Pathogenic role of complement in renal ischemia/reperfusion injury

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CHAPTER 9

General discussion and summary

9.1 INTRODUCTION

Ischemia/reperfusion (I/R) is an inevitable and injurious event in clinical conditions such as infarction, sepsis and solid organ transplantation. Ischemia occurs after insufficient local blood supply. Depletion of cellular energy (ATP) is the most prominent cause of cellular injury during ischemia. Reperfusion of ischemic tissue e.g. following transplantation provides oxygen as well as substrates that are necessary for tissue regeneration, restoration of energy levels and concurrent removal of toxic metabolites. Nevertheless, restoration of blood flow to ischemic tissue paradoxically exacerbates tissue damage by initiating a cascade of inflammatory events including release of reactive oxygen species (ROS), pro-inflammatory cytokines and chemokines, recruitment of leukocytes and activation of the complement system (1-4). Such deterioration of tissue function and integrity after reperfusion is defined as ischemia/reperfusion injury (IRI). Renal I/R is the major cause of acute kidney injury (AKI). AKI is a clinical syndrome characterized by a rapid decrease in renal function and plasma accumulation of creatanine, urea, metabolic acids, potassium and phosphate. AKI may not only occur in the context of kidney transplantation, in which I/R is inevitable, but is also a consequence of impaired kidney perfusion e.g. during major surgery or sepsis. Incidence of ischemic AKI varies from more than 5000 cases per million people per year for non-dialysis-requiring AKI, to 295 cases per million people per year for dialysis-requiring disease (5). AKI has a frequency of 1,9% in hospitalized patients (6) and is especially common in critically ill patients, in whom the prevalence of AKI is greater than 40% at admission to the intensive-care unit if sepsis is present.

In the renal transplant setting, ischemia during the transplant procedure, under toxic therapautical conditions or immunological injury, compromise tissue viability and promote alloimmunity. Therefore, AKI not only has a major impact on short-term but also on long-term graft survival following kidney transplantation and is strongly associated with delayed graft function (DGF), clinical morbidity and mortality (7-11). Ischemic AKI following renal I/R is characterized by injury to the proximal tubular epithelial cells (PTEC) mostly in the cortico-medullary junction. This process is called acute tubular necrosis (ATN). The injured tubular cells are important for tubular reabsorption, the process by which filtered metabolites e.g. salts, proteins and glucose, are reabsorbed from the primary filtrate via the brush border of these cells and transported back into the blood. During an ischemic event, there is shedding of the proximal tubular brush border within several minutes (13) and loss of polarity with mislocalization of adhesion molecules, complement regulators and other membrane proteins (12;13). In advanced ischemic injury, viable and necrotic tubular epithelial cells detach

from the basement membrane. These sloughed tubular cells, brush-border vesicle remnants and cellular debris form characteristic tubular casts, which might activate complement. The complement system is therefore an important contributor to inflammation after IRI. As a consequence of renal ischemia, there is an altered localization and expression of complement regulators on tubular cells (14), which makes these cells prone for complement activation. In addition, apoptotic as well as necrotic cells and tubular casts generated during I/R are potent activators of the complement system (15-18).

9.1.1 Scope of this thesis

Several lines of evidence support an important role for complement in renal IRI. Most of this evidence however, is based on studies performed in mice, which demonstrate a predominant role for the alternative pathway of complement (19-21). In the kidney, the cortico-medullary junction harboring the proximal tubular cells (PTEC) is the region, where most complement activation and deposition of C3 and C5b-9 occurs. The loss of complement regulators at the basolateral surface of tubular cells allows complement activation following I/R on these cells. (14). Although complement activation is a clear hallmark of renal IRI in mice, the involvement of complement activation in human IRI remains to be explored. In addition, the contribution of the different complement pathways in human IRI has not been delineated.

In the past, it has been shown that high levels of MBL are associated with worse allograft survival following clinical kidney transplantation, suggesting a harmful role for MBL and the lectin pathway of complement. Whether MBL might play a harmful role in the early phase of kidney transplantation during renal I/R, remains to be explored. Together, these subjects form the scope of this thesis. In the first part of the thesis (chapter 2-4) we focused on the role of complement and the activation pathways involved in human IRI. In addition, we made an approach to measure human complement activation locally in the renal tissue, in circulation and in the urine early following clinical kidney transplantation. In the second part (chapter 5-7) we focused on the role of MBL and a therapeutic approach to target MBL in the setting of renal IRI. Finally, we describe (chapter 8) how I/R and the mediators involved affect the endothelial compartment, which enables MBL to reach the tubular compartment and injure tubular cells.

9.2 COMPLEMENT IN HUMAN IRI

Most of the IRI studies performed in mice show a predominant role for the alternative pathway of complement (19-21). However, direct and in-depth

studies in humans confirming these findings are lacking and so far in human renal IRI, the activation pathways of complement by ischemic PTEC are incompletely elucidated. To successfully develop therapeutic interventions targeted towards complement activation in human, it is essential to establish the validity of murine data relative to what takes place in the human situation.

9.2.1 Human complement activation *in vitro*

Thus far, it was unknown whether experimental data obtained in mice might be extrapolated to humans. This prompted us to study whether complement is activated and which pathways of complement activation are initiated by human PTEC following I/R and to compare these findings to mouse PTEC (chapter 2). For this purpose, we developed an *in vitro* model to induce hypoxia and subsequent reoxygenation to simulate I/R on human and mouse PTEC. Following reoxgygenation, we studied the role of the different pathways of complement activation. Exposure of human or mouse PTEC to hypoxia followed by reoxygenation in serum as a source of complement, resulted in extensive complement activation. Mouse PTEC exclusively activated complement via the alternative pathway, which was completely in line with *in vivo* IRI studies previously performed in mice (19;21). In contrast, complement activation by human PTEC was exclusively dependent on the classical pathway, supported by studies in C1q-depleted serum and use of blocking antibodies to human C1q. The activation of the classic pathway was mediated by IgM through interaction with modified phosphomonoesters exposed on human hypoxic PTEC. It is known from several studies that late apoptotic and necrotic cells bind natural IgM antibodies, which will lead to activation of the CP (22;23). We could demonstrate a similar binding of IgM to hypoxic human PTEC. Using IgM-deficient serum we showed that complement activation by hypoxic cells also occurs via binding of IgM, probably to phosphorylcholine residues exposed on hypoxic cells, as shown by inhibition of complement activation using phosphorylcholine. In line with these findings, it was recently shown that cultured pancreatic islets bind natural IgM and also activate the CP, probably caused by hypoxia or injury during the culture or harvesting procedure.

Data from studies in mice but also human indicate that complement activation following I/R in skeletal muscle (24), heart (25), intestine (26) and limb (27) *in vivo* also occurs through binding of natural IgM. However, in renal IRI in mice it has been shown that immunoglobulins do not play such a role (28). These findings are in line with reports showing that C4-deficient mice were not protected against renal IRI (21), so it appears that in mice, renal IRI can proceed independently of C4 and immunoglobulins. The reason for this difference compared to human PTEC is unknown so far.

In a pig model however, it was recently shown that the classical and lectin pathways of complement were involved in renal IRI (29). Importantly, this study demonstrated reduced ischemic injury when pigs were treated with C1 inhibitor, a specific inhibitor of both the classical and lectin pathway (30;31). These porcine data are in line with our findings on human tubular cells, which activated the classical pathway of complement. Interestingly, this study (29) also demonstrated that the classical and lectin pathway were activated in human renal transplant recipients suffering from delayed graft function (DGF). The co-localization of activated C4 with both C1q and MBL in graft biopsies obtained from these patients indicated that both these pathways were activated on peritubular capillaries, within the interstitium, and on the glomerular endothelium. However, in this study no C4 staining on tubular epithelial cells was observed, which could be explained by the fact that only cortical biopsies were taken, whereas tubular injury following reperfusion is mostly observed in the cortico-medullary junction of the kidney. In addition, biopsies were taken at 7-15 days after transplantation, in which tubular injury most probably has been cleared and repaired. Due to safety concerns however, biopsying early after reperfusion and specifically in the corticomedullary region is difficult. Previous studies on IRI in humans mostly relied on peripheral blood measurements, which lack sensitivity and do not discriminate between the release of factors from the allograft and systemically released factors. For these reasons, in-depth studies on the role and kinetics of reperfusion-induced complement activation in human have been hampered sofar.

9.2.2 Human complement activation in circulation

Recently, a renewing approach was established (32), which enabled us to measure circulating complement activation products directly released from the reperfused kidney and thereby avoiding the limitations of systemic measurements in human (chapter 3). Via a small catheter placed in the renal vein, blood aliquots were sampled at consecutive time points after reperfusion. In addition, paired arterial blood samples were collected. We concentrated on measurement of sC5b-9 because it is the common endpoint of all three pathways. Furthermore, it is suggested that in mice specifically C5b-9 is essential in the induction of tubular damage in renal IRI (21). For these reasons, arteriovenous concentration differences of terminal sC5b-9 were assessed directly over the reperfused organ to reveal the local activation of complement in human IRI.

Release of sC5b-9 in living donor (LD), brain-dead donor (BDD), and cardiac dead donor (CDD) kidney transplantation were compared. A significantly higher rate of delayed graft function (DGF) was observed in BDD and CDD kidneys compared with LD kidneys. We demonstrated that mostly from CDD grafts, which are most

severely affected by I/R, sC5b-9 was released directly after reperfusion, indicative of intravascular terminal complement activation. sC5b-9 was released only very transiently, directly after reperfusion. This may represent a washout effect. The complement system may be triggered on encounter with intravascular cellular debris accumulated during the cold ischemic period or by encounter with hypoxic or injured endothelium (33;34). Intravascular thrombosis and clot formation during warm ischemia, especially in CDD kidneys might occur. The clotting and complement system are tightly intertwined (35-39). Activated thrombin as well as formed fibrin clots have been shown to activate complement leading to C5b-9 formation. Indeed, potent thrombin activation was demonstrated directly following reperfusion by measuring prothrombin fragment (F)1 and 2 levels, indicative of conversion of prothrombin into thrombin by factor Xa (32). This thrombin activation might also be the cause of the transiently released sC5b-9 observed directly after reperfusion.

A limitation of our study was the fact that the sampling time was restricted to maximally 30 minutes following reperfusion. Except for the very transient release of C5b-9 instantly after reperfusion, there was no release of C5b-9 during the entire sampling period of 30 minutes. The absence of complement activation is in line with mouse IRI experiments, in which the first membrane attack complex elements C6 and C9 are observed only after 12 and 24 hours of reperfusion, respectively (40). Also in our rat IRI model, we observed the first signs of C5b-9 deposition after 24 hours of reperfusion (41), indicating that terminal complement activation is a relatively late event following reperfusion in human as well as in rodents.

Finally, the possibility remains that the complement cascade was transiently activated in living donor kidneys as well, without leading to terminal complement activation. Therefore, release of C5a from the reperfused kidney was assessed, because C5a is more upstream in the complement cascade than the terminal complex C5b-9 is. In agreement with C5b-9 measurements, there was no C5a release from living donor kidneys. This excludes early complement activation after reperfusion and also excludes early involvement of C5a, which has also been ascribed as harmful role in IRI (42).

9.2.3 Human complement activation in renal tissue

Besides the intravascular sC5b-9 formation, C5b-9 could be also formed locally in the kidney without any release into the circulation. To assess local complement activation on tubular cells, tissue content and distribution of C5b-9 in needle biopsies collected before and after reperfusion was assessed. Surprisingly, the tubular surface, where C5b-9 is expected to deposit (21), showed no deposition of C5b-9 in LD, BDD or CDD kidneys after reperfusion. In contrast, renal tissue

of a patient with acute graft rejection showed extensive C5b-9 deposition in the tubular compartment. These findings were supported by a study of Haas et al. (43), where in 1 hour post-transplantation biopsies, no complement deposition as a consequence of reperfusion was detected either. However in this study, only cortical biopsies were collected, whereas complement activation on tubular cells deeper in the cortical-medullary region might have been missed. Therefore, in our study needle biopsies were taken, which make it possible to sample deeper into the kidney. Because these needle biopsies are relative thin, we cannot fully exclude a sampling error in which the most affected area still have been missed. Nevertheless, in more than thirty needle biopsies collected no C5b-9 deposition could be detected, making these findings quite reliable.

9.2.4 Human complement activation in urine

The third approach we applied to study the role of complement in human renal IRI, was to assess complement activation products in the urine in the early post-transplant period. Increased glomerular permeability to large plasma proteins (proteinuria) is common in the early period following renal transplantation, with a prevalence of 15% to 30% at 1 year post-transplantation (44). Complement activation at the tubular epithelial surface of the kidney, which lacks several important complement regulators (CD46, CD55) (45), is considered to be a mediator of tubular injury in the proteinuric condition. In proteinuria, complement proteins, which normally are retained in circulation, are able to pass the glomerular filter barrier, end up in the tubular lumen and are activated by the unprotected epithelial surface of the tubuli (46-48). To this end, the detection of soluble (s)C5b-9 in urine is considered as a clinical indicator of tubular complement activation (49-51), however in the setting of renal IRI in human this has not been assessed before. Therefore, we collected urinary samples at consecutive days following kidney transplantation in a cohort of deceased cardiac dead donors and assessed protein and sC5b-9 content (chapter 4). In line with proteinuria studies, we were able to confirm the relation of urinary sC5b-9 and proteinuria. In the early post-transplant period we found high urinary levels of sC5b-9, significantly correlating with the degree of proteinuria, suggesting activation of filtered complement components at the tubular epithelial surface of the kidney. However, when mimicking proteinuria *in vitro* by exposing serum (or blood) to urine (both negative for sC5b-9), we found extensive generation of sC5b-9 in urine. This process was inhibited by EDTA, confirming activation of the complement system. Removal of remaining cells and cellular debris by filtering the urine reduced the sC5b-9 generation in the urine by half, suggesting that sC5b-9 generation in proteinuric urine is partially caused by cellular debris and dead epithelial cells. This implies that following kidney

transplantation, proteinuria but also procedure-related (microscopic) hematuria may cause urinary complement activation resulting in high urinary sC5b-9 levels. Centrifugation or filtration following collection of patient urines would not prevent this, since urinary complement activation is rapid and most probably already occurs in the urinary tract. So, although sC5b-9 is an attractive urinary biomarker, one should be aware of the risk of extra-renal complement activation independent of a renal contribution. Although urinary sC5b-9 was detected in the majority of renal allograft recipients and significantly correlates with the degree of proteinuria, the implications and clinical relevance of measured sC5b-9 are rather unpredictable and therefore could not be used to draw any further conclusions from the urinary sC5b-9 measurements.

9.3 MBL IN RENAL IRI

Besides a predominant role for the alternative pathway of complement, also involvement of the lectin pathway, in particular by MBL, in the course of renal IRI in mice has been demonstrated. Mice subjected to renal I/R display evident renal MBL deposition on tubular cells which co-localizes with depositions of C3, C6 and C9. The degree of early deposition of MBL is associated with later complement activation, neutrophil influx and organ failure (40). Therefore, a role for MBL in complement activation following I/R was suggested.

Also in human biopsies, deposition of MBL have been observed after kidney transplantation (29) and low pretransplantation levels of MBL are associated with better graft survival after deceased-donor kidney transplantation at the long term (52). Of interest, MBL-deficient mice are protected against renal IRI and MBL-deficient mice reconstituted with recombinant human MBL show a dose-dependent increase of renal injury comparable to the severity in wild-type mice (53).

Remarkably in mice, the downstream classical and lectin pathway component C4 is not involved in renal IRI, given that C4-deficient mice are not protected (21). These findings from MBL- and C4-defiicient mice have been a discrepancy for many years and suggest that other effector functions of MBL besides activation of the lectin pathway of complement might exist. Recently however, it was demonstrated that MBL is able to bypass C2 and C4 and directly activates C3. In different IRI models in mice it was shown that MBL/MASP-1 complexes directly might activate C3 in the absence of C2 and C4 (54), though this was not shown for renal IRI. In the mouse kidney, interaction of MBL with highly glycosylated meprins expressed and secreted by mouse PTEC has been suggested to be the initial step for MBL binding and subsequent complement activation (55), as

these meprins *in vitro* are able to bind MBL and activate complement. Although MBL/MASP-1 complexes are able to bypass C2 and C4 (56), its contribution to the degree of complement activation *in vitro* has been shown to be modest. Since MBL is a highly versatile protein, we cannot exclude that other effectors functions of MBL besides activation of the lectin pathway of complement might play a role in renal IRI.

9.3.1 MBL-mediated tubular injury

In chapter 5 we describe a novel role for MBL in the pathogenesis of renal IRI. Remarkably, this novel role was completely independent of complement activation. In a rat model of renal IRI, we demonstrated that therapeutic inhibition of MBL is protective against renal IRI and prevents kidney dysfunction, tubular damage, neutrophil and macrophage accumulation, and expression of proinflammatory cytokines and chemokines. Following reperfusion, vascular leakage resulted in extravasation of circulation-derived MBL in the interstitial space. Subsequent exposure of tubular epithelial cells to MBL resulted in internalization of MBL followed by the rapid induction of tubular epithelial cell death. This MBL-mediated tubular injury was completely independent of complement activation since inhibition of C3 or C5 was not protective against renal IRI. MBL-mediated cell death preceded complement activation, strongly suggesting that exposure of epithelial cells to MBL immediately following reperfusion is the primary culprit of tubular injury, and not the lytic C5b-9 complex.

Several studies, mainly performed in mice, have shown an important role for complement in the induction of renal IRI. A study using C3, C5 and C6 deficient mice showed a predominant role for C5b-9 in renal IRI (21), and also inhibition of C5 with monoclonal antibodies was protective (57). In contrast to these data, others have shown that gene knock-out (58), inhibition (58) or depletion of C3 (59) was not protective against renal IRI. In our rat IRI model, deposition of C3 and C5b-9 following reperfusion was observed, although this was in a relatively late phase (24 hours onwards). A similar late kinetics of C5b-9 deposition has also been shown in mouse IRI (57), although this mouse model was characterized by an early increase in deposition of C3 (2 hours). We did not observe early C3 deposition, indicating possible differences in kinetics and pathogenesis of renal IRI among species. Differences in organ size and metabolism between species could play a role in the observed differences, given that in mice clamping times of usually 20-25 minutes are needed to induce renal injury, while in rats 45-60 minutes of clamping time is needed to induce substantial IRI. Also, difference in relative strength of complement activation pathways could be of influence. In this respect, it has been shown that serum complement activity even among

rat strains are varying (60), explaining why in certain rat strains complement activation might be more involved. Indeed, the rat strain we used (Lewis), has a relatively low serum complement activity.
Despite the fact that complement activation was not involved in the induction of renal IRI in our rat model, we did find a pivotal role for MBL in the pathogenesis of renal IRI, since transient inhibition of MBL in the early phase following reperfusion was completely protective. It is tempting to speculate that these mechanisms might contribute to a previous observation that genetically determined high levels of MBL are an important risk factor for renal graft loss following clinical kidney transplantation (52;61).
In human intestinal IRI, an association of MBL null alleles with preserved epithelial cell integrity was shown (62). Interestingly, also here no signs of complement activation were observed, suggesting that also in intestinal IRI, MBL might have a cytotoxic effect on epithelial cells. Moreover, MBL has been shown to bear anti-tumor activity to colorectal carcinoma cells, which has been termed MBL-dependent cell-mediated cytotoxicity (63) .
Exposure of tubular epithelial cells to circulation-derived MBL following renal I/R was shown to mediate tubular injury. In normal physiologic conditions, these cells are not exposed to MBL (from the basolateral surface). However, during I/R there is an increased vessel destabilization, endothelial injury and leakage of plasma components in the interstitial compartment. In chapter 8 we demonstrated the presence of such vessel destabilization and loss of endothelial integrity shortly after I/R which was associated by a disturbance in the angiopoietin-1 and -2 balance. This condition enables MBL to diffuse from circulation and reach the epithelial cells from the basolateral side. A therapeutic approach aimed to stabilize endothelial integrity might therefore be successful in several ways. Firstly, leakage of MBL from the vasculature is reduced, thereby preventing MBL to interact with tubular cells. Secondly, a stable endothelium prevents formation of edema and will consolidate the local blood flow. Thirdly, leukocyte interaction and infiltration might be reduced. Therefore, next to blocking MBL following renal I/R, a therapy targeted towards endothelial stabilization might be promising as well.

9.3.2 MBL and TLR signaling

Stressed or dying cells release damage associated molecular pattern molecules (DAMPs), which can signal through pattern recognition receptors (PRR), a class of innate immune response-expressed proteins that respond to pathogen-associated molecular patterns (PAMPs) and DAMPs. A subfamily of these membrane-associated PRR are the Toll-like receptors (TLRs), which sense PAMPs or DAMPs extracellularly or in endosomes and may link innate and

adaptive immune responses. TLRs are present on a variety of cell types including leukocytes, dendritic cells as well as epithelial and endothelial cells. Especially TLR-2 and 4 are highly expressed on PTEC. Interestingly, mice deficient for TLR-2, and -4 are protected against renal IRI (64;65) as shown by a reduction in kidney dysfunction, tubular injury and proinflammatory cytokines, and a concomitant decrease in infiltration by leukocytes. TLR4 signaling through the MyD88-dependent pathway is required for the full development of kidney IRI (65), as both TLR4 and MyD88 deficient mice were protected. It is known that there is a powerful crosstalk between complement and TLR activation in renal IRI (66). Recent studies have shown that there is tight and direct collaboration between MBL and other host defense pathways and receptors to both specify and amplify the immune response following TLR signaling. This is illustrated by the observation that MBL enhances TLR-2 and -6 signaling (67) and by a study showing a tight interaction of MBL with TLR-4 (68). Several endogenous ligands for TLRs like high-mobility group protein B1, hyaluronan and biglycan have been implicated in the pathophysiology of renal IRI (69-74), of which biglycan have been described to interact with MBL (75). It is therefore tempting to speculate about a possible role for MBL in TLR signaling following renal IRI, which might contribute to the protective effect observed in MBL-deficient mice or following therapeutic inhibition of MBL.

9.3.3 MBL and coagulation

Upon ligand binding by MBL, the MBL-associated serine proteases have been shown to not only cleave complement components, but also coagulation factors. For instance, MBL/MASP-1 has been shown to exert thrombin-like activity, to interact with plasma clot formation on different levels and to drive the formation of cross-linked fibrinogen (36;38;39) in a murine model of occlusive thrombosis. MASP-1 is therefore a significant contributor to coagulation and plays a key role in thrombus formation. Also MASP-2 is capable of promoting fibrinogen turnover and generation of a fibrin clot by cleavage of prothrombin, generating active thrombin (37). In addition, MBL is able to bind to fibrinogen and fibrin thereby augmenting clot formation by additional cleavage and activation of fibrinogen and prothrombin (37;76). Several studies have pointed out the relationship between IRI and the coagulation system. Treatment with Antithrombin III (77), tissue factor pathway inhibitor (78) or soluble thrombomodulin (79) following reperfusion in rats reduced renal dysfunction, tubular injury, microvascular leukocyte rolling and attachment and endothelial permeability, indicating that altered coagulation contribute to the pathogenesis of ischemic renal injury. De Vries et al (32) recently observed a release of prothrombin fragment 1 and 2, which are formed when prothrombin is converted to thrombin, shortly after living

donor transplantation. The rapid activation of coagulation by the transplanted kidney may lead to the generation of fibrin, which might damage the graft by clot formation in the microvasculature (32;80). The parallel expression of activation products of the coagulation, fibrinolysis, and complement systems has long been observed in both clinical and experimental settings. In man, enhanced thrombin generation after reperfusion of deceased donor kidneys might be a part of the explanation of the poorer outcome of transplantation observed in deceased compared to living donor kidneys (81). High pretransplantation levels of MBL, which is associated with patient and graft survival after kidney transplantation, might predispose to enhanced microvascular thrombosis through MBL/MASP-mediated activation of coagulation. Therefore, thrombus formation and coagulopathy might be an additional way in which MBL is involved in renal IRI.

9.3.4 Mechanism of MBL-mediated tubular injury

PTEC that encounter nutrient and oxygen deprivation during ischemia use various adaptive responses to maintain homeostasis and survival, including the initiation of macroautophagy and the activation of the ER stress or unfolded protein response (UPR). These adaptive responses decrease energy expenditure, increase nutrient availability and promote cell survival. However, these biological processes also lead to the generation of proinflammatory cytokines and chemokines, indicating that stressed tissues generate alarm signals that produce an inflammatory microenvironment (82). In chapter 6, the thesis reports a possible mechanism by which MBL mediates direct tubular injury following renal I/R. Vascular leakage results in exposure of tubular cells to MBL, which was shown to be the primary culprit of tubular injury. We demonstrated that inhibition of MBL *in vivo* protected tubular cells from loss of vital GRP78 and subsequent tubular injury. GRP78 is a constitutively expressed molecular chaperone, however expression is enhanced under a variety of stressful conditions including hypoxia, glucose deprivation, alterations in intracellular calcium and oxidative stress (83), events that take place during renal I/R. Induction of GRP78 is critical for maintaining viability of cells that are subjected to such stresses and loss of GRP78 would therefore be detrimental (84;85). We demonstrated that renal I/R is accompanied by ER-stress and loss of GRP78 in the cortico-medullary region of kidney, the region that is mostly affected. Importantly, therapeutic inhibition of MBL protected tubular cells located in this region from loss of vital GRP78 and consequent tubular injury, indicating that MBL might be injurious to tubular cells by interfering with GRP78. Expression of proinflammatory IL-6, a consequence of GRP78 degradation (86) and ER-stress (82;87), was also significantly reduced by inhibition of MBL following reperfusion.

Internalization of circulation-derived MBL *in vivo* was shown to be the process

by which tubular epithelial cells following renal I/R might be injured (41). By exposing tubular cells *in vitro* to MBL, we demonstrated that basolateral internalization of MBL by tubular cells induced a potent ER-stress response which was accompanied by an excessive ATP production, a subsequent loss of the mitochondrial membrane potential, production of mitochondrial superoxide followed by induction of cell death. Internalized MBL colocalized with mitochondria and GRP78. Although GRP78 is mostly known from its function as molecular chaperone in the ER, GRP78 has also been shown to be present, although in low amounts, on mitochondria, in the cytoplasm, on the cell membrane and as a secreted form (88-93). Membrane-bound GRP78 might be a possible candidate as a cellular receptor by which MBL is internalized.

It has been shown that ligation of cell-surface GRP78 on cancer cells causes release of calcium from ER stores mediated via phospholipase C, thereby increasing cytosolic calcium (94). In line with this, it is known that especially tumor cells have increased levels of GRP78 on the cell surface. Importantly, is has been shown that MBL bears antitumor effects, which has been termed MBL-dependent cell-mediated cytotoxicity (63). Since MBL is able to interact with GRP78, we speculate that, next to killing of tumor cells, the cytotoxic effect of MBL on tubular cells might be mediated by signaling through cell surface GRP78. Under physiologic conditions GRP78 is hardly present on normal cells, however induction of ER-stress has been shown to promote GRP78 localization on the surface of kidney cells (93). ER-stress induced by glucose deprivation during renal ischemia might be the initial trigger for GRP78 to traffic to the basolateral membrane of the tubular cells. Especially tubular cells in the cortico-medullary border are subjected to low oxygen and glucose levels during I/R, making these cells particular sensitive to ER-stress. Vascular leakage following reperfusion enables circulation-derived MBL to enter the interstium and there might bind to GRP78 on the basolateral surface of tubular cells. Signaling to GRP78 might then have detrimental effects. Next to signaling, internalization of MBL might also be mediated by GRP78, since evidence has emerged that GRP78 also serves as a receptor for viral entry into host cells (95). Since the ligand for MBL on tubular cells has not been found thus far, these speculations still need to be confirmed.

These findings until now suggest that initially ER-stress is induced following MBL exposure, followed by disturbances in mitochondrial homeostasis. Release of calcium from ER stores might be the main cause of MBL-mediated ER-stress. An increase in cytosolic calcium in tubular cells then would lead to enhanced calcium influx into mitochondria, disrupting mitochondrial metabolism eventually leading to tubular cell death.

9.3.5 Therapeutic inhibition of MBL

Upon reperfusion of the ischemic kidney, MBL from circulation leaks into the interstitium where it is internalized by tubular cells and induces tubular injury. Unfortunately, to date no anti-MBL therapy or other treatments for renal IRI in the clinic exists. Recently however, several clinical possibilities of pharmacologic inhibition of complement, including C1 inhibitor (C1INH) and Eculizumab (anti-C5 monoclonal antibody) have become available (31).

C1INH, a physiologic serine protease inhibitor belonging to the serpin superfamily, acts as a major inhibitor of complement system activation and prevents uncontrolled complement activation. C1INH might be a possible therapeutic candidate to prevent renal IRI given that C1INH regulates vascular permeability (96) and might prevent leakage of MBL in the interstitial compartment following reperfusion. Secondly, C1INH targets the MASPs, which might be involved in the cytotoxic effect of MBL on tubular cells. Thirdly, complement that is activated at a later stage following reperfusion leading to exacerbation of tissue injury might be inhibited. Recombinant human C1INH, which recently has been registered for treatment of hereditary angioedema, is differentially glycosylated (97;98) with high oligo-mannose structures and therefore might include an additional inhibitory effect towards MBL recognizing mannose residues. We explored the therapeutic application of rhC1INH (chapter 7) in a rat model of renal IRI and studied whether rhC1INH is able to attenuated MBL-mediated tubular injury. We demonstrated that rhC1INH is functional active in rat serum and blocks both classical and lectin pathway activation *in vitro*. However, the therapeutic administration of rhC1INH *in vivo* did not attenuate renal dysfunction, tubular injury, inflammatory cell influx and complement activation following reperfusion in rats. Kinetic studies revealed that rhC1INH was cleared from circulation within 2 hours, complement activity was only shortly reduced and circulating MBL levels were unaffected. In addition, we demonstrated that rhC1INH was not able to block the MBL-mediated cytotoxicity towards human tubular cells *in vitro*.

Recently, a clinical trial for the prevention and treatment of reperfusion injury in kidney transplantation has been started (NCT01756508). In this trial, C5 is targeted using the humanized monoclonal antibody (Eculizumab). This antibody binds to C5 and prevents cleavage of C5 and formation of C5b-9 and C5a. Our studies in rats and human however do not support a key role for C5 in renal IRI, given that inhibition of C5 using an identical antibody targeting rat C5 was not protective a no release of human sC5b-9 and C5a or local C5b-9 deposition could be detected early after clinical reperfusion. Importantly, our data demonstrate that therapeutic targeting of MBL following reperfusion might be very promising. Currently, a mouse monoclonal blocking antibody to human MBL (3F8) is available and in the near future will be humanized for therapeutic application in humans.

When clinically available, short interference with MBL using this antibody may have important therapeutical implications. In our rat model, we demonstrated that a single injection of anti-MBL antibody shortly before reperfusion provides an almost complete protection against renal IRI. In the clinical setting of kidney transplantation, a short treatment of the recipient with an anti-MBL antibody therefore could significantly alter the extent of kidney damage following renal IRI.

9.4 CONCLUSIONS

Although the main function of complement is to clear pathogens and maintaining homeostasis by clearing apoptotic cells and cellular debris, complement activation might also contribute to renal IRI by exacerbating local inflammation and tubular injury through release of C5a and formation of C5b-9. Studies mainly performed in mice have demonstrated an important role for the alternative pathway. Nevertheless, in this thesis we show that human hypoxic PTEC predominantly activates the classical pathway of complement, demonstrating important species-specific differences in complement activation. In addition we showed that early following reperfusion in human there is only a very transient activation of complement in circulation, which is virtually absent at the tissue level. We also demonstrated that it is not reliable to assess sC5b-9 in urine, given that spontaneous C5b-9 generation can occur, independent of a renal contribution. From these findings and kinetic data obtained from our rat IRI model, we conclude that complement activation following reperfusion is merely a secondary process activated by injured and apoptotic tubular cells in a relatively late phase following reperfusion (Fig 1). In contrast, early after reperfusion circulation-derived MBL, independent of complement activation, exerts cytotoxic effect to tubular cells mediating initial tubular injury. Although not shown in this thesis, MBL might also be involved in potentiating TLR signaling, which have been implicated in the pathophysiology of renal IRI. Furthermore, a crosstalk between MBL and coagulation might be involved, facilitating coagulation and clot formation in the renal microvasculature and contributing to the coagulopathy in renal IRI.

In short, MBL is a highly versatile protein which, in addition to potent complement activation, also exerts direct cytotoxic effect, amplifies TLR signaling and promotes coagulation. In conclusion, this thesis documents the important role of MBL in the early pathogenesis of renal IRI and shows that complement activation is a relatively late process in the pathophysiology of renal IRI. Treatment with rhC1INH, or targeting C3 and C5 was not protective against renal IRI. In contrast,

interfering with MBL was highly effective in reducing renal IRI, indicating that MBL has a pivotal role in the pathophysiology of renal IRI. We demonstrate a crucial role for MBL in renal IRI and identify MBL as a novel therapeutic target in kidney transplantation. We therefore suggest that a short therapeutic intervention at the level of MBL might significantly alter the extent of kidney damage following renal I/R. The development and application of a humanized anti-MBL antibody might be a promising step forward in the treatment of renal IRI following kidney transplantation.

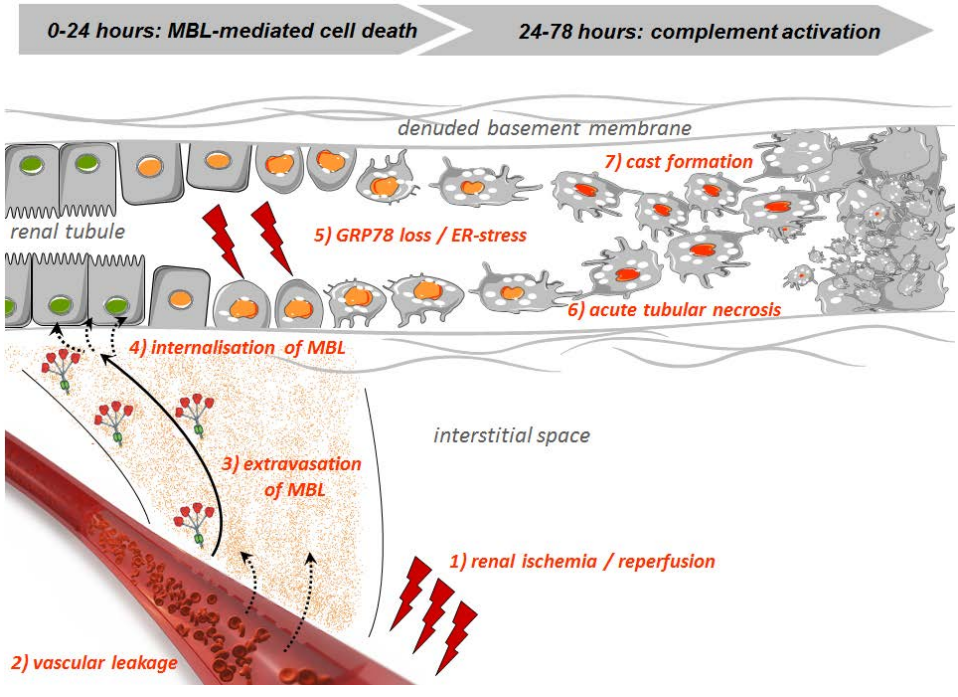

Figure 1. Role of complement and MBL in renal ischemia/reperfusion injury. Renal I/R is associated with a dysbalance of angiopoietins and vessel destabilization (**chapter 8**), resulting in vascular leakage, edema and extravasation of MBL and other complement molecules like C1q in the interstitial space (**chapter 5**). Basolateral exposure of tubular epithelial cells to MBL results in binding and internalization of MBL, which is accompanied by loss of ER-stress sensor GRP78, induction of endoplasmic reticulum-stress (chapter 6) and subsequent tubular cell death within several hours of reperfusion (**chapter 6**). Injury to the tubular cells lead to activation of the complement system in the later phase of reperfusion and in human is mediated by natural IgM and C1q (**chapter 2**). In circulation or at the tissue level, no complement activation products could be detected early after reperfusion (**chapter 3**), while in the urine complement measurement is not reliable (**chapter 4**). Therapeutic targeting of MBL was highly protective against renal IRI, whereas blocking of complement activation at the level of C3 and C5 (**chapter 5, 7**) was not, indicating that MBL play a pivotal role in renal IRI, independent of complement activation.

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