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

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General introduction and outline of this thesis

Kidney structure and function

Kidneys are two bean-shaped organs localized in the retroperitoneal space that serve an essential role in the maintenance of body homeostasis by regulating body fluid volume, osmolarity, electrolytic content and acidity. They function as a natural filter of blood by removing waste products (e.g. urea), and foreign substances (toxins and drugs). The nephron is the functional unit of the kidneys and is composed of a glomerulus and a long tubule segmented into proximal tubule, loop of Henle, distal tubule, and collecting duct. The human kidneys contain approximately 1 million nephrons. The process of urine formation begins in the glomerular capillary network, where ultrafiltrate of plasma is formed. Blood enters the glomerular capillaries via an afferent arteriole and leaves via an efferent arteriole. The glomerular filtration barrier is composed by the fenestrated endothelium, the glomerular basement membrane and the foot process of podocytes. The glomerular barrier is permeable to water and small solutes, but not to large molecules (larger than about 50KDa), negatively charged molecules, and blood particles. The glomerular capillaries are supported by mesangial cells. Glomeruli account for 2-5% of the total kidney volume, whereas the rest of the kidney is occupied by tubules and interstitial space.

Kidneys receive through the renal artery the highest tissue-specific blood flow of all organs (20% of the cardiac output, about 350mL/min per 100g tissue), and one fourth of the plasma that enters the glomerulus is filtered by the glomerular barrier. Therefore, it is not surprising that glomeruli are often affected by systemic diseases such as autoimmune complex- and antibody-mediated autoimmune diseases, hypertension and diabetes.

After the ultrafiltrate is collected in the Bowman's capsule, it enters the renal tubule, which is composed by a single layer of epithelial cells responsible for absorption and secretion of solutes and fluids to create the final urine.

Proximal tubules absorb most of the filtered small solutes: 60% of filtered Na^+, Cl^-, K^+, Ca^{2+} and H_2O, more than 90% of filtered HCO_3-. They reabsorb almost all filtered glucose and amino acids via the Na^+-dependent cotransport. Proximal tubules are characterized by a prominent brush border, basolateral inflodings, which are lined with large mitochondria that supply energy for the active pumps (Na^+/K^+-ATPase). Because of their high demand of energy (ATP) and oxygen, tubular epithelial cells (TEC) are highly susceptible to injury upon hypoxia, especially the proximal TEC of the S3 segment present in the cortico-medullary region.

The loop of Henle makes a hairpin loop into the medulla. It is composed by the terminal straight portion of the proximal tubule, the thin descending and ascending limbs, and the thick ascending limb. It is important for maintaining the medullary hypertonicity and for concentrating urine. The thin limbs comprise epithelial cells with no prominent mitochondria; in this segment, H_2O is reabsorbed via medullary hypertonicity. The thick limb is water impermeable and it is thicker; cells have big mitochondria and reabsorb the final Na^+, $2Cl^-$, K^+, Mg^{2+}.

The distal segment of the nephron comprises the distal tubule and the collecting duct and lacks brush borders. It functions in regulating the acid-base balance and the final adjustments in urine volume. Furthermore, the collecting ducts are the site of action of the hormones aldosterone, vasopressin and atrial natriuretic factor.

After passing the collecting ducts, urine enters the ureter and finally the urinary bladder. Besides serving the body as a natural filter of blood, kidneys also function as an endocrine organ producing erythropoietin, which is fundamental for erythrocyte maturation, and 1,25-dihydroxy-vitamin D3, which regulates calcium and phosphate balance, hence bone structure.
The common indexes of kidney function used in the clinic comprise: glomerular filtration rate (GFR), plasma concentration of urea and creatinine, and proteinuria. In the last years novel genes have been identified that are normally expressed at low levels in kidneys and are upregulated upon kidney injury. Among the front-runners are genes, such as osteopontin, clusterin, glutathione S-transferase, neutrophil gelatinase–associated lipocalin (NGAL), kidney injury molecule-1 (KIM-1), tissue inhibitor of metalloproteinase-1 (TIMP-1), interleukin-18 (IL-18), and cystatin C (1).

Renal diseases relevant for this thesis

Here, I summarize the cellular pathophysiological mechanisms underlying acute kidney injury (AKI), endotoxic shock-induced AKI, and chronic kidney disease (CKD). In the studies presented in this thesis, two murine models have been used: AKI induced by lipopolysaccharide (LPS) intraperitoneal injection and unilateral ureteral obstruction (UUO), which resemble, respectively, the human clinical situation of septic shock-mediated AKI and CKD-associated renal fibrosis.

Acute Kidney Injury

Acute kidney injury has been defined by the Acute Kidney Injury Network (AKIN) as an "abrupt (within 48 hours) reduction in kidney function" reflected by the increase in serum levels of waste products such as creatinine and urea.[2] The concentration of creatinine and urea nitrogen in circulation represents the standard metrics used to define and monitor the progression of AKI. However, these parameters are suboptimal markers of kidney injury as they change significantly only after pronounced kidney damage and with a substantial delay in time.[3] In the past years, new urinary biomarkers have been identified as earlier and more sensitive indicators of renal damage, including kidney injury molecule-1 (KIM-1) and neutrophil gelatinase-associated lipocalin (NGAL). Both KIM-1 and NGAL are markedly induced in proximal and distal tubules, respectively, soon after injury and can be detected in urine preceding any changes in the conventional biomarkers (e.g. serum creatinine).[4]
Despite advances in understanding the pathophysiology of AKI and in patient clinical care, AKI continues to be associated with high morbidity and mortality; generally the mortality rates are reported to be in the 30-70% range. AKI affects 2-7% of the hospitalized patients, with a higher incidence (up to 25%) in patients admitted to the intensive care unit.[5] The most common causes of AKI are decreased renal perfusion (heart failure, cardiac arrest, hypovolemia), sepsis, drug nephrotoxicity, and major surgery.[6]
The pathophysiology of AKI is complex and is further complicated by the fact that often AKI occurs in concomitance with multi-organ failure. Hemodynamic and tubular factors, together

with inflammation, contribute to the dysfunction of the kidney. In response to ischemic or toxic insults, the common pathomechanims contributing to AKI development include: (a) reduction in renal blood flow with endothelial vasoconstriction, small vessel occlusion, compromised microcirculation and hence regional ischemia particularly in the outer medulla, (b) tubular dysfunction, cell death and cell desquamation contributing to intratubular obstruction, (c) tubular transport abnormalities and alteration of the tubuloglomerular balance leading to feedback pre-glomerular arteriolar vasoconstrictive responses, and (d) intrarenal production of inflammatory mediators resulting in interstitial inflammation and vascular congestion.[3, 4]

At a tubular cell level, injury causes a rapid loss in cytoskeletal integrity and cell polarity with mislocalization of adhesion molecule and membrane transport protein, and loss of brush border by proximal tubular cells. Cells may die by apoptosis and necrosis. With the progression of injury, cells are desquamated allowing backleak of filtrate into the interstitium through the basement membrane and resulting in intratubular obstruction by cellular debris.[3]

The tubular epithelium is not merely a passive victim of injury but an active participant in the inflammatory responses. Damaged and activated tubular cells generate inflammatory and vasoactive mediators and upregulate cell adhesion molecules, which in turn worsen the inflammation and vasoconstriction. In addition to secreting proinflammatory and chemotactic cytokines such as monocyte chemoattractant protein-1 (MCP-1), interleukin (IL)-6, IL-8, IL-1β, tumor necrosis factor-α (TNF-α), ENA-78, and RANTES, which activate inflammatory cells, tubular cells also express Toll-like receptors (TLR), complement (e.g.C3) and complement receptors, and costimulatory molecules (CD40, B7-1, B7-2), which regulate T lymphocyte activity.[1, 7-9] Thus, inflammation contributes in a critical manner to the pathophysiology of AKI.

The kidneys have the potential to recover from ischemic or toxic insult by efficiently restoring the cells lost and regaining, in large part, the pre-insult renal function. Whether the source of regeneration is to be ascribed to a subpopulation of renal stem/progenitor cells or to dedifferentiated surviving cells is still matter of debate.[10-12] The contribution of bone marrow-derived cells to the direct replacement of cells seems to be minimal, even though bone marrow cells may have paracrine effects that stimulate proliferation of surviving cells or resident stem cells and/or favor repair by suppressing inflammation.[12, 13]

AKI in Systemic Inflammatory Response Syndrome

Systemic inflammatory response syndrome (SIRS) is a potentially deadly clinical condition that often leads to severe organ dysfunction and/or failure, including AKI. SIRS frequently results from a harmful host response to infection (sepsis).[14, 15] The proportion of septic patients developing acute renal failure increases with the severity of clinical parameters: approximately 19% in moderate sepsis, 23% in severe sepsis, 51% in septic shock. Furthermore, the combination of sepsis and AKI raises the mortality rate to 70%.[16]

Other non-infectious causes of SIRS include acute intestinal ischemia, pancreatitis, gut bleeding, autoimmune diseases, burns, adverse reaction to drugs, cocaine, amphetamines, myocardial

infarction, and trauma. Independently of the etiology, SIRS has the same pathophysiologic properties, with minor differences in inciting cascades. Infectious insults (endotoxins, exotoxins, cell wall components of gram positive bacteria, viruses, fungi) as well as non-infectious insults (cellular debris, complement products, immune complexes) are capable of stimulating the host immune system to produce a number of important mediators such as cytokines, eicosanoids, complement and coagulation components, oxygen radicals, and nitric oxide (NO) that can have profound effects on vascular tone and permeability, resulting in microcirculatory disturbances and finally shock and organ dysfunction.[17]

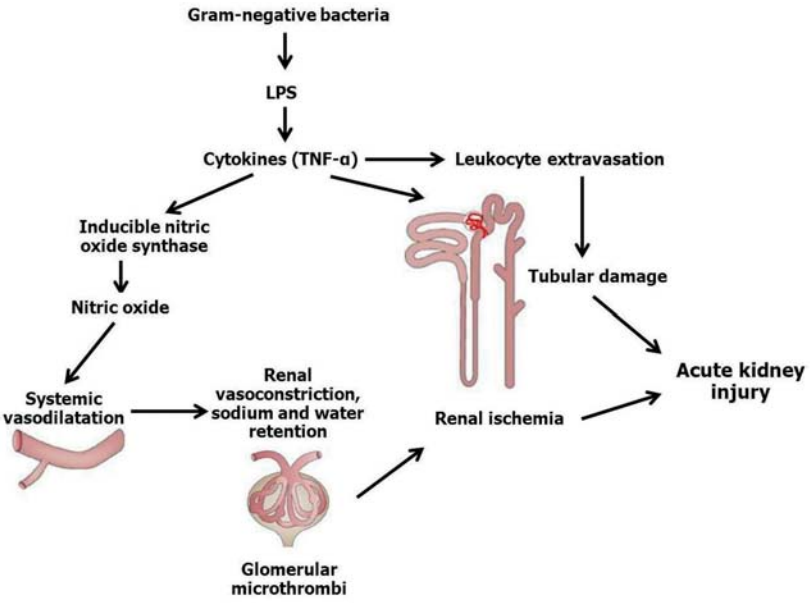
Gram-negative bacteria account for about 60% of septic cases with a microbiological diagnosis, and the lipopolysaccharide released from their outer membrane has a dominant role in initiating the inflammatory responses. LPS is mainly sensed via the TLR4/MD-2 complex after binding LPS-binding protein (LBP) and CD14; the signaling cascade downstream of TLR4 leads to the activation of the transcription factors NF-κB, AP-1 and IRF3 resulting in proinflammatory cytokine gene expression.[15, 18-21] Being a potent inducer of SIRS, LPS has been largely used in animal models to study the pathophysiology of SIRS and SIRS-induced organ dysfunction, including AKI. In this setting, extrarenal TLR4 activation, consequential production of cytokines and NO, sympathetic dysregulation, alterations of endothelium and leukocyte organ infiltration were proposed to contribute to the induction of AKI.[16, 22-24] Cytokines such as TNF contribute to kidney injury directly or through the action of NO, reactive oxygen/nitrogen species, and caspases.[16, 25-27] Renal cells damaged in the first "cytokine storm" release stress ligands and cytokines that account for a second "wave" of inflammation/injury.[28, 29]

Endotoxemia produces a peripheral vasodilatation but a renal vasoconstriction. This paradox can be explain by the cytokine-mediated induction of NO synthase (NOS), which leads to NO-mediated arterial vasodilatation. As a feedback mechanism, the renin-angiotensin-aldosterone system is activated, the sympathetic tone is increase and vasopressin is released. Overall, these effects lead to reduced kidney perfusion and renal vasoconstriction with water and sodium retention, which is the predominant pathogenic factor in early sepsis-related AKI.[16, 24, 25]

Although renal dysfunction is evident in septic AKI, only mild morphological changes are observed in human and animal renal tissues, including tubular cell sloughing, loss of brush border and apoptosis of few tubular cells.[16, 24, 30]

The proinflammatory phase in sepsis and SIRS is followed by an anti-inflammatory response, also known as compensatory anti-inflammatory response syndrome (CARS), characterized by increased levels of anti-inflammatory cytokines such as IL-10, impaired response to endotoxin and reduce lymphocyte activity. It has been suggested that CARS is not a global defect of the immune status of circulating cells, rather an adapted reprogramming of leukocytes to dampen an excessive inflammatory response.[31]

Figure 1. LPS-induced AKI.



LPS from Gram-negative bacteria is recognized by TLR4 on circulating mononuclear cells resulting in hypercytokinemia. Cytokines mediate leukocyte organ infiltration, contribute directly to kidney injury, and induce the production of NO. Nitric oxide mediates arterial vasodilatation, which by a feedback mechanism (activation of the renin-angiotensin-aldosterone system) causes renal vasoconstriction with water and sodium retention and reduced renal perfusion. Adapted and modified from Schrier *et al.*: Acute renal failure and sepsis.[16]

Chronic Kidney Disease

Chronic kidney disease (CKD) is a general term for heterogeneous disorders affecting the structure and the function of the kidney. CKD are defined on basis of kidney damage (e.g. albuminuria) and renal dysfunction (e.g. glomerular filtration rate - GFR < 60 mL/min/1.73 m^2) for 3 months or for a longer period. The disease is classified in 5 stages according to the GFR. The only treatment in end-stage renal disease (ESRD) is dialysis and transplantation.[32]

In many countries, the incidence of CKD is high as 200 cases per million per year. Common causes of CKD include glomerular and tubulointerstitial diseases arising from infection and exposure to drugs and toxins, and more importantly diabetic nephropathy and hypertensive nephrosclerosis. Indeed, chronic kidney diseases are generally associated with old age, diabetes, hypertension, obesity, and cardiovascular diseases.[32] Another risk factor for CKD is represented by AKI; recent epidemiological studies (2009) show that patients who suffered from AKI have a marked increase in risk for ESRD development.[33]

Regardless of the etiology, CKD are characterized by progressive scarring that ultimately leads to an irreversible loss of kidney parenchyma and end-stage renal failure. Kidney fibrosis is set up by non-resolving inflammation after a sustained injury. In contrast to wound healing, the length of the post-inflammatory phase exceeds and continues unchecked triggering the activation and expansion of matrix-producing cells and resulting in scar formation.[34, 35]

Major cellular events in tubulointerstitial fibrosis include: tubular cell damage, macrophage and T lymphocyte infiltration, myofibroblast activation and expansion from multiple sources, and eventually tubular atrophy, microvascular rarefaction, and excessive extracellular matrix (ECM) deposition.[34, 36]

Tubular epithelial cells (TEC) actively participate in the kidney scarring process. They can release cytokines or damage associated molecular pattern molecules (DAMPs) such as high-mobility group-1 (HMGB-1) and heat-shock proteins (HSP60, HSP70) stimulating an inflammatory response. The potential of TEC as a source of myofibroblasts through epithelial-to-mesenchymal transition (EMT) is still a matter of debate. Whereas a broad agreement exists on TEC undergoing EMT *in vitro*, especially under transforming growth factor-β1 (TGF-β1) treatment, the results of *in vivo* studies using genetic lineage tracking are controversial. Nevertheless, damaged proximal tubular cells have been shown to have a direct role in the fibrosis process by generating profibrotic cytokines. Utilizing several murine models of renal disease, Yang *et al.* demonstrated a causal association between cell cycle arrest in G2/M of proximal TEC and renal interstitial fibrosis. These G2/M-arrested cells were shown to activate the JNK signaling cascade, which upregulates profibrotic cytokines production; inhibition of JNK or p53 (to bypass the G2/M arrest) was sufficient to rescue injured kidneys from fibrosis.[33, 34, 37]

Inflammation has a crucial role in the initiation of renal fibrogenesis after injury. For instance, RAG1 knockout (KO) mice lacking mature B and T lymphocytes are protected against fibrosis after obstructive injury, whereas reconstitution with purified $CD4^+$ T cells in RAG1 KO mice restores fibrogenesis after injury.[38] Similarly, depletion of macrophages is beneficial and ameliorates renal fibrosis after various injuries,[39] while adoptive transfer of macrophages aggravates the fibrotic lesions.[40] It is thought that inflammatory cells promote fibrosis in a paracrine fashion by secreting profibrotic cytokines that act on resident fibroblasts and tubular cells to promote fibrosis.[34] Although inflammation typically precedes fibrosis, different subpopulation of the same cell type may have antagonizing effects on fibrogenesis. For instance, Th-2 responses have profibrotic effects, whereas Th-1-related cytokines have antifibrotic activities; similarly different macrophage subtypes differentially promote repair or fibrosis.[41]

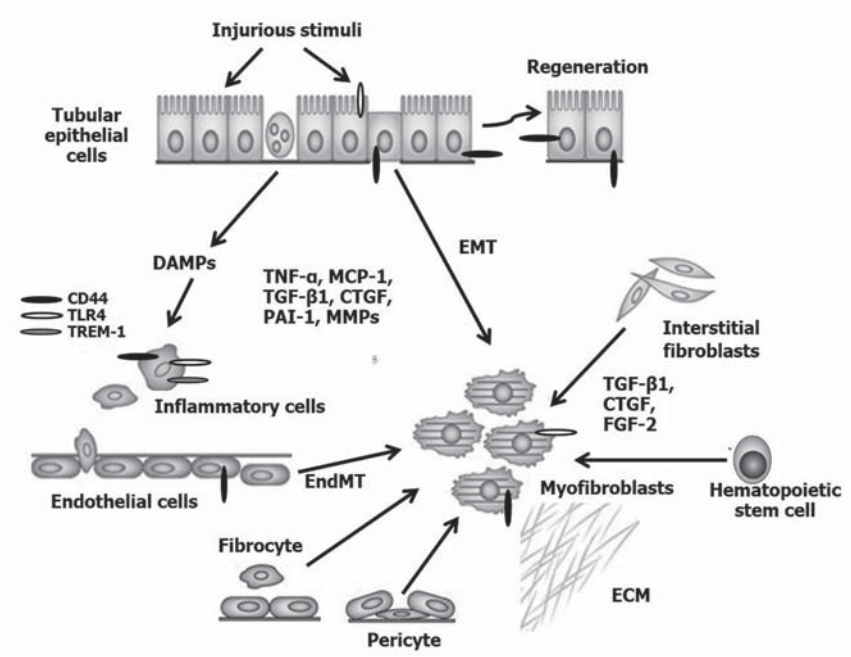
Activated fibroblasts or myofibroblasts are generally accepted as the key effectors in the pathogenesis of fibrosis in kidneys and other organs. Fibroblasts are spindle-shaped cells of mesenchymal origin; their activation and transition to myofibroblasts is classically marked by de novo expression of α-smooth muscle actin (α-SMA) in rodents.[42] The origin of myofibroblasts has long been a subject of investigation and debate. It is believed that myofibroblasts are a heterogeneous population originated from multiple sources through diverse mechanisms, including activation of interstitial fibro-

blasts and pericytes, phenotypic conversion of tubular epithelial and endothelial cells and recruitment of circulating fibrocytes.[34] The first evidence of EMT *in vivo* was published in 2002 by Iwano *et al.* using bone marrow chimeras and transgenic reporter mice in a unilateral ureteral obstruction (UUO) model. The authors showed that after UUO circulating precursors and EMT contribute, respectively, to circa 12% and 36% of the interstitial kidney fibroblasts, identified as FSP-1 (fibroblast specific protein-1) positive cells.[43] However, the use of FSP-1 as marker of myofibroblasts has been reevaluated by the findings that both fibroblasts and leukocytes express this marker during progressive renal injury.[44] The study of Iwano and co-authors utilize Cre/Lox technology to track proximal tubular cells as the Cre recombinase expression is under regulation of the promoter of γ-glutamyl transferase (γ-GT), which is specifically expressed in proximal tubules.[43] In a more recent study, lineage tracing by Cre/Lox techniques did not identify epithelial cells as source of renal myofibroblasts, but rather interstitial pericytes/perivascular fibroblasts following ureteral obstruction. In this study, both FSP-1 and α-SMA were utilized as markers of activated fibroblasts and the Cre drivers were set under the control of Six2 to label nephron epithelial, HoxB7 to label collecting duct epithelia and FoxD1 to label renal stromal cells.[45] Finally, in 2013 Wu *et al.* proposed a causal link between pericyte-derived myofibroblasts and injured epithelium since TGF-β1 causes renal epithelial cell injury, cell cycle arrest and production of profibrotic cytokines, which in turn can mediate pericyte transformation.[46]

Endothelial-to-mesenchymal transition (EndMT) is another mechanism by which myofibroblasts can originate through a dedifferentiation process. Zeisberg *et al.* revealed a contribution of EndMT to approximately 30-50% of the fibroblast population using genetic lineage tracing of endothelial cells (Tie2-Cre-EYFP) and double labelling of tissue for endothelial (CD31) and fibroblast markers (α-SMA, FSP-1).[47]

Once activated fibroblasts secrete abundant extracellular matrix causing architectural remodeling in parallel with functional deterioration.[35] Numerous cytokines/growth factors modulate the progression of glomerular and tubulointerstitial scarring. According to current understanding, fibroblast activation and proliferation in the kidney is triggered by locally secreted fibrogenic cytokines including TGFβ-1,[48, 49] platelet derived growth factor (PDGF),[50] connective tissue growth factor (CTGF),[49] basic fibroblast growth factor (bFGF/FGF-2),[51] TNF-α [52] and MCP-1.[53] Many studies highlighted the central role of TGF-β in the pathological cellular mechanisms underlying the progression of chronic renal diseases. TGF-β family members are important modulators of tubular cell apoptosis or G2/M cell cycle arrest, fibroblast proliferation, myofibroblast activation, EMT and EndMT (*in vitro*), collagen deposition, and hence fibrosis formation.[34] Fibroblast activation and collagen deposition are antagonized by hepatocyte growth factor (HGF),[54, 55] bone morphogenic protein-7 (BMP-7),[56] and interferon-γ (IFN-γ).[57] Both HGF and BMP-7 have been linked to decreased TGF-β1 signaling, preserved renal function and prevention of fibrosis development; thus, their exogenous administration represents a potential therapeutic intervention.[58, 59]

Figure 2. Renal fibrosis.



Upon injury, damaged tubular epithelial cells can release cytokines or DAMPs that contribute to the recruitment and activation of inflammatory cells, which in turn amplify inflammation and hence damage. Myofibroblasts can be derived from different sources through various mechanisms: activation of interstitial fibroblasts, differentiation from vascular pericytes, phenotypic transition of capillary endothelial cells or tubular epithelial cells, recruitment from circulating fibrocytes or hematopoietic stem cells. Epithelial and inflammatory cells can also produce paracrine factors that favor the expansion of the myofibroblast population. The relative contribution of each source to the myofibroblast pool is still controversial. Resident fibroblasts and pericytes appear to be the major route for the generation of myofibroblasts in kidneys. Adapted and modified from Liu *et al.*: Cellular and molecular mechanisms of renal fibrosis.[34]

Besides cytokines, dying/damaged renal cells release several DAMPs such as HMGB-1, heat-shock proteins, histones, hyaluronan (HA), biglycan, fibronectin, and ATP that activate TLR2, TLR4, and NLRP3 inflammasome in parenchymal and infiltrating inflammatory cells.[60] In the setting of chronic injury, DAMPs and their receptors may contribute to maladaptive wound healing by triggering chronic inflammation and amplifying fibrosis via activation of fibrogenic cell populations.[61]

Unilateral Ureteral Obstruction (UUO)

Because chronic renal disorders (whether glomerular or interstitial, congenital or acquired) lead to renal fibrosis, there is a great interest in identifying factors to prevent the fibrotic outcome by means of animal models and transgenic mice.

Unilateral ureteral obstruction was initially performed in rabbits in 1978 resulting in proliferation

of renal interstitial fibroblasts, their transformation in myofibroblasts, and progressive increase in interstitial collagen fibers.[62] Since then, animal models of UUO have been extensively used to elucidate the pathogenesis of obstructive nephropathy, which is the most common cause of renal failure in infants and children, as well as mechanisms responsible for progressive renal fibrosis. The use of UUO as a model of renal fibrosis provides the advantages of the lack of exogenous toxins and of uremic environment, and the availability of the contralateral kidneys as control. Complete ureteral obstruction leads within 24 hours to reduced renal blood flow and GFR, and within days to hydronephrosis, interstitial inflammatory cell infiltration, and tubular cell death. One/ two weeks after UUO, kidneys are severely hydronephrotic with marked loss of renal parenchyma and evident scarring.[63]

Toll-Like Receptor 4

Toll-like receptors (TLRs) were initially identified as proteins that recognize specific molecular patterns of pathogens and are expressed on cells of the innate immune system. It was later established that TLRs can also interact with a variety of endogenous ligands (DAMPs) and their expression is not confined to innate immune cells. TLRs have been found to reside in parenchymal cells of different organs such as the heart, lungs, intestines, liver and kidneys.[60] The intracellular signalling cascades initiated by activated TLR homo- or heterodimers leads to the activation of transcription factors that regulate the expression of proinflammatory cytokines and chemokines and are required for the development of adaptive immune responses. The proximal events of TLR4-mediated intracellular signaling are initiated by the cytosolic adapters MyD88 or Trif, which, respectively, require TIRAP/Mal and TRAM for their activation. The MyD88-dependent pathway results in phosphorylation of the inhibitor of NF-κB (IκB) targeting IκBs for ubiquitination and proteasomal degradation, and allowing NF-κB nuclear translocation. The MyD88-dependent pathway also activates the MAP kinase pathway, which results in phosphorylation and activation of AP-1. NF-κB and AP-1 induce proinflammatory cytokine expression. TLR4 also recruits TRAM and Trif, leading to the phosphorylation of IRF3 by TBK1 and IKKi kinase and, hence, dimerization and translocation into nucleus, where IRF3 induces type I interferon expression.[18]
TLRs exert an important role in the pathogenesis of renal diseases: their exaggerated activation is associated with ischemic kidney damage, acute kidney injury, end-stage renal failure, acute tubulointerstitial nephritis, acute renal transplant rejection and delayed allograft function.[61, 64] Injury-induced release of intracellular molecules from dying cells and ECM remodelling initiate sterile renal inflammation through TLRs. The TLR4 ligands released/accumulated in the damaged kidneys include: HMGB-1, heat-shock proteins, S100 proteins, uric acid crystals, Tamm-Horsfall glycoprotein, neutrophil-derived alarmins (defensins, lactoferrin), and ECM components such as hyaluronan, biglycan, fibrinogen, heparan sulfate, and fibronectin extra domain A.[65]
Distinct studies highlighted the importance of TLR4 activation in acute renal ischemic injury as reflected by the preserved renal function and tubular morphology, reduced accumulation of

neutrophils and macrophages, expression of proinflammatory cytokines and chemokines in absence of TLR4.[66, 67] In support of these findings, administration of the TLR4 endogenous ligand HMGB-1 after reperfusion exacerbates AKI, whereas treatment with neutralizing antibodies to HMGB-1 affords significant protection in terms of reduced renal dysfunction, damage, tubulointerstitial inflammatory infiltrate and tubular apoptosis.[68] Together with HMGB-1, hyaluronan and biglycan expression increases after renal injury in wild-type and TLR4-null mice, suggesting that these DAMPs may activate TLR4 in ischemia-induced AKI.[66]

In chronic renal injury, TLRs also regulate inflammation and fibrosis; the latter appears to be largely modulated by TLR4, and not by other TLRs.[61] In a murine model of obstructive nephropathy, TLR4-null mice had more tubular damage soon after ureteral obstruction, but develop considerably less fibrosis at later stage of obstruction. Despite lower collagen accumulation, TLR4-deficient mice showed no reduction in myofibroblast accumulation, but rather a decrease in MMP-9 activity. Interestingly, *in vitro* TGF-β-mediated upregulation of collagen type I gene expression was absent in primary TLR4 KO TEC and reduced in renal TLR4 KO myofibroblasts as compared to the wild-type cells (chapter 3[69]). These data were confirmed in a second study, in which TLR4 hyporesponsive mice exhibited a significant reduction in obstruction-induced α-SMA expression, fibroblast accumulation, and renal fibrosis.[70] Another group demonstrated that the profibrotic changes triggered by IL-18 on tubular epithelial cells depend on the induction by IL-18 of TLR4 expression and thus signaling, independently of TGF-β activity.[71]

TLRs and their endogenous ligands may also be important in chronic allograft dysfunction. In human kidney transplants, increased expression of TLR4 and HMGB-1 is found in deceased donor kidneys, while kidneys from patients with a TLR4 loss-of-function mutation have lower levels of inflammatory markers and improved graft function post-transplant.[72] Furthermore, expression of DAMPs, including biglycan, HSPs, fibrinogen, and HMGB-1, increases in the acute and chronic phases after kidney transplantation and mice studies sustain the role of TLRs in allograft dysfunction.[73] Kidney transplants to mice lacking TLR2, TLR4, or the adaptor proteins MyD88 and TRIF showed improved graft function and morphology and decreased leukocyte infiltration and expression of fibrotic markers, cytokines, and chemokines.[73]

Besides TLR functions in inflammation and fibrosis, activation of TLRs also has been implicated in epithelial repair.[74] The work of Kulkarni *et al.* links TLR4 activation to the regenerative outgrowth of surviving TEC. The authors found that IL-22 production by intrarenal mononuclear phagocytes accelerates tubular regeneration after ischemia-reperfusion injury and showed that DAMP-mediated activation of TLR4 signaling induces IL-22 expression.[75] Interestingly, in renal biopsies taken in the acute phase after transplant, higher levels of the TLR4 endogenous ligands S100A8 and S100A9[76] are predictive of favourable graft outcome.[77]

Conclusively, TLRs act as gatekeepers for several response systems sensing infectious agents and alterations in tissue homeostasis; however, if the damaging stimulus cannot be eliminated they may trigger maladaptive responses such as fibrosis.

Triggering Receptor Expressed by Myeloid cells (TREM)-1

TREMs are transmembrane glycoproteins of the immunoglobulin gene family that constitutively associate with intracellular DAP12 for induction of signaling. Human TREM-1 mainly controls inflammatory responses in neutrophils and monocytes, whereas TREM-2 regulates the development and function of dendritic cells, as well as microglia and osteoclasts.[78, 79] An alternative mRNA splice variant of TREM-1 has been detected; this variant lacks the transmembrane and cytoplasmic domains and might generate a secreted TREM-1 decoy with the potential to modulate TREM-1 activation and signaling.[78] TREMs have also been identified in the mouse. Besides TREM-1 and-2, mice express an additional TREM, TREM-3, which exists in human as a pseudogene.[80, 81] Cross-linking of TREM-1 leads to tyrosine phosphorylation of several proteins including DAP12, phospholipase Cγ, ERK1/2, and NTAL (non-T cell activation linker).[79, 82] The latter appears to negatively regulate TREM-1/DAP12-induced signaling.[82] Overall, engagement of TREM-1 ultimately leads to Ca^{2+} mobilization, actin cytoskeleton rearrangement, and activation of the transcription factors p65/RelA, Stat3 and Stat5, resulting in production of proinflammatory chemokines/cytokines (e.g. MCP-1, IL-8, MIP-1α, TNF-α) and upregulation of adhesion molecules (e.g. CD86).[78, 79, 83, 84]

The role of TREM-1 *in vivo* as amplifier of inflammation has been shown in murine model of LPS-induced shock and models of septic shock by intraperitoneal injection of Escherichia coli and by caecal ligation and puncture. Moreover, blocking signaling through TREM-1 with a soluble TREM- 1/immunoglobulin fusion protein protected mice from shock and death.[85]

Importantly, TREM-1 engagement and TLR stimulation synergize in the production of proinflammatory cytokines. Indeed, stimulation of monocyte with anti-TREM-1 monoclonal antibodies together with the TLR2 ligand Mycobacterium tuberculosis or the TLR4 ligand LPS enhances proinflammatory cytokine secretion, while TREM-1 engagement in combination with LPS or the TLR3 ligand poly(I:C) inhibits production of the anti-inflammatory IL-10. Furthermore, expression of TREM-1 is upregulated upon TLR activation.[83] Altogether, these data suggest that the synergy of TREM-1 and TLR results in more effective inflammatory reactions.

The nature of TREM-1 ligands is still illusive, although putative ligands have been proposed, including soluble or cell-surface proteins that are upregulated upon inflammation or tissue damage. For instance, HMGB-1 and HSP70 have been suggested as TREM-1 ligands, since the cytokine production induced by these molecules is reduced after blocking TREM-1.[86] Additionally, soluble TREM-1 binds resting human platelets and murine granulocytes during sepsis and peritonitis, indicating the presence of TREM-1 ligands on the outer membrane of platelets and granulocytes.[87, 88]

In kidneys, we found TREM-1 being expressed in tubulointerstitial cells during human and murine hydronephrosis, but not in healthy kidneys (chapter 4[89]). Campanholle and co-authors confirmed the upregulation of TREM-1 in mouse damaged kidneys utilizing UUO and ischemia/reperfusion models and identified the TREM-1 positive cells as M1 type macrophages.[90] Altogether, these studies showed no significant contribution of TREM-1 in renal injury, macrophage recruitment, inflammation or fibrosis *in vivo*. The results of Campanholle *et al.* also suggest that

TREM-1 is not a major target for endogenous renal DAMPs, as kidney DAMPs did not activate DAP12 pathway in NFAT-LacZ reporter cells expressing TREM-1 and DAP12 and neither activation nor blockage of TREM-1 affected DAMP-mediated IL-1β expression by macrophages *in vitro*.[90] A soluble form of TREM-1 (sTREM-1) appears after its cleavage from the cell surface through the action of matrix metalloproteinase-9.[91] sTREM-1 has been studied as a sepsis biomarker, but investigations of its plasma concentration have had contrasting results.[92] In the study by Su and colleagues, the urinary sTREM-1 concentration had a positive predictive value for the early diagnosis of sepsis. More interestingly, Su *et al.* found that urinary sTREM-1 measurement is able to predict the development of sepsis-associated acute kidney injury within 48 hours before AKI diagnosis. This poses the question whether urinary sTREM-1 can become a new sensitive biomarker for sepsis-associated AKI.[93]

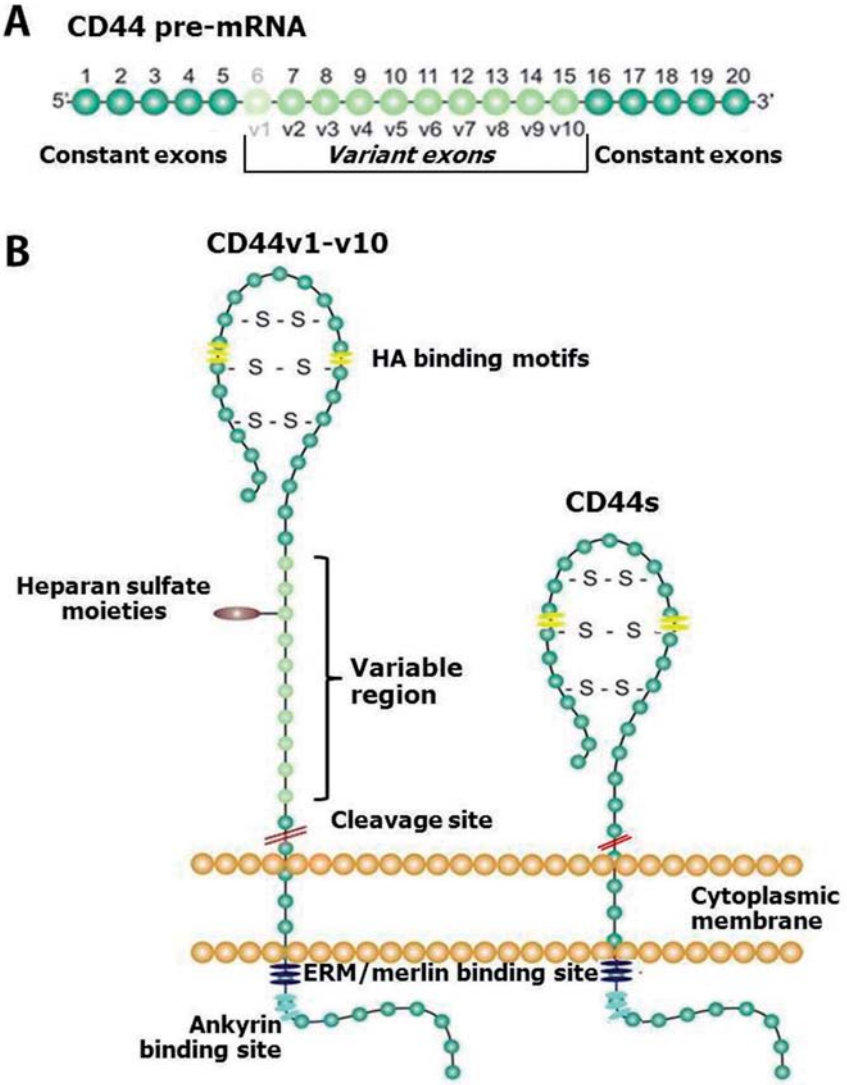
CD44

CD44 represents a polymorphic group of proteins (80–200 kDa in size) present on the surface of most vertebrate cells,[94] including hematopoietic, mesenchymal, epithelial and endothelial cells. CD44 was first described in 1983 as a lymphocyte homing receptor.[95] Since then, the number of studies to understand CD44 structure, function and implications in normal and pathological conditions has increased annually. CD44 involvement has been established in many diseases such as cancer, atherosclerosis, autoimmune diseases, endotoxic shock, chronic fibrotic disorders and acute organ failure, as well as physiological processes including organ development (ureteric bud, prostate gland, limb bud development), neuronal axon guidance, skin homeostasis, numerous immune functions (lymphocyte homing, rolling, and activation, immune responses to infections, interaction between bacteria and host cells) and hematopoiesis.[96]

CD44 Structure

All CD44 proteins are encoded by a single, highly conserved gene, on chromosome 11p13 in humans and chromosome 2 in mice.[94] The heterogeneity of the CD44 isoforms is generated by alternative splicing of the pre-mRNA and post-translational modifications, which differ depending on the cell type and growth conditions. The CD44 pre-mRNA is encoded by 20 exons, 10 of which are called variant (v) exons and can undergo alternative splicing. Exons 6 to 15 (v1 to v10) are variably expressed and inserted by alternative splicing; in humans, the variant v1 is not expressed due to a stop codon in exon 6a. Exons 1 to 5 represent the constant amino-terminal globular domain of the extracellular region, where the binding sites for hyaluronic acid (HA/hyaluronan) are contained.[97, 98] Hyaluronan is a main polysaccaridic component of the extracellular matrix and a regulator of inflammation.[99] Inclusion of the variant exons lengthens the extracellular domain, creating larger isoforms and exposing sites for additional post-translational modifications and ligand-binding sites. The transmembrane region is encoded by exon 17 in humans and exons 17-18 in mice and contributes to CD44 oligomerization and association with membrane glycolipid-enriched

Figure 3. CD44 structure.



A. Schematic representation of human CD44 mRNA. The CD44 gene comprises 20 exons: exons 1-5 and 16-20 are constant exons; exons 6-15 are variant exons, which can undergo alternative splicing. B. Protein structure of (left) CD44 variant 1-10 and (right) CD44 standard (lacking all variant exons). The binding sites for hyaluronan are included in the constant (exons 1-5) globular extracellular region. Three disulphide bonds (S–S) confer stability to the amino-terminal domain and maintain the correct folding necessary for the HA binding. The variant exon products are inserted in the extracellular region in proximity to the transmembrane segment. Exon v3 is modified by the addition of heparan sulfate moieties. The cytoplasmic tail has binding motifs for cytoskeletal linker proteins (ankyrin, ezrin-radixin-moesin, merlin). Adapted and modified from Hertweck *et al.*: CD44 in hematological neoplasias.[115]

microdomains, which harbor in the inner side adaptor and signal-transducing molecules important for CD44-mediated signal transduction.[97, 100-102] In mice, exon 19 encodes the cytoplasmic tail. In humans, CD44 can have a short or long (most common) cytoplasmic tail; the coding region and the 3'-untranslated regions (UTR) of the long tail are carried on exon 19, while exon 18 carried the 3'-UTR associated with the short-tail CD44 variant.[94, 103] The cytoplasmic tail interacts with many signaling mediators and has binding sites for the actin-cytoskeleton adaptor proteins ankyrin and ezrin, radixin, and moesin (ERM proteins), which are engaged in regulation of cell adhesion, motility and shape (actin cytoskeleton reorganization).[104-106] Direct splicing from constant exon 5 to constant exon 16 generates the smallest isoform, CD44 standard (CD44s), which is expressed on the membrane of most vertebrate cells, with the highest expression on hematopoietic cells.[102] Expression of CD44 variant (CD44v) isoforms is tissue-specific and regulated by extracellular stimuli, and is most frequent in proliferating cells.[96]
Besides mRNA alternative splicing, post-translational modifications of the extracellular domain, such as N-/O-linked glycosylation and addition of glucosaminoglycan (GAG) chains including heparan sulfate and chondroitin sulfate,[107-109] contribute to the structural heterogeneity of CD44 and determine its ligand-repertoire and ligand binding affinity. The CD44v segments carry some specific post-translational modifications. For instance, isoforms containing variant 3 exon have a heparan sulfate site, which enables the binding to heparin-binding proteins such as hepatocyte growth factor (HGF), heparin-binding epidermal growth factor (HB-EGF), fibroblast growth factor-2 (FGF-2), and vascular endothelial growth factor (VEGF).[97]

CD44 Interaction with Ligands/ Growth Factors/ Growth Factor Receptors and associated Functions

The list of molecules interacting with CD44 is continuously expanding. Here, I describe the CD44-mediated cellular effects of a few molecules that are relevant and upregulated in kidney pathologies.

Hyaluronan

Hyaluronan is the principal ligand of CD44; its binding motifs are included in the constant amino-terminal globular extracellular region (amino acids 32-132 and amino acids 150-158) of CD44.[98, 110] The HA/CD44 binding affinity can be regulated by cytokines and is affected by the molecular structure of CD44 (variant exon inclusion, post-translational modification), CD44 clustering, and cellular activation state.[97, 111-114] The binding of HA to CD44 results in cytoplasmic signaling that increases cell motility, proliferation, survival, and is involved in migration and extravasation of hematopoietic cells, as well as metastasizing tumor cells.[102, 115-117] The signaling/cellular effects induced by HA/CD44 binding are affected by the molecular weight (MW) of HA: low (L)MW HA (abundant at inflammation site) and high (H)MW HA exert opposite effects on cell growth and motility, with LMW-HA promoting and HMW-HA inhibiting both processes.[118, 119] HA markedly accumulates at sites of injury/inflammation; low and intermediate MW fragments are generated via the action of hyaluronidases, oxygen free radicals, or peroxynitrites.[117]
In the kidneys, hyaluronan is normally expressed at low levels: it is absent in the renal cortex and

present in the medullary and papillary interstitium, where it takes part in the urinary concentrating process.[117] In contrast, a pronounced deposition of HA in the tubulointerstitial and glomerular spaces is found in kidneys affected by ischemic injury, glomerulonephritis, tubulointerstitial nephritis, lupus nephritis, endotoxemic injury, or chronic damage with progressive fibrosis.[120-124]
Renal tubular epithelial cells constitutively express HA synthases and hyaluronidases genes, suggesting their involvement in both synthesis and degradation of HA. The production of HA by TEC is induced during proliferation, after mechanical injury and upon proinflammatory cytokine stimulation.[117, 125] Furthermore, proximal TEC acquire an inflammatory phenotype in response to LMW-HA, but not intact HA, by secreting proinflammatory cytokines and, upon HA stimulation, TEC upregulate intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) through a mechanism involving NF-κB and AP-1.[126, 127]
Finally, the prominent expression of HA during renal diseases provides an adhesive vascular and interstitial matrix that enables $CD44^+$ leukocyte infiltration[128] and retention through tubular expression of cytokines and adhesion molecules. Hence, HA/CD44 interactions dictate the extension of inflammation in the renal pathology.
CD44 does not only function as a HA-receptor activating proinflammatory pathways, but it is also important for HA uptake and clearance from the ECM in the resolution phase. Indeed, lack of CD44 is associated with persistent HA accumulation in injured kidneys and lungs.[120, 124, 129]
Besides hyaluronan, CD44 binds to other ECM components, such as collagens, fibronectin, osteopontin, chondroitin sulfate and heparan sulfate proteoglycans.[97, 108, 109, 130]

Osteopontin

Osteopontin (OPN), also known as early T-lymphocyte activation-1 (ETA-1), is a secreted glycoprotein in both phosphorylated and unphosphorylated forms.
OPN acts as a potent chemoattractant, as it can promote the migration of macrophages, dendritic cells (DC) and T cells. On the other hand, OPN also promotes survival in cells that have undergone injury such as ischemia/reperfusion or physical/chemical damage. Osteopontin acts through two different receptor families: integrins and CD44.[131, 132] The phosphorylated form of OPN interacts with CD44 isoforms that contain exons v6 and v7; this interaction has been proposed as a mechanism to maintain tissue integrity during inflammation.[96]
In normal kidneys, OPN is primary localized in the loop of Henle and the distal nephrons. After renal damage (AKI, CKD, allograft rejection), OPN is strongly upregulated in glomeruli and proximal and distal tubules in animals and humans.[120, 124, 133] Its expression can be induced by proinflammatory cytokines, such as TNF-α and IL-1β, and other factors as angiotensin II, TGF-β, hyperglycemia and hypoxia.[134]
Animal studies have demonstrated that OPN expression correlates with accumulation of macrophages and the severity of tubulointerstitial damage.[135, 136] Osteopontin may play a role in macrophage-mediated renal injury, but it also exerts renoprotective actions. The work of Noiri *et al.* demonstrated that OPN increases tolerance to acute ischemia, promotes proximal tubular cell survival,

and decreases cell apoptosis after ischemic injury.[137] In bone marrow cells, osteopontin has been shown to promote, via binding to CD44, proliferation and survival, mainly through the phosphatidylinositol 3-kinase (PI3K)/Akt pathway.[138] The same pathway was also shown to act upstream of OPN, inducing OPN expression in human renal proximal tubular cells.[139]
CD44 could also be involved in the anti-apoptotic actions of OPN. Lee *et al.* showed that the OPN-elicited anti-apoptotic effects depend on its interaction with CD44v. Indeed, osteopontin treatment and ectopical expression of CD44v6 in human embryonic kidney HEK293 cells could rescue cells from ultraviolet radiation-induced apoptosis, whereas irradiated parental cells, which exclusively express CD44s, were sensitive to apoptosis.[140]
Alternative translation of osteopontin generates an intracellular form with different biological activities from the secreted isoform.[141] The intracellular OPN is an integral component of a CD44-ezrin/radixin/moesin attachment complex, and it modulates cytoskeletal-related functions including cell motility, cell fusion and survival.[142-144] As the intracellular domain of CD44 is encoded by the constant exons, it is reasonable to assume that all CD44 isoforms may interact with the intracellular form of osteopontin, in contrast to the soluble form that binds CD44v.

Transforming Growth Factor-β

Many studies highlighted TGF-β central role in the pathological cellular mechanisms underlying the progression of chronic renal diseases.[145]
The interaction of CD44 with TGFβ has been investigated by several groups, particularly in cancer cells. Bourguignon *et al.* showed that the cytoplasmic domain of CD44 binds to TGFβ receptor type I (TGF-βRI) in metastatic breast tumor cells with high affinity. Moreover, the authors reported that HA binding to CD44 stimulated TGF-βRI serine/threonine kinase activity, resulting in increased phosphorylation of Smad2/Smad3 signaling molecules and production of parathyroid hormone-related protein (downstream effector of TGFβ signaling).[146]
TGF-β is secreted as a latent inactive form complexed with two other polypeptides; proteinases catalyze the release of active TGF-β from the complex. Yu *et al.* demonstrated that CD44 can localize proteolytically active MMP-9 to the surface of murine mammary carcinoma and human melanoma cells.[146] In addition, hyaluronan-mediated cross-linking of CD44 induced co-clustering of CD44 and MMP-9 in mammary carcinoma cells and promoted MMP-9 proteolytic activity.[147] In a later work, the same authors provided evidence for requirement of CD44 in MMP-9-mediated cleavage of latent TGF-β. Indeed, the activation of TGF-β and of its signaling occurred only when MMP-9 was anchored to the cell surface by CD44 in normal as well as in malignant cells.[148] Consistent with these findings, loss of CD44 resulted in impaired TGF-β signaling activation in murine hydronephrotic kidneys. CD44 KO mice, subjected to UUO, presented a remarkable attenuation of renal fibrosis in parallel with reduced intrarenal levels of phosphorylated and nuclear Smad2/3 in contrast to the wild-type littermates.[124]
The studies presented in this thesis suggest that CD44s is the major CD44 isoform participating in the activation of TGF-β signaling pathway (chapter 5 and 6). Indeed, primary renal tubular

epithelial cells overexpressing CD44s at their surface were more sensitive to TGF-β treatment by augmenting Smad-signaling activation and expression of TGF-β target genes in comparison to TEC overexpressing CD44v3 (chapter 5[149]). In a second study (chapter 6) utilizing CD44 KO/knockin (KI) mice that express either CD44s or CD44v3 on a CD44-null background, expression of solely CD44s in obstructed kidneys led to increased tubular cell damage and apoptosis, and interstitial fibrosis together with higher levels of phosphorylated Smad-3 and TGF-βRI, compared to the injured kidneys expressing solely CD44v3. These results suggest that CD44s interacts with TGF-βRI and, thereby, enables TGF-β signaling.
In support of this, Mima *et al.* reported that high expression of CD44s associates with high percentage of phosphorylated Smad-2-positive nuclei in hepatocellular carcinoma cells and that CD44s regulates the TGFβ-mediated mesenchymal phenotype, characterized by low E-cadherin and high vimentin expression.[150] An independent study showed that an isoform switch from CD44v to CD44s is required for the epithelial-to-mesenchymal transition triggered by TGF-β.[151]

Hepatocyte Growth Factor

An increasing body of evidence indicate that hepatocyte growth factor (HGF) is a renoprotective multifunctional cytokine. HGF acts, by binding to its specific tyrosine kinase receptor c-Met, as a promoter of cell proliferation, regeneration, differentiation and migration. HGF is upregulated in the kidneys after injury and its beneficial effects have been shown in models of toxic/ischemic acute renal injury as well as in chronic renal disease models, where it counteracts tubulointerstitial fibrosis development.[152]
The importance of CD44 in c-Met interaction and HGF-signaling has been showed by Orian-Rousseau and colleagues. Their work demonstrates that CD44 isoforms bearing the exon v6 sequence act as essential co-receptor for c-Met activation, since the formation of the CD44-HGF-c-Met complex and the HGF signal transduction require, respectively, the extracellular v6 region and the cytoplasmic tail of CD44.[153]
Other CD44 isoforms involved in HGF binding are the ones containing the v3 sequence. This region is modified by addition of heparan sulfate (HS) chains, converting the CD44v3 isoforms in heparan sulfate proteoglycans capable of binding heparin-binding growth factors such as bFGF, HB-EGF, and HGF.[107, 154] In a Burkitt lymphoma cell line lacking CD44 and c-Met, the importance and specificity of HS-modified CD44 in promoting HGF binding/signaling was evidenced by several approaches: (i) transfection with CD44v3 cDNA, but not CD44s and CD44v8 cDNAs, allowed efficient cell binding to HGF, (ii) co-transfection with c-Met and CD44v3 cDNAs induced HGF-mediated signaling, whereas co-transfection with c-Met and CD44s cDNAs resulted in weak signaling, (iii) treatment with heparitinase or a HGF mutant, with decreased affinity for HS, suppressed the enhancement of c-Met signal transduction induced by CD44v3.[154]
In the murine ureteral obstruction model presented in this book (chapter 5 and 6), HGF expression was induced in kidneys soon after injury, but declined with the progression of the renal pathology. Importantly, in CD44 KO/KI mice expressing solely CD44v3 retained high renal

levels of HGF even in advanced phase of nephropathy. In these mice, the phosphorylation rate of renal c-Met was elevated at late stage of obstruction as compared to CD44 KO/KI expressing solely CD44s (chapter 6). In addition, *in vitro* HGF stimulation of primary and immorto TEC confirmed a greater enhancement in c-Met signaling when CD44v3 was expressed instead of CD44s (chapter 5[149] and 6).

Bone Morphogenetic Protein-7

Bone morphogenetic proteins (BMP) are members of the TGF-β superfamily known for their capacity of inducing tissue development, regeneration, and repair. In chronic kidney diseases with progressive tissue scarring, BMP-7 has received particular attention due to its anti-fibrotic effects by inhibiting TGF-β/Smad signaling. Intracellularly, BMP-7 signals through the Smad proteins 1, 5 and 8.[155] Peterson *et al.* detected, by yeast two-hybrid analysis, binding between the Smad1 protein and a full-length cytoplasmic domain construct of CD44. This interaction was confirmed in CD44-negative COS-7 cells transfected with cDNAs encoding full-length CD44 or a CD44 truncated form lacking the cytoplasmic domain; the results showed that only intact CD44 binds Smad1. Furthermore, BMP-7 treatment resulted in a clear translocation of the signaling protein Smad-1 to the nucleus in $CD44^+$ COS-7 transfectants, but not in parental COS-7 cells.[156] Recently, the same group showed that expression of CD44 in chondrocytes promotes BMP-7 signaling, since CD44 KO cells were less responsive to BMP-7 in terms of Smad1 phosphorylation and aggrecan upregulation. In addition, HA was shown to participate in the cellular responses to BMP-7, which were inhibited by hyaluronidase treatment or HA synthesis inhibition.[157]

In our murine model of obstructive nephropathy, we found increased synthesis of BMP-7 and expression of phosphorylated Smad-1 in the ligated kidneys expressing CD44v3 on proximal tubules. In agreement, higher protein levels of the BMP-7 target ID-3 were detected in primary TEC overexpressing CD44v3 in respect to CD44s-overexpressing cells, in response to BMP-7 stimulation (chapter 5[149]).

Interestingly, in osteoblastic cells BMP-7 was shown to bind cell surface heparan sulfates and this interaction was required for BMP-7 signaling.[158] Further studies are necessary to assess whether CD44 isoforms containing the v3 sequence play a role in BMP-7 binding and signaling and whether this putative interaction contributes to the BMP-7 mediated renoprotective effects in kidney diseases.

CD44 in Kidneys

In normal kidneys, CD44 expression is restricted to few interstitial cells (passenger leukocytes) and urothelial cells.[159] However, in inflammatory renal diseases, CD44 expression is markedly enhanced, particularly in glomerular crescents and injured tubules as documented in human diseases and in several animal models: human IgA nephropathy and renal transplants, murine renal ischemia-reperfusion injury, unilateral ureteral obstruction injury, interstitial nephritis, and lupus nephritis.[120, 124, 159-163] Moreover, the degree of CD44 expression on tubules closely correlates

with inflammation in renal transplantation[162] and with the rate of tubular damage and interstitial fibrosis in IgA nephropathy[160] and renal transplantation.[163] Upregulation of CD44 in the kidneys upon tissue damage/inflammation is concomitant to the increase in expression of its ligands, suggesting that the CD44/ligands interactions contribute to the cellular pathomechanisms underlying the renal pathology.[120, 124]

Utilizing CD44-null mice, the potential role of CD44 in the pathogenesis/prevention of renal diseases have been previously studied in several murine models, namely, ischemia/reperfusion injury, ureteral obstruction and pyelonephritis.[120, 124, 164]

In the ischemia/reperfusion model, CD44 was detected early after reperfusion (one day) on peritubular endothelium and interstitial cells, whereas at later time-points (3, 7, 14 days) its expression was also detected on TEC of the cortico-medullary region. Importantly, the rapid upregulation of CD44 on renal capillary endothelial cells at day 1 led to a massive recruitment of neutrophils and, to a lower extent, of macrophages into the wild-type kidneys, whereas the renal inflammatory infiltrates were modestly increased in CD44 KO mice. This resulted in a better preserved renal function and morphology in CD44-deficient mice during the acute and recovery phases. The neutrophil influx in the injured kidney parenchyma was mediated by renal CD44 and not by CD44 on neutrophils, which expressed membrane-bound HA. More interestingly, the renal endothelilal-CD44/neutrophil-HA interactions could be inhibited by administration of anti-CD44 antibodies prior ischemia causing a marked reduction in neutrophil influx and protection of renal function.[120] Due to its contribution in the onset of renal inflammation, CD44 appears to promote the pathogenesis of ischemia-induced AKI.

In the model of chronic injury and progressive fibrosis instead, CD44 expression by TEC appears to be protective against tubular injury and TEC apoptosis and associated with an early induction of proliferation by TEC (day 3). On the other hand, renal CD44 upregulation in wild-type mice might contribute to tubulointerstitial fibrosis development, since in the CD44-null littermates collagen deposition was attenuated and myofibroblast accumulation was delayed after ureteral obstruction. This could not be explained by diverse extracellular matrix degradation by MMP-2 or MMP-9, but rather by reduced TGF-β1 signaling (reflected by the levels of phosphorylated Smad-2 and -3) in total kidneys and in tubular cells in absence of CD44.[124] As mentioned above, CD44 can interact with the TGF-β receptor type I in mammary carcinoma cells[146] and the standard isoform plays a downstream role in TGFβ-mediated signaling in hepatocellular carcinoma cells.[150] Additionally, CD44 was shown to co-localize with TGF-β receptor type I and II also in human proximal tubular HK2 cells under resting conditions.[165] The study of Webber *et al.* demonstrated that endogenous hyaluronan mediates and is necessary for myofibroblast differentiation.[166] Altogether, these reports provide a plausible explanation for the reduced TGF-β1 signaling and myofibroblast accumulation and, hence, attenuated fibrosis in mice lacking CD44. Similarly, TEC of CD44-deficient mice exhibited an impaired activation of the HGF receptor c-Met upon UUO.[124] The lessening of HGF-mediated renoprotective activities is likely to endorse exaggerated tubular damage and cell apoptosis. The lack of CD44 isoforms bearing the v3 and/or v6 regions explains the

markedly reduced c-Met phosphorylation in obstructed CD44-deficent kidneys. Controversially, upon UUO renal CD44 partially protected TEC from damage and apoptosis, but contributed to degeneration of the renal capillary network. Indeed, in CD44-null mice, the capillary network was still preserved in late stage of obstructive nephropathy and the endothelial cell apoptosis rate was significantly attenuated. The apoptosis of the peritubular capillary endothelial cells was further shown to be mediated by TGF-β1 in synergy with HA, and not by TGF-β1 alone or thrombospondin-1, suggesting that the CD44/HA interaction amplifies TGF-β1 pro-apoptotic effects.[167]
CD44 has also been largely studied in the inflammatory responses set up to fight infections. The results of these studies are quite controversial and highlight the fact that CD44 involvement in host defence can vary depending on pathogen species/derivatives and primary infection site.
We found that, in LPS-induced shock, lack of CD44 attenuates renal inflammation, leukocyte recruitment, and endothelial activation, delaying the onset of kidney dysfunction (chapter 2[123]).
In agreement, in other murine models the absence of CD44 resulted in fewer macrophages migration into lungs in response to inhaled LPS,[168] less macrophage and T lymphocyte recruitment into Mycobacterium tuberculosis-infected lungs,[169] and less pulmonary recruitment of neutrophils in polymicrobial sepsis. In the latter model, lung damage could be inhibited by antibodies targeting CD44 expressed on neutrophils.[170, 171] In contrast, CD44-deficient mice displayed more lung inflammation and more proinflammatory cytokine release in Escherichia coli-induced pneumonia and peritonitis, respectively.[172] Yet, in a pyelonephritis murine model caused by Escherichia coli infection of the urinary tract, CD44-deficiency limited bacterial outgrowth without affecting neutrophils recruitment or cytokine production in the kidneys. This study provided evidences for facilitation of Escherichia coli trans-epithelial migration by CD44 and HA, which are both expressed on urothelial cells after experimental pyelonephritis in wild-type mice, but absent in CD44 KO mice.[164] Thus, CD44 expression on urothelium can be used by E. coli to invade the host.
CD44 expression might be indicative of an attempted tubular cell regeneration after injury as some studies identified $CD44^+$ tubular cells as dedifferentiated proliferating/regenerating cells.[173, 174] New evidences are given that the extraordinary regenerative potential of renal tubules after acute injury has to be attributed to surviving proximal TEC with an adaptive phenotype (co-called "scattered tubular cells"). This TEC population is phenotypically characterized by signs of dedifferentiation and expression of stem cell markers (including CD44) and show a high proliferative index.[11, 12, 175] The expression of CD44 by regenerating cells is in line with the notion that CD44 is a downstream target Wnt/β-catenin pathway.[176-178] The Wnt/β-catenin signaling is activated by wounding and participates in the healing process,[179] including tubular regeneration after ischemic injury.[180]

Outline of this Thesis

In the following chapters the relative contribution of CD44, TLR4, TREM-1 and DAP12 in renal inflammation, tubular injury and interstitial fibrosis will be addressed.
In **chapter 2**, the involvement of CD44 in the systemic and renal inflammatory responses to LPS injection was assessed. Comparing wild-type mice with CD44 knockout littermates, we found that CD44 partially contributes to the initial systemic "cytokine storm" and to the consequent leukocyte influx, cytokine release, and endothelial activation in the kidneys. These effects explain the delayed increase in plasma urea concentrations in CD44 KO mice. *In vitro* assays showed the importance of CD44 in the prominent proinflammatory cytokine response by bone marrow-derived macrophages, early after LPS exposure, and in the chemotaxis of blood monocytes.
Chapter 3 addresses the question whether TLR4 activation by DAMPs, released upon kidney damage, contributes to the extent of inflammation and fibrosis in the unilateral ureteral obstruction murine model. TLR4, which is constitutively express in the kidneys, was upregulated during UUO in a time-dependent manner together with its danger ligands. TLR4 expression promoted renal fibrosis development without affecting matrix metalloproteinase activity or myofibroblast accumulation, but preventing upregulation of Bambi, a pseudoreceptor for TGF-β and inhibitor of TGF-β signaling. In agreement, type-I collagen gene expression was induced in a TLR4-dependent manner in primary renal tubular cells and myofibroblasts. Nevertheless, TLR4 also exhibits some renoprotective effects soon after injury as wild-type mice at day 1 of UUO showed attenuated tubular damage together with higher tubular cell proliferation rate as compared to TLR4 KO animals.
TREM-1 belongs to a new family of innate sensors expressed on myeloid cells that rely on the transmembrane adapter molecule DAP12 for cell signaling. TREM-1/DAP12 pathway cross-talks and amplifies the intracellular signaling pathways of several TLRs. Given the role of TLR4 in renal fibrosis development, TREM-1 expression and the putative contribution of the TREM-1/DAP12 pathway in the evolution of obstructive nephropathy were examined in **chapter 4**. TREM-1 was detected on tubulointerstitial cells in human and murine hydronephrotic kidneys, but not in healthy kidneys. Gene expression of TREM-1 and DAP12 progressively increased during obstructive nephropathy in mice; however, their expression did not influence renal scarring nor induction of TLR4 expression. Upon obstruction, mice lacking DAP12 displayed less renal MCP-1, KC and TGF-β1 levels and less macrophage influx, as well as diminished TLR2 expression at late stage of UUO (day 7 and 14). Surprisingly, in absence of TREM-1 solely MCP-1 and TLR2 levels of, respectively, day 14 and day 7 obstructed kidneys were diminished, whereas the other parameters measured were similar to the wild-type situation. In conclusion, TREM-1 appears not to be a major determinant of the magnitude of inflammation during obstructive nephropathy, possibly because its function is overwhelmed by the activation of TLRs, as TLR2 and -4, which are also expressed on tubular cells besides infiltrating inflammatory cells. DAP12, instead, partially modulates inflammation in late stages of UUO, when leukocyte accumulation become abundant.
In **chapters 5** and **6**, we aim to unravel the functional properties of the CD44 isoforms standard and v3-v10 in the progression of UUO-induced renal pathology. Previous studies from our group

highlighted the role of CD44 in fibrogenesis upon UUO and the positive correlation between its expression levels and the degree of interstitial fibrosis in human IgA nephropathy and renal transplants. CD44 expression is rapidly induced after renal damage, especially on injured tubular cells; yet, the effects of CD44 de novo expression and the functions of each isoform in renal diseases remain largely unknown.

For the study of **chapter 5**, we generated new transgenic mice in which the transgene (CD44s/CD44v3 gene) expression was driven by the γ-glutamyl transpeptidase type-1 (γGT-1) gene promoter resulting in an overexpression of CD44s or CD44v3 specifically and solely on renal proximal tubular epithelial cells. *In vitro* stimulation of primary TEC derived from these transgenic mice and wild-type littermates with TGF-β1, HGF, and BMP-7 showed different cellular responses between TEC overexpressing either CD44s or CD44v3. The TGF-β1-induced upregulation of the profibrotic genes was more pronounced in CD44s overexpressing TEC and weaker/absent in CD44v3$^+$ TEC. Upon TGF-β1, CD44v3$^+$ TEC displayed also a weaker activation of TGF-β1 signaling as reflected by less Smad-3 phosphorylation. On the contrary, cell stimulation with HGF led to an increased HGF intracellular pathway activation in CD44v3 overexpressing TEC in respect to WT and CD44s$^+$ TEC. Similarly, BMP-7 induced greater effects in CD44v3$^+$ TEC than in CD44s$^+$ TEC (e.g. upregulation of ID-3, downregulation of MCP-1). One day after UUO, obstructed kidneys from CD44s$^+$ transgenic mice showed more tubular damage and interstitial edema, whereas CD44v3$^+$ obstructed kidneys presented less myofibroblast accumulation, increased BMP-7 synthesis/signaling and reduced HGF levels. Despites lower renal HGF expression, c-Met activation was not reduced in CD44v3 kidneys. Nonetheless, the differences in tubular damage seen at day 1 among the genotype-groups, disappeared at later time-points. Similarly, the proportion of tubules expressing CD44 was equal among the strains in advance stages of UUO. Since the transgenes are overexpressed in proximal TEC on a wild-type background, the upregulation of the "wild-type CD44" by TEC and other cell types might overwhelm the transgene functions.

In the study described in **chapter 6**, we used wild-type and transgenic mice expressing solely CD44s or CD44v3 on a CD44 knockout background, and subjected them to ureteric ligation. After UUO, the presence of CD44v3 diminished tubular damage, decreased apoptosis and increased proliferation of tubular epithelial cells, and prevented renal fibrosis development. In contrast, expression of CD44s led to increased tubular damage and TEC apoptosis and more renal fibrosis. This was accompanied by a relative increase in renal β-catenin expression, HGF production and HGF/c-Met signaling, a relative inhibition of TGF-β1 down-stream signaling and TGF-β type I receptor expression in CD44v3 knockin mice in respect to CD44s knockin mice. Further *in vitro* experiments were performed utilizing immorto tubular cells and mouse embryonic fibroblasts (MEF) from wild-type or CD44 KO mice; CD44s$^+$ and CD44v3$^+$ cells were obtained by transfecting CD44 KO cells with plasmids encoding CD44s or CD44v3, respectively. Wnt3a/HGF-treatment of tubular cells resulted in higher β-catenin/p-AKT levels in CD44v3$^+$ TEC than in CD44s$^+$ TEC, sustaining the relative higher β-catenin expression and HGF signaling seen in

CD44v3$^+$ obstructed kidneys. In agreement with the mild fibrosis observed in CD44v3$^+$ kidneys, TGF-β1 induced a mild type-I collagen upregulation in CD44v3$^+$ MEF as compared to CD44s$^+$ cells suggesting that fibroblasts expressing solely CD44v3 are less effective in producing collagen. Conclusively, CD44s and CD44v3 differentially modulate regenerative and fibrotic stimuli.

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