CHAPTER 2

The Lectin Pathway in Renal Disease: Old Concept and New Insights

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Abstract

The complement system is composed of a network of at least 40 proteins, which significantly contributes to health and disease. The lectin pathway (LP) is one of three pathways that can activate the complement system. Next to protection of the host against pathogens, the LP has been shown to play a crucial role in multiple renal diseases as well as during renal replacement therapy. Therefore, several complement-targeted drugs are currently being explored in clinical trials. Among these complement inhibitors, specific LP inhibitors are also being tested in renal abnormalities such as in immunoglobulin A nephropathy and lupus nephritis. Using various in vitro models, Yaseen et al. (Lectin pathway effector enzyme mannan-binding lectin-associated serine protease-2 can activate native complement component 3 (C3) in absence of C4 and/or C2. FASEB J 2017; 31: 2210–2219) showed that Mannan-associated serine protease 2 can directly activate C3 thereby bypassing C2 and C4 in the activation of the LP. These new findings broaden our understanding of the mechanisms of complement activation and could potentially impact our strategies to inhibit the LP in renal diseases. In support of these findings, we present data of human renal biopsies, demonstrating the occurrence of the LP bypass mechanism in vivo. In conclusion, this review provides a detailed overview of the LP and clarifies the recently described bypass mechanism and its relevance. Finally, we speculate on the role of the C4 bypass mechanism in other renal diseases.
Introduction

The complement system is a major pillar of our innate immune system and additionally plays a vital role in renal diseases. The complement system can be activated via three different pathways: the classical pathway (CP), the lectin pathway (LP) and the alternative pathway (AP). Activation of any of these pathways leads to the cleavage of complement component 3 (C3) and subsequently activation of the terminal pathway of complement. The LP has been shown to be involved in the pathogenesis of various renal diseases, and will be discussed in further detail.

Brief description of the LP

In hindsight, the first article published about the LP was in The Lancet in 1968, when Miller et al. reported on patient with a familial plasma-associated defect in phagocytosis. However, in 1978 mannose-binding lectin (MBL) was first discovered and isolated from the rabbit liver. Yet, it took another 9 years for the study by Ikeda et al. to demonstrate the ability of MBL to activate the complement system. Finally, the landmark study by Super et al. in 1989 linked the presence of low levels of MBL to a defect in phagocytosis. Moreover, Sumiya et al. published in 1991 the genetic basis for the low levels of MBL in children with recurrent infections. Ultimately, Matsushita and Fujita added the missing piece of the puzzle in 1992. Initially, the view of the LP consisted of MBL binding to sugars on pathogens, leading to Mannan-associated serine protease (MASP)-2 activation and subsequent cleavage of C4 and C2, and generation of C4bC2a, the C3-convertase. Finally, cleavage of C3 results in the generation of C5a and C5b-9. However, new findings have transformed our view of the LP from a simple route to a vastly complex one involved in health and disease. First of all, in addition to MBL other pattern recognition molecules of the LP have been discovered, namely the Ficolins (Ficolin-1, Ficolin-2 and Ficolin-3) and the Collectins (CLs) (CL-10 and CL-11). Binding of these initiators (MBL/ Ficolins/CLs) to molecular patterns (e.g. sugars) leads to activation of MASP-1, which thereafter activates MASP-2. Collectively, the MASPs cleave C4 and C2 into the C4bC2a, the C3-convertase. This convertase further cleaves C3 into C3a and C3b. Furthermore, novel regulators of the LP have also been described, more specifically MAp19 and MAp44. These molecules are competitive antagonists of the MASPs and thereby prevent complement activation via the LP. In addition, the LP has been linked to the AP. Recently, MASP-3 was discovered, an alternative splicing product of MASP-1. The function of this serine protease was unknown for a long time until Dobó et al. revealed that MASP-3 cleaves pro-factor D into factor D, thereby establishing a crucial link between the LP and the AP. Additionally, Yaseen et al. described an exciting new finding about the LP, named the MASP-2-dependent bypass. Using various in vitro models with purified or recombinant complement components and normal serum or specific complement deficient serum, the authors established that activated MASP-2 can also directly cleave native C3. This means that MASP-2 can support LP activation without previous cleavage of C4 and/or C2. Furthermore, the C4 and/or C2 bypass mechanism is only present for the LP and not for the CP. These new insights inspired us to re-evaluate previous findings about the LP in experimental and translational studies.
Moreover, this new mechanism could have implications for therapeutic strategies of the LP in tissue injury and disease (Figure 1).

Figure 1 | Overview of the lectin pathway of the complement system. The lectin pathway (LP) is one of the activation pathways of the complement system. The LP consists of three types of pattern recognition molecules (PRM): Ficolins, Mannan-binding lectin (MBL) and Collectins. These initiators form complexes with the MBL-associated serine proteases (MASP-1, MASP-2, MASP-3). In brief, Ficolins bind with high affinity to sugars or acetylated compounds, while MBL recognizes predominantly polysaccharides. Moreover, the PRMs of the LP can recognize these molecules on pathogens but also on apoptotic and stressed cells. Additionally, other molecules such as immunoglobulin A are also able to activate the LP. The main regulators of the LP are MAp19 and MAp44, which are competitive antagonists of the MASPs. Once the LP is initiated, C3 activation occurs by the C3-convertase C4bC2a. The formation of the C3-convertase depends on previous cleavage of C4 and C2. The C4 bypass proposed by Yassen et al. forms an additional route for C3 activation. In the C4 bypass, MASP-2 directly leads to C3 activation independent of previous C4 and C2 activation. Next, C3 activation leads to C5 cleavage, forming C5a and C5b. Finally, C5b merges with C6-C9 forming the C5b-9, also called membrane attack complex.
The LP in renal transplantation

Renal transplantation was one of the first clinical entities in which the LP was shown to be involved in complement-mediated injury.\textsuperscript{11,12} In hindsight, the description of the C4/C2 bypass is crucial to understand experimental findings of LP involvement in renal ischemia reperfusion injury (IRI). IRI is characterized by a temporary halt of blood flow to an organ. Originally, Zhou et al. demonstrated that C3-deficient mice were protected from renal IRI whereas C4-deficient mice were not.\textsuperscript{13} The authors then concluded that the AP must have been responsible for complement-mediated injury in IRI, and not the LP or CP. To test this hypothesis, Asgari et al. subjected MASP-2-deficient mice to renal IRI and found that the lack of MASP-2 was protective.\textsuperscript{14} Moreover, MASP-2 deficiency led to decreased complement activation thereby preserving renal function after IRI. The initial notion that the LP was not involved in renal IRI was, therefore, rejected. However, this finding led to many new questions, such as which LP initiator was responsible for MASP-2 activation. To further investigate the role of the LP in IRI, Farrar et al. induced renal IRI in mice deficient for the pattern recognition molecule CL-11.\textsuperscript{15} In accordance to MASP-2 -/- mice, CL-11 deficiency ameliorated renal function. Furthermore, Farrar et al. also investigated the molecular pattern responsible for activating the LP. In the mouse model, the main mechanism of complement activation in renal IRI is the induction of L-fucose by cell stress leading to the binding of CL-11 and subsequent activation of MASP-2, thereby cleaving C3 and as a result the formation of C5a.\textsuperscript{13-16} However, the lack of protection seen in C4 -/- mice remained an unsolved enigma. Correspondingly, similar results were obtained in rodent models of myocardial and gastrointestinal IRI, where MASP-2 deficiency was protective as well but C4 deficiency was not.\textsuperscript{17} The recent observation by Yaseen et al. of the C4/C2 bypass mechanism of the LP, leading to direct C3 activation without involvement of C4 and C2, provides an explanation for this long-standing paradox of the LP in IRI.

Despite these novel findings, human proof for the C4/C2 bypass phenomenon is absent. In the past years, we investigated the role of complement in renal transplantation, with special interest in deceased-donor invoked-complement activation.\textsuperscript{18} We wanted to evaluate the role of the LP and, therefore, performed double staining for MASP-2/C4d and MASP-2/ C3d in three human kidney biopsies of non-heart-beating donors prior to transplantation. As depicted in Figure 2, MASP-2 deposition co-localizes with C3d but not with C4d. Overall, C3d deposition was present in the renal medulla and cortex and MASP-2 was seen in renal medulla. On the contrary, C4d was only deposited in glomeruli. Hence, MASP-2 seems to be involved in C3 activation in the medulla of deceased organ donors without C4 activation. However, we cannot exclude the possibility of MASP-2 synthesis, making the deposition of MASP-2 in the medulla of the kidney a sign of production rather than activation. Nonetheless, the liver predominantly produces MASP-2 and the production of MASP-2 by the kidney has thus far not been demonstrated, making it less likely.\textsuperscript{19} Moreover, in a transcriptomic analysis of non-heart-beating deceased donors' biopsies, MASP-2 expression was not upregulated when compared with living donors, supporting that MASP-2 production by the kidney is unlikely.\textsuperscript{20} Since MASP-2 is reported to be predominantly expressed in the liver, no MASP-2 deposition would be expected in the kidney, suggesting that the MASP-2 depositions seen in the biopsies might be due to complement activation. Our data indicate the existence of complement activation via the
Figure 2 | Immuno-histochemical analysis of complement activation in renal tissue of human non-heart beating donors. Confocal microscopy of a human kidney of a non-heart beating (NHB) organ donor. Immunofluorescent staining was performed using a polyclonal antibody against C3d (A0063, Dako, Carpinteria, CA, USA), polyclonal against C4d (BI-RC4D, Biomedica, Vienna, Austria) and a monoclonal antibody specific for MASP-2 (HM2191, Hycult, Uden, The Netherlands). Staining for C3d and C4d was developed with an fluorescein isothiocyanate (FITC)-labelled anti-rabbit IgG (green). Tetramethylrhodamine isothiocyanate (TRITC)-labelled anti-mouse IgG (red) was used for MASP-2. DAPI was used to counterstain nuclei (blue). Negative controls, without primary antibodies, showed no positive staining for TRITC or FITC (data not shown). Overlayed images were obtained with the Leica Confocal Software (Leica Microsystems Heidelberg GmbH, Mannheim, Germany). (A) Image of the renal medulla. (1) Double staining for C3d and MASP-2. Co-localization of C3d and MASP-2 was seen in the tubuli. (2) Double staining for C4d and MASP-2. No C4d deposition was seen in the tubuli, only MASP-2 depositions. (B) Image of the renal cortex. (1) Double staining for C3d and MASP-2. C3d depositions were seen periglomerular. (2) Double staining for C4d and MASP-2. C4d deposition were only seen in the glomeruli. Magnifications (A) 200x and (B) 400x.

C4 bypass mechanism in human tissue, which would be in agreement with results from animal studies. Although the current finding implies the presence of the C4 bypass in human settings, further research is needed. Previously, complement activation in deceased organ donors has been shown to affect outcome after transplantation. More specifically, Damman et al. described that high C5b-9 levels in deceased donors are associated with acute rejection after renal transplantation.21
Additionally, in a genetic analysis of C3 allotypes, the association with primary non-function was only seen in non-heart-beating donors. Together, these results suggest an important role for the complement system in renal injury prior to transplantation.

In transplantation, the role of the LP has also been investigated in rejection after kidney transplantation. However, conflicting data exist, since LP activation has been associated with either a protective or deleterious effect on the occurrence of acute rejection. Previously, Berger et al. demonstrated that high MBL levels were associated with a more severe rejection leading to graft loss. However, recently Golshayan et al. reported in a larger cohort that low MBL levels were associated with a higher occurrence of acute rejection. Moreover, the LP has also been suggested to be responsible for the C4d deposition seen in antibody-mediated rejection. In summary, there is growing evidence of the role of the LP in renal transplantation. However, with the current new findings about the C4 bypass mechanism, it is important to re-evaluate the LP in other complement-related disease.

The LP in renal disease, a fresh look at old data

Deregulation of the complement system plays a major role in several renal diseases. Moreover, a particular interest has arisen in the LP since this pathway has been linked to harmful effects in kidney transplantation and other complement-related renal diseases. With the recent discovery of the C4 bypass, previous reports and studies about the LP in renal pathology should be re-evaluated.

Primary, immunoglobulin A (IgA) nephropathy (IgAN) is a glomerular nephropathy characterized by deposition of IgA and complement proteins in the kidney. Initially, complement activation in this disease was thought to arise from AP activation because of the finding of properdin and C3 depositions in IgAN biopsies. However, Roos et al. later demonstrated a role for the LP in IgAN. More specifically, the presence of MBL, C4d and ficolin-2 depositions in the renal biopsies was associated with worse renal function and more severe progression of disease. In support of this, C4d deposition, used as a marker for LP activation, was again shown to be a good predictor of disease progression. Additionally, a new study in a large cohort of IgAN patients recently showed that high serum levels of MBL are associated with accelerated IgAN progression. Thus, in addition to the AP, a role has been demonstrated for the LP in the progression and prognosis of IgAN. Whether both the LP and AP are primarily involved in the disease or whether the AP merely functions as an amplification loop, remains to be investigated. Moreover, the recently described link between AP and LP could also explain the presence of AP depositions in IgAN, since activation of pro-factor D by MASP-3 critically affects AP activation.

Another important complement-related renal disease is lupus nephritis (LN). In the majority of patients with systemic lupus erythematosus (SLE), there is histological proof of LN. The role of the complement system in lupus is dual. On the one hand, complement deficiencies are associated with the occurrence of SLE, but on the other hand complement activation is linked to disease activity. In LN, complement activation is thought to be triggered by the CP and the AP, since depositions of IgG, C1q, C3d, properdin and C5b-9 are seen in renal biopsies. However, several studies have also
demonstrated the involvement of the LP activation. In LN, renal biopsies displayed depositions of MBL and Ficolin-2.\textsuperscript{32,33} In addition, MBL depositions as well as MBL serum levels have been shown to be a predictor of disease activity of SLE.\textsuperscript{34} Moreover, plasma levels of Ficolin-1 and Ficolin-3 also form valuable biomarkers to monitor disease activity of SLE.\textsuperscript{34} In accordance, results from murine models indicated LP involvement in LN, by showing co-localization of MBL and C3 depositions in diseased kidneys.\textsuperscript{35} To summarize, the CP (and AP) have a major role in the initiation of the disease, while LP seems to be involved in the progression of the disease. Remarkably, it has previously been demonstrated that renal C4d deposition in LN cannot be attributed to the LP.\textsuperscript{36} Furthermore, C4 deficiencies are associated with the development of SLE. Interestingly, this deficiency would prevent CP activation in SLE individuals, whereas complement activation via the LP would still be possible via the C4 bypass mechanism.\textsuperscript{37} Future studies should, therefore, explore the possible contribution of this new C4 bypass mechanisms in LN and SLE. In diabetic nephropathy (DN), evidence for a role of the complement system comes from experimental and clinical studies. Two different mechanisms have been proposed for the role of complement in the pathogenesis of DN: (I) LP activation by sugars and (II) hyperglycemia-induced dysfunction of complement regulators.\textsuperscript{38} Both mechanisms are related to LP activation, subsequently leading to complement activation. Fittingly, MBL and Ficolin-3 have been shown to be reliable biomarkers in the prediction and progression of DN in both Type 1 and Type 2 diabetes.\textsuperscript{39,40} Additionally, MASP-1 and MASP-2 levels were shown to be higher in diabetic patients, although this finding was not restricted to patients with DN.\textsuperscript{41} Recently, renal depositions of complement components were analyzed in a cohort of diabetic patients with and without DN. Patients with DN showed significantly increased amounts of C4d depositions when compared with patients without DN, whereas healthy controls exhibited no C4d deposition.\textsuperscript{42} In contrast, low C4 plasma levels were reported in diabetic patients and associated with microvascular disease.\textsuperscript{43} In conclusion, complement activation via the LP is well established in diabetic individuals. Besides the role of the complement system in renal diseases, complement activation is also a key mediator of inflammation during hemodialysis (HD).\textsuperscript{44,45} The LP has been shown to be involved in HD-induced complement activation.\textsuperscript{46,47} Proteomic studies by Mares et al. revealed that Ficolin-2 and MBL bind to the HD membrane leading to LP activation.\textsuperscript{48} The latter is supported by previous studies, showing that systemic C4d levels increase during HD and correlate with C3d levels. Furthermore, these results also imply a lesser role of the C4 bypass mechanisms in HD. However, reports by Lhotta et al. indicate that C4-deficient patients still exhibited complement activation.\textsuperscript{49} The authors concluded that the remaining complement activation must have been due to AP activity. However, another explanation could be that the C4 bypass mechanism led to the observed complement activation in these patients.

**Future perspectives on LP-related diseases**

An important aspect that remains unanswered is the purpose of the C4 bypass mechanism. The answer possibly lies in the fact that C4 and C2 form the rate-limiting step in CP and LP activation.\textsuperscript{50} However, for systemic complement activation, this is not a problem since there is an abundant
amount of C4 and C2 present in serum. Nevertheless, during local complement activation, such as occurs in the renal interstitium in IRI, the amount of C4 and/or C2 could potentially limit the immune activation. We therefore speculate that this bypass mechanism enables LP activation in remote tissue areas with reduced perfusion and, therefore, limited amounts of C4/C2. In accordance with this hypothesis, we observed that the C4 bypass mechanisms occurred in the renal medulla and not the cortex. Fittingly, the renal medulla is less perfused than the renal cortex. Altogether, we speculate that the C4 bypass mechanism would be predominantly important for local complement activation (solid phase) and less for systemic complement activation (fluid phase) (Figure 3).

Figure 3 | The proposed importance of the C4 bypass mechanism to local complement activation. The importance of the C4 bypass mechanisms remains to be investigated. However, we propose that this bypass mechanism is essential to enable local complement activation. In general, C4 and C2 could form a rate-limiting step for complement activation of the classical pathway and LP. However, in blood there is an abundance of C4 and C2 and complement activation via the LP occurs via the C4b2a also known as the C3-convertase (left side). The role of C4 bypass mechanisms in the circulation is most likely limited. In contrast, in tissue with low perfusion there is a little presence of C4 and C2, thereby limiting LP-mediated complement activation. Under these conditions, the C4 bypass mechanism enables local complement activation of LP. Damaged renal tubule due to ischemia represents an example of local complement activation where the C4 bypass mechanisms is essential (right side). To conclude, we hypothesize that the C4 bypass mechanism is mainly important for local LP-mediated complement activation under condition with reduced availability of C2/C4.
A better understanding of the LP is relevant for the design and implementation of complement-targeted therapies. Different targets can be used to inhibit complement activation.\textsuperscript{51,52} Possible strategies include (I) blockade of the initiators of the complement pathway, (II) blockade of the C3- and/or C5-convertases, (III) blocking the terminal pathway of the complement system and finally (IV) enhancing the capacity of complement inhibitors present in serum. Currently, eculizumab and C1-inhibitor are the only Food and Drug Administration (FDA)-approved drugs for complement inhibition. Eculizumab inhibits C5, thereby blocking the terminal part of the complement cascade, thus inhibiting the final part of all three pathways. Alternatively, C1-inhibitor is an inhibitor of the initiators of the CP and also for the LP, especially by binding of MASP s. Novel approaches for complement inhibition are under development. A MASP-2 inhibitor (OMS721-Omeros) is currently being tested in a phase II clinical trial for IgAN. Inhibition of MASP-2 is a suitable way to fully stop LP activation, and would also impair the C4 bypass mechanism. This novel drug could, therefore, be a promising new therapeutic approach for LP-mediated renal diseases. Furthermore, other specific LP inhibitors have also been described, however, only in in vitro or in preclinical models. For instance, low-molecular weight heparinoids, which are already widely used in clinic for their anticoagulant effects, are also known to inhibit the LP.\textsuperscript{54} Alternatively, Keizer et al. showed that tissue factor pathway inhibitor is another selective inhibitor of the LP.\textsuperscript{55} Another approach to inhibit the LP could be blockade of the C3-convertase by using a monoclonal antibody against C2 or C4. However, this strategy would also inhibit CP activation and would not prevent LP activation via the C4 bypass.

**Conclusion**

In summary, in vitro studies as well as animal models of ischemia reperfusion have demonstrated a mechanism of LP activation without the use of C2 and/or C4. However, these findings warrant additional investigations in humans. We have provided first evidence supporting the presence of the C4 bypass mechanism of direct activation of C3 in humans as well. Therefore, a possible role for this new bypass mechanism in other complement-related diseases should be considered. The findings by Yaseen et al. could be of major importance for the development and implementation of new complement therapies in nephrology. In addition, the recent success of complement inhibitors in clinical trials and the common off-label use of these drugs support the concept that new complement-targeted therapies could be useful in clinical practice.\textsuperscript{53} More specifically, current trials investigating new LP inhibitors in kidney diseases might change the treatment and prognosis of multiple renal diseases.
References
