasmic accumulation of lysosomal proteases (e.g. cathepsin B) could then either directly or indirectly activate the inflammasome. Lastly, the third model proposes that different kind of ligands eventually trigger the production of intracellular reactive oxygen species (ROS) who are subsequently linked, via yet unidentified routes to inflammasome activation. The relevance of each of the proposed model pathways remains unclear. It seems however likely, that these models function next to each other and that several ligands collectively cause effective NLRP3 activation, subsequent inflammasome formation and eventually IL1β release.

Figure 4: This picture (adapted from: Schroder K. et al. 2013) schematically presents the formation of the intracellular multi-protein complex called the inflammasome, consisting of a NLRP protein (e.g. NLRP3), the ASC adaptor protein and (pro)caspase-1. Inflammasome activation results in the catalytic cleavage and release of precursor cytokines, such as IL1β, whose precursor-protein synthesis first requires a priming signal that can be exerted by TLR signaling. Currently, three different activation mechanisms are proposed that could result in assembly and activation of the NLRP3 inflammasome. The first represents the ‘channel’ model that enables activation via the entrance of small molecules through membranous pores that directly interact and activate the NLRP3 inflammasome. The formation of these membrane pores is linked to entrance of ATP through its purinergic receptor (P2X7 receptor) or an efflux of intracellular potassium. The second model is the ‘lysosomal rupture’ model, hypothesizing that inefficient clearance of large endocytosed particles leads to phagosomal destabilization and lysosomal rupture. The resultant cytoplasmic accumulation of lysosomal proteases (e.g. cathepsin B) could then either directly or indirectly activate the inflammasome. The third ROS model proposes that all NLRP3 activators that have been studied eventually trigger the production of ROS who are subsequently linked to NLRP3 inflammasome activation.
General Introduction

RAGE
Besides the classical five families of PRRs, the innate immune system contains additional receptors that sense tissue and cellular damage and induce inflammation. One of these is the immunoglobulin superfamily member Receptor for Advanced Glycation End products (RAGE) that was initially identified as a receptor for advanced glycation endproducts (AGEs). In addition, RAGE also recognizes the nuclear protein high-mobility group box1 (HMGB1). HMGB1 can be released either passively by necrotic cells or can be actively secreted by cells in response to injury and functions as a danger signal. In certain cases RAGE transduces signals by acting cooperatively with TLRs, like HMGB1-bound DNA that forms a complex with TLR9 and RAGE to induce proinflammatory cytokines. Furthermore, RAGE can be activated by several molecules that belong to the family of calcium-binding S100/calgranulin proteins, that accumulate at high concentrations in inflamed tissues.

Formyl-peptide Receptors (FPRs)
Members that belong to the family of formyl-peptide receptors (FPRs) are able to sense and detect N-terminal formylated peptides. Currently, there are three human and seven murine FPRs identified and described. Upon ligand sensing and binding, FPRs mediate downstream signaling via G-protein-coupled pathways. Eventually, their activation can result in different responses, including intracellular calcium mobilization, cell migration and release of inflammatory mediators. Most FPRs were initially characterized in phagocytic leukocytes. At this time, it is known that these are expressed on many other different cell types as well, including non-immune cells like epithelium.

As bacteria are the only extrinsic sources that synthesize formylated peptides, FPRs are clearly capable of detecting the presence of proteins derived from microbial origin. Moreover, mitochondria, which are intrinsically present in almost all eukaryotic cells, initiate their peptide chain synthesis by using formylated methionyl-tRNA. As a consequence, mitochondria may be regarded as an additional, intrinsic source of formylated peptides, albeit that these mitochondrial proteins can only act as FPR ligands and chemoattractants once released by damaged or necrotic cells into the extracellular milieu. Hence, in a sterile injury setting FPRs contribute to proinflammatory responses upon activation by mitochondrial-derived formylated peptides that are normally hidden intracellularly.
3.2 Endogenous danger molecules

Since Polly Matzinger introduced the danger-hypothesis, in which she suggested that besides PAMPs, several DAMPs could also be sensed by and in turn activate individual PRRs \(^{72}\), a lot of effort is put into the identification and characterization of endogenous molecules with such an immuno-potency.

The potential of individual TLRs to sense and become activated by a certain DAMP largely depends on their subcellular distribution. TLRs present on the cell surface, like TLR2 and TLR4, were primarily identified as being responsible for the recognition of extracellular microbial membrane components such as LPS (TLR4) and peptidoglycans (TLR2) \(^{130-132}\).

Currently, it is widely accepted that these TLRs are also activated by an expanding list of DAMPs, including breakdown molecules of the extracellular matrix such as biglycan \(^{133}\), hyaluronan \(^{134}\), heparan sulfate \(^{135}\) and also fibrinogen \(^{136}\) and Tamm-Horsfall glycoprotein \(^{137}\). In addition, nuclear proteins such as heat shock proteins (e.g. Gp96) \(^{138-140}\) or HMGB1 \(^{141,142}\) can be sensed by these TLRs when released from dying cells.

The endosomal compartment-associated TLRs like TLR3, TLR7, TLR8 and TLR9 however, are capable of detecting microbial or self nucleic acids (e.g. double-stranded DNA and single- or double-stranded RNA motifs) \(^{86,143,144}\). In particular, TLR9 was found to detect unmethylated CpG motifs, abundantly present in foreign DNA structures, but also in mitochondrial DNA \(^{145}\). Additionally, these TLRs are important in recognition of the nuclear protein HMGB1 \(^{120}\) and dying cells \(^{146}\).

With respect to the NLRP3-inflammasome, many ligands are shown to trigger its activation including PAMPs and environmental irritants such as silica, asbestos and alum \(^{147,148}\). In addition, several DAMPs are potent NLRP3 activators such as extracellular matrix components biglycan \(^{149}\) and hyaluronan \(^{150}\), cholesterol crystals \(^{112}\), uric acid crystals \(^{151}\), reactive oxygen species (ROS) \(^{114}\), extracellular ATP \(^{152}\) and an efflux of intracellular potassium \(^{153}\). Moreover, it was shown that necrotic, but not apoptotic cells can release endogenous danger signals that results in mature cytokine release via the NLRP3-inflammasome \(^{154}\). Notably, it has been shown that some NLRP3 ligands such as biglycan can activate the NLRP3-inflammasome with subsequent IL1β release without the need for an additional priming step \(^{149}\).

All data available at present demonstrated that many endogenous molecules with varying structure, size, physiological function and availability can all be sensed by specific PRRs and in turn may induce a proinflammatory response. Likely, the current list of DAMPs will further expand and extended with additional endogenous molecules of which their (abundant) presence may reflect a state of cellular and/or tissue stress and injury.
3.3 Mitochondria

Nowadays, the potential of mitochondria as inducers or enhancers of sterile inflammatory responses has been commonly recognized. Mitochondria are intracellular organelles present into almost all cell types of eukaryotic organisms, except erythrocytes\(^{155}\). They appear as double-membraned structures with inner cristae, in which their own ribosomes and DNA are present. The number of mitochondria present per cell demonstrates a tissue-specific distribution pattern that is mainly dependent on cellular energy demand and metabolism. As such, liver tissue contains for example relatively high numbers of mitochondria per cell\(^{156}\).

The primary function of a mitochondrion is the generation of ATP, the main organic energy molecule. This aerobic metabolism is exerted through a process of oxidative phosphorylation via the electron transport chain by five different multiprotein complexes that are embedded in a mitochondrion’s inner membrane structure. As a consequence of mitochondrial-dependent ATP production, a cell’s total energy supply profoundly increases. Moreover, mitochondria exert additional biological processes, including fatty acid metabolism, regulation of apoptotic and proliferation processes and calcium storage and signaling\(^{157-159}\).

Interestingly, mitochondria that are locked up in eukaryotic cells have many features in common with prokaryotic cells. This supports the theory of endosymbiosis that hypothesizes the prokaryotic ancestry of mitochondria\(^{160}\). This is strengthened by the fact that mitochondrial ribosomes are similar in size and structure to those of bacteria\(^{161}\), and that mitochondria contain their own DNA (mtDNA) and are therefore the only cellular source of genomic DNA, besides the nucleus. Like prokaryotes, a mitochondrion contains one single circular genome that lacks histones and is enriched in unmethylated CpG motifs, that has been shown to contain immunostimulatory properties\(^{162,163}\). Indeed, purified mtDNA has been shown to be very immunopotent and capable to induce arthritis due to its high levels of CpG motifs and oxidative status\(^{164,165}\). The mtDNA encodes for several ribosomal and transfer RNAs and 13 characterized peptides that all are part of the oxidative phosphorylation enzyme complexes\(^{166}\). Importantly, it has been demonstrated that initiation of mitochondrial peptide synthesis depends on the presence of a formylated tRNA and as a consequence results in formation of N-terminal formylated peptides\(^{128}\). As already mentioned before, these so-called formylated peptides can be derived from only two different natural sources, either from bacterial origin or the ones encoded by the mitochondrial genome, while both provide similar chemotactic signals for leukocytes due to the presence of the formylated group\(^{127,129,167}\). These formylated peptides are sensed and in turn activate the highly sensitive FPRs present on leukocytes\(^{79,126,168}\).
Collectively, mitochondria can be regarded as an additional intracellular source of DAMPs that are normally locked inside the cell. In case of cellular injury or necrosis, or once a mitochondrion’s integrity is disturbed, mitochondria-derived DAMPs become available and are exposed to the immune system 169, 170. Moreover, as a consequence of its physiological function a mitochondrion already contains a certain level of immunopotency such as the production of ATP and the substantial formation of ROS that is as a direct ‘byproduct’ of the oxidative phosphorylation process within mitochondria. As already mentioned in a previous paragraph, ATP as well as ROS were shown to be activating ligands for e.g. the NLRP3 inflammasome and hence induce inflammation 113, 114, 152. As such, mitochondria continuously produce molecules that in certain circumstances may function as a DAMP and may initiate inflammatory cascades.

3.4 Regulation of PRR activity

Excessive or continuing stimulation of the innate immune system (e.g. by DAMP-mediated activation of PRRs) may cause collateral damage and as a result be detrimental to the host. Hence, tight control of receptor activation, downstream signaling cascades and cytokine/chemokine production are essential. As such, the immune system has evolved several means of regulation in order to prevent excessive activation and subsequent detrimental effects. An initial level of regulation is mediated by reduction of receptor expression via protein degradation and/or lowering gene transcription and protein synthesis. Next, soluble decoy proteins (e.g. sTLR2, sTLR4 and sRAGE) can catch away potential ligands and subsequently reduce the total amount of available activators of immune receptors in an environment. In addition, alternatively spliced proteins that lack an essential component and/or have an altered confirmation can block the recruitment of a subsequent protein so that downstream signaling cascades are impaired. Likewise, splice variants are described from proteins of the intracellular signaling cascades of TLRs, including MyD88s and IRAK-M 171, 172. In addition, to prevent overwhelming cytokine stimulation, expression of receptor antagonists can be enhanced like the IL1-receptor antagonist (IL1-RA) 173. Furthermore, receptors belonging to the TLR/IL1 superfamily can negatively modulate immune responses through binding to TLR or IL1R signaling components. These include the receptor T1/ST2 174-176 and the orphan receptor Toll-IL-1 receptor 8 (TIR8), also known as Single Ig IL-1-related receptor (SIGIRR) 177-179. Like TLRs, TIR8 contains an extracellular Ig domain and an intracellular TIR domain. Although no ligands are described to bind and/or activate TIR8, it has been demonstrated that
TIR8 can negatively regulate IL1 and TLR signaling pathways by transient interaction to the TLR4 or IL1 receptor complex, thereby interfering with heterodimerization of the receptor. In turn, the intracellular TIR domain then attenuates the recruitment of proximal signaling components of these receptors. As a result, stimulated cells demonstrate a reduced NFκB and/or JNK activation. Under normal tissue homeostasis activity of innate immune receptors is tightly regulated. In several pathological conditions a dysbalance between activation and inhibition of PRRs or a shortcoming of inhibitory signals may however contribute to an overshooting and detrimental inflammatory response and as such may lead to the development of pathological processes.

4. Expression PRRs in the kidney

As described in the previous section, different subsets of PRRs (TLRs and NLRs including NLRP3) play a prominent role in the induction of a proinflammatory response upon infection, and following sterile tissue injury when activated by several DAMPs. Initially, these receptors and their associated intracellular signaling proteins were thought to be expressed exclusively by various immune cells. Hence, attention was primarily paid to inflammatory cells such as professional antigen-presenting cells, leukocytes and lymphocytes in order to investigate the role and function of individual PRRs. The classical primary focus on inflammatory cells as (part of) the innate immune system is slightly broadening towards a role of intrinsic parenchymal cells of solid organs, like the kidney. Indeed, last decades it has been demonstrated that several PRRs, including TLRs, NLRs and their associated proteins are also present within solid organs, and expressed by parenchymal cells of heart, liver, lungs and kidneys.

In the kidney TLR expression, including TLR2 and TLR4, was particularly observed on the tubular epithelium. Currently, much more insight is gained about the distribution and expression pattern of individual TLRs on intrinsic renal cells. Different non-immune renal cells, including mesangial cells, podocytes, endothelial cells and TECs are shown to express TLRs. In particular renal TECs express several TLRs like TLR1, TLR2, TLR3, TLR4 and TLR6, suggesting that they might actively contribute to immune responses. Indeed, renal TLRs were shown to functionally respond to several different stimuli. In particular renal-associated TLR2 and TLR4 were shown to sense bacterial products, indicating that tubular epithelial cells may functionally contribute as cellular sentinels to detect pathogens or foreign bacterial products following upper urinary tract infections. The observation that TLRs can also be activated by various DAMPs,
as indicated in the previous paragraph, suggests that renal-associated TLRs can actively sense tissue damage and in turn induce a sterile inflammatory response. Interestingly, renal-associated TLR2 has already been demonstrated to mediate ischemia/reperfusion injury in the kidney. Part of this thesis will address the role TLR4 has in sterile renal injury models.

More recent effort has shown that NLRP3 is highly expressed in epithelial structures, while it is also expressed by leukocytes and macrophages. In addition, the adaptor protein ASC is found at high expression levels in renal epithelium. Again, this implicates that cytosolic NLRs among which NLRP3, may play an apparent role in sensing (renal) tissue damage and in turn contribute to establish an effective inflammatory response by cytokine maturation like IL1β.

Furthermore, in line with several individual members of the PRR families, the RAGE receptor is expressed on a wide range of renal cells, including mesangial cells, (proximal) TECs, podocytes and epithelial cells of the Bowman’s capsule. In line with inflammatory cells, non-immune cell types like epithelial cells do have mechanisms to prevent uncontrolled excessive activation of the receptors belonging to the innate immune system. Likely, TIR8 has been demonstrated to be expressed in epithelial tissues like the lung, gut and predominantly the kidney of human and mice.

Together, these results suggest that renal parenchymal cells (e.g. renal TECs) might play an active and crucial role in sensing the presence of a diverse array of DAMPs that reflect (renal) tissue damage and/or cellular stress, and hence may contribute to an inflammatory response.
5. Outline of this thesis

In this thesis, the contribution of different PRRs to the development and progression of renal pathology is investigated using a model of either acute kidney injury or chronic kidney disease. Sterile tissue injury induces the release and accumulation of multiple endogenous DAMPs that have the capacity to alert the (innate) immune system. Receptors belonging to the innate immune system, like TLRs and NLRs can be activated by these DAMPs and subsequently initiate an inflammatory response. This may be either beneficial in terms of clearing damaged cells and secreting essential factors that stimulate tissue repair or detrimental when excessive and causing collateral tissue damage. The specific objectives of each individual investigation in this thesis are delineated in the respective chapters. In chapter 2 the role of TLR4 and its intracellular downstream signaling cascades in acute kidney injury are examined using an experimental model of renal I/R injury. In the study described in chapter 3 we investigated the function of the intracellular NLRP3 inflammasome in sensing necrosis and inducing inflammatory responses following acute tubular necrosis. In chapter 4 we investigated the role of mitochondria and mitochondrial-derived DAMPs in the induction of systemic inflammation and their contribution to the development of acute kidney injury. The well-described and highly potent DAMPs HMGB1 and S100 proteins that are released following tissue injury activate several immune receptors, including TLR4 and RAGE. In chapter 5 we examined the therapeutic potential of neutralizing HMGB1 and the role of the HMGB1-RAGE axis in inducing renal inflammation following acute kidney injury, while in chapter 6 we looked into more detail into the role of the s100A8/9 protein in activating renal inflammation following acute kidney injury.

Once tissue injury progresses into a more chronic state, different underlying pathological processes become apparent. Hence, in chapter 7 and chapter 8 we respectively investigated the role of TLR4 and the NLRP3 inflammasome in the pathological processes and inflammation underlying progressive renal injury.
6. Abbreviations:

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AGE</td>
<td>Advanced Glycation End products</td>
</tr>
<tr>
<td>AKI</td>
<td>Acute kidney injury</td>
</tr>
<tr>
<td>ASC</td>
<td>Apoptosis-associated speck-like protein containing a CARD</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>CKD</td>
<td>Chronic kidney disease</td>
</tr>
<tr>
<td>DAMP</td>
<td>Damage Associated Molecular Pattern</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EMT</td>
<td>Epithelial to mesenchymal transition</td>
</tr>
<tr>
<td>FPR</td>
<td>Formyl Peptide Receptor</td>
</tr>
<tr>
<td>HMGB1</td>
<td>High Mobility Group Box-1</td>
</tr>
<tr>
<td>IFN-β</td>
<td>Interferon-β</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>I/R</td>
<td>Ischemia/Reperfusion</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>LRR</td>
<td>Leucin-rich Repeats</td>
</tr>
<tr>
<td>MAL</td>
<td>Myeloid differentiation factor-88 adaptor-like protein</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metallo proteinase</td>
</tr>
<tr>
<td>MyD88</td>
<td>Myeloid Differentiation primary response gene 88</td>
</tr>
<tr>
<td>NFκB</td>
<td>Nuclear Factor-κB</td>
</tr>
<tr>
<td>NLR</td>
<td>Nucleotide-binding domain, Leucine-Rich repeat containing protein</td>
</tr>
<tr>
<td>NLRP3</td>
<td>NACHT, LRR and PYD domains-containing protein 3</td>
</tr>
<tr>
<td>PAMP</td>
<td>Pathogen Associated Molecular Pattern</td>
</tr>
<tr>
<td>PRR</td>
<td>Pattern Recognition Receptor</td>
</tr>
<tr>
<td>PYD</td>
<td>Pyrin domain</td>
</tr>
<tr>
<td>RAGE</td>
<td>Receptor for Advanced Glycation Endproducts</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>SARM</td>
<td>sterile α- and armadillo motif-containing protein</td>
</tr>
<tr>
<td>SIGIRR</td>
<td>Single Ig IL-1-related receptor</td>
</tr>
<tr>
<td>T1/ST2</td>
<td>Interleukin-1 receptor-like 1</td>
</tr>
<tr>
<td>TEC</td>
<td>Tubular Epithelial Cell</td>
</tr>
<tr>
<td>TGFβ</td>
<td>Transforming growth factor-β</td>
</tr>
<tr>
<td>TIR</td>
<td>Toll/IL1-Receptor domain</td>
</tr>
<tr>
<td>TIR8</td>
<td>Toll-IL-1 receptor 8</td>
</tr>
<tr>
<td>TIRAP</td>
<td>Toll-interleukin-1 receptor (TIR) domain containing adaptor protein</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like Receptor</td>
</tr>
<tr>
<td>TRAM</td>
<td>TRIF-related adapter molecule</td>
</tr>
<tr>
<td>TRIF</td>
<td>TIR domain-containing adapter-inducing interferonβ</td>
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