Tissue-specific roles of the pattern recognition receptors NLRP3, NLRX1 and TLR9 in sterile inflammatory kidney disease
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Chapter 1

General introduction and outline of this thesis
Introduction

1. The kidney
The kidney is vital to maintain body homeostasis through regulation of multiple important functions. First, waste products produced in the body are taken up in the bloodstream and subsequently removed by the kidney. Second, volume and electrolyte homeostasis are maintained within strict boundaries by sensing osmolality, reabsorption of water and secretion of hormones by the renin-angiotensin-aldosterone system. And third, filtered molecules important to the body, are reabsorbed such as glucose.

The kidney consists of 1.2 million nephrons, the smallest unit of which a kidney consists. A nephron consists of a glomerulus, the proximal tubule which transfers to the loop of Henle and the distal tubule. The juxtaglomerular apparatus senses osmolality and adjusts reabsorption of ions and proteins if necessary. The last unit of the nephron is the collecting duct which connects to the urethra and finally to the bladder.

Blood enters the glomerulus via the afferent artery where molecules are filtered based on charge and size. The endothelial glycocalyx together with the glomerular basement membrane are negatively charged leading to the retention of negatively-charged molecules. Slits created by foot processes of podocytes create a size-based filter. The kidney filters 180 litres of blood daily. Filtered molecules, electrolytes and water create the ultrafiltrate or pro-urine. Larger molecules such as albumin, glucose and $\beta_2$-microglobuline and also salts, urea and uric acid are actively reabsorbed from the ultrafiltrate in the proximal tubule. Water is also reabsorbed in the proximal tubule to achieve iso-osmolality.

Using the counterflow principle, water and salts are reabsorbed in the loop of Henle and distal tubule. The juxtaglomerular apparatus senses osmolality and regulates filtration and osmolality through restriction or dilatation of the afferent and efferent artery. Finally, ‘fine-tuning’ of water and electrolyte transport is achieved by the collecting duct cells: principal and intercalated cells. Principal cells are under the influence of aldosterone and resorb sodium, chloride and potassium. Intercalated cells secrete hydrogen ions. Water permeability is regulated by vasopressin or anti-diuretic hormone.
2. Renal disease relevant for this thesis

Renal disease can be characterised by disease progression: acute or chronic. Acute renal failure is characterised by a sudden loss of renal function whereas chronic renal disease is a slow, gradual loss of renal function. Acute renal disease, if treated accordingly, is a reversible disease whereas chronic renal failure is not. Treatment is aimed at preventing lasting damage to the body. In contrast, chronic renal disease slowly progresses and is often advanced when clinical symptoms appear. Treatment is aimed at stopping progression to preserve renal function.

An important cause of acute renal disease is ischemia reperfusion (IR)-induced acute kidney injury (AKI) whereas with the epidemic rise in obesity, metabolic syndrome-associated chronic kidney disease (CKD) poses a challenge to healthcare and society.

2.1 Acute kidney injury

Acute kidney injury is the sudden loss of kidney function, measured by serum creatinine and urine output, occurring within 48 hours and resulting in the retention of metabolic waste products and dysregulation of fluid, electrolyte, and acid-base homeostasis. A recent study using multiple large cohort studies showed pooled incidence rates for AKI of 21.6% in adults and 33.7% in children in a hospital setting. The AKI-associated mortality rates were 23.9% and 13.8% respectively and declined over time (1). This translates to 1 in 5 adults and 1 in 3 children that experience AKI during a hospital stay.

Causes of AKI can be divided into three categories: pre-renal, intrinsic or post-renal. In case of pre-renal causes, glomerular and tubular function is intact however renal function is decreased due to factors limiting blood flow to the kidney. Examples of pre-renal causes are shock, diarrhoea or poor fluid intake resulting in hypovolemia and subsequent reduced renal perfusion. Renal dysfunction due to obstruction of the urinary outflow tract is categorised as post-renal which can occur due to cancer or prostatic hypertrophy. Often renal dysfunction is reduced due to processes that occur within the kidney itself and are termed intrinsic causes. The intrinsic cause can be localised to a specific compartment: the interstitium, glomeruli or tubules. Tubular damage is often ischemic or nephrotoxic. Here, we will focus on ischemic AKI.

Ischemia is the restriction of blood supply to an organ causing oxygen and glucose shortage. This is followed by restoration of the blood supply and concomitant re-oxygenation (2). Tissue re-oxygenation is accompanied by an increase in tissue
injury and a profound inflammatory response termed ‘reperfusion injury’. Ischemia reperfusion-induced injury is not limited to the ischemic organ since ischemia in the liver can also induce intestinal and kidney tissue injury leading finally to multi organ failure (3). Ischemia-reperfusion (IR)-induced AKI can be characterised by two phases (Fig 1): i) renal injury where cell death and inflammation are profound to clear any invading pathogen and ii) renal repair where fibrosis and proliferation are dominant processes in order to restore renal function.

![Figure 1. Renal injury and repair. Following IR-induced injury, tubular epithelial cells lose their polarity and brush border. With increasing time/severity of ischemia, there is cell death by necrosis or apoptosis. Necrotic debris is released into the lumen where it obstructs the lumen. Repair is initiated when viable epithelial cells dedifferentiate and migrate. These cells undergo division and replace lost cells. Ultimately, cells go on to differentiate and re-establish the normal polarity of the epithelium. (Figure adapted from (4))](image-url)
2.1.1 Renal injury

Characteristic morphological changes upon ischemia reperfusion injury are loss of brush border of proximal tubular epithelial cells (PTEC) and detachment of tubular cells exposing the basement membrane resulting in tubular dilatation. Cast formation is frequently observed due to detached tubular cells or cellular debris. ATP depletion also leads to disruption of the cytoskeleton which results in loss of cellular polarity and tight junctions. Tight junctions are important in preventing back leak, cellular polarity and form a trafficking and signalling platform.

Tissue injury is dominated by epithelial and endothelial cellular injury. Ischemic epithelial cells deplete intracellular ATP levels leading to cell injury and finally cell death. All tubular cells are subject to ischemia however the PTEC is most vulnerable because of its function and position. PTECs have a high metabolic rate due to the many transport functions it has. Second, PTECs are located at the S3 segment of the tubule and here relative hypoxia is present increasing their vulnerability to ischemia. PTECs damage leads to luminal obstruction and backleak of filtrate across injured epithelial cells resulting in ineffective glomerular filtration and thereby a decreased glomerular filtration rate (GFR).

IR-induced tubular necrosis leads to the release of pro-inflammatory ligands which will trigger i) tubular apoptosis, ii) the secretion of chemokines such as keratinocyte chemo-attractant (KC or CXCL1) and monocyte chemotactic protein-1 (MCP-1 or CCL2) by epithelial cells and iii) upregulation of adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1 or CD54) by endothelial and mesangial cells. In addition, ischemia leads to stabilisation of hypoxia-inducible factor (HIF) which will amplify inflammation (5).

Subsequently, chemokine release in combination with upregulation of adhesion molecules will induce granulocyte and macrophage extravasation into the renal tissue. Granulocytes will infiltrate the tissue and release reactive oxygen species (ROS) thereby damaging surrounding tissue. Infiltrating macrophages have a pro-inflammatory M1 phenotype and release pro-inflammatory cytokines such as TNF-α which stimulates tubular necrosis and apoptosis. Summarised, the initial IR-induced tubular injury triggers a positive feedback loop where cell death leads to inflammation and subsequently enhances cell death (6). If the original IR-induced insult is limited, the kidney is able to recover however if the extent of IR-induced injury is major, the resulting inflammation and accompanying
cell death will damage the entire kidney thereby inducing renal dysfunction and eliminating the potential for recovery.

2.1.2 Renal repair

In contrast to the heart and brain, renal function can completely recover from IR-induced AKI on the short-term. In contrast, long-term effects of AKI are an increased risk to develop CKD (7–9) which suggests an unresolved pathology following AKI. Renal tubular epithelium proliferates slowly under normal conditions but upon injury, proliferation increases dramatically as indicated by the proliferation marker Ki67. A specific subpopulation of tubular cells is responsible for proliferation (termed progenitor or scattered tubular cells) and a transcriptional program is initiated upon injury to dedifferentiate surviving tubular cells into proliferating cells (10). This mechanism was confirmed using irreversible genetic tagging and cell-fate tracking, and shown to be not present under physiological growth (11, 12).

Macrophages play a dual role following IR since early M1 macrophages are detrimental to the kidney whereas M1 macrophages are replaced by the anti-inflammatory, M2 macrophages to stimulate proliferation (13). The same article showed that both in vitro and in vivo, pro-inflammatory macrophages convert into M2 macrophages in the presence of renal tubular cells.

Finally, fibrosis is often observed in IR-induced AKI however not too such an extent as in the unilateral ureteral obstruction (UUO)-induced model of CKD. Fibrosis is characterised by an accumulation of α-smooth muscle actin (α-SMA)+ myofibroblasts, fibronectin, collagen type I and the growth factors connective tissue growth factor (CTGF), transforming growth factor-β (TGF-β). Myofibroblasts originate from local proliferating fibroblasts, endothelium and epithelium through a process termed endothelial- or epithelial-to-mesenchymal transition (EMT) (14). Fibrosis is the adaptive process to injury to maintain organ integrity however it can become maladaptive when fibrosis induces an abnormal accumulation of extracellular matrix, tubular atrophy and renal dysfunction. Fibrosis is limited in a model of IR-induced AKI and seen as an contributor to long-term development of CKD (15).

Summarised, IR-induced injury is followed by renal repair which consists of proliferation of surviving epithelial cells to restore renal function combined with the initiation of fibrosis to replace degraded extracellular matrix and maintain organ integrity.
2.2 Chronic kidney injury

CKD is defined as the presence of kidney damage for more than 3 months, i.e. structural or functional abnormalities which manifests itself either by markers of kidney damage or a GFR of less than 60 ml/min/1.73m² (16). Individuals with established kidney damage and a GFR >60 ml/min/1.73m² are at an increased risk of two major outcomes of CKD: loss of kidney function and cardiovascular disease. The prevalence of CKD (stages 1-4) increased from 10.0% in 1988-1994 to 13.1% in 1999-2004 (17). Stage 1-2 CKD is present in 38% of CKD patients (5% of 13.1%) however these individuals don’t experience renal dysfunction. Currently, there is an emphasis on early diagnosis and prevention to reduce risks of cardiovascular events, kidney failure and death (18). Therefore, biomarkers are developed to diagnose CKD at an early stage and prevent progression towards stage 3, 4 and finally end-stage renal disease (ESRD) (19).

CKD is a common term for a range of heterogeneous disorders which affect renal function and structure. CKD is generally associated with old age, diabetes, hypertension or obesity however the exact diagnosis is often difficult except in case of exposure to nephrotoxic drugs. In addition, AKI can lead to the development of CKD (15). Diabetes, hypertension and obesity are traits of the metabolic syndrome together with dyslipidemia. Indeed, more traits of the metabolic syndrome associate with an increasing incidence of CKD (20). Also, all traits by itself associate significantly with the development of an estimated GFR (eGFR) of <60 ml/min/1.73m² such as impaired fasting glucose and abdominal obesity (21). Due to its heterogeneous nature, the pathology can be diverse but several hallmarks can be identified: a systemic, low-grade inflammation in combination with glomerulosclerosis and tubular atrophy. In addition, proteinuria by itself is a strong predictor for CKD or in combination with other markers an indicator of CKD.

The mechanisms through which metabolic syndrome induces CKD are diverse. Hemodynamic factors, inflammation and metabolic effects are important for the development of metabolic syndrome-associated CKD. Type of diet is also important in the initiation and progression of metabolic syndrome-associated CKD. In a prospective study with 9514 participants, it was established that the consumption of a western dietary pattern, meat and fried foods promotes the incidence of metabolic syndrome (22). Similarly, a meta-analysis of 11 prospective cohort studies showed that sugar-sweetened beverages increased the relative risk for developing type 2 diabetes or metabolic syndrome (23).
General introduction

Line with the association between metabolic syndrome and CKD, a western diet was found to increase the risk for CKD (24). High fructose corn syrup is often used as a sweetener in beverages and is has steadily increased in parallel with the growth of the metabolic syndrome incidence. Fructose induces renal injury, inflammation and CKD (25–27). In case of fructose, uric acid, an important metabolite of fructose, is thought to contribute to renal injury (28–30). In this thesis, either fructose- or Western diet-induced CKD will be investigated.

2.2.1 Dyslipidemia

Several studies have documented a relation between dyslipidemia and the progression of CKD: men with low high density lipoprotein (HDL) levels and increased non-HDL cholesterol levels were at an increased risk to develop a reduced GFR (31). Vice versa, increased HDL levels associated with a decreased risk to progress towards CKD (32).

The association with lipids and renal disease was already proposed in 1858 by Virchow. Observations about dyslipidemia in CKD were unified in the ‘lipid nephrotoxicity hypothesis’ in 1982 (33) and updated in 2009 (34) which states that several pathophysiological changes drive disease progression: inflammation, oxidative stress, endoplasmatic reticulum stress, endothelial dysfunction and activation of the renin-angiotensin system (Fig 2). Inflammation can modify renal lipid homeostasis through changes in i) lipoprotein composition and ii) cholesterol distribution leading to cellular stress and enhanced inflammation. This positive feedback loop will stimulate disease progression and ultimately lead to ESRD.

Cholesterol is needed for basic physiological functions as can be seen by cholesterol

Figure 2. Proposed mechanisms by which dyslipidemia drives renal pathophysiological changes. Cardiovascular disease (CVD), endoplasmic reticulum (ER). [Adapted from (34)]
accumulation following IR-induced AKI in the renal cortex which contributed to repair of damaged cell membranes (35, 36). SREBP-2 was shown to regulate repair of damaged plasma membranes (37) and both SREBP-2 activation and cholesterol accumulation were observed in kidneys in a model of type 2 diabetes (38) and diet-induced obesity (39) indicating both a beneficial and detrimental role for cholesterol in renal disease.

The detrimental role of cholesterol was expanded through studies where cholesterol accumulation led to the accumulation of foam cells and lipid-mediated changes in kidney cells, glomerulosclerosis and interstitial fibrosis (40) possibly through alteration of lipid rafts and subsequent protein signalling (41, 42). Furthermore, studies of patients with renal disease using statins to inhibit cholesterol synthesis showed a reduced loss of kidney function and reduced proteinuria (43).

3. The immune system

The immune system is a diverse set of physiological mechanisms that humans and other animals use to protect us from a variety of detrimental causes such as pathogenic organisms, harmful substances or tumour cells. Cells and proteins dedicated to orchestrate this defence system are collectively called the immune system. The immune system protects us from infections such as influenza which would be lethal to us without an immune system.

3.1 Innate and adaptive immunity

When faced with an infection, the body generates a response aimed at specifically targeting and deleting the invading pathogens. This response involves T cells, B cells and antibodies and generally takes days to weeks. We call this part of the immune system the adaptive immune system and it is characterised by its specificity and memory. During development of the adaptive immune response, the body is exposed to the infection and a response is needed to control this outbreak of pathogens while the adaptive immune response is formulated. The answer to bridge this period is the innate immune response: a rapid response that recognises conserved motifs of pathogens. Conserved motifs found on pathogens are called pathogen-associated molecular patterns (PAMP) which are detected by pattern recognition receptors (PRR).
3.2 Pattern recognition receptors and the Danger model

The body uses PRRs in combination with the detection of PAMPs to detect invading pathogens. However, no progressive inflammatory disorder is present in the intestine where around two kilograms of bacteria are present. A new conceptual framework for immune regulation was proposed by Polly Matzinger in 2002 to reconcile various unexplained observations with the role of PRRs and PAMPs which is called the Danger model (44). Shortly, it states that not ‘self’ vs. ‘non-self’ discrimination is important in initiating an immune response but rather the presence of danger, defined as alarm signals released by injured tissue. PRRs were first proposed by Janeway to allow antigen-presenting cells (APC) to detect infectious non-self from non-infectious non-self. The extra layer added by Matzinger tells us that PRRs can also detect danger signals derived from injured cells exposed to pathogens, toxins and mechanical damage. This indicates an additional function for PRRs in detecting ‘self’ proteins (derived from injured cells) next to ‘non-self’ (bacteria). The main message here is that healthy cells or cells dying by regulated cell death (apoptosis) do not send danger signals. Nowadays, we designate these danger or alarm signals as damage-associated molecular patterns (DAMP).

3.2.1 The PRR family: Toll-like receptors

PRRs are currently categorised into four classes: i) Toll-like receptors (TLR), ii) C-type lectin receptors (CLR), iii) retinoic acid-inducible gene-I (RIG-I)-like receptor (RLR) known as RIG-I helicases (RLH) and iv) the nucleotide binding and oligomerisation domain (NOD)-like receptors (NLR) (45). Within the TLR sub-family, ten human and twelve murine TLRs have been discovered which bind a wide range of PAMPs and DAMPs and will be discussed later on. Toll and its receptor (Toll receptor) were first identified as an essential molecule in the development of Drosophila and later in antifungal immunity (46). A homologous family was later found in humans and termed Toll-like receptors (47). TLR family members are differentially expressed among immune cells and are also present on renal epithelial cells (48). TLRs are characterised by their leucine-rich repeats and share a similar downstream activation cascade as the IL-1 receptor: these include MyD88, IL-1R-associated protein kinase (IRAK) and tumour necrosis factor receptor-activated factor 6 (TRAF6). TLRs are located either at the cell surface (TLR1, TLR2, TLR4-6) or in endosomes (TLR3, TLR7-9) (49).
3.2.1a The TLR sub-family: Toll-like receptor 9

Endosomal TLRs (TLR3, 7, 9) recognise nucleic acids whereas TLRs found at the cell surface detect a wide range of ligands. TLR9 is located in the endolysosome where it detects bacterial DNA and oligonucleotides containing unmethylated CpG motifs whereas eukaryotic DNA and methylated oligonucleotides do not activate TLR9 (50). In a sterile inflammatory setting, mitochondrial DNA can activate TLR9 (51). Inappropriate TLR9 activation is limited through autophagy of ‘self’ nucleic acids (52) and receptor compartmentalization (53). Carrier proteins are needed to guide TLR9 ligands towards the designated compartment, i.e. phagosome. Examples of carrier proteins are antibodies (54), histones (55) or HMGB1 (56) which can also act as DAMPs in sterile inflammatory disease.

The role of TLR9 in renal disease is diverse. TLR9 deficiency was protective in case of sepsis-induced AKI (57) and activation of TLR9 worsened renal disease in systemic lupus erythematosus (58) or immune complex-driven glomerulonephritis (58). TLR9 inhibition conferred protection following ischemia reperfusion in the liver (59) but not in de kidney (60). Similarly, TLR9 did not regulate fibrosis following UUO (61). We explored the role of TLR9 further following moderate and severe IR-induced AKI in this thesis.

3.2.2 The PRR family: NOD-like receptors

NLRs together with RLRs make up the cytoplasmic part of the PRR family. NLRs share partly the same structure as TLRs since both members possess one or multiple leucine-rich repeats. NLRs in addition have a nucleotide-binding domain and an ATPase domain dubbed the NACHT domain. The N-terminal effector domain of the NLR family is heterogeneous and can generally be divided into one that has a caspase recruitment domain (CARD) termed NLRC or a pyrin domain (PYD) termed NLRP. The CARD domain facilitates binding to RIP2 whereas the PYD domain binds to caspase-1 (62).

3.2.2a The NLR sub-family: NOD-like receptor protein 3

A well-studied NLR protein is the Nod-like receptor protein 3 (NLRP3). NLRP3 connects to caspase-1 via apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC). ASC interacts with NLRP3 through the pyrin domain (63). Using its CARD, it brings monomers of caspase-1 into close proximity which leads to self-cleavage and subsequent activation of caspase-1. Active caspase-1 proteolytically cleaves a number of proteins such as pro-IL-1β
and pro-IL-18 (64). Release is through a non-classical secretion pathway (65). NLRP3 is regulated through a two-step mechanism, i.e. gene expression alone is not sufficient (66). NLRP3 needs to be primed first followed by inflammasome formation. Caspase-1 activation is in case of live Gram-negative bacteria also dependent on caspase-11 and leads to pyroptosis (inflammatory cell death) (67). Three main activating mechanisms have been proposed for NLRP3 inflammasome formation: i) K+ efflux, ii) mitochondrial dysfunction and generation of mitochondria-derived reactive-oxygen species (ROS), and iii) phagolysosomal destabilization in response to particulates (68). A recent study aimed to provide an explanation for these diverse NLRP3-activating mechanisms, by demonstrating that all tested NLRP3-activating stimuli act through promoting K+ efflux and subsequent Na+ influx (69). Ligands able to activate NLRP3 are ATP (70), uric acid crystals (71), cholesterol crystals (72), pore-forming toxins such as nigericin or oxidized mitochondrial DNA (73) and saturated fatty acids (74).

Other factors regulate down-stream effects of NLRP3 inflammasome activation. Autophagy limits IL-1β activation or release as shown by increased IL-1β levels in mice that were deficient for autophagy (ATG16L1 -/- mice) (75). In addition, in vivo data shows that caspase-1 is dispensable for IL-1β activation (76). Non-caspase proteases such as proteinase 3 have the ability to activate IL-1β in an inflammasome-independent manner (65). In addition, IL-1α is partly regulated through the NLRP3 inflammasome but is able to signal through the same receptor as IL-1β does, i.e. IL-1 receptor (IL-1R) (77, 78).

NLRP3 is intensely investigated in the context of obesity and insulin resistance (74, 79–82). With respect to renal disease, it was shown that NLRP3 is detrimental in case of IR-induced AKI (70, 83), unilateral ureteral obstruction (84, 85), oxalate nephropathy (86) and diet-induced CKD (87). In addition, it was shown that NLRP3 reduces the responsiveness to TGF-β induced α-SMA, MMP2 and MMP9 expression (88). We explored the tissue-specific role of NLRP3 following IR-induced AKI and determined the role on renal homeostasis in a model of diet-associated CKD.

3.2.2b The NLR sub-family: NOD-like receptor X 1

Nod-like receptor X-1 (NLRX1) has an unique N-terminal domain, which accounts for the ‘X’ in its acronym. The ‘X’ was found to contain a mitochondrial addressing sequence (89, 90) which localizes NLRX1 to the mitochondrial
matrix (91). NLRX1 was linked to the production of ROS and amplification of NF-κB (90). It has an interaction with UQCRC2, a matrix-facing protein of the mitochondrial respiratory chain complex III which suggests a possible route to produce ROS (91). NLRX1 was also found to interact with the mitochondrial antiviral signalling adaptor (MAVS) on the outside of mitochondria (89) and to negatively regulates the virus-induced RIG-I MAVS interaction and type I interferon production (92). In addition, NLRX1 targets the IκB kinase (IKK) complex resulting in impaired downstream NF-κB activation (93). These data point towards a role of NLRX1 as a negative regulator of cytokine responses while enhancing ROS production. A unifying hypothesis stated that NLRX1 suppresses spontaneous antiviral signalling in unstimulated cells but on activation of a RIG-I-like helicase, NLRX1 turns into a ROS enhancer (94). Its ligand is thought to be intracellular viral RNA since it interacts directly with RNA (95). Since no studies have been performed on the role of NLRX1 with respect to kidney disease, we set out to determine the effect of NLRX1 deficiency in a model of IR-induced AKI.

4. The role of the innate immune system in sterile inflammatory kidney disease

Originally, the role of the innate immune system is to detect invading pathogens and initiate an immune response to combat the infection. However, PRRs do not only detect PAMPs but also DAMPs which expands their function from pathogen detection towards surveillance of cellular homeostasis (Fig 3). Sterile inflammation is characterised by activation of the immune system without the presence of pathogens. Evaluating the innate immune system and specifically the PRRs NLRP3, NLRX1 and TLR9 in this thesis, will tell us more about the role of these receptors on the induction and resolution of general cellular homeostatic pathways such as inflammation, fibrosis, proliferation and cell death. We use two models of sterile inflammation in this thesis: a model of IR-induced AKI and of diet-associated CKD. A prerequisite for innate immune activation in these models is the presence of PRRs different cellular subsets and the availability of DAMPs to these PRRs.
4.1 Danger-associated molecular patterns in sterile inflammatory kidney disease

DAMPs are a heterogeneous group of ligands which are expressed constitutively in different biological compartments. They are normally hidden from the immune system and, upon injury or stress, are passively exposed. DAMPs can be derived from several sources which influences the DAMP repertoire to be released and subsequent immune activation. The extracellular space can release biglycan, fibrinogen, hyaluronan, heparan sulphate, oxalate crystals or uromodulin (6). Here, we will discuss cell death and dietary intake as a the source for DAMPs in respectively IR-induced AKI and diet-associated CKD.

4.1.1 Cell death

The fate of the epithelial cell is dependent on the extent of ischemia (97). Limited ischemia results in injury of which a tubular cell can recover whereas severe ischemia induces lethal injury leading to cell death. Traditionally, cells die through
either apoptosis or necrosis. Apoptosis is regarded a ‘silent’ mode of cell death and characterised by cellular and nuclear shrinkage whereas necrotic cells show swelling of the cell and organelles. Plasma membrane integrity is quickly lost in necrotic cells which allows the efflux of intracellular proteins into the extracellular space and trigger inflammation whereas in apoptotic cells plasma membrane integrity is preserved until late in the apoptotic process. Apoptosis is considered a regulated mode of cell death but only recently, necrosis is now also considered a regulated form of cell death (6, 98). Pyroptosis, necroptosis and ferroptosis are three newly discovered regulated forms of necrosis which occur following renal IR (99–101). Research on the precise role of these modes of cell death and their contribution to inflammation is still ongoing but involves the release of IL-1β (101). Ligands released following tubular necrosis are HMGB1 (102), histones (103), ATP (70), mitochondrial DNA (51) and uric acid (71). TLR2, TLR4, NLRP3 and TLR9 mediate the detection and effector mechanism upon detection of these DAMPs.

4.1.2 Nutrients

Lipids are also able to active the innate immune system in addition to previously mentioned proteins (e.g. HMGB1), crystals (e.g. uric acid crystals) and nucleic acids (e.g. mitochondrial DNA). A diet rich in free fatty acids (FFA), cholesterol or fructose is able to activate the immune system. Dietary FFAs are able to activate TLR2, TLR4 (104, 105) and NLRP3 (74). NLRP3 can also be activated by a variety of ligands such as cholesterol crystals (72), the free fatty acid palmitate (74), ceramide (79) and the oxysterol 7-ketocholesterol (106). A positive feedback loop can be created by initial cholesterol accumulation which enhances the secretion of inflammatory cytokines (40). Subsequently, pro-inflammatory cytokines disrupt LDL receptor (LDLR) feedback causing unrestrained LDLR-mediated cholesterol uptake (107). Fructose can also be detrimental to the kidney through the induction of MCP-1 in renal tubular epithelial cells (27) and ICAM-1 on endothelial cells (108).

4.2 PRR expression and function in sterile inflammatory kidney disease

The original role of PRRs was to detect invading pathogens and therefore, PRR expression and function was investigated primarily in leukocytes. Previous work done in our group and by others shows that renal parenchymal cells and more specifically tubular epithelial cells express PRRs and are contributors rather than simple bystanders in the innate immune response following IR or a Western diet (96).
4.2.1 Ischemia reperfusion-induced acute kidney injury
The kidney expresses PRRs such as TLR2 (109), TLR4 (110, 111), NLRP3 (70, 83, 88) and expression is increased following IR indicating a role during disease. TLR2 was shown to be most prominently expressed in renal epithelium and increased upon renal ischemia (48). Corresponding to this observation, Leemans and colleagues showed a detrimental role for renal-associated TLR2 (109). Here, TLR2KO mice showed reduced tubular injury, less apoptotic tubuli, reduced inflammation and an improved renal function. Mice lacking TLR2 in the renal parenchymal compartment showed improved renal function compared to mice with TLR2 deficiency in the bone marrow compartment and wild type mice. This indicates that the effects of TLR2 are solely mediated by renal parenchymal cells. The mechanism of action behind TLR2 was later discovered by Allam and colleagues (108). TLR2/4 deficiency prevented histone-induced tubular necrosis and granulocyte influx in vivo. Similarly, histone neutralisation reduced IR-induced injury and inflammation. Using in vitro experiments, they showed that histones from dying renal epithelial cells activate NF-κB in leukocytes and decreased viability of endothelial and epithelial cells. In contrast, TLR2 also mediates regenerative effects. Renal progenitor cells were shown to increase proliferation, branching morphogenesis and differentiation into renal epithelial cells upon TLR2 activation (112). TLR2 therefore functions both as a mediator of repair, inflammation and cell viability depending on the stage of disease and localisation.

TLR4 is also strongly expressed by renal tubular epithelium and increased upon renal ischemia (48). Wu and colleagues showed that TLR4 was predominantly expressed by infiltrating cells after one day of reperfusion whereas tubules expressed TLR4 mostly after three days (111). Similar to TLR2, TLR4 deficiency protected against IR-induced tubular injury, renal dysfunction and inflammation. TLR4KO tubular epithelium secreted less cytokines and chemokines and decreased apoptosis in vitro following ischemia. Both leukocyte- and renal-associated TLR4 mediated tubular injury and renal dysfunction after one day of reperfusion. These results were confirmed by Pulsens et al. (110). Histones contributed to NF-κB activation in leukocytes and decreased cellular viability in renal tubular epithelium via TLR2/TLR4 (103). In addition to histones, HMGB1 induces renal dysfunction, inflammation and tubular injury through TLR4 (102). These data support a detrimental role for TLR4 however it also plays a beneficial role during the regenerative phase of IR (113). Necrotic cells released TLR4
ligands which activated TLR4 on bone marrow-derived DCs triggering IL-22 release. IL-22 blockade significantly decreased tubular proliferation indicating a protective role for TLR4 on intrarenal leukocytes through IL-22. TLR4 therefore plays a dual role following IR as TLR2 does.

The first observation on NLRP3 in a model of IR-induced AKI was made by our group in cooperation with Iyer et al. (70). Here, NLRP3 deficiency improved survival following lethal IR. In addition, NLRP3 deficiency improved renal function and reduced inflammation after one day of reperfusion. Shigeoka and colleagues showed subsequently that NLRP3 mediated IL-18 and IL-1β secretion after one day of reperfusion however IL-1R- and IL-18-deficient mice showed no improved renal function thereby suggesting non-canonical (NLRP3-dependent, IL-1β-, IL-18-independent) effects (83). Indirectly, renal-associated NLRP3 was shown to mediate the detrimental effects of NLRP3 after one day of reperfusion through reduced tubular cell death. Extending this hypothesis, the NLRP3 ligands nigericin and ATP were shown to induce necrosis in tubular epithelium in vitro (114). Thus, NLRP3 also has a direct effect on tubular epithelium through the regulation of cell viability.

Besides a role during the injury phase, NLRP3 was also shown to play a role during EMT, a process linked to tubular repair after IR. Injured tubular epithelium contributes to fibrosis through the release of TGF-β mRNA-containing exosomes which stimulate fibroblast proliferation and collagen type 1 expression (115). NLRP3 deficiency decreased the responsiveness to TGF-β and subsequent EMT in primary tubular epithelial cells, independent of IL-1β, IL-18 or caspase-1 (88).

Summarised, PRRs such as TLR2, TLR4 and NLRP3 play an important role during both injury and repair and their detrimental or beneficial role is dependent on the cellular subset and time of reperfusion. We set out to expand the knowledge on NLRP3 and investigate the role of NLRX1 and TLR9 on renal injury and repair with respect to their localisation and time of disease.

4.2.2 Diet-associated chronic kidney disease

Few studies have been published to date on the role of PRRs in diet-associated CKD (96). One study reports increased NLRP3 expression in fructose-fed rats however no NLRP3 inhibition was studied here (116). A second study by Kuwabara and colleagues used a high-fat diet (HFD) to induce nephropathy in WT and TLR4KO mice (117). Here, TLR4 deficiency reduced high fat diet-induced glomerular macrophage accumulation.
Diabetes is a risk factor for the development of metabolic syndrome and secondary CKD. The renal pathological manifestation of diabetes is diabetic nephropathy (DN) which is characterised by high plasma glucose levels, hypertension, glomerulosclerosis and tubulointerstitial inflammation. TLR4 was upregulated in biopsies of human DN patients and correlated with infiltrating macrophages (118). The authors show that high glucose induced TLR4 expression and subsequent TLR4-dependent IL-6 and MCP-1 secretion in tubular epithelium. Finally, TLR4 deficiency reduced renal dysfunction, albuminuria and renal inflammation in a mouse model of DN (118). The detrimental role of TLR4 was confirmed in a type 2 diabetes model using db/db mice (120). TLR2 also played a detrimental role in a model of streptozotocin-induced DN (119). Here, TLR2 deficiency reduced renal inflammation, TGF-β levels and the expression of the slit membrane proteins nephrin and podocin. In addition, ICAM-1 was found to mediate renal dysfunction in db/db mice indicating a role for infiltrating leukocytes in diabetic nephropathy (121). The NLR-family member NLRP3 was also studied in the context of DN. ATP was found to signal through the P2X4 receptor in HK2 cells to induce IL-1β and IL-18 (122). In addition, P2X4 expression was increased in tubules of diabetic nephropathy patients and correlated with urinary IL-1β and IL-18 levels (122). NLRP3 was also found to be increased in streptozotocin-treated rats together with IL-1β, IL-18 and increased renal cholesterol levels (123). Overall, we can conclude that PRRs play a detrimental role in models of DN through the induction of inflammation.}

Proteinuria is a hallmark for CKD development and is believed to contribute to the pathology of CKD. Only low molecular weight proteins such as albumin are selectively filtered by the glomerulus and subsequently reabsorbed by the proximal tubules. Evidence of proteinuria therefore indicates a decreased glomerular filtration barrier or a decreased capacity of tubular epithelium to reabsorb proteins. Albumin overload in WT mice induced loss of the renal cellular tight junctions ZO-1 and claudin-1 which was dependent on NLRP3 and caspase-1 (124). A similar observation was made by in the model of unilateral ureteral obstruction-induced CKD by Pulskens et al. indicating a conserved mechanism (85). The mechanism behind albumin-induced NLRP3 activation was discovered to be mediated by megalin and cubilin (125). Both receptors endocytosed albumin which triggered NF-κB activation and induced lysosomal rupture, indicative for NLRP3 activation. The role of IL-1β or IL-18 was not investigated here.
CHAPTER 1

In conclusion, the PRRs TLR2, TLR4 and NLRP3 show a pro-inflammatory role in models of metabolic syndrome-associated CKD, i.e. diabetic nephropathy and albumin overload. We investigated the role of NLRP3 in a model of diet-associated CKD with respect to inflammation, fibrosis and lipid homeostasis in this thesis.
5. Outline of this thesis

In this thesis, the role of PRRs in sterile inflammatory kidney disease is investigated in models of either IR-induced AKI or diet-associated CKD. Leukocytes infiltrating the kidney upon tissue damage and renal parenchymal cells express TLRs and NLRs. In both models, DAMPs are present either released by necrotic cells or through dietary intake. In line with the acknowledged role of PRRs, TLRs and NLRs were found to be pro-inflammatory and thus detrimental to the kidney. However, research into the role of PRRs on other physiological processes such as cell death, proliferation and fibrosis revealed novel non-inflammatory, tissue-specific effects. Here, we investigated the tissue-specific role of PRRs on inflammation, fibrosis, proliferation, lipid homeostasis and cell death using the model of IR-induced AKI and diet-associated CKD.

In chapter 2, we investigated the role of the Nod-like receptor NLRP3 on the development of fructose water- or Western diet-induced metabolic syndrome and subsequent CKD characterised by albuminuria, renal inflammation and fibrosis. Furthermore, we analysed renal lipid homeostasis as a detrimental factor linked to NLRP3 and diet-associated nephropathy. We continued in chapter 3 by investigating the role of the same receptor NLRP3 but here in a model of acute kidney injury (AKI), i.e. ischemia reperfusion (IR)-induced injury. We performed bone marrow transplantations and applied ischemia to the kidneys of these chimeric mice to investigate the contribution of leukocyte- versus renal-associated NLRP3 on renal function, repair and injury. In chapter 4, we examined the role of the mitochondrial-associated NLRX1 on renal dysfunction, cell death and inflammation. In addition, the contribution of leukocyte- versus renal-associated NLRX1 on renal dysfunction and tubular apoptosis and necrosis was analysed. Finally, the role of Toll-like receptor TLR9 was studied following moderate and severe renal ischemia reperfusion in relation to the release of its ligand, mitochondrial DNA and the induction of remote liver injury in chapter 5.
CHAPTER 1

References


General introduction


CHAPTER 1


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CHAPTER 1


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