Mechanisms of renal injury and repair: role of stem cells, chemokines and the nodosome

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General Introduction
The kidney

In order to excrete excessive fluid and waste products, blood is filtrated by the kidney. However, the main function of the kidney is the maintenance of the organism’s homeostatic balance by regulating, among other things, the electrolyte balance, blood pressure and production of several hormones. The smallest functional unit of the kidney is the nephron, which consists of the glomerulus, proximal tubule, Henle’s loop, distal tubule, and collecting duct. Macroscopically, the kidney is divided in the cortex and medulla. Whereas human kidneys are composed of about a dozen lobes consisting of a pyramid of medullary tissue plus the cortical tissue overlying its base and covering its sides, murine kidneys are unilobular and are composed of a continuous medullary region surrounded by cortex.

Filtration of the blood occurs in the glomerulus, which is a network of capillaries surrounded by visceral epithelial cells called podocytes. The capillaries form loop-like structures that are supported by mesangial cells. Blood is supplied to the glomerulus via an afferent arteriole, and leaves the glomerulus via an efferent arteriole. The Bowman’s capsule, consisting of parietal epithelial cells, surrounds the glomerulus. This capsule connects to the tubule and is intersected by the efferent and afferent arterioles.

The filtrate passes through the renal tubule before entering the collecting ducts. The tubule can be divided into distinct segments (proximal tubule, Henle’s loop, and distal tubule) with specific functions including reabsorption of water, salts, and organic solutes, primarily glucose and amino acids. After leaving the glomerulus the filtrate first enters the proximal tubule which can be further subdivided into the S1, S2, and S3 segments. The S3 segment, which is particular vulnerable to oxygen deprivation, is located in the area where cortex and medulla meet, the corticomedullary region.

Finally the filtrate enters the collecting duct, the last component of the kidney to influence the body’s electrolyte and fluid balance. Urine leaves the collecting duct system through the renal papillae, and passes through the ureter before entering the urinary bladder.

Renal diseases relevant for this thesis

In the studies presented in this thesis two different models for acute kidney injury (AKI), i.e. ischemia/reperfusion and sepsis-induced, and one model for chronic kidney injury, i.e. unilateral ureter obstruction, are used to investigate the mechanisms taking place during various renal diseases.

Acute kidney injury

AKI is defined as an abrupt decrease in kidney function that leads to the accumulation of waste products such as urea and creatinine in the blood. Next to elevated plasma levels of urea and creatinine, the urinary biomarkers kidney injury molecule-1
(KIM-1) and neutrophil gelatinase-associated lipocalin (NGAL) are currently used as more sensitive predictors of renal damage. Despite the progresses made in health care, AKI is still a major clinical problem that affects up to 5% of hospitalized patients and has a mortality rate of 50-80% in these patients. The most common causes of AKI are sepsis, major surgery, low cardiac output, hypovolemia, and medications. Several models are developed to mimic the events taking place during AKI. Underneath the 2 models used in this thesis are described in more detail.

Renal ischemia/reperfusion injury
A major initiator of AKI is ischemia/reperfusion (I/R) injury which occurs in shock, vascular surgery and during renal transplantation procedures. I/R injury results from a sudden transient drop in blood flow to the kidney leading to deprivation of oxygen and nutrients and an accumulation of waste products in the kidney. Once the blood flow is restored, the reperfusion phase, inflammatory cells are able to infiltrate the injured kidney and initiate collateral damage. Renal I/R injury affects endothelial cells of the renal vasculature, however, the most pronounced effects are observed in the tubular epithelial cells (TEC) of the corticomedullary region. TEC actively participate in the early inflammatory response following an ischemic episode via the production of several pro-inflammatory mediators (figure 1). These include chemokines (e.g. KC/CXCL1 and MCP-1/CCL2), pro-inflammatory cytokines (e.g. IL-6 and IL-1β), and complement (e.g. C3). Additionally, damaged tubular epithelial cells release danger ligands (e.g. high mobility group box 1 (HMGB1) and heat shock proteins) which can activate innate immune receptors and thereby induce production of pro-inflammatory mediators. The functions of chemokines will be discussed in more detail later on in this chapter, and the role of chemokines in renal I/R injury is addressed in chapter 2-5.

Figure 1. Following renal ischemia/reperfusion (I/R) injury tubular epithelial cells produce several pro-inflammatory mediators.
Morphological alterations of the TEC upon I/R include loss of brush border and disruption of cell polarity and the cytoskeleton. If the injury is mild at this stage, complete recovery of the TEC can occur. If the TEC is irreversibly damaged, cells die either by necrosis or apoptosis and can obstruct the tubular lumen. Depending on the severity of injury, renal tubules have the capacity to regenerate and eventually restore the lost TEC resulting in functional recovery of the kidney. In the mouse model of I/R injury, tubular repair is observed several days after the initial ischemic insult. This reparative phase is characterized by chaotic arrangement of TEC which are dedifferentiated (absence of polarization, absence of brush border, high nuclear/cytoplasm ratio) and the presence of numerous mitotic figures, indicating high proliferation and hence reparative processes within the tubules. In addition to dedifferentiation of viable TEC, hematopoietic stem cells (HSC) are suggested to be involved in this regenerative process as well. In figure 2 the structural alterations of the tubular epithelium following renal I/R injury are depicted. Several studies have found Y-chromosome-positive TEC in renal female grafts transplanted into male recipients, and in kidneys of female mice transplanted with male bone marrow. However, this was a rare event, occurring only in a small percentage of tubules thereby making the functional contribution questionable. Therefore the role of HSC in renal disease has gained a lot of attention in the past.

Figure 2. Upon ischemia/reperfusion (I/R) injury, proximal tubular epithelial cells (TECs) lose their brush border and polarity, followed by necrosis or apoptosis of the damaged cells. The tubular epithelium has the capacity to restore lost cells either via dedifferentiation and proliferation of surviving TEC or via the incorporation of stem cells. Adapted from: Krause et al. J Clin Invest 2005;115:1705-8
decade. Although initial reports showed a high number of HSC with an epithelial phenotype after renal I/R injury\textsuperscript{12,13}, careful designed experiments by the same and other groups could only observe a few epithelial-like HSC in renal tubules after ischemic injury\textsuperscript{14-16}. Since the levels of HSC that engraft the injured tubules and develop into functional TEC are very low, their contribution to renal repair is thought to be minor. Enhancing the migration of HSC to the injured kidney may result in a significant contribution of these cells to renal repair, and hence has therapeutic potential. In addition to HSC, the bone marrow contains multipotent mesenchymal stromal cells (MSC) that are capable of multilineage differentiation. Besides their presence in the bone marrow, MSC reside in virtually all organs and tissues including the kidney\textsuperscript{17} where they might contribute to local tissue repair most likely via paracrine mechanisms. Indeed, administration of MSC ameliorates tissue damage and enhances anti-inflammatory mediators upon renal I/R injury\textsuperscript{18-20}. However, in a large-animal model of renal I/R injury, infusion of MSC did not show protective effects\textsuperscript{21}. Renal recovery after I/R injury by MSC might involve several mechanisms including engraftment into damaged tubules, release of paracrine and/or endocrine signals, and stimulation of endogenous repair by regenerating local resident stem cells (reviewed in \textsuperscript{22}). Of these three mechanisms, engraftment of exogenous MSC is thought to have a minor contribution to renal repair. Currently, the paracrine and/or endocrine actions of MSC are believed to be predominantly responsible for the protective effect. Although the mechanism of MSC-related renal repair is not completely clear, the therapeutic potential of MSC is currently investigated in clinical trials.

In addition to enhancing tubular regeneration and/or incorporation of stem cells, strategies to prevent initial damage upon renal I/R injury has gained a lot of attention. Since the infiltrating leukocytes not only clean up dead cells but also induce collateral damage, dampening the inflammatory response might be protective. Approaches to dampen the inflammatory response include reducing leukocyte influx by blocking chemokines that are involved in leukocyte migration, and inhibiting innate immune receptors in order to reduce production of pro-inflammatory mediators.

\textit{Sepsis-induced kidney injury}

Sepsis is a characteristic set of systemic reactions to overwhelming infection that causes multiple organ failure, including AKI. Between 45\% and 70\% of all AKI cases is associated with sepsis\textsuperscript{23-26}. Different pathophysiological mechanisms of sepsis-induced AKI are proposed including vasodilatation-induced glomerular hypoperfusion, dysregulated circulation within the peritubular capillary network, inflammatory reactions by systemic cytokine storm or local cytokine production, and tubular dysfunction induced by oxidative stress (reviewed in \textsuperscript{27,28}). Although renal dysfunction is evident in septic AKI, only mild, non-specific renal histological changes in human and animal sepsis are observed\textsuperscript{29}. On the contrary, non-septic causes of AKI (e.g. I/R) induce apparent histological changes. Therefore it is now
believed that the pathogenesis of septic AKI involves distinct mechanisms as compared to non-septic causes of AKI.

The inflammatory response taking place during sepsis is initiated by recognition of the pathogen by so-called pattern recognition receptors such as toll-like receptors (TLRs) and nucleotide-binding domain and leucine-rich repeat containing receptors (NLRs). TLRs and NLRs are expressed in many cell types among which are inflammatory cells and various renal cells including TEC. Signaling via TLRs and NOD1 and NOD2, both members of the NLR family, leads to the activation of NF-κB and subsequent production of pro-inflammatory cytokines. The renal cells that are damaged upon this first inflammatory response, release stress ligands (e.g. high mobility group box-1, heat shock proteins) that can signal via TLRs and NLRs to induce a second wave of injury. This pro-inflammatory phase is followed by a compensatory anti-inflammatory immune response characterized by increased production of pro-inflammatory cytokine IL-10, impaired chemotaxis, and increased lymphocyte apoptosis. Apart from signaling via TLRs and NLRs, sepsis also affects several other pathways including complement cascade, coagulation pathway activation, and vascular injury.

Animal models to mimic sepsis can be divided into three categories: 1) injection of an exogenous toxin (e.g. the Gram- bacterial cell wall component lipopolysaccharide (LPS)); 2) alteration of the animal’s endogenous protective barrier, such as intestinal leakage (e.g. cecal ligation and puncture); and 3) infusion or instillation of exogenous bacteria. A widely used model for sepsis is LPS administration, which induces systemic inflammation that mimics many of the initial clinical features of human sepsis including increases in proinflammatory cytokines such as TNFα and IL-1β without bacteremia. Furthermore, LPS administration causes renal injury as indicated by increased blood urea and influx of leukocytes. One major drawback of LPS as a model for sepsis is that only Gram- bacteria possess LPS. Especially since 2 decades the most common organisms responsible for the development of sepsis are Gram- bacteria (~50% of all cases), while Gram- bacteria account for ~40% and polymicrobial infections are responsible for only 5% of all cases of sepsis. The major component of Gram- bacterial cell walls is peptidoglycan (PGN) which can also be found as a thin layer in Gram- bacteria. Whether PGN alone is able to induce sepsis is still a matter of debate, and of course is dependent on the dosage. A few studies have shown organ injury, including renal dysfunction, and an inflammatory response upon administration of purified PGN. Others have reported a synergistic effect of PGN and LPS, whereas PGN alone does not induce an inflammatory response and/or organ dysfunction. Therefore we co-administered LPS and PGN in mice to mimic the events taking place during sepsis, and additionally determined the role of NOD1 and NOD2 during the systemic inflammatory response and development of sepsis-induced AKI (chapter 7).
Chronic kidney injury

Tissue fibrosis is a leading cause of morbidity and mortality; nearly 45% of all deaths in the Western world are attributed to some type of chronic fibroproliferative disease. Renal fibrosis is regarded as the final common pathway for almost all forms of chronic renal disease, and involves interstitial fibrosis and glomerular sclerosis. Clinically, progression of renal insufficiency is much better correlated with renal interstitial fibrosis and inflammation than with glomerular sclerosis. Renal fibrosis is defined by the accumulation of interstitial leukocytes and myofibroblasts that contribute to abnormal accumulation of extracellular matrix (ECM) and eventual tubular atrophy and loss of renal function.

A widely-used animal model to study renal fibrosis is unilateral ureter obstruction (UUO) which generates progressive renal injury. Complete UUO initiates a rapid sequence of events in the obstructed kidney, leading within 24 hours to reduced renal blood flow and glomerular filtration rate. This is followed within several days by hydronephrosis, interstitial infiltration of leukocytes (mainly macrophages) that produce cytokines responsible for tubular damage and fibroblast proliferation and activation, tubular cell death by apoptosis and necrosis leading to tubular atrophy, and finally phenotypic transition of resident renal cells. The influx of leukocytes is preceded by local expression of chemokines, chemokine receptors and adhesion molecules. Among the inflammatory cells, macrophages are the most numerous, moreover they play a dual role during the development of progressive renal injury; they can either promote fibrosis via the production of TGFβ or attenuate inflammation and induce tissue repair by phagocytosis of ECM fragments. Additionally, macrophages promote apoptosis of TEC which together with a decreased TEC proliferation shifts the balance between apoptosis and proliferation towards cell death. The development of tubulointerstitial fibrosis is characterized by the appearance of activated (α-SMA-positive) fibroblasts, also known as myofibroblasts, these cells are mainly responsible for ECM deposition. Although the UUO model has provided many new insights into the pathogenesis of obstructive nephropathy and of progressive renal fibrosis in general, the complex mechanisms taking place during chronic renal failure are not fully elucidated. In this thesis we investigated the contribution of the innate immune receptors NOD1 and NOD2, also known as the nodosome, to renal injury and fibrosis during obstructive nephropathy.

Chemokines

In all three models for renal disease studied in this thesis, expression of several chemokines was increased following injury. Chemokines are chemotactic cytokines that are well-known for their ability to direct the migration and activation of inflammatory cells. Chemokines can be divided into four families based on their
amino acid sequence in relation to their cysteine moieties/differences in structure: CC, CXC, CX3C and XC (reviewed in53). The CXC chemokine subfamily can be further divided based on the presence of a glutamate-leucine-arginine (ELR) motif that precedes the first cysteine residue in the primary amino acid sequences of these chemokines. Currently 28 CC, 17 CXC, 1 CX3C, and 2 XC mammalian chemokines are known. Within these chemokine families functional homology exists: CC chemokines attract one or more classes of mononuclear cells, eosinophils and basophils; ELR+ CXC chemokines attract mainly granulocytes; ELR- CXC chemokines attract lymphocytes; CX3C chemokines act on T cells; and XC chemokines attract lymphocytes, natural killers cells and granulocytes53;54. ELR+ CXC chemokines (e.g. CXCL1/KC and CXCL8/IL-8) orchestrate the early phases of the inflammatory response, their key function is to attract granulocytes to sites of inflammation and induce granule exocytosis and the respiratory burst53. On the other hand, ELR- CXC chemokines (e.g. CXCL10/IP-10 and CXCL11/I-TAC) and CC chemokines (e.g. CCL2/MCP-1 and CCL5/RANTES) attract lymphocytes and monocytes and hence might play a role during chronic inflammation. In addition, chemokines can be further classified as either homeostatic or inflammatory based on their expression pattern and function in the immune system. The homeostatic chemokines are generally those that are constitutively expressed, e.g. CXCL12/SDF-1. Inflammatory chemokines are upregulated by pro-inflammatory stimuli and help orchestrate innate and adaptive immune responses. Chemokines exert their biological effect by binding to G protein-coupled cell surface receptors. In line with the nomenclature for chemokines, the receptors are designated CCR1-11, CXCR1-7, CX3CR1, and XCR1 according to the binding of respectively CC, CXC, CX3C, or XC chemokines. Chemokine and receptor interactions vary widely in terms of selectivity. A few receptors bind exclusively to one receptor and vice versa. However, there is also redundancy in chemokine and receptor interactions since several chemokines bind more than one receptor and many receptors recognize more than one chemokine.

Originally studied for their role in inflammation, it has now become clear that chemokines are also involved in other processes including angiogenesis, homeostasis, development, migration of stem cells and wound healing54;55. During renal I/R injury, infiltrated leukocytes56-58 as well as all types of renal cells including TEC3 start to produce chemokines. In different experimental models of renal injury the functional role of several of these chemokines and chemokine receptors has already been investigated (reviewed by Anders et al.59). In lupus nephritis for example, blocking the interaction between CCL2/MCP-1 and CCR260-62 or neutralizing CX3CR163 ameliorated the initiation and progression of renal damage. In experimental diabetic nephropathy, CCL2/MCP-1 deficiency64;65 or neutralizing CXCL10/IP-1066 decreased renal damage by reducing the accumulation of macrophages or T cells, respectively. In ischemic renal injury a protective role of inhibiting CCL2/MCP-1 signaling67;68, neutralizing CXCL1/KC69 or CXCL2/MIP-269, and blocking the chemokine receptor CX3CR170;71 during the inflammatory response
has been reported. However, a deficiency for CCR5, the receptor for 8 different CC chemokines, did not impair interstitial macrophage accumulation in renal interstitial fibrosis and augmented macrophage accumulation in glomerulonephritis probably due to compensatory signaling via other CC chemokine receptors. The large redundancy and possible compensatory mechanisms are challenging when considering one specific chemokine or chemokine receptor as a therapeutic target. In addition, as tissue damage and repair is dependent on timely induction and suppression of chemokines and is modulated by the influx of leukocytes it is important to identify the spatial and temporal expression of chemokines during renal disease. By means of correlating leukocyte influx with chemokine expression, it has been proposed that temporal expression of chemokines is a crucial factor in the regulation of I/R injury and repair as has been demonstrated by a biphasic expression of several CXC chemokines after renal I/R injury coinciding with the acute inflammatory and the later reparative phase (chapter 4). In addition, the high therapeutic potential of stem cells has boosted the research for CXCL12/SDF-1 and its receptor CXCR4, the chemokine axis thought to be responsible for stem cell migration. However, recently it has become clear that CXL12 is also an important factor in the survival of cells including TEC. Overall, it is essential to dissect the role of individual chemokines carefully before considering it as a therapeutic target due to time and spatial dependent expression and to elucidate whether they have additional biological effects besides chemotaxis of leukocytes or stem cells. In this thesis the role of CXCL12/SDF-1 (chapter 2, 3) and CCL2/MCP-1 (chapter 5) during renal I/R injury has been investigated. Therefore, these 2 chemokines are described in more detail below.

**SDF-1/CXCL12**
The chemokine stromal cell-derived factor-1 (SDF-1, also known as CXCL12) and its receptor CXCR4 have been identified as the central signaling axis regulating the trafficking of hematopoietic stem cells (HSC) and progenitor cells. In immune-deficient mice the SDF-1/CXCR4-axis is essential for efficient homing of human stem cells as indicated by the impaired repopulation of the bone marrow after blocking stem cell-associated CXCR4. In addition, local administration of recombinant SDF-1 results in an increased homing of stem cells to the liver, spleen and bone marrow. SDF-1 is upregulated after injury in diverse experimental models including skin ischemia, toxic liver injury, myocardial infarct and DNA damage. In the kidney we and others have shown that after I/R injury SDF-1 protein levels are upregulated. In addition, SDF-1 is increased in kidneys from patients with various renal diseases including chronic allograft nephropathy and acute tubular necrosis. Tögel et al. also observed a selective homing of exogenous administered bone marrow cells to the injured kidney and could inhibit this by blocking bone marrow-associated CXCR4. We, however, showed that SDF-1 does not play a significant role...
in the migration of purified HSC during renal I/R injury. Although HSC preferentially migrate to the injured kidney following renal ischemia, local administration of SDF-1 recombinant protein did not increase HSC migration to the kidney during I/R injury. Using the opposite approach, neutralization of SDF-1 or blocking of HSC-associated CXCR4 with neutralizing antibodies also did not inhibit HSC migration.

Hence, the functional significance of the increased post-ischemic SDF-1 expression by TEC might not be related to tubular repair involving HSC incorporation. Recent studies implicate that besides regulating the migration of cells, SDF-1 also has other functional activities. SDF-1 can enhance cell survival by inhibiting apoptosis in a wide variety of cells. We have shown that SDF-1 provides morphological and functional protection against I/R injury. Overall, although postulated to induce migration of renoprotective cells from the bone marrow, renal SDF-1 seems more important for the induction of resistance of TEC to apoptosis.

The chemokine receptor CXCR7, initially identified as an orphan receptor, was recently described as second receptor for SDF-1 by Balabanian et al. Although CXCR7 binds the chemokines SDF-1 and I-TAC/CXCL11 with high affinity, characteristic chemokine responses like chemotaxis could not be assessed. Instead, SDF-1/CXCR7 interactions are involved in the survival of various cell types. However, others have shown that the role of SDF-1 in survival is not regulated via CXCR7 but via CXCR4.

Initial research regarding the role of SDF-1 in renal disease focused on the migration of HSC and progenitor cells and their role in repair of damaged kidney. The discovery of a second receptor for SDF-1 with distinct function as CXCR4 has revealed a new role for SDF-1 during renal disease. Since the role of HSC during renal repair is still under debate (see above) it may well be that the production of SDF-1 upon injury serves as a survival factor via the interaction with CXCR7 which we have shown to be expressed by TEC (unpublished data). Since the discovery of CXCR7 as a second receptor for SDF-1, unexpected results might be explained by the interaction of SDF-1 and CXCR7 and their role in cell survival. The differential role of CXCR4 and CXCR7 is also underscored by the viability of knockout mice, while CXCR4 deficient mice are viable and hence CXCR7 does not play a crucial role in HSC migration.

**CCL2/MCP-1**

The chemokine CCL2, also known as monocyte chemoattractant protein-1 (MCP-1), is a potent chemoattractant for monocytes and in addition for T cells and natural killer cells. In vivo MCP-1 is the main chemoattractant for monocytes; MCP-1 deficiency results in a decreased and delayed macrophage accumulation in various inflammatory models. As discussed earlier, macrophages can play various roles which can be described as either pro-inflammatory or anti-inflammatory. Moreover, macrophages can be roughly divided into either classical (M1) or alternate (M2) activated, possessing a pro-inflammatory and an anti-inflammatory or tissue...
remodeling phenotypic function respectively (reviewed in 109). There is a tight balance between the detrimental and beneficial effects of macrophages; reducing macrophage accumulation does not necessarily lead to better outcome after an inflammatory event. Indeed, in a model of skeletal muscle I/R107 and myocardial infarct105 reduced numbers of macrophages are accompanied by an accumulation of (apoptotic) granulocytes and damaged cells in MCP-1+/− mice suggesting defective phagocytosis of dead cells and impaired tissue remodeling.

Moreover, the role of MCP-1 extends beyond its monocyte chemoattractant properties. MCP-1 activates the respiratory burst of monocytes and induces the expression of the pro-inflammatory cytokines IL-6 and IL-1β110. In addition, MCP-1 may exert direct effects on TEC; in vitro MCP-1 triggers TEC leading to activation of NF-κB, a transcription factor commonly involved in inflammatory responses, without interaction with its chemokine receptor CCR2111. This is supported by a study describing significantly reduced mean graft survival of recipients carrying a MCP-1 polymorphism resulting in increased MCP-1 production, while carriers of a CCR2 polymorphism, which alters CCR2 expression and function, had no effect on graft survival112. Based on these properties MCP-1 can be regarded as pro-inflammatory. On the other hand, MCP-1 may also function as a survival factor since it inhibits apoptosis of T cells113, cardiomyocytes114,115, neurons and astrocytes116, alveolar epithelial cells117, prostate cancer cells118, and TEC (chapter 5). Recently it was suggested that MCP-1 itself may also play a role in the polarization of macrophages. Blocking MCP-1 reduced M2 macrophage number in tumors in vivo119. Moreover, Roca et al. demonstrate that MCP-1 induces the activation of M2 macrophages120. Interestingly, upon renal I/R injury there was a shift in macrophage type towards a more pro-inflammatory (M1) phenotype in MCP-1−/− mice compared with MCP-1+/+ (chapter 5). This suggests that MCP-1 deficiency skews the macrophage polarization towards M1 due to a reduced ability to differentiate into M2 macrophages. Overall, the versatile functions of MCP-1 may indicate that time and spatial factors are involved in determining whether MCP-1 might function as a mediator of inflammation or survival.

MCP-1 is produced by TEC in response to various stimuli (reviewed in 3). As early as 1991, Safirstein et al. reported an increased and prolonged expression of MCP-1 after renal ischemia121. Importantly, MCP-1 expression was reported to correlate with monocyte infiltration in the post-ischemic kidney122. Furthermore, in vitro studies using various human TEC cell lines have shown that the majority of monocyte chemoattractant activity produced by these cells is accounted for MCP-1123;124. Studies exploring the role of MCP-1 in renal I/R injury have focused on the interaction between MCP-1 and its chemokine receptor CCR2. Decreased renal damage and reduced influx of macrophages and granulocytes was observed after blocking CCR2 in renal I/R injury67,68. However, in addition to MCP-1, MCP-2, -3, -4, and -5 are also chemokine ligands for CCR254. Therefore, blocking CCR2 does not discriminate between the different MCP chemokines. In addition, since recent
data indicates that MCP-1 has versatile functions which might be independent of interaction with CCR2, it is important to elucidate the role of MCP-1 during renal disease. A deficiency for MCP-1 augments renal damage by increased apoptosis of TEC (chapter 5) during the acute inflammatory phase following I/R injury. It may well be that blockage of MCP-1 at later time point following I/R injury has a different outcome and might impede macrophage accumulation as has been shown in a model of nephrotoxic serum nephritis\textsuperscript{125}. Even if macrophage accumulation is decreased by blocking MCP-1 the outcome is not clear; macrophages are involved in progression of disease towards a chronic phenotype by enhancing fibrosis, but on the other hand macrophages are required to clean up inflammatory cells such as granulocytes and dead cells from the injured tissue. Before considering MCP-1 as a therapeutic target its role during the various phases of renal injury has to be determined thoroughly to avoid detrimental effects.

**Role microbiota in systemic immune response**

The role of microbiota in gut homeostasis and gut immune development is well established. However, systemic effects of the microbiota are less well investigated. Previous studies have demonstrated a role for microbiota in modulating the adaptive immunity. Mice lacking an intestinal microbiota develop less severe symptoms in autoimmune models for arthritis\textsuperscript{126} and encephalomyelitis\textsuperscript{127} (i.e. a mouse model for multiple sclerosis), both studies showed altered T cell populations. On the contrary, in other autoimmune conditions lack of microbiota increases disease severity as demonstrated in non-obese diabetes mice deficient for the toll-like receptor adaptor molecule Myd88 where normal intestinal microbiota alleviated spontaneous progression of type 1 diabetes\textsuperscript{128}. However, not only the adaptive immunity is shaped by microbiota, but evidence is accumulating that there is also a direct effect on innate immunity as shown by Clarke et al\textsuperscript{129}. In this study granulocytes isolated from antibiotic-treated or germ-free mice were less efficient in killing of bacteria \textit{ex vivo}. Moreover, they show that the bacterial cell wall component PGN, which is constantly turned over in the gut and either excreted or translocated across the gut mucosa into the circulation, primes granulocytes\textsuperscript{129}. It is believed that microbial products derived from the intestinal microbiota are found systemically where they can modulate both the innate and adaptive immune system, thereby facilitating a rapid inflammatory response upon infection.

The question was raised whether the intestinal microbiota plays also a role in sterile inflammation, such as renal I/R injury, where a fast responding immune system, and more specific primed granulocytes, may be detrimental. Upon inducing renal I/R injury, granulocytes are the first inflammatory cells infiltrating the damaged kidney\textsuperscript{8}. These recruited granulocytes can clear dead cells and debris but also amplify renal damage. Moreover, renal I/R injury is exacerbated\textsuperscript{130} or attenuated\textsuperscript{131-133}
by experimentally increasing or reducing granulocyte infiltration, respectively. Interestingly, depletion of the intestinal microbiota by broad-spectrum antibiotic treatment did preserve renal function with concomitant impaired granulocyte influx following renal I/R injury (chapter 6). Although depletion of microbiota in adult mice did protect against renal I/R injury, a study using germ-free mice, that lack microbial communities from birth, revealed that they are more prone to renal I/R injury. Germ-free mice had greater renal function impairment compared with control mice, moreover, conventionalized germ-free mice showed less tubular damage as compared with germ-free mice upon renal I/R injury. The discrepancy between both studies may be explained by the role of the gut microbiota in postnatal development of the immune system. During the early postnatal period, the intestinal microbiota stimulates the development of both local and systemic immunity, while later on these components evoke inhibitory mechanisms intended to keep both mucosal and systemic immunity in check. Overall, the sterile inflammatory response taking place upon renal I/R injury is influenced by the intestinal microbiota.

Various studies in mice without or with altered microbiota have revealed that the intestinal microbiota can shape the development, distribution, activation level, differentiation status and inflammatory response of many inflammatory cells both in the gut and at peripheral sites within the host. Next to this priming effect of the microbiota on the immune system, the microbiota may also elicit an inflammatory response directly by translocation of bacteria from the intestine into the systemic circulation. Bacterial translocation is a well known phenomenon during intestinal and liver ischemia. Moreover, in a rat model of 5/6 nephrectomy bacterial translocation occurred in 40% of the animals. However we could not detect an effect on intestinal permeability and no evidence of bacterial translocation was found following renal ischemia (chapter 6). Consequently, there is no evidence of a direct role of the intestinal microbiota in renal I/R injury, suggesting that the role of the microbiota following I/R of the kidney is limited to an immunomodulatory effect.

The nodule: NOD1 and NOD2

NOD1 and NOD2 are members of the nucleotide-binding domain and leucine-rich repeat containing receptor (NLR) family which are intracellular pattern recognition receptors (PRRs) involved in the rapid response against invading pathogens. High structural homology is found among NLR members, i.e. a C-terminal LRR domain, and a central NOD (also known as NACHT) domain. The N-terminal region of NOD1 and NOD2 is composed of one or two CARD (caspase-recruitment domain) respectively. Different sub-structures of PGN, a component of bacterial cell wall, can signal via NOD1 and NOD2 to induce the production of pro-inflammatory
cytokines via NF-κB activation\textsuperscript{140-142}. NOD1 senses DAP (diaminopimelic acid) which is found only in Gram\textsuperscript{-} bacteria\textsuperscript{143;144}, while NOD2 senses MDP (muramyl dipeptide) a component of PGN present in Gram\textsuperscript{-} and Gram\textsuperscript{+} bacteria\textsuperscript{145;146}. In addition to bacterial structures, Sabbah \textit{et al.} reported the activation of NOD2 by single-stranded RNA viruses\textsuperscript{147}. Recently, activation of NOD1 and NOD2 by the non-pathogenic derived cell permeable small molecule DMXAA was reported\textsuperscript{148}. As far as we know, no endogenous stress ligands that are released upon tissue injury for NOD1 and NOD2 are described. However, based on their structural and functional similarities with other NLR family members and TLRs it is conceivable that NOD1 and NOD2 are also activated by currently unknown endogenous ligands. Expression of both NOD1 and NOD2 is found in various cells and because of their high similarity, it is not surprising that NOD1 and NOD2 contribute in a redundant manner to the immune response following infection\textsuperscript{149;150}. NOD1 is widely expressed in many cell types and organs including TEC in the kidney\textsuperscript{140;151-153}. Although NOD2 expression is believed to be more restricted, it has been described in leukocytes and various epithelial cells including TEC\textsuperscript{141;142;152-154}. The expression of NOD1 and NOD2 in the kidney and more specific in TEC, that are vulnerable and actively participate in the immune response following kidney injury, indicates that these PRRs may participate in the inflammatory response observed following renal damage. This hypothesis is addressed in chapter 6-8.

NLRs are linked genetically to inflammatory disorders. A well known association is the one between NOD2 and Crohn’s disease (CD), a chronic idiopathic inflammatory bowel disorder. Reduced or lost ability to sense MDP and subsequently activate NF-κB has been reported in CD-associated NOD2 mutations\textsuperscript{146;155}. In addition, several missense mutations of NOD2, resulting in constitutive NF-κB activation and enhanced response to MDP, cause Blau syndrome\textsuperscript{156} and early-onset sarcoidosis\textsuperscript{157}, two autosomal dominant disorders characterized by early-onset granulomatous inflammation involving the skin, eyes, and joints. Graft-versus-host disease has also been reported to be more severe in patients bearing certain NOD2 polymorphisms\textsuperscript{158}, however this could not be confirmed in a Japanese population\textsuperscript{159}. Several NOD1 polymorphisms have been associated with susceptibility to asthma\textsuperscript{160}, atopic eczema\textsuperscript{161}, and allergy\textsuperscript{162}. These associations suggest that NOD1 and NOD2 play an important role in regulating the immune system.

To unravel the role of NOD1 and NOD2 in the above mentioned inflammatory disorders and during for instance bacterial infection, mice deficient for NOD1 and/or NOD2 were generated. Prior to subjecting knockout mice to disease models, the phenotype must be described in order to distinguish between the primary and secondary effects of genetic changes. The analysis of phenotypic abnormalities in these mice already provided some clues to the physiological role of NOD1 and NOD2. NOD1\textsuperscript{-/-} mice have reduced numbers of isolated lymphoid follicles in the distal ileum and colon, and additionally the total bacterial flora was expanded 100-fold in the ileum\textsuperscript{163}. Recently, Clarke \textit{et al.} observed that granulocytes from
NOD1<sup>−/−</sup> mice showed defects in basal level of killing bacteria<sup>129</sup>. Although NOD2<sup>−/−</sup> mice displayed macroscopically no abnormalities and no overt symptoms of intestinal inflammation<sup>164-166</sup>, abnormal development and function of the Peyer’s patches, characterized by an exaggerated immune response and increased permeability, was observed<sup>164;167</sup>. In addition, increased bacterial loads were present in the terminal ileum of NOD2<sup>−/−</sup> mice<sup>166</sup>. These results indicate that normal functionality of NOD1 and NOD2 in the intestine is important in maintaining the homeostasis between microbiota and the host immune system.

Since NOD1 and NOD2 contribute in a redundant manner to bacterial infection<sup>149;150</sup>, mice deficient for both receptors (NOD1/2 DKO) were generated to investigate the role of the complete nodosome in various conditions. Although in the Salmonella colitis model mice deficient for NOD1 or NOD2 did not show significant changes in pathology, NOD1/2 DKO mice had reduced inflammation and cytokine production coinciding with an increased bacterial burden in the mucosal tissue<sup>168</sup>. A role for the nodosome in metabolic disease was demonstrated by Schertzer et al. They showed that NOD1/2 DKO mice are more insulin tolerant and have reduced lipid accumulation and lower inflammation in adipose and hepatic tissues after a high fat diet<sup>169</sup>. Phenotypic analysis of naïve NOD1/2 DKO mice revealed a possible role for NOD1/2 in metabolic processes and confirmed a role for NOD1/2 in protecting intestinal integrity (chapter 7).

The ability of NOD1 or NOD2 agonist to induce an inflammatory response <em>in vivo</em> is under debate. Several studies reported the production of pro-inflammatory cytokines upon administration of PGN, DAP, MDP or synthetic NOD1 or NOD2 agonists which was abolished in NOD1 and/or NOD2 deficient mice<sup>170-173</sup>. However, others have shown that stimulation with NOD1 or NOD2 agonist alone does not induce the production of cytokines, but co-administration of endotoxin and an agonist for NOD1 or NOD2 enhances TLR-mediated responses<sup>149;150;165;174-176</sup>. NOD1/2 DKO mice subjected to septic shock by co-administration of PGN with LPS had similar systemic response as WT mice, however the renal response was altered as NOD1/2 DKO mice showed increased inflammatory response and preserved renal damage and function (chapter 7). These results indicate a role for NOD1 and/or NOD2 in sepsis-induced acute renal disease.

**Outline of this thesis**

In chapter 2 we investigated the role of the chemokine CXCL12/SDF-1 in directing HSC to the ischemic damaged injury. HSC preferentially migrate towards the ischemic damaged kidney. Manipulation of the SDF-1/CXCR4-axis by neutralizing endogenous SDF-1 or HSC-associated CXCR4 or by local administration of SDF-1 did not significantly alter the migration of HSC towards the ischemic kidney. Since SDF-1 is significantly increased upon renal I/R, we wondered whether there was a
role for SDF-1 independent of HSC trafficking in this model. To examine the role of SDF-1 in the pathogenesis of renal I/R injury, renal SDF-1 protein was decreased by antisense treatment in chapter 3. We observed that antisense treatment resulted in a reduction of corticomedullary SDF-1 expression which was accompanied by severely increased tubular injury and decreased renal function without affecting CXCR4-positive HSC migration. These data suggest that renal SDF-1 is not crucial for HSC migration, but rather protects the TEC against ischemic injury by enhancing its survival.

Chemokines are important players in the migration of leukocytes to sites of injury. In chapter 4 we used microarray analysis to determine the expression of chemokines, chemokine receptors and related genes during the different phases following renal I/R injury. Strikingly, chemokine expression was highest during the reparative phase. Additionally we observed a biphasic expression of several CXC chemokines coinciding with the early inflammatory phase and late repair phase. Of the upregulated chemokines, CCL2/MCP-1 was chosen to determine its role during renal I/R in more detail. In chapter 5 we observed that MCP-1 deficient mice had significant worse survival upon renal I/R injury concomitant with increased TEC apoptosis. Although MCP-1 is mainly known as a monocyte chemoattractant, we did not observe altered macrophage influx, however, we did see a shift in macrophage phenotype towards the more pro-inflammatory (M1) macrophage in MCP-1 deficient mice.

In chapter 6 we determined the role of the intestinal microbiota during renal I/R injury. Broad-spectrum antibiotic treatment resulted in depletion of intestinal microbiota and markedly protected wildtype mice against renal I/R injury. Renal ischemia did not increase intestinal permeability and no evidence of bacterial translocation due to renal I/R injury was observed. To determine whether the role of microbiota during renal I/R injury was regulated via NOD1/2, we subjected NOD1/2 DKO with intact or depleted intestinal microbiota to renal I/R injury. In chapter 6-8 NOD1/2 DKO mice were used. Since these mice are not well described, we first analysed the phenotype of NOD1/2 DKO mice under naïve and inflammatory conditions. In chapter 7 we show a possible role for NOD1/2 in metabolic processes under physiological conditions, and in the development of renal disease during systemic inflammation induced by co-administration of LPS and PGN.

In chapter 8 we determined whether NOD1/2 play a role in inflammation and fibrosis during obstructive nephropathy. Minor changes in inflammatory response were observed in NOD1/2 DKO mice, while no effect was seen on renal injury or development of fibrosis.
References

15. Lin F, Moran A, Igarashi P: Intrarenal cells, not bone marrow-derived cells, are the major source for regeneration in postischemic kidney. J Clin Invest 115:1756-1764, 2005
32. Wray GM, Foster SJ, Hinds CJ, Thiemermann C: A cell wall component from pathogenic and non-pathogenic gram-positive bacteria (peptidoglycan) synergises with endotoxin to cause the release of tumour necrosis factor-alpha, nitric oxide production, shock, and multiple organ injury/dysfunction in the rat. Shock 15:135-142, 2001
Introduction and outline of this thesis


58. Yang L, Mosmann T: Synthesis of several chemokines but few cytokines by primed uncommitted precursor CD4 T cells suggests that these cells recruit other immune cells without exerting direct effector functions. Eur J Immunol 34:1617-1626, 2004


78. Ceradini DJ, Gurtner GC: Homing to hypoxia: HIF-1 as a mediator of progenitor cell recruitment to injured tissue. Trends Cardiovasc Med 15:57-63, 2005


89. Liu Z, Habener JF: Stromal cell-derived factor-1 promotes survival of pancreatic beta cells by the stabilisation of beta-cellin and activation of transcription factor 7-like 2 (TCF7L2). Diabetes 52:1589-1598, 2009


118. Roca H, Varsos ZS, Jiang JJ, CCL2 is a negative regulator of AMP-activated protein kinase to sustain mTOR complex-1 activation, survivin expression, and cell survival in human prostate cancer PC3 cells. Neoplasia 11:1309-1317, 2009


154. Ekman AK, Cardell LO: The expression and function of Nod-like receptors in neutrophils. Immunology 130:55-63, 2010


