View metadata, citation and similar papers at core.ac.uk

brought to you by CORE

provided by Leiden University Scholary Publications

Innate Immune Functions in Kidney Transplantation

Stefan Philip Berger

The printing of this thesis was financially supported by Amgen, Astellas Pharma, Genzyme, Shire, Novartis and Wyeth

Lay-out: Legatron Electronic Publishing, Rotterdam
Printer: PrintPartners Ipskamp, Enschede (www.ppi.nl)

Copyright © 2009 S.P. Berger
All rights reserved. No part of this thesis may be reproduced or transmitted in any form, by any means, electronic or mechanical, without the prior written permission of the author, or where appropriate, of the publisher of the articles.

Innate Immune Functions in Kidney Transplantation

Proefschrift

ter verkrijging van
de graad van Doctor aan de Universiteit Leiden,
op gezag van Rector Magnificus prof.mr. P.F. van der Heijden,
volgens besluit van het College voor Promoties
te verdedigen op woensdag 28 januari 2009
klokke 16.15 uur

door
Stefan Philip Berger

geboren te Johannesburg
in 1969

Promotiecommissie

Promotores:	Prof.Dr. M.R. Daha Prof.Dr. F.J. de Fijter
Co-promotor:	Dr. A. Roos
Referent:	Prof.Dr. I. J. ten Berge Academisch Medisch Centrum, Universiteit van Amsterdam
Overige leden:	Prof.Dr J.A. Bruijn, Prof.Dr. F.H.J. Claas Dr. L.B. Hilbrands, Universitair Medisch Centrum St Radboud, Nijmegen Prof.Dr. T.W.J. Huizinga, Dr. C. van Kooten Prof.Dr. G.J. Navis, Universitair Medisch Centrum Groningen

Voor Tanja

Contents

Chapter 1	Introduction and scope of the thesis	9
Chapter 2	Complement and the Kidney *Nephrology Dialysis Transplantation 2005; 20:2613-2619*	15
Chapter 3	Association between Mannose-binding Lectin Levels and Graft Survival in Kidney Transplantation *American Journal of Transplantation 2005; 5:1361-1366*	31
Chapter 4	Low Pre-transplantation Mannose-binding Lectin Levels Predict Superior Patient and Graft Survival after Simultaneous Pancreas-kidney Transplantation *Journal of the American Society of Nephrology 2007; 18:2416-2422*	47
Chapter 5	Infectious complications after simultaneous pancreas-kidney transplantation: A role for the lectin pathway of complement activation *Transplantation 2008; 85:75-80*	65
Chapter 6	Properdin binds to late apoptotic and necrotic cells independently of C3b, and regulates alternative pathway complement activation *Journal of Immunology 2008; 180(11):7613-7621*	83
Chapter 7	Complement activation by tubular cells is mediated by properdin binding *Am J Physiol Renal Physiol 2008; 295(5):F1397-403*	111
Chapter 8	General Discussion	131
	Nederlandse samenvatting	145
	Curriculum vitae	149
	Publications	150
	Nawoord	152
	Color figures	153

Chapter 1

Introduction and scope of the thesis

Complement research is currently experiencing a renaissance. The discovery of the role of complement in diseases such as the hemolytic uremic syndrome and age related macular degeneration have lead to a new appreciation of the role of complement in human disease and will have an important impact on the management of these patients [1-4]. The role of the innate immune system and specifically complement is also increasingly being recognized in transplantation medicine which has traditionally been dominated by research into the role of the adaptive immune system. Animal studies have demonstrated that complement plays an important role in the initial ischemia-reperfusion injury [5]. The finding that transplanted organs from C3-deficient mice are protected against acute rejection has lead to a whole new area of research into the role of complement in the regulation of the adaptive immune response [6-8]. The detection of the complement split product C4d in transplant biopsies has lead to an appreciation of the role of humoral rejection and points towards complement-mediated damage pathways in allograft rejection [9].

Complement activation involves three pathways. This thesis focuses on the lectin and alternative pathways and their possible role in kidney transplantation and chronic renal disease.

The pathways of complement activation and their role in renal disease are reviewed in **chapter 2**. The lectin pathway of complement activation is initiated by binding of its recognition molecules mannose-binding lectin (MBL) and the ficolins to carbohydrate structures on a wide variety of microorganisms or on injured tissue. MBL is a multimeric C-type lectin consisting of collagenous tails similar to C1q. Circulating MBL levels are determined by frequently occurring polymorphisms (SNPs) of the MBL gene (*mbl2*). These SNPs are locatied in codon 54 (B genotype), codon 57 (C genotype), and codon 52 (D genotype) of the first exon of the MBL gene, which encodes the collagenous region of the MBL molecule [10-12]. The presence of these SNPs interferes with the polymerization of the MBL molecule resulting in low levels of functional MBL [13;14]. Furthermore, polymorphisms in the promoter region lead to reduced circulating MBL levels [15]. The resulting low MBL levels are associated with an increased risk for infectious complications in situations of impaired adaptive immunity such as early infancy and immunosupression [16-18]. Next to its interaction with microorganisms MBL may also interact with immunoglobulins [19;20] and altered host tissue for example in the setting of ischemia/reperfusion damage [21]. MBL is deposited in mouse and human kidneys in the setting of ischemia/reperfusion injury [22] and mice deficient for both MBL-A and MBL-C are partially protected against renal ischemia-reperfusion injury [23].

In view of the role of MBL in ischemia-reperfusion injury and the interaction of MBL with immunoglobulins it seemed conceivable that MBL contributes to tissue damage in the setting of solid organ transplantation. We questioned whether recipient MBL participates in organ damage in the setting of human renal allograft transplantation. In **chapter 3** we first studied the relationship between MBL levels and outcome after deceased donor kidney transplantation. MBL levels were measured in serum samples obtained directly before transplantation and related to outcome parameters including delayed graft function, rejection, and patient and graft survival.

MBL has also been shown to contribute to micro and macro-vascular damage in both type 1 and type 2 diabetes [24-26]. With the harmful effects of MBL in diabetes in mind we were specifically interested in the role of MBL after simultaneous pancreas-kidney transplantation. This type of transplantation is characterized by a high rate of infectious complications, rejection and cardiovascular morbidity. In **chapter 4** we studied the association of MBL levels and MBL genotypes causing these low MBL levels with organ and patient survival after simultaneous pancreas kidney transplantation.

Since MBL recognizes microorganisms and is thought to be an important component of the innate immune response we studied the role of MBL in infectious complications after transplantation. In **chapter 5** the thesis reports our findings concerning the role of MBL in infectious complications after simultaneous pancreas kidney transplantation and demonstrates a particular role for MBL in the protection against urosepsis.

The alternative pathway is constantly activated at a low rate by spontaneous hydrolysis of C3 which leads to the association with factor B and formation of the alternative pathway C3 convertase $C3(H_2O)Bb$. The C3 convertase cleaves additional C3b. If surfaces favoring alternative pathway activation such as bacterial walls are present C3b is protected against inactivation by factor I and H and more C3bBb is formed which is a highly efficient C3 convertase, particularly upon its stabilization by properdin (see chapter 2). However, recent work has reemphasized that properdin may not only bind to C3bBb once it has been formed on a bacterial surface but it may actually play a role in the initiation of the alternative pathway by the means of its pattern recognition capacity. This concept was originally suggested by Pillemer in 1954 [27] and has now been rediscovered 50 years later [28].

The clearance of apoptotic cells plays an important role in the initiation of the immune response in both transplantation and autoimmunity. Both MBL and C1q recognize apoptotic cells and contribute to their clearance [29;30]. We questioned whether properdin interacts with apoptotic cells and whether this interaction leads

to activation of the alternative pathway of complement. In **chapter 6** this thesis describes our studies on the interaction of properdin with apoptotic cells and its contribution to the immune regulation by phagocytic cells.

In **chapter 7** we further focus on the capacity of properdin to target alternative pathway activation to cellular surfaces. Complement activation on tubular cells is thought to be an important mediator of damage in proteinuric renal disease [31]. However, until now it was not clear how tubular cells activate complement molecules which are present in proteinuric urine. We show that properdin binds to the apical surface of viable tubular cells leading to activation of the alternative pathway of complement. This interaction between tubules and properdin may be a crucial step in the initiation of tubulo-interstitial damage in proteinuric renal diseases. Complement molecules entering the tubular lumen in proteinuric states will be targeted to the brush border by properdin resulting in activation of the alternative pathway with production of the anaphylatoxins C3a and C5a and the membrane attack complex.

Finally, in **chapter 8** the findings presented in this thesis are critically discussed and the possible implications for transplantation and the understanding of progressive renal disease are presented.

Reference List

1. Caprioli J, Bettinaglio P, Zipfel PF, Amadei B, Daina E, Gamba S et al. The molecular basis of familial hemolytic uremic syndrome: mutation analysis of factor H gene reveals a hot spot in short consensus repeat 20. *J Am Soc Nephrol* 2001; 12(2):297-307.
2. Caprioli J, Noris M, Brioschi S, Pianetti G, Castelletti F, Bettinaglio P et al. Genetics of HUS: the impact of MCP, CFH, and IF mutations on clinical presentation, response to treatment, and outcome. *Blood* 2006; 108(4):1267-1279.
3. Edwards AO, Ritter R, III, Abel KJ, Manning A, Panhuysen C, Farrer LA. Complement factor H polymorphism and age-related macular degeneration. *Science* 2005; 308(5720):421-424.
4. Warwicker P, Goodship TH, Goodship JA. Three new polymorphisms in the human complement factor H gene and promoter region. *Immunogenetics* 1997; 46(5):437-438.
5. Zhou W, Farrar CA, Abe K, Pratt JR, Marsh JE, Wang Y et al. Predominant role for C5b-9 in renal ischemia/reperfusion injury. *J Clin Invest* 2000; 105(10):1363-1371.
6. Pratt JR, Basheer SA, Sacks SH. Local synthesis of complement component C3 regulates acute renal transplant rejection. *Nat Med* 2002; 8(6):582-587.
7. Heeger PS, Lalli PN, Lin F, Valujskikh A, Liu J, Muqim N et al. Decay-accelerating factor modulates induction of T cell immunity. *J Exp Med* 2005; 201(10):1523-1530.
8. Bohmig GA, Exner M, Habicht A, Schillinger M, Lang U, Kletzmayr J et al. Capillary C4d deposition in kidney allografts: a specific marker of alloantibody-dependent graft injury. *J Am Soc Nephrol* 2002; 13(4):1091-1099.
9. Herzenberg AM, Gill JS, Djurdjev O, Magil AB. C4d deposition in acute rejection: an independent long-term prognostic factor. *J Am Soc Nephrol* 2002; 13(1):234-241.
10. Lipscombe RJ, Sumiya M, Hill AV, Lau YL, Levinsky RJ, Summerfield JA et al. High frequencies in African and non-African populations of independent mutations in the mannose binding protein gene. *Hum Mol Genet* 1992; 1(9):709-715.
11. Sumiya M, Super M, Tabona P, Levinsky RJ, Arai T, Turner MW et al. Molecular basis of opsonic defect in immunodeficient children. *Lancet* 1991; 337(8757):1569-1570.
12. Madsen HO, Garred P, Kurtzhals JA, Lamm LU, Ryder LP, Thiel S et al. A new frequent allele is the missing link in the structural polymorphism of the human mannan-binding protein. *Immunogenetics* 1994; 40(1):37-44.
13. Roos A, Garred P, Wildenberg ME, Lynch NJ, Munoz JR, Zuiverloon TC et al. Antibody-mediated activation of the classical pathway of complement may compensate for mannose-binding lectin deficiency. *Eur J Immunol* 2004; 34(9):2589-2598.
14. Larsen F, Madsen HO, Sim RB, Koch C, Garred P. Disease-associated mutations in human mannose-binding lectin compromise oligomerization and activity of the final protein. *J Biol Chem* 2004; 279(20):21302-21311.
15. Madsen HO, Garred P, Thiel S, Kurtzhals JA, Lamm LU, Ryder LP et al. Interplay between promoter and structural gene variants control basal serum level of mannan-binding protein. *J Immunol* 1995; 155(6):3013-3020.
16. Mullighan CG, Heatley S, Doherty K, Szabo F, Grigg A, Hughes TP et al. Mannose-binding lectin gene polymorphisms are associated with major infection following allogeneic hemopoietic stem cell transplantation. *Blood* 2002; 99(10):3524-3529.

17. Peterslund NA, Koch C, Jensenius JC, Thiel S. Association between deficiency of mannose-binding lectin and severe infections after chemotherapy. Lancet 2001; 358(9282):637-638.
18. Koch A, Melbye M, Sorensen P, Homoe P, Madsen HO, Molbak K et al. Acute respiratory tract infections and mannose-binding lectin insufficiency during early childhood. *JAMA* 2001; 285(10):1316-1321.
19. Arnold JN, Wormald MR, Suter DM, Radcliffe CM, Harvey DJ, Dwek RA et al. Human serum IgM glycosylation: identification of glycoforms that can bind to mannan-binding lectin. *J Biol Chem* 2005; 280(32):29080-29087.
20. Roos A, Bouwman LH, Gijlswijk-Janssen DJ, Faber-Krol MC, Stahl GL, Daha MR. Human IgA activates the complement system via the mannan-binding lectin pathway. *J Immunol* 2001; 167(5):2861-2868.
21. Collard CD, Vakeva A, Morrissey MA, Agah A, Rollins SA, Reenstra WR et al. Complement activation after oxidative stress: role of the lectin complement pathway. *Am J Pathol* 2000; 156(5):1549-1556.
22. de Vries B, Walter SJ, Peutz-Kootstra CJ, Wolfs TG, van Heurn LW, Buurman WA. The mannose-binding lectin-pathway is involved in complement activation in the course of renal ischemia-reperfusion injury. *Am J Pathol* 2004; 165(5):1677-1688.
23. Moller-Kristensen M, Wang W, Ruseva M, Thiel S, Nielsen S, Takahashi K et al. Mannan-binding lectin recognizes structures on ischaemic reperfused mouse kidneys and is implicated in tissue injury. *Scand J Immunol* 2005; 61(5):426-434.
24. Hovind P, Hansen TK, Tarnow L, Thiel S, Steffensen R, Flyvbjerg A et al. Mannose-binding lectin as a predictor of microalbuminuria in type 1 diabetes: an inception cohort study. *Diabetes* 2005; 54(5):1523-1527.
25. Hansen TK, Tarnow L, Thiel S, Steffensen R, Stehouwer CD, Schalkwijk CG et al. Association between mannose-binding lectin and vascular complications in type 1 diabetes. *Diabetes* 2004; 53(6):1570-1576.
26. Hansen TK, Gall MA, Tarnow L, Thiel S, Stehouwer CD, Schalkwijk CG et al. Mannose-binding lectin and mortality in type 2 diabetes. *Arch Intern Med* 2006; 166(18):2007-2013.
27. Pillemer L, Blum L, Lepow IH, Ross OA, Todd EW, Wardlaw AC. The properdin system and immunity. I. Demonstration and isolation of a new serum protein, properdin, and its role in immune phenomena. *Science* 1954; 120(3112):279-285.
28. Hourcade DE. The role of properdin in the assembly of the alternative pathway C3 convertases of complement. *J Biol Chem* 2006; 281(4):2128-2132.
29. Nauta AJ, Castellano G, Xu W, Woltman AM, Borrias MC, Daha MR et al. Opsonization with C1q and mannose-binding lectin targets apoptotic cells to dendritic cells. *J Immunol* 2004; 173(5):3044-3050.
30. Nauta AJ, Daha MR, van Kooten C, Roos A. Recognition and clearance of apoptotic cells: a role for complement and pentraxins. *Trends Immunol* 2003; 24(3):148-154.
31. Abbate M, Zoja C, Remuzzi G. How does proteinuria cause progressive renal damage? *J Am Soc Nephrol* 2006; 17(11):2974-2984.

Chapter 2

Complement and the kidney

Stefan P. Berger,
Anja Roos and
Mohamed R. Daha

Department of Nephrology, Leiden University Medical Center

Nephrology Dialysis Transplantation 2005; 20:2613-2619

Introduction

The renewed appreciation of the role of the complement system as a mediator and marker of renal damage has led to numerous novel investigations in the field of complement and renal disease. The aims of the present review are to recapitulate the pathways of complement activation with an emphasis on the more recently described lectin pathway of complement activation, to discuss some of the new data on the role of complement in renal disease and to briefly provide information about new diagnostic techniques in the field of complement.

Pathways of complement activation

The complement system is not only an important component of the innate immune system but also plays an essential role in the initiation and control of the adaptive immune response. The three pathways of complement activation converge at the level of C3. Activation of C3 leads to the formation of the membrane attack complex (MAC) on complement-activating surfaces (Figure 1).

The classical pathway of complement activation is initiated via binding of its recognition molecule C1q to immune complexes or charged molecules. This leads to a conformational change resulting in activation of the C1q-associated serine proteases C1r and C1s. Activated C1s cleaves both C4 and C2 which associate to form the classical pathway C3 convertase, the C4b2a enzyme complex. Next to activation by IgG and IgM immune complexes, C1q may also be activated by apoptotic and necrotic cells and by acute phase proteins such as CRP [1].

The lectin pathway of complement utilizes the same C3 convertase as the classical pathway. It is initiated by binding of mannose-binding lectin (MBL) or ficolins which recognize patterns of carbohydrate ligands that are found on the surface a wide variety of microorganisms [2]. MBL consists of up to six trimeric subunits and its structure resembles a bouquet-like shape similar to that of C1q. The plasma concentrations can vary up to 1000-fold. This variation is largely explained by single nucleotide polymorphisms within exon 1 of the MBL-2 gene. Polymorphisms of the promoter region contribute further to the variation in MBL levels.

Binding of MBL to its ligands results in the activation of the associated serine protease MASP-2 and subsequent cleavage of C4 and C2 leading to the formation of C4b2a.

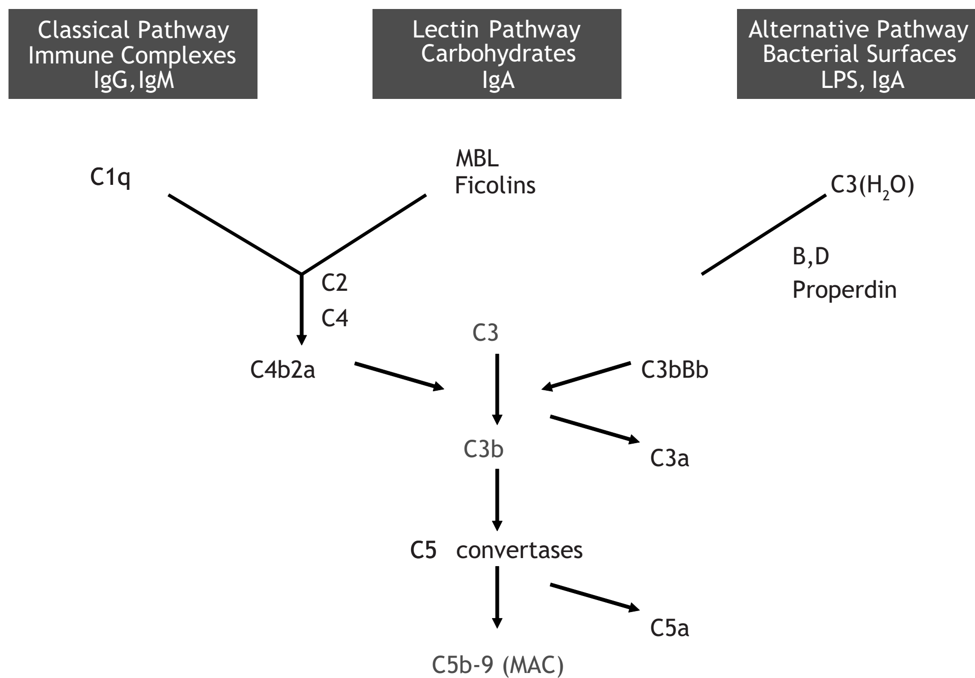


Figure 1. Overview of the three pathways of complement activation

The alternative pathway depends on spontaneous hydrolyzation of C3 in plasma leading to the formation C3(H_2O). This molecule binds to factor B and subsequent activation by factor D results in the formation of C3(H_2O)Bb. This complex cleaves additional C3 to C3a and C3b constantly and at a low rate. In the presence of an activating surface (e.g. a bacterial wall) C3b is protected from inactivation by regulatory proteins like factor I and H and the more active alternative pathway C3 convertase C3bBb is formed, which is further stabilized by properdin.

The common terminal pathway is similar for the classical, lectin and alternative pathways. The incorporation of C3b in the C3 convertases results in the formation of C3bBbC3b for the alternative pathway and C4b2a3b for the classical and lectin pathway. These C5 convertases initiate the formation of the membrane attack complex by cleavage of C5 to C5a and C5b. C5b forms a trimolecular complex with C6 and C7. After insertion in a cell membrane C8 and multiple molecules of C9 bind and the pore-forming MAC is assembled. In sublytic doses insertion of MAC in the cell membrane may lead to cell activation [3] and enhancement of the innate immune

responses.
Next to the production of MAC with resulting lysis or activation of cells, complement activation can also lead to the production of the chemo attractive anaphylatoxins C3a and C5a. Complement split products such as C3b and C4b associate with immune complexes increasing their solubility and facilitating their clearance. Both MBL and C1q may bind to apoptotic cells and aid in their clearance [4-6[.

Role of complement in renal disease

Glomerulonephritis

Complement may play both a beneficial as well as a harmful role in renal disease. Complement deposition is detected in kidney biopsies obtained from patients with various forms of renal disease. Except for type II membranoproliferative glomerulonephritis complement deposition is usually accompanied by the deposition of immunoglobulins. In the following section we will discus some of the new data on the role of complement in lupus nephritis and IgA nephropathy as examples for glomerular disease

Lupus nephritis

The deposition of IgG, IgM IgA, C3, C4 together with C1q is the hallmark of lupus nephritis and is referred to as the full house pattern of immune deposition. The complement deposition in kidneys with lupus nephritis and the marked reduction of complement levels in most of these patients suggest an important role for classical pathway-mediated damage in lupus nephritis. The manipulation of the complement system in various mouse models has shed light on the complex role of complement in this disease. The disruption of both the C1q or the C4 gene in mice with a 129 x C57BL/6 genetic background leads to the spontaneous development of glomerulonephritis with the production of autoantibodies and accumulation of apoptotic cells [7;8]. In line with these findings inherited deficiencies of C1q and C4 are strongly associated with the development of SLE in humans. Interestingly C1q deficiency did not significantly influence the development of glomerulonephritis in the spontaneously lupus developing MLR/lpr mice [9]. If on the other hand lupus prone NZB/W mice were treated with an anti-C5 antibody the development of glomerulonephritis could be prevented [10]. Similar protective results were obtained when MLR/lpr mice were treated with the soluble rodent complement inhibitor rCrry-Ig [11]. Considering the data obtained

both from humans and the animal models it seems that the beneficial role of the early components of the classical pathway in opsonisation and clearance of apoptotic cells and immune complexes override the possible damaging role mediated by downstream complement activation products. These considerations have important implications for the possible role of therapeutic interventions in the complement system. Complement inhibition further downstream may inhibit the production of the powerful anaphylatoxin C5a and MAC without impairing the protective role of the upstream components of the complement pathway.

Antibodies directed against C1q are detectable in 30 to 40% of SLE patients [12]. These antibodies correlate with the presence of active lupus nephritis with a sensitivity of 87% and a specificity of 92% [13]. The generation of homologous mouse anti-mouse C1q antibodies has provided a tool to study whether these antibodies actually play a role in the pathogenesis of lupus nephritis. Administration of these antibodies alone led to deposition of C1q in kidneys of naive mice with granulocyte influx without clinical expression of renal disease such as albuminuria. However, when mice were pre-treated with a subnephritiogenic dose of rabbit anti-GBM antibodies, administration of mouse anti-C1q antibodies resulted in increased deposition of immunoglobulins and complement as well as marked renal damage [14]. Application of this model to mice genetically deficient for C4, C3 or all three Fc-γ receptors demonstrated that anti-C1q-mediated renal damage was dependent on both complement activation and the contribution of Fc-γ receptors.

IgA-nephropathy

Deposition of predominantly polymeric IgA of the IgA1-subclass is the hallmark of IgA nephropathy. Co deposition of C3 is usually detected in renal biopsies. This is thought to result from alternative pathway activation since IgA does not activate the classical pathway of complement. But as C4 deposition is detected in 30% of biopsies from kidneys with IgA nephropathy [15] complement activation via the MBL pathway has been suggested. Indeed co-deposition of IgA with MBL has been demonstrated in biopsies from patients with IgA nephropathy [16]. In line with these findings our group has shown that MBL binds to IgA resulting in complement activation [17].

Ischemia Reperfusion damage

Several studies have underscored the role of complement in ischemia reperfusion damage. Zhou et al. studied mice deficient for C3, C4, C5 or C6 in a kidney ischemia reperfusion model [18]. C3, C5 and C6 deficiency was associated with marked

protection from ischemia/reperfusion damage, whereas C4-deficient mice were not protected. These findings suggest an important role of C5b-9 activated by the alternative pathway in ischemia/reperfusion damage. Classical pathway activation did not seem to play a role in this model. This concept has been supported by a study showing protection from renal ischemia reperfusion damage in mice deficient for factor B [19]. The same group has demonstrated C3b deposition without evidence of C4b deposition in human kidneys with acute tubular necrosis [(20], showing that the alternative pathway may also be the dominant route of complement activation in ischemia reperfusion damage of the human kidney. Complement may also cause damage due to the formation of chemotactic molecules such as C5a [21].

An important role of MBL has recently been demonstrated in ischemia reperfusion damage of the heart and intestine [22;23]. Mice deficient for MBL-A and MBL-C were protected from cardiac and gastrointestinal ischemia/reperfusion injury whereas C1q-deficient mice were not protected. MBL deposition has been detected in mouse and human kidneys with ischemia reperfusion damage [24] and a possible contribution of MBL to ischemia reperfusion injury of the kidney has recently been proposed in a study using mice deficient for MBL A and C [25].

Kidney Transplantation

The introduction of C4d staining in biopsies obtained from renal transplants has led to a new appreciation of the role of humoral rejection in renal transplantation. C4d binds covalently to basement membranes and therefore may remain detectable for weeks. The presence of C4d in the peritubular capillaries indicates humoral rejection as shown by the strong correlation with panel-reactive [26] or donor-specific antibodies [27]. Staining for C4d has been shown to predict poorer graft survival in several studies [28;29]. These findings have resulted in the addition of antibody-mediated rejection to the Banff '97 classification of renal allograft rejection [30]. Numerous treatment modalities including intravenous immunoglobulins, plasmapheresis and anti-CD 20 have been tried successfully in patients with humoral rejection. No randomized trials are available at this moment. Next to the obvious clinical implications of a timely diagnosis of humoral rejection the detection of C4d in as many as 30% of kidney transplant biopsies has triggered an increased interest in the role of complement in mediating renal damage in rejection. The presence of C4d in renal biopsies suggests complement activation by the classical pathway. However, the lectin pathway may also interact with immunoglobulins as has been shown for IgM and IgA [17;31]. With these findings in mind our group questioned whether MBL levels influence outcome

in kidney transplantation. Indeed, higher pre-transplant MBL levels were associated with poorer graft survival [32]. The superior graft survival in patients with low MBL levels was explained by a lower rate of treatment-resistant rejection. These findings suggest that MBL plays an unfavorable role in renal transplantation.

Rejection and damage to renal allografts may not only be influenced by circulating complement, but also by complement produced locally in the kidney. Pratt et. al studied the role of locally produced C3 in a mouse kidney transplantation model [33]. Whereas graft survival was not influenced when kidney donors were C3 deficient, survival was markedly improved if the transplanted organ was obtained from C3-deficient mice. Possibly locally produced C3 functions as a costimulator in the interaction between antigen presenting cells (APCs) and T-cells. This concept is supported by the recent report, that APCs lacking the complement inhibitor DAF (decay-accelerating factor) led to enhanced T-cell responses when compared with wild type APCs [34].

Atypical Hemolytic Uremic Syndrome

Recent data suggest an important role for complement in atypical hemolytic uremic syndrome (HUS). Mutations in the complement regulatory protein factor H have been described in patients with sporadic and familial HUS in several studies [35-37]. The described mutations interfere with the capacity of factor H to control alternative pathway activation on cellular surfaces. Determination of factor H serum levels is not sufficient to detect factor H mutations since mutant, dysfunctional factor H may circulate at normal concentrations [38]. A functional assay for factor H mutations has been described, which may facilitate screening for factor H mutations in patients with HUS [39]. More recently mutations of the complement regulators factor I and MCP have been proposed as predisposing factors in patients with atypical HUS [40-42]. Screening for these mutations may provide important information for risk assessment since these patients have a high incidence of disease recurrence after renal transplantation.

Progression of chronic renal disease

As complement molecules are detectable in urine from patients with non-selective proteinuria it has been suggested, that these components contribute to the tubulointerstitial damage in proteinuric renal disease [43]. Urinary C5b-9 excretion has been described in both animal models of membranous nephropathy and humans with this disease [44;45]. Interestingly high levels of C5b-9 excretion have also been

detected in patients with diabetic nephropathy whereas low levels were detected in the relatively benign condition of minimal change disease [46].

Use of the C6-deficient PVG rat in various models of proteinuria-associated interstitial damage has provided strong evidence for a harmful role of complement in the progression of renal disease. Complement-sufficient animals developed more severe tubulointerstitial damage than C6-deficient rats [47] in the puromycin model of proteinuric renal damage. A similar protective role of C6-deficiency was demonstrated in the remnant kidney model. Once complement has entered the tubuli in the setting of unselective proteinuria it may be activated on the tubular brush border by the high local ammonia concentrations [48].

Diabetic Nephropathy

As mentioned above high concentrations of C5b-9 are also found in the urine obtained from patients with diabetic nephropathy [46]. MAC deposition has been described in kidneys [49], nerves [50] and retinas [51] from patients with diabetes mellitus. Inactivation of the complement regulatory protein CD 59 by glycation has been suggested as a possible mechanism underlying complement activation in diabetes [52]. A role for lectin pathway mediated damage in diabetic nephropathy is suggested by the association between high levels of MBL and microalbuminuria in diabetic subjects ([53;54].

Taken together these studies strongly suggest a role for complement in the amplification of vascular and tissue injury in diabetes.

Measurement of complement pathway activity: methods and indications

Circulating complement can be measured by both functional assays and the measurement of antigen concentrations [55]. Functional assays of the complement pathway include the CH50 to assess the classical pathway and the AP50 to assess the alternative pathway. The CH50 determines the capacity of patient serum to lyse sheep erythrocytes coated with rabbit antibodies. It is a useful initial screening tool for the classical pathway, since an intact functional capacity of all 9 components of the classical pathway is required for a normal result. The AP50 measures lysis of unsensitized rabbit erythrocytes. Recently a simple and standardized ELISA based assay of all three pathways of complement activation including the lectin pathway

has been developed [56] and shown to be valuable for the detection of primary and secondary complement deficiencies.

C4 and C3 levels are usually measured by radial immunodiffusion or nephelometry using poyclonal antibodies. Decreased levels of circulating C3 and C4 can be detected in several renal diseases and may help to narrow the differential diagnosis. Renal immune complex diseases associated with hypocomplementemia include SLE, MPGN (all three types), cryoglobulinemia, post-streptococcal glomerulonephritis and glomerulonephritis associated with chronic infection (e.g. endocarditis or abdominal abcesses). In post-streptococcal glomerulonephritis and MPGN type II C3 is usually decreased more than C4 while a proportionate reduction in both C3 and C4 is generally detected in the classical pathway mediated complement consumption of SLE and cryoglobulinemia.

We recommend the determination of both the classical pathway activity and alternative pathway activity next to C3 and C4 levels for the initial screening of patients with a suspected complement deficiency. The combination of these determinations will help to identify the nature of complement consumption and to detect rare inherited complement deficiencies (e.g. C1q or C4 deficiency in SLE). In the setting of an unexplained propensity for infections or an increased risk for infections [57], measurement of the lectin pathway may be appropriate.

In situations of increased complement catabolism in which complement depletion is not detected due to the replenishment by increased synthesis, complement turnover may de detected by the measurement of complement activation products such as C3a, C3d or C5a. Determination of factor H and I levels and function can be useful in atypical HUS.

To monitor patients with SLE serial determination of either C3 or C4 levels is sufficient. It is not clear whether one of both determinations is preferable above the other. Following either C3 or C4 may be helpful to monitor the response to treatment and to detect changes in activity. The interpretation of complement levels should always be done with consideration of the clinical context [58]. The addition of an anti-C1q antibody assay may help to predict the presence of nephritis in patients with SLE [13;59;60].

Inhibitors of complement activation in the treatment of renal disease

Following the increasing knowledge about the role of complement in the pathophysiology of various diseases, numerous options for therapeutic manipulation of the complement system have been proposed [61]. Therapeutic complement inhibition may be approached at various levels of the complement cascade. Inhibition at the initiation level may allow specific regulation of one of the three pathways without interfering with the protective function of the other pathways. An intervention at the level of C3 inhibits the entire complement system with the possibility of high efficacy but the drawback of an increased risk of infections. Inhibition at the level of C5b-9 would prevent MAC mediated tissue damage without preventing complement-mediated clearance of immune complexes and apoptotic cells. Additionally the anaphylatoxins C3a and C5a could be inhibited directly.

Many of these possible approaches have been tested in animal models of renal disease. The rodent C3 convertase inhibitor Crry has similarity with the human complement receptor 1 (CR-1). Both the overexpression of Crry and the application of recombinant Crry confer protection in a mouse model of anti-GBM glomerulonephritis [62;63]. Administration of soluble Crry to MLR/lpr mice resulted in a marked reduction in renal damage in this model of SLE [11]. A soluble form of human CR1 (sCR1) was protective in glomerular disease in rats [64]. Treatment with a membrane-binding complement regulator based on CR1 resulted in amelioration of ischemia/reperfusion damage and rejection in a rat model of kidney transplantation [65]. A pharmaceutical preparation of sCR1 (TP-10; Avant Immunotherapeutics Inc., Needham, MA) has been developed but has not been tested in human renal disease.

Anti-C5 antibodies have been demonstrated to ameliorate lupus like disease in mice [10] and pharmaceutical C5-inhibitors have been developed for use in humans. Results from ongoing trials with the fully humanized C5-inhibitor Eculizumab (Alexion Pharaceuticals, Cheshire, CT) in patients with membranous glomerulonephritis are being awaited. The efficacy of this antibody has been documented in paroxysmal nocturnal hemoglobinuria [66;67].

Concluding Remarks

The complement system contributes to renal damage in many of the disease entities encountered by the nephrologist. Sound understanding of the complement system will aid the nephrologist in understanding the pathophysiology of renal disease and provide support in making the correct diagnosis. Monitoring complement may offer guidance in therapeutic decisions if interpreted with prudence in the clinical context. Whether therapeutic interventions in the complement system will result in meaningful improvements for our patients remains to be established. A skeptical position is justified in view of the large discrepancy between the huge volume of laboratory results and the meager progress in terms of clinical implication.

Reference List

1. Nauta AJ, Daha MR, van Kooten C, Roos A. Recognition and clearance of apoptotic cells: a role for complement and pentraxins. *Trends Immunol* 2003; 24(3):148-154.
2. Turner MW. The role of mannose-binding lectin in health and disease. *Mol Immunol* 2003; 40(7):423-429.
3. Adler S, Baker PJ, Johnson RJ, Ochi RF, Pritzl P, Couser WG. Complement membrane attack complex stimulates production of reactive oxygen metabolites by cultured rat mesangial cells. *J Clin Invest* 1986; 77(3):762-767.
4. Nauta AJ, Trouw LA, Daha MR, Tijsma O, Nieuwland R, Schwaeble WJ et al. Direct binding of C1q to apoptotic cells and cell blebs induces complement activation. *Eur J Immunol* 2002; 32(6):1726-1736.
5. Nauta AJ, Raaschou-Jensen N, Roos A, Daha MR, Madsen HO, Borrias-Essers MC et al. Mannose-binding lectin engagement with late apoptotic and necrotic cells. *Eur J Immunol* 2003; 33(10):2853-2863.
6. Nauta AJ, Castellano G, Xu W, Woltman AM, Borrias MC, Daha MR et al. Opsonization with C1q and mannose-binding lectin targets apoptotic cells to dendritic cells. *J Immunol* 2004; 173(5):3044-3050.
7. Botto M, Dell'Agnola C, Bygrave AE, Thompson EM, Cook HT, Petry F et al. Homozygous C1q deficiency causes glomerulonephritis associated with multiple apoptotic bodies. *Nat Genet* 1998; 19(1):56-59.
8. Paul E, Pozdnyakova OO, Mitchell E, Carroll MC. Anti-DNA autoreactivity in C4-deficient mice. *Eur J Immunol* 2002; 32(9):2672-2679.
9. Mitchell DA, Pickering MC, Warren J, Fossati-Jimack L, Cortes-Hernandez J, Cook HT et al. C1q deficiency and autoimmunity: the effects of genetic background on disease expression. *J Immunol* 2002; 168(5):2538-2543.
10. Wang Y, Hu Q, Madri JA, Rollins SA, Chodera A, Matis LA. Amelioration of lupus-like autoimmune disease in NZB/WF1 mice after treatment with a blocking monoclonal antibody specific for complement component C5. *Proc Natl Acad Sci U S A* 1996; 93(16):8563-8568.
11. Bao L, Haas M, Boackle SA, Kraus DM, Cunningham PN, Park P et al. Transgenic expression of a soluble complement inhibitor protects against renal disease and promotes survival in MRL/lpr mice. *J Immunol* 2002; 168(7):3601-3607.
12. Seelen MA, Trouw LA, Daha MR. Diagnostic and prognostic significance of anti-C1q antibodies in systemic lupus erythematosus. *Curr Opin Nephrol Hyperten*s 2003; 12(6):619-624.
13. Moroni G, Trendelenburg M, Del Papa N, Quaglini S, Raschi E, Panzeri P et al. Anti-C1q antibodies may help in diagnosing a renal flare in lupus nephritis. *Am J Kidney Dis* 2001; 37(3):490-498.
14. Trouw LA, Groeneveld TW, Seelen MA, Duijs JM, Bajema IM, Prins FA et al. Anti-C1q autoantibodies deposit in glomeruli but are only pathogenic in combination with glomerular C1q-containing immune complexes. *J Clin Invest* 2004; 114(5):679-688.
15. Miyazaki R, Kuroda M, Akiyama T, Otani I, Tofuku Y, Takeda R. Glomerular deposition and serum levels of complement control proteins in patients with IgA nephropathy. *Clin Nephrol* 1984; 21(6):335-340.

16. Endo M, Ohi H, Ohsawa I, Fujita T, Matsushita M, Fujita T. Glomerular deposition of mannose-binding lectin (MBL) indicates a novel mechanism of complement activation in IgA nephropathy. *Nephrol Dial Transplant* 1998; 13(8):1984-1990.
17. Roos A, Bouwman LH, Gijlswijk-Janssen DJ, Faber-Krol MC, Stahl GL, Daha MR. Human IgA activates the complement system via the mannan-binding lectin pathway. *J Immunol* 2001; 167(5):2861-2868.
18. Zhou W, Farrar CA, Abe K, Pratt JR, Marsh JE, Wang Y et al. Predominant role for C5b-9 in renal ischemia/reperfusion injury. *J Clin Invest* 2000; 105(10):1363-1371.
19. Thurman JM, Ljubanovic D, Edelstein CL, Gilkeson GS, Holers VM. Lack of a functional alternative complement pathway ameliorates ischemic acute renal failure in mice. *J Immunol* 2003; 170(3):1517-1523.
20. Thurman JM, Lucia MS, Ljubanovic D, Holers VM. Acute tubular necrosis is characterized by activation of the alternative pathway of complement. *Kidney Int* 2005; 67(2):524-530.
21. de Vries B, Matthijsen RA, Wolfs TG, van Bijnen AA, Heeringa P, Buurman WA. Inhibition of complement factor C5 protects against renal ischemia-reperfusion injury: inhibition of late apoptosis and inflammation. *Transplantation* 2003; 75(3):375-382.
22. Walsh MC, Bourcier T, Takahashi K, Shi L, Busche MN, Rother RP et al. Mannose-Binding Lectin Is a Regulator of Inflammation That Accompanies Myocardial Ischemia and Reperfusion Injury. *J Immunol* 2005; 175(1):541-546.
23. Hart ML, Ceonzo KA, Shaffer LA, Takahashi K, Rother RP, Reenstra WR et al. Gastrointestinal ischemia-reperfusion injury is lectin complement pathway dependent without involving C1q. *J Immunol* 2005; 174(10):6373-6380.
24. de Vries B, Walter SJ, Peutz-Kootstra CJ, Wolfs TG, van Heurn LW, Buurman WA. The mannose-binding lectin-pathway is involved in complement activation in the course of renal ischemia-reperfusion injury. *Am J Pathol* 2004; 165(5):1677-1688.
25. Moller-Kristensen M, Wang W, Ruseva M, Thiel S, Nielsen S, Takahashi K et al. Mannan-binding lectin recognizes structures on ischaemic reperfused mouse kidneys and is implicated in tissue injury. *Scand J Immunol* 2005; 61(5):426-434.
26. Feucht HE, Schneeberger H, Hillebrand G, Burkhardt K, Weiss M, Riethmuller G et al. Capillary deposition of C4d complement fragment and early renal graft loss. *Kidney Int* 1993; 43(6):1333-1338.
27. Collins AB, Schneeberger EE, Pascual MA, Saidman SL, Williams WW, Tolkoff-Rubin N et al. Complement activation in acute humoral renal allograft rejection: diagnostic significance of C4d deposits in peritubular capillaries. *J Am Soc Nephrol* 1999; 10(10):2208-2214.
28. Bohmig GA, Exner M, Habicht A, Schillinger M, Lang U, Kletzmayr J et al. Capillary C4d deposition in kidney allografts: a specific marker of alloantibody-dependent graft injury. *J Am Soc Nephrol* 2002; 13(4):1091-1099.
29. Herzenberg AM, Gill JS, Djurdjev O, Magil AB. C4d deposition in acute rejection: an independent long-term prognostic factor. *J Am Soc Nephrol* 2002; 13(1):234-241.
30. Racusen LC, Colvin RB, Solez K, Mihatsch MJ, Halloran PF, Campbell PM et al. Antibody-mediated rejection criteria - an addition to the Banff 97 classification of renal allograft rejection. *Am J Transplant* 2003; 3(6):708-714.
31. Arnold JN, Wormald MR, Suter DM, Radcliffe CM, Harvey DJ, Dwek RA et al. Human serum IgM glycosylation: identification of glycoforms that can bind to mannan-binding lectin. *J Biol Chem* 2005; 280(32):29080-29087.

32. Berger SP, Roos A, Mallat MJ, Fujita T, de Fijter JW, Daha MR. Association between mannose-binding lectin levels and graft survival in kidney transplantation. *Am J Transplant* 2005; 5(6):1361-1366.
33. Pratt JR, Basheer SA, Sacks SH. Local synthesis of complement component C3 regulates acute renal transplant rejection. *Nat Med* 2002; 8(6):582-587.
34. Heeger PS, Lalli PN, Lin F, Valujskikh A, Liu J, Muqim N et al. Decay-accelerating factor modulates induction of T cell immunity. *J Exp Med* 2005; 201(10):1523-1530.
35. Caprioli J, Bettinaglio P, Zipfel PF, Amadei B, Daina E, Gamba S et al. The molecular basis of familial hemolytic uremic syndrome: mutation analysis of factor H gene reveals a hot spot in short consensus repeat 20. *J Am Soc Nephrol* 2001; 12(2):297-307.
36. Perez-Caballero D, Gonzalez-Rubio C, Gallardo ME, Vera M, Lopez-Trascasa M, Rodriguez dC et al. Clustering of missense mutations in the C-terminal region of factor H in atypical hemolytic uremic syndrome. *Am J Hum Genet* 2001; 68(2):478-484.
37. Warwicker P, Goodship TH, Donne RL, Pirson Y, Nicholls A, Ward RM et al. Genetic studies into inherited and sporadic hemolytic uremic syndrome. *Kidney Int* 1998; 53(4):836-844.
38. Neumann HP, Salzmann M, Bohnert-Iwan B, Mannuelian T, Skerka C, Lenk D et al. Haemolytic uraemic syndrome and mutations of the factor H gene: a registry-based study of German speaking countries. *J Med Genet* 2003; 40(9):676-681.
39. Sanchez-Corral P, Gonzalez-Rubio C, Rodriguez dC, Lopez-Trascasa M. Functional analysis in serum from atypical Hemolytic Uremic Syndrome patients reveals impaired protection of host cells associated with mutations in factor H. *Mol Immunol* 2004; 41(1):81-84.
40. Fremeaux-Bacchi V, Dragon-Durey MA, Blouin J, Vigneau C, Kuypers D, Boudailliez B et al. Complement factor I: a susceptibility gene for atypical haemolytic uraemic syndrome. *J Med Genet* 2004; 41(6):e84.
41. Noris M, Brioschi S, Caprioli J, Todeschini M, Bresin E, Porrati F et al. Familial haemolytic uraemic syndrome and an MCP mutation. *Lancet* 2003; 362(9395):1542-1547.
42. Kavanagh D, Kemp EJ, Mayland E, Winney RJ, Duffield JS, Warwick G et al. Mutations in complement factor I predispose to development of atypical hemolytic uremic syndrome. *J Am Soc Nephrol* 2005; 16(7):2150-2155.
43. Hsu SI, Couser WG. Chronic progression of tubulointerstitial damage in proteinuric renal disease is mediated by complement activation: a therapeutic role for complement inhibitors? *J Am Soc Nephrol* 2003; 14(7 Suppl 2):S186-S191.
44. Schulze M, Baker PJ, Perkinson DT, Johnson RJ, Ochi RF, Stahl RA et al. Increased urinary excretion of C5b-9 distinguishes passive Heymann nephritis in the rat. *Kidney Int* 1989; 35(1):60-68.
45. Schulze M, Donadio JV, Jr., Pruchno CJ, Baker PJ, Johnson RJ, Stahl RA et al. Elevated urinary excretion of the C5b-9 complex in membranous nephropathy. *Kidney Int* 1991; 40(3):533-538.
46. Morita Y, Ikeguchi H, Nakamura J, Hotta N, Yuzawa Y, Matsuo S. Complement activation products in the urine from proteinuric patients. *J Am Soc Nephrol* 2000; 11(4):700-707.
47. Nangaku M, Pippin J, Couser WG. Complement membrane attack complex (C5b-9) mediates interstitial disease in experimental nephrotic syndrome. *J Am Soc Nephrol* 1999; 10(11):2323-2331.
48. Nath KA, Hostetter MK, Hostetter TH. Pathophysiology of chronic tubulo-interstitial disease in rats. Interactions of dietary acid load, ammonia, and complement component C3. *J Clin Invest* 1985; 76(2):667-675.

49. Falk RJ, Sisson SP, Dalmasso AP, Kim Y, Michael AF, Vernier RL. Ultrastructural localization of the membrane attack complex of complement in human renal tissues. *Am J Kidney Dis* 1987; 9(2):121-128.
50. Rosoklija GB, Dwork AJ, Younger DS, Karlikaya G, Latov N, Hays AP. Local activation of the complement system in endoneurial microvessels of diabetic neuropathy. *Acta Neuropathol* (Berl) 2000; 99(1):55-62.
51. Zhang J, Gerhardinger C, Lorenzi M. Early complement activation and decreased levels of glycosylphosphatidylinositol-anchored complement inhibitors in human and experimental diabetic retinopathy. *Diabetes* 2002; 51(12):3499-3504.
52. Qin X, Goldfine A, Krumrei N, Grubissich L, Acosta J, Chorev M et al. Glycation inactivation of the complement regulatory protein CD59: a possible role in the pathogenesis of the vascular complications of human diabetes. *Diabetes* 2004; 53(10):2653-2661.
53. Hansen TK, Tarnow L, Thiel S, Steffensen R, Stehouwer CD, Schalkwijk CG et al. Association between mannose-binding lectin and vascular complications in type 1 diabetes. *Diabetes* 2004; 53(6):1570-1576.
54. Hovind P, Hansen TK, Tarnow L, Thiel S, Steffensen R, Flyvbjerg A et al. Mannose-binding lectin as a predictor of microalbuminuria in type 1 diabetes: an inception cohort study. *Diabetes* 2005; 54(5):1523-1527.
55. Hebert LA, Cosio FG, Neff JC. Diagnostic significance of hypocomplementemia. *Kidney Int* 1991; 39(5):811-821.
56. Seelen MA, Roos A, Wieslander J, Mollnes TE, Sjoholm AG, Wurzner R et al. Functional analysis of the classical, alternative, and MBL pathways of the complement system: standardization and validation of a simple ELISA. *J Immunol Methods* 2005; 296(1-2):187-198.
57. Bouwman LH, Roos A, Terpstra OT, de Knijff P, van Hoek B, Verspaget HW et al. Mannose binding lectin gene polymorphisms confer a major risk for severe infections after liver transplantation. *Gastroenterology* 2005; 129(2):408-414.
58. Walport MJ. Complement and systemic lupus erythematosus. *Arthritis Res* 2002; 4 Suppl 3:S279-S293.
59. Trendelenburg M, Marfurt J, Gerber I, Tyndall A, Schifferli JA. Lack of occurrence of severe lupus nephritis among anti-C1q autoantibody-negative patients. *Arthritis Rheum* 1999; 42(1):187-188.
60. Siegert CE, Daha MR, Tseng CM, Coremans IE, van Es LA, Breedveld FC. Predictive value of IgG autoantibodies against C1q for nephritis in systemic lupus erythematosus. *Ann Rheum Dis* 1993; 52(12):851-856.
61. Quigg RJ. Use of complement inhibitors in tissue injury. *Trends Mol Med* 2002; 8(9):430-436.
62. Quigg RJ, He C, Lim A, Berthiaume D, Alexander JJ, Kraus D et al. Transgenic mice overexpressing the complement inhibitor crry as a soluble protein are protected from antibody-induced glomerular injury. *J Exp Med* 1998; 188(7):1321-1331.
63. Quigg RJ, Kozono Y, Berthiaume D, Lim A, Salant DJ, Weinfeld A et al. Blockade of antibody-induced glomerulonephritis with Crry-Ig, a soluble murine complement inhibitor. *J Immunol* 1998; 160(9):4553-4560.
64. Couser WG, Johnson RJ, Young BA, Yeh CG, Toth CA, Rudolph AR. The effects of soluble recombinant complement receptor 1 on complement-mediated experimental glomerulonephritis. *J Am Soc Nephrol* 1995; 5(11):1888-1894.

65. Pratt JR, Jones ME, Dong J, Zhou W, Chowdhury P, Smith RA et al. Nontransgenic hyperexpression of a complement regulator in donor kidney modulates transplant ischemia/reperfusion damage, acute rejection, and chronic nephropathy. *Am J Pathol* 2003; 163(4):1457-1465.
66. Hill A, Hillmen P, Richards SJ, Elebute D, Marsh JC, Chan J et al. Sustained response and long-term safety of eculizumab in paroxysmal nocturnal hemoglobinuria. *Blood* 2005.
67. Hillmen P, Hall C, Marsh JC, Elebute M, Bombara MP, Petro BE et al. Effect of eculizumab on hemolysis and transfusion requirements in patients with paroxysmal nocturnal hemoglobinuria. *N Engl J Med* 2004; 350(6):552-559.

Chapter 3

Association between Mannose-binding lectin levels and graft survival in kidney transplantation

Stefan P. Berger[1],
Anja Roos[1],
Marko J.K. Mallat[1],
Johan W. de Fijter[1],
Teizo Fujita[2],
Mohamed R. Daha[1]

[1]Department of Nephrology, Leiden University Medical Center, Leiden, The Netherlands
[2]Department of Biochemistry, Fukushima Medical University, Fukushima, Japan

American Journal of Transplantation 2005; 5:1361-1366

Summary

The mannose-binding lectin (MBL) pathway of complement is activated by pattern recognition. Genetic MBL variants are frequent and are associated with low MBL serum levels. We hypothesized that higher MBL levels may be associated with more complement-mediated damage resulting in inferior graft survival.

Pretransplant serum samples collected from 266 consecutive deceased donor kidney transplant recipients were analyzed for MBL concentration by ELISA. Subsequently the cohort was analyzed for transplant-related outcome.

There was no significant difference in the incidence of delayed graft function in recipients with a low MBL level (≤ 400 ng/ml) compared to those with a higher MBL level (> 400 ng/ml) (37.1 vs. 34.9%). At 10-years, the death censored graft survival was 89.9% in patients with an MBL level below 400 ng/ml compared with 78.8% in patients with a higher MBL level ($P < 0.02$). Multivariate analysis including traditional risk factors for graft loss showed an independent risk of 2.7 (95% CI 1.2-6.3) for death censored graft loss if pretransplant MBL levels were above 400 ng/ml. This difference was almost entirely explained by rejection-associated graft loss (2.4 vs. 12,4%, $P < 0,01$).

In our cohort higher MBL levels seem to be associated with a more severe form of rejection leading to treatment failure and graft loss. If these data can be confirmed pretransplant MBL levels may provide additional information for risk stratification prior to kidney transplantation.

Introduction

Recently the interest in the role of the innate immune system in organ transplantation has increased. Within the innate immune system complement is thought to be one of the major inflammatory mediators particularly in the setting of ischemia/reperfusion injury [1;2]. In a mouse model of acute kidney rejection, disruption of the gene encoding for the complement component C3 in the transplanted kidney led to marked improvement of organ survival. In human transplantation a role for complement activation has been established by showing the presence of the complement split product C4d as a marker of acute humoral rejection [3;4] and its association with chronic transplant glomerulopathy [5].

Next to activation of the complement system via the classical or alternative pathway, the lectin complement pathway may play a role in renal transplantation. The collectin mannose-binding lectin (MBL) binds via its carbohydrate recognition domain to saccharides such as D-mannose, L-fucose and N-acetylglucosamine [6] on various microorganisms. This interaction leads to the activation of the MBL-associated serine proteases (MASP) and cleavage of C4 and C2 followed by formation of the C3 convertase C4b2a. In addition to activating the lectin complement pathway, MBL can mediate phagocytosis of opsonized organisms.

The serum MBL concentration shows a large inter-individual variation due to common mutations in the structural as well as the regulatory part of the MBL gene. Several studies have related MBL deficiency with an increased rate of infection in early childhood [7] and other conditions characterized by disturbed host defense [8;9]. Experimental data have shown that the lectin complement pathway contributes to activation of the complement cascade in the context of ischemia/reperfusion damage. Endothelial cells exposed to oxidative stress activate the lectin complement pathway *in vitro* [10]. A recent publication indicates that MBL also binds to both late apoptotic and necrotic cells [11]. *In vivo* studies show that inhibition of MBL with monoclonal antibodies leads to reduction of damage in a rat model of cardiac ischemia/reperfusion injury [12].

We hypothesize that MBL binding to injured tissue may lead to additional inflammation, thereby aggravating tissue damage and potentiating antigen presentation. Based on this hypothesis and the recent findings concerning the role of complement in ischemia/reperfusion damage and rejection we questioned whether higher recipient MBL levels might be associated with inferior outcome in the setting of kidney transplantation.

Methods

Study population

Pretransplant sera of 266 consecutive deceased donor kidney transplant recipients routinely collected at our institution from January 1990 to December 1994 were utilized for this study. Thirty-one recipients of the total number of kidney transplants performed during this period were excluded due to missing serum samples. Pretransplantation sera were routinely collected since 1989 and stored at –80°C. They had not been subjected to freeze/thaw cycles before analysis in our study. Sera from MBL-genotyped healthy controls (n = 70) [13] were used for comparison.

Data analysis was done using the Leiden Kidney Transplantation Database. This database contains donor variables (gender, age at time of death), recipient variables (age at time of transplantation, gender, panel reactive antibodies, CMV status), transplantation related factors (human leukocyte antigen-A [HLA-A], -B, and -DR mismatches; cold ischemia time; warm ischemia time), and post-transplantation features including immunosuppressive regimen, delayed graft function, rejection history, rejection treatment, dipstick proteinuria and serum creatinine values. Graft histology was evaluated retrospectively according to the Banff '97 classification [14]. After transplantation patients were followed until death, reinitiation of dialysis or December 2002. Delayed graft function was defined as the need for dialysis for more than 7 days post transplantation. Rejection-associated graft loss was defined as histologically proven acute rejection with ongoing functional deterioration despite antithymocyte treatment or chronic rejection leading to the reinitiation of dialysis treatment. Acute rejection episodes were treated according to a standard protocol consisting of methylprednisolone 1 g intravenously for three consecutive days; a 10d course of antithymocyte globulin at a dose 5mg/kg guided by absolute lymphocyte counts; or again methylprednisolone for the first, second (or steroid-resistant), or third rejection episode, respectively. An MBL concentration of 400 ng/ml was chosen as a cut-off to define individuals with normal and low MBL levels respectively. The higher and lower MBL groups were analyzed for differences in known predictors of transplantation outcome, such as the incidence of delayed graft function or acute rejection. Patients who lost their grafts within 3 months after transplantation were excluded from analysis for patient and graft survival.

ELISA

Serum MBL levels were assessed by sandwich ELISA as described previously [15]. In short 96-well ELISA plates (Greiner, Germany) were coated with 3E7 (mouse IgG1 anti-MBL at 2.5 µg/ml). After blocking residual binding sites with PBS containing 1% BSA and washing, serum samples were diluted 1/50 and 1/250 and incubated. Dig-conjugated 3E7 was added as detecting antibody. After washing detection of binding of Dig-conjugated antibodies was performed using HRP-conjugated rabbit anti-Dig Abs (Fab, from Boehringer Mannheim). Enzyme activity was detected using 2,2'-azino-bis(3-ethybenzthiazoline-6-sufonic acid)(Sigma). The optical density (OD at 415nm) was measured using a microplate biokinetics reader (EL312e; Biotek Instruments, Winooski, VT). A calibration line was produced using human serum from a healthy donor with a known concentration of MBL.

Statistical analysis

Categorical characteristics among MBL-groups were compared using cross-tables with calculation of the exact p-values. Continuous variables were analyzed using the Student t-test, when test assumptions were met, and otherwise with the Mann-Whitney test. Patient and graft survival was estimated using the Kaplan-Meier product-limit method and the curves were compared with the Log-Rank test. For analysis of differences in survival among MBL-groups, at individual time points, z-scores were calculated and p-values estimated using the standard normal distribution (Z-test).

To identify risk factors for graft loss and to adjust for potential confounding factors Cox Proprotional Hazards Regression was used.

P-values < 0.05 were considered to be statistically significant. All analyses were performed with SPSS Statistical Software Package (Version 10.07; SPSS, Inc., Chicago, Ill.).

Results

Follow up data were available for all transplanted patients. The mean MBL concentration of the 266 available sera was 1112 ng/ml (median 691 ng/ml; IQR 270-1697). These results were very similar to the levels we measured in healthy donors with a mean MBL level of 1054 ng/ml (median 679 ng/ml) [13]. The distributions of both the transplant recipients and the healthy donors are shown in figure 1. For analysis the patients were divided into groups with MBL levels below 400 ng/ml and

above 400 ng/ml. Using this cut-off 97 kidney recipients (36.5%) had a low MBL-level, which is comparable to our group of healthy donors (35.7% below 400 ng/ml) and to the frequency of variant alleles as defined in other populations [16]. In our genotyped control population 75% of those with an MBL level below 400 ng/ml have a variant MBL genotype (A/O or O/O) whereas 89% of those with an MBL level above 400 ng/ml have the wildtype MBL genotype (A/A) [13], showing a close association between MBL variant alleles and MBL levels below 400 ng/ml (P = 0.0001).

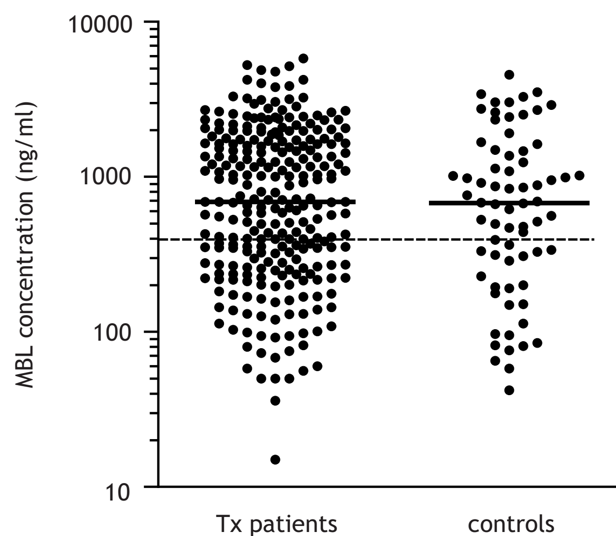


Figure 1. MBL concentration in pre-transplant sera and healthy controls. Horizontal solid lines indicate the median. The dashed line indicates the cutoff level used in the present study (400 ng/ml).

Between the two MBL groups there were no significant differences in recipient or donor age, years on dialysis, cold ischemia time, CMV serotype or sex distribution (Table 1). There was no difference in the distribution of the dialysis modality prior to transplantation.

The normal and low MBL groups were also compared for transplantation outcome. No significant difference in the incidence of delayed graft function (37.1% vs. 34.9%) or the incidence of first acute rejection episodes was found between the groups, illustrated in figure 2. Equally there was no difference if vascular and interstitial rejection or severity of rejection were analyzed separately (data not shown).

Table 1. Characteristics of study population according to MBL levels [a]

	Acceptor MBL-level (ng/ml)		
	MBL < 400	MBL ≥400	P-value
n	82	153	
Recipient age (yrs)	45	46.51	0.40
Donor age (yrs)	40.05	37.37	0.20
Hemodialysis (%)	48.1	48.3	0.98
Years on dialysis	4.78	3.8	0.09
CIT (h)	28.28	29.44	0.54
CMV sero-positive	59.3	48.4	0.11
Female (%)	41.5	32	0.10

[a] MBL, mannose-binding lectin; CIT, cold ischemia time; CMV, cytomegalovirus

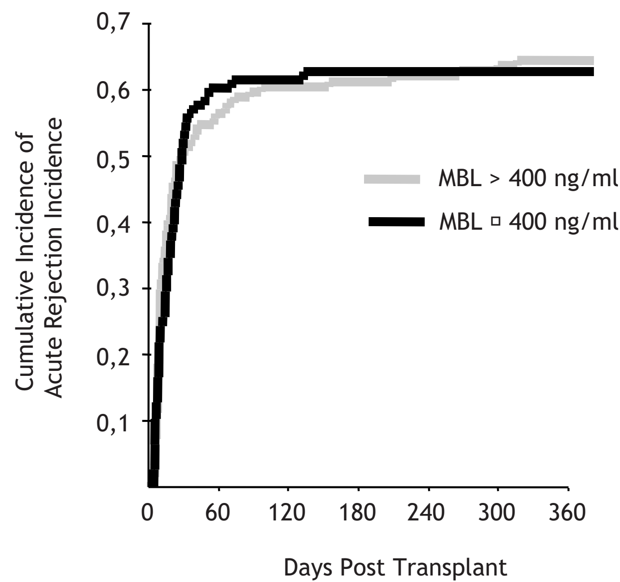


Figure 2. Cumulative incidence of first acute rejection according to MBL level.

For survival analysis all grafts that functioned for less than 3 months were excluded. This was done to exclude graft loss due to technical complications. At 3 months mean creatinine levels were the same in both the higher and lower MBL groups (168.8 μmol/l vs. 166.6 μmol/l, P = 0.82). There was a non-significant tendency towards

better patient survival in the group with a lower MBL level (P = 0.103) (figure 3). However overall graft survival was superior in acceptors with a low MBL level as shown in figure 4A (log-rank, P = 0.017). A similar difference was found when graft survival was censored for patient death with a functioning graft (log-rank, P = 0.028), (figure 3B). The 5-year death censored graft survival was 93.3% and 87.3% in the lower and the higher MBL group, respectively (P = 0.067). The 10-year death censored graft survival was 89.8% in the lower MBL group and 78.8% in the higher MBL group (P = 0.018).

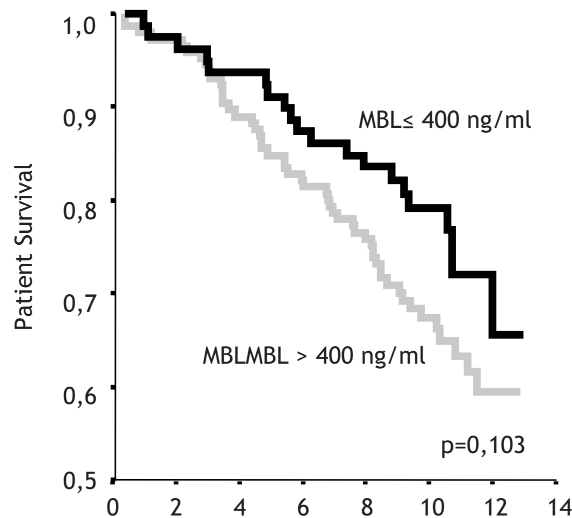


Figure 3. Patient survival according to MBL level.

As shown in table 2 the risk for death censored graft loss was significantly increased if the donor age was above 50 years (RR = 2.08; 95% CI, 1.1-4.04) or if acute rejection occurred (RR 4.18, 95% CI, 1.6-10.8). Neither HLA mismatches, a negative acceptor CMV serology nor cold ischemia time were significant risk factors for graft loss in our well-matched cohort. An MBL level above 400 ng/ml was associated with a 2.5 fold relative risk for death-censored graft loss (95% CI 1.1-1.57). When adjusted for other risk factors in a multivariate model, an MBL level above 400 ng/ml was shown to be an independent risk factor for graft loss. If the pre-transplantation MBL level was entered as a continuous parameter every 100 ng/ml concentration increase was associated with a RR of 1.03 (95% CI, 1.001-1.052). In the multivariate model the MBL level also proved to be an independent risk factor if analyzed as a continuous parameter (P = 0.009).

Table 2. Risk factors of death censored graft loss[a]

	Univariate		Multivariate	
	RR	95% CI	RR	95% CI
MBL> 400 ng/ml	2.50	1.1-5.7	2.76	1.2-6.32
Donor age >50 yr	2.08	1.1-4.04	2.23	1.14-4.3
Acute Rejection	4.18	1.6-10.8	4.26	1.64-11.00
CIT	1.03	0.98-1.08		
Acceptor CMV neg.	0.34	0.6-2.49		
HLA A-B ≥ 1mm	1.124	0.82-1.53		
HLA DR ≥1 mm	1.90	0.96-3.8		

[a] RR, relative risk; CI, confidence interval; CIT, cold ischemia time; HLA, human leukocyte antigen; mm, mismatch

To study the mechanism underlying MBL associated graft loss, we analyzed the reasons for graft loss in the low and high MBL groups (table 3). The excess graft loss in patients with a MBL above 400 ng/ml was almost entirely explained by an increased incidence of rejection associated graft loss (P = 0.01).

There was no difference in the need of a first or second treatment for acute rejection between both groups, whereas 22.9% of the kidney recipients with a MBL-level above 400 ng/ml did not adequately respond to antithymocyte treatment and thus recieved 3 or more courses of rejection treatment compared with 12.2% of the recipients with an MBL-level below 400 ng/ml (P = 0.03). (Data not shown)

Table 3. Reason for graft loss later than 3 months after transplantation according to MBL levels [a]

	Acceptor MBL-level (ng/ml)		
	MBL < 400	MBL ≥ 400	P-value
n	82	153	
No graft loss	58	84	0.02
Rejection associated graft loss	2	19	0.01
Recurrent disease	2	4	0.98
Death with functioning graft	17	40	0.42
Other	3	5	0.97

[a] MBL, mannose-binding lectin

Discussion

In the present study we analyzed the interaction between pre-transplantation MBL levels and outcome after deceased donor kidney transplantation. This is the first report showing that higher MBL levels are significantly associated with increased overall and death-censored graft loss. This difference was almost entirely explained by rejection-associated graft loss and coincided with a greater need for additional treatment for acute rejection after a course of antithymocyte globulin in the group with higher MBL-levels. The relative risk for graft loss with a pre-transplantation MBL levels above 400 ng/ml was comparable with the effect of receiving a graft from a donor over 50 years of age. Higher MBL levels were not associated with an increased incidence of delayed graft function or first acute rejection episodes.

The serum MBL concentrations in our study population were comparable with the levels found in other populations [13;17;18]. The inter-individual differences in MBL levels are largely explained by polymorphisms within the promoter region and in exon 1 of the *MBL2* gene. As a consequence the variation of MBL levels within individuals are small. Levels are only increased two to three-fold during acute phase responses in the critically ill and changes in MBL levels do not correlate with changes in C-reactive protein (CRP) during treatment on an intensive care unit [17]. In addition strong acute phase reactions in our transplant recipients are very unlikely since patients with evidence of acute infections or active inflammatory disease are not accepted for transplantation. With this in mind, it can be assumed that the MBL levels measured in pretransplantation samples reliably represent the mean MBL level in these individuals.

A single study has evaluated MBL levels in patients with advanced renal failure and hemodialysis treatment [19]. This study found significantly increased MBL levels in Japanese patients approaching end stage renal failure (4343 ng/ml) and on hemodialysis treatment (8879 ng/ml) as compared with normal individuals (1452 ng/ml). All except one patient in our cohort were undergoing renal replacement treatment prior to transplantation. The MBL levels in our patients were similar to those found in normal controls. The reason for this difference is unclear. Since MBL is too large in size to be cleared by glomerular filtration in the non-proteinuric kidney, renal insufficiency by itself would not explain accumulation of MBL.

Genotyping our kidney acceptors for MBL mutations would have allowed classifying patients independently of external factors influencing MBL levels and would have made the use of a somewhat arbitrary MBL cut-off level unnecessary. Unfortunately

DNA was not available for MBL-genotyping of our recipients. On the other hand, although largely genetically determined, MBL levels can vary considerably within one genotype and the actual phenotype probably is functionally more important than the genotype. A comparison with healthy MBL-genotyped donors revealed a close association between MBL variant alleles and MBL serum concentrations below 400 ng/ml. Furthermore recent data from our group indicate that serum MBL levels closely correlate with MBL pathway function [13].

MBL may influence the outcome of a kidney transplant by various mechanisms. The complement system is known to contribute to organ damage in the setting of ischemia/reperfusion [1;2;20]. Inhibition of the lectin complement pathway with an antibody directed against MBL reduces C3 deposition and organ damage in a rat model for myocardial ischemia/reperfusion injury [12]. A recent study has shown MBL to be co-deposited with C6 in both a murine model of ischemia/reperfusion injury and human transplant kidneys after reperfusion [21]. We did not find an association of the MBL levels with delayed graft function as a marker for ischemia/reperfusion damage. This does not exclude that MBL contributes to more subtle forms of ischemia/reperfusion damage than overt acute tubular damage.

Next to ischemia/reperfusion injury the complement system is also involved in the context of acute and chronic rejection. Inhibition of complement activation by administering a membrane-binding complement regulator based on complement receptor type 1 resulted in amelioration of ischemia/reperfusion damage in a rat model of kidney transplantation. This intervention also led to a reduction of acute and chronic rejection [22]. Transplantation of kidneys obtained from C3 knockout mice leads to marked improvement of graft survival when compared with kidneys obtained from wild type mice [23]. In our study higher MBL levels seem to be associated with more severe and possibly treatment-resistant forms of rejection Damage resulting from acute rejection may be enhanced in the presence of high circulating levels of MBL by interaction of MBL with damaged tissue. MBL can bind to necrotic and late apoptotic cells, resulting in enhanced phagocytosis of these cells by macrophages and dendritic cells [11;24;25]. Phagocytosis of necrotic cells may induce dendritic cell maturation and macrophage activation [26-28]. It is conceivable that high MBL levels may increase immune reactivity and cell damage via binding to damaged tissue and enhancing activation of antigen presenting cells. The observation that higher MBL levels are associated with more treatment-resistant forms of rejection necessitating additional courses of rejection treatment after application of antithymocyte globulin may point to a higher prevalence of humoral rejection in high MBL individuals.

Initially the interest in the lectin complement pathway was directed towards beneficial effects in the setting of infectious diseases. The high frequency of polymorphisms in the MBL gene however points towards a possible advantageous effect of low MBL levels [29]. This concept is strengthened by recent data. In a mouse model of acute septic peritonitis MBL-A deficient mice had enhanced survival [30]. MBL mutations associated with low MBL serum levels may protect against the development of ulcerative colitis in humans [31]. In type 1 diabetics high MBL genotypes have been associated with an increased frequency of diabetic nephropathy [32]. In the same population the presence of cardiovascular disease was associated with higher MBL levels. The lectin complement pathway has also been linked to renal damage in IgA nephropathy and Henoch-Schonlein purpura [15,33,34].

In conclusion our study suggests that higher MBL levels are associated with poorer graft survival due to rejection-associated graft loss in deceased donor kidney transplantation. Obviously this observation will need confirmation in other transplantation cohorts. At this point of time we can only speculate about the mechanisms responsible for this deleterious effect of MBL. Further studies into the role of MBL in ischemia/reperfusion injury of the kidney and the interaction of the complement system with cellular immune mechanisms may help to understand this interesting finding. In addition to the potential pathophysiological implications of these findings our data suggest that determining MBL levels prior to transplantation may serve as a prognostic marker in kidney transplantation.

Acknowledgements

Funding sources: This study was supported in part by the Dutch Kidney Foundation, (grant number: CO3-6014) and the European Union (grant numbers: EU-QLG1-CT 2001-10139 and EU-QLG1-CT 2002-01215)

Reference List

1. Zhou W, Farrar CA, Abe K, Pratt JR, Marsh JE, Wang Y et al. Predominant role for C5b-9 in renal ischemia/reperfusion injury. *J Clin Invest* 2000; 105(10):1363-1371.
2. Bonventre JV. Complement and renal ischemia-reperfusion injury. *Am J Kidney Dis* 2001; 38(2):430-436.
3. Bohmig GA, Exner M, Habicht A, Schillinger M, Lang U, Kletzmayr J et al. Capillary C4d deposition in kidney allografts: a specific marker of alloantibody-dependent graft injury. *J Am Soc Nephrol* 2002; 13(4):1091-1099.
4. Collins AB, Schneeberger EE, Pascual MA, Saidman SL, Williams WW, Tolkoff-Rubin N et al. Complement activation in acute humoral renal allograft rejection: diagnostic significance of C4d deposits in peritubular capillaries. *J Am Soc Nephrol* 1999; 10(10):2208-2214.
5. Regele H, Bohmig GA, Habicht A, Gollowitzer D, Schillinger M, Rockenschaub S et al. Capillary deposition of complement split product C4d in renal allografts is associated with basement membrane injury in peritubular and glomerular capillaries: a contribution of humoral immunity to chronic allograft rejection. *J Am Soc Nephrol* 2002; 13(9):2371-2380.
6. Turner MW. The role of mannose-binding lectin in health and disease. *Mol Immuno*l 2003; 40(7):423-429.
7. Koch A, Melbye M, Sorensen P, Homoe P, Madsen HO, Molbak K et al. Acute respiratory tract infections and mannose-binding lectin insufficiency during early childhood. *JAMA* 2001; 285(10):1316-1321.
8. Garred P, Pressler T, Madsen HO, Frederiksen B, Svejgaard A, Hoiby N et al. Association of mannose-binding lectin gene heterogeneity with severity of lung disease and survival in cystic fibrosis. *J Clin Invest* 1999; 104(4):431-437.
9. Mullighan CG, Heatley S, Doherty K, Szabo F, Grigg A, Hughes TP et al. Mannose-binding lectin gene polymorphisms are associated with major infection following allogeneic hemopoietic stem cell transplantation. *Blood* 2002; 99(10):3524-3529.
10. Collard CD, Montalto MC, Reenstra WR, Buras JA, Stahl GL. Endothelial oxidative stress activates the lectin complement pathway: role of cytokeratin 1. *Am J Pathol* 2001; 159(3):1045-1054.
11. Nauta AJ, Raaschou-Jensen N, Roos A, Daha MR, Madsen HO, Borrias-Essers MC et al. Mannose-binding lectin engagement with late apoptotic and necrotic cells. *Eur J Immunol* 2003; 33(10):2853-2863.
12. Jordan JE, Montalto MC, Stahl GL. Inhibition of mannose-binding lectin reduces postischemic myocardial reperfusion injury. *Circulation* 2001; 104(12):1413-1418.
13. Roos A, Garred P, Wildenberg ME, Lynch NJ, Munoz JR, Zuiverloon TC et al. Antibody-mediated activation of the classical pathway of complement may compensate for mannose-binding lectin deficiency. *Eur J Immunol* 2004; 34(9):2589-2598.
14. Racusen LC, Solez K, Colvin RB, Bonsib SM, Castro MC, Cavallo T et al. The Banff 97 working classification of renal allograft pathology. *Kidney Int* 1999; 55(2):713-723.
15. Roos A, Bouwman LH, Gijlswijk-Janssen DJ, Faber-Krol MC, Stahl GL, Daha MR. Human IgA activates the complement system via the mannan-binding lectin pathway. *J Immunol* 2001; 167(5):2861-2868.
16. Kilpatrick DC. Mannan-binding lectin and its role in innate immunity. *Transfus Med* 2002; 12(6):335-352.

17. Hansen TK, Thiel S, Wouters PJ, Christiansen JS, Van den BG. Intensive insulin therapy exerts antiinflammatory effects in critically ill patients and counteracts the adverse effect of low mannose-binding lectin levels. *J Clin Endocrinol Metab* 2003; 88(3):1082-1088.
18. Hansen TK, Thiel S, Knudsen ST, Gravholt CH, Christiansen JS, Mogensen CE et al. Elevated levels of mannan-binding lectin in patients with type 1 diabetes. *J Clin Endocrinol Metab* 2003; 88(10):4857-4861.
19. Satomura A, Endo M, Ohi H, Sudo S, Ohsawa I, Fujita T et al. Significant elevations in serum mannose-binding lectin levels in patients with chronic renal failure. *Nephron* 2002; 92(3):702-704.
20. de Vries B, Matthijsen RA, Wolfs TG, van Bijnen AA, Heeringa P, Buurman WA. Inhibition of complement factor C5 protects against renal ischemia-reperfusion injury: inhibition of late apoptosis and inflammation. *Transplantation* 2003; 75(3):375-382.
21. de Vries B, Walter SJ, Peutz-Kootstra CJ, Wolfs TG, van Heurn LW, Buurman WA. The mannose-binding lectin-pathway is involved in complement activation in the course of renal ischemia-reperfusion injury. *Am J Pathol* 2004; 165(5):1677-1688.
22. Pratt JR, Jones ME, Dong J, Zhou W, Chowdhury P, Smith RA et al. Nontransgenic hyperexpression of a complement regulator in donor kidney modulates transplant ischemia/reperfusion damage, acute rejection, and chronic nephropathy. *Am J Pathol* 2003; 163(4):1457-1465.
23. Pratt JR, Basheer SA, Sacks SH. Local synthesis of complement component C3 regulates acute renal transplant rejection. *Nat Med* 2002; 8(6):582-587.
24. Ogden CA, deCathelineau A, Hoffmann PR, Bratton D, Ghebrehiwet B, Fadok VA et al. C1q and mannose binding lectin engagement of cell surface calreticulin and CD91 initiates macropinocytosis and uptake of apoptotic cells. *J Exp Med* 2001; 194(6):781-795.
25. Nauta AJ, Castellano G, Xu W, Woltman AM, Borrias MC, Daha MR et al. Opsonization with C1q and mannose-binding lectin targets apoptotic cells to dendritic cells. *J Immunol* 2004; 173(5):3044-3050.
26. Gallucci S, Lolkema M, Matzinger P. Natural adjuvants: endogenous activators of dendritic cells. *Nat Med* 1999; 5(11):1249-1255.
27. Li M, Carpio DF, Zheng Y, Bruzzo P, Singh V, Ouaaz F et al. An essential role of the NF-kappa B/Toll-like receptor pathway in induction of inflammatory and tissue-repair gene expression by necrotic cells. *J Immunol* 2001; 166(12):7128-7135.
28. Roos A, Xu W, Castellano G, Nauta AJ, Garred P, Daha MR et al. Mini-review: A pivotal role for innate immunity in the clearance of apoptotic cells. *Eur J Immunol* 2004; 34(4):921-929.
29. Casanova JL, Abel L. Human Mannose-binding Lectin in Immunity: Friend, Foe, or Both? *J Exp Med* 2004; 199(10):1295-1299.
30. Takahashi K, Gordon J, Liu H, Sastry KN, Epstein JE, Motwani M et al. Lack of mannose-binding lectin-A enhances survival in a mouse model of acute septic peritonitis. *Microbes Infect* 2002; 4(8):773-784.
31. Rector A, Lemey P, Laffut W, Keyaerts E, Struyf F, Wollants E et al. Mannan-binding lectin (MBL) gene polymorphisms in ulcerative colitis and Crohn's disease. *Genes Immun* 2001; 2(6):323-328.
32. Hansen TK, Tarnow L, Thiel S, Steffensen R, Stehouwer CD, Schalkwijk CG et al. Association between mannose-binding lectin and vascular complications in type 1 diabetes. *Diabetes* 2004; 53(6):1570-1576.

33. Endo M, Ohi H, Ohsawa I, Fujita T, Matsushita M, Fujita T. Glomerular deposition of mannose-binding lectin (MBL) indicates a novel mechanism of complement activation in IgA nephropathy. *Nephrol Dial Transplant* 1998; 13(8):1984-1990.
34. Endo M, Ohi H, Ohsawa I, Fujita T, Matsushita M. Complement activation through the lectin pathway in patients with Henoch-Schonlein purpura nephritis. *Am J Kidney Dis* 2000; 35(3):401-407.

Chapter 4

Low pre-transplantation Mannose-binding lectin levels predict superior patient and graft survival after simultaneous pancreas-kidney transplantation

Stefan P. Berger[1],
Anja Roos[1,2],
Marko J.K. Mallat[1],
Alexander F.M. Schaapherder[3],
Ilias I. Doxiadis[4],
Cees van Kooten[1],
Friedo W. Dekker[5],
Mohamed R. Daha[1] and
Johan W. de Fijter[1]

Departments of [1]Nephrology, [2]Clinical Chemistry, [3]Surgery, Immunohematology and Bloodtransfusion[4] and Clinical Epidemiology[5], Leiden University Medical Center, Leiden, The Netherlands

Journal of the American Society of Nephrology 2007; 18:2416-2422

Summary

Simultaneous pancreas-kidney transplantation (SPKT) is the treatment of choice for type 1 diabetics with renal failure. However, this procedure is characterized by a high rate of post-operative infections, acute rejection episodes and cardiovascular mortality. The lectin pathway of complement activation contributes to cardiovascular disease in diabetes and may play an important role in inflammatory damage after organ transplantation. We therefore studied how mannose-binding lectin (MBL), a major recognition molecule of the lectin pathway of complement activation, influences outcome after SPKT.

MBL serum levels were determined in 99 and MBL genotypes were determined in 97 consecutive patients who received a SPKT from 1990 through 2000, and related to patient and graft survival.

At 12 years, cumulative death-censored kidney graft survival was 87.5% in patients with an MBL level below 400 ng/ml and 74.8% in the group with MBL levels above 400 ng/ml ($p = 0.021$). Pancreas graft survival was significantly better in patients with low MBL-levels ($p = 0.016$). MBL levels above 400 ng/ml were associated with a hazard ratio of 6.28 for patient death (95% CI 1.8-20.3 $p = 0.003$). Accordingly, survival was significantly better in recipients with MBL gene polymorphisms associated with low MBL levels.

Our findings identify MBL as a potential risk factor for graft and patient survival in SPKT. We hypothesize that MBL contributes to the pathogenesis of inflammation-induced vascular damage both in the transplanted organs as well as in the recipient's native blood vessels.

Introduction

Simultaneous pancreas-kidney transplantation (SPKT) is the preferred treatment option for patients with long standing type 1 diabetes and end-stage renal failure. The major arguments favoring SPKT in these patients rather than renal transplantation alone include improved quality of life, prevention of recurrent diabetic nephropathy, and stabilization of diabetic neuropathy and retinopathy. Recent studies demonstrated that SPKT, compared to kidney transplantation alone, leads to improved allograft survival [1] and improved patient survival [2;3]. In spite of these benefits, mortality after SPKT transplantation remains high with 10 year patient survival rates of less than 70% [2;4].

The complement system contributes to tissue damage at various stages of the transplantation process. An important role in ischemia/reperfusion injury and acute rejection has been demonstrated in various animal models [5;6]. Recently, the F/F and F/S donor allotypes of the C3 complement molecule have been associated with better long term outcome after kidney transplantation [7].

Mannose-binding lectin (MBL) is the major recognition molecule of the lectin pathway of complement activation. In host defense wildtype MBL binds to carbohydrate moieties leading to complement deposition, opsonisation and elimination of pathogens. Single nucleotide polymorphisms (SNPs) in the structural as well as regulatory parts of the MBL gene lead to large interindividual variations in the concentration of functional MBL in serum [8].

Various studies showed an association of low serum MBL levels and MBL SNPs with decreased host defense against various infectious agents. This is especially apparent in situations of impaired adaptive immunity such as early childhood or prolonged immunosuppression [9-12].

However, wildtype MBL may also interact with tissue and lead to complement-mediated enhancement of damage in various non-infectious inflammatory settings. In ischemia/reperfusion damage MBL may contribute to tissue injury by binding to host cells exhibiting a modified surface [13;14]. Recently, high MBL levels have been related to an increased risk of vascular disease and diabetic nephropathy in both patients with type 1 and type 2 diabetes [15-17].

Our group has shown that low pre-transplantation MBL levels are associated with better graft survival after deceased donor kidney transplantation [18]. In view of the role of MBL in diabetes and transplantation, we hypothesize that MBL could be major determinant of outcome in SPKT which is characterized by a high rate of infectious complications, acute graft rejection and cardiovascular mortality.

Methods

Study Population

Between January 1990 and December 2000, 114 SPKTs were performed in the Leiden University Medical Center. All patients had diabetes mellitus type 1. Pre-transplantation serum was available from 99 and DNA was available from 97 of these consecutive recipients. Both pre-transplant serum and DNA was available from 87 of these patients. Pre-transplantation sera were routinely obtained at the time of admission for transplantation and stored in aliquots at -80°C. All measurements of MBL were performed in sera that had been frozen and thawed only once. All included patients were regularly followed at our center. None of the 99 patients were lost to follow up. The study was performed according to the guidelines of the ethics committee of the Leiden University Medical Center and patient anonymity was maintained.

The following clinical data were collected using the Leiden Transplant Database: donor variables including gender and age at time point of death, recipient variables (age at time of transplantation, gender, panel-reactive antibodies, CMV status, duration of diabetes and dialysis treatment, smoking status and cholesterol levels), transplantation-related factors (human leukocyte antigen-A [HLA-A], -B, and –DR mismatches; cold ischemia time), and post-transplantation features including immunosuppressive regimen, occurrence of delayed graft function, acute rejection history, rejection treatment, status of both the kidney and pancreas allograft, cause of allograft loss, vital status and cause of death. Rejection was defined as either biopsy proven rejection or clinical rejection of the kidney with a favorable response to anti-rejection treatment. Since pancreas rejection is difficult to diagnose and isolated rejection of the pancreas is a rare event this was not analyzed separately in this study. After transplantation, patients were followed until death or until January 1st 2006. Until May 1995 standard maintenance immunosuppression consisted of prednisone, cyclosporine and azathioprine. All recipients transplanted after May 1995 received prednisolone, cyclosporine and mycophenolate mofetil. Eighteen patients received induction treatment with OKT-3 between 1991 and 1994. From 1999 onwards, induction treatment was reinitiated and consisted of either polyclonal antithymocyte globulin (ATG-Fresenius) or Daclizumab (n = 19). Acute rejection episodes were treated according to a standard protocol consisting of methylprednisolone 1 g intravenously for three consecutive days; a 10 day course of antithymocyte globulin at a dose of 5 mg/kg guided by absolute lymphocyte counts; or again methylprednisolone for the first, second (or steroid-resistant), or third rejection episode, respectively.

ELISA

Serum MBL levels were assessed by sandwich ELISA as described previously (18). In brief 96-well ELISA plates (Greiner, Frickenhausen, Germany) were coated with the monoclonal antibody 3E7 (mouse IgG1 anti-MBL at 2.5 µg/ml), kindly provided by Dr. T. Fujita (Fuhushima, Japan). Serum samples were diluted 1/50 and 1/500 and incubated in the coated wells. MBL was detected with Dig-conjugated 3E7. Detection of binding of Dig-conjugated antibodies was performed using HRP-conjugated sheep anti-Dig Abs (Fab fragments, Roche, Mannheim, Germany). Enzyme activity was detected using 2,2'-azino-bis(3-ethybenzthiazoline-6-sufonic acid) (Sigma Chemical Co., St. Louis, MO)). The optical density was measured at 415 nm using a microplate biokinetics reader (EL312e; Biotek Instruments, Winooski, VT). A calibration line was produced using human serum from a healthy donor with a known concentration of MBL. Earlier studies indicated that this assay primarily detects wildtype MBL in serum and plasma and that there is a direct association with the MBL genotype and with MBL function [19].

Genotyping

DNA from 97 SPKT recipients was isolated routinely from blood. MBL single nucleotide polymorphisms (SNPs) at codons 52, 54 and 57 of the *mbl2* gene were typed by high resolution DNA melting analysis [20]. The detailed methodology will be published separately (A. Roos and R.H. Vossen et al., manuscript in preparation). The MBL genotype of only wildtype allele carriers is designated as A/A and the presence of 1 or 2 variant allele(s) (B, C, or D) is designated as A/O or O/O. In the survival analysis carriers of A/O and O/O MBL genotype were considered as one group.

Statistical analysis

Categorical characteristics were compared using cross-tables with calculation of the exact p-values. Interval variables were analyzed using the Independent-Samples T-test when assumptions for parametric testing were met. Otherwise the Mann-Whitney U test was used. Patient and graft survival was estimated using the Kaplan-Meier product-limit method and the curves were compared with the Log-Rank test. For both pancreas and renal allograft survival the analysis was censored for patient death. Organs lost due to technical failure or thrombosis within one week after transplantation were excluded from survival analysis.

Cox Proportional Hazards Regression was used to identify possible confounders influencing baseline MBL levels. In the multivariate model, MBL was adjusted for

recipient age, sex and baseline CRP level. MBL was tested both as a dichotomous (MBL below or above 400 ng/ml) and a continuous factor (after log transformation). P-values < 0.05 were considered to be significant. Data analysis was performed with SPSS Statistical Software Package (Version 11.0.1; SPSS, Inc., Chicago, Ill.).

Results

The mean MBL concentration in the 99 available sera obtained directly prior to transplantation was 1053 ng/l. The median concentration was 694 ng/ml. A cut-off of 400 ng/ml was used to discriminate between high and low MBL levels. This cut-off correlates with the presence of single nucleotide polymorphisms (SNPs) in the first exon of the MBL gene in both a control population [19] and the recipients studied here (Figure 1). The median MBL concentration in SPKT recipients with only wild-type MBL alleles (A/A) was 1493 ng/ml (n = 54). In recipients with the A/O (n = 29) or O/O (n = 4) genotypes the median MBL concentrations were 245 ng/ml and 166 ng/ml, respectively. Of the patients with an MBL level above 400 ng/ml, 89.3% had only wild-type MBL alleles (A/A) whereas 90% of the patients with an MBL level below 400 ng/ml had at least one of the exon 1 MBL polymorphisms (A/O or O/O). To assess whether pre-transplant MBL levels are representative for the levels after transplantation we determined the MBL concentrations one year after SPKT in 30 patients and compared them with the levels measured in the pre-transplant sample. We found a high intra-individual correlation of MBL levels over time (r = 0.87, P < 0.0001).

Thirty-four (34.3%) SPKT recipients had a low MBL level and 65 (65.6%) recipients had high MBL levels. Table 1 shows the characteristics of the high and low MBL recipients. No significant difference between both groups concerning demographic and clinical characteristics including donor and recipient age, CMV status and sex distribution was noted. Both groups had a comparable proportion of patients undergoing SPKT before initiation of dialysis treatment. Both the high and low MBL-groups had a comparable proportion of patients receiving triple immunosuppression including mycophenolate mofetil. The proportion of patients with at least one significant coronary stenosis was 27.3% in the low MBL-group and 22.3% in the high MBL-group (p = 0.58). Of note, there was no difference in the baseline CRP levels between the two MBL groups. The majority of patients required treatment for acute rejection, 88.2% and 86.2% in the low and high MBL groups, respectively (p = 0.99). Likewise the number of rejection treatments per patient was comparable in both groups (1.85 vs. 1.68, p = 0.49).

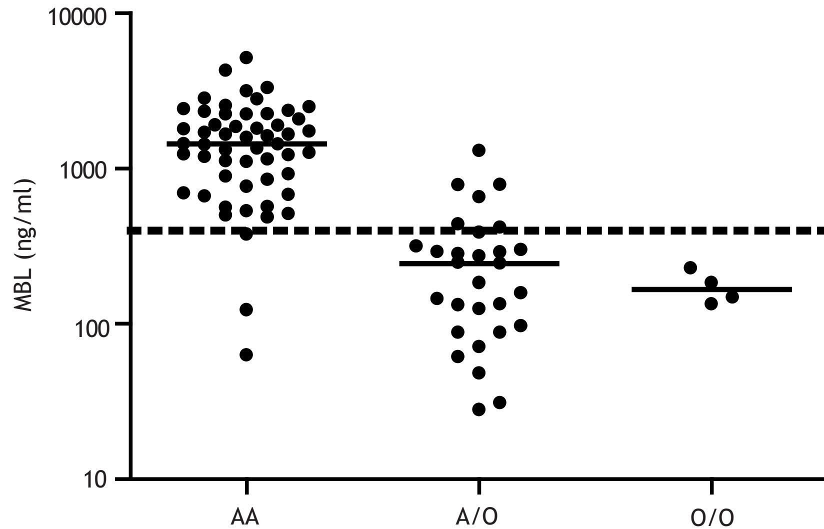


Figure 1. Pre-transplantation MBL levels stratified according to MBL genotype. The dashed line represents the cut-off level of 400 ng/ml. MBL levels are represented on a log scale.

Analysis for death-censored graft survival revealed a significant survival advantage for both the renal and pancreas allografts in favor of the low MBL recipients. At 12 years after transplantation, cumulative death-censored pancreas graft survival was 100% in the low MBL-group vs 82% in the high MBL-group (p = 0.016 by the log-rank test with grafts lost within 1 week excluded) (Figure 2A). Death-censored renal allograft survival at 12 years after transplantation was 87.5% in patients with an MBL level below 400 ng/ml and 74,8% in patients with an MBL level above 400 ng/ml (p = 0.021 by the log-rank test) (Figure 2B).

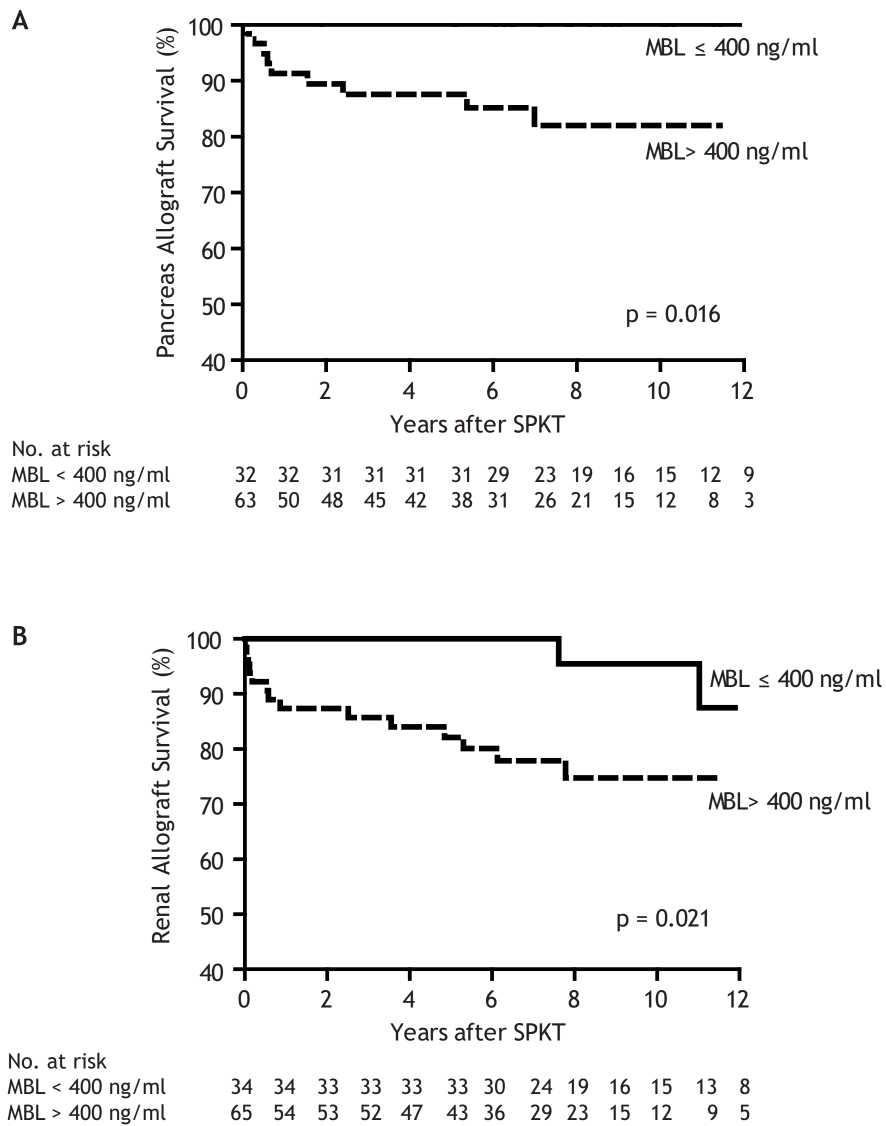


Figure 2. Unadjusted Kaplan-Meier survival curves according to pre-transplantation MBL-level. (A) Death-censored survival of pancreas allografts, (B) death-censored survival of kidney allografts

Subsequently, the MBL status was related to patient survival. Twelve years after transplantation cumulative patient survival was 86.9% in the low MBL group and 49.1% in the high MBL group (p = 0.001 by the log rank test) (Figure 3A). To examine whether

the inferior patient survival in high MBL recipients was a mere consequence of graft loss we repeated the survival analysis after excluding the patients who lost either the kidney or pancreas allograft. In the group with functioning allografts patient survival remained inferior in those with MBL levels above 400ng/ml (p = 0.02).

Table 1. Characteristics of study population according to MBL levels[a]

	Acceptor MBL level[a] (ng/ml)		
	MBL ≤ 400	**MBL > 400**	**P-value**
n	34	65	
Recipient age (yrs)	39.9 ± 7.8	40.8 ± 6.8	0.89
Female recipient (%)	32.4	38.5	0.55
Years diabetes	27.1 ± 6.2	26.2 ± 6.3	0.65
Active smoking (%)	32.4	19.7	0.17
Significant stenosis in Pre-tx CAG (%)	27.3	22.2	0.58
Baseline CRP (mg/l)	4.3 ±6.4	4.45 ± 7.4	0.96
Baseline cholesterol (mmol/l)	5.11 ±1.2	5.27 ± 1.3	0.57
Pre-emptive SPKT (%)	44.1	35.4	0.40
CMV sero-positive (%)	41.2	35.9	0.61
Donor age (yrs)	33.0 ± 9.1	29.8 ± 11.8	0.17
Cold ischemia time (hrs)	14.8 ± 2.9	15.2± 3.9	0.59
Rejection episodes	1.85 ± 0.9	1.69 ± 1.13	0.49
HLA DR mismatches	1.32 ± 0.64	1.29 ± 0.63	0.81
Mycophenolate (%)	47.1	47.6	0.98

[a] MBL, mannose-binding lectin; CIT, cold ischemia time; CMV, cytomegalovirus; tx, transplantation; CAG, coronary angiogram,CRP, C-reactive protein, where appropriate values are given as mean ± s.d.

To confirm these findings we also analyzed recipient survival according to the MBL genotype. Superior survival was found in patients with a variant MBL genotype when compared with recipients with only wildtype MBL alleles (p = 0.026) (Figure 3B).

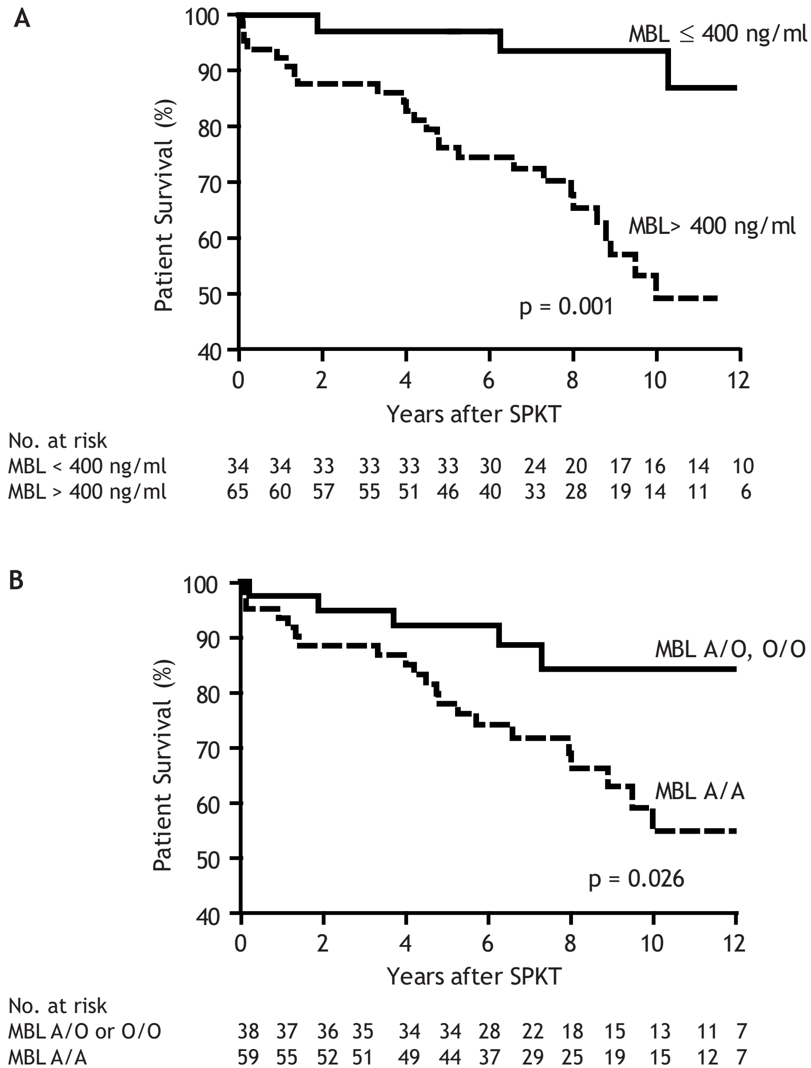


Figure 3. Unadjusted Kaplan-Meier survival curves of patient survival and cardiovascular survival according to MBL status. (A) Patient survival according to pre-transplantation MBL level, (B) Patient survival according to recipient MBL genotype, (A/A= wildtype MBL genotype; A/O or O/O= variant MBL genotype)

We analyzed various characteristics in relation to patient survival (Table 2). An MBL level above 400 ng/ml was associated with a strongly increased mortality (HR 6.28; 95% CI 1.89-20.87, p = 0.003). Accordingly, the presence of wild-type MBL alleles

was associated with an increased risk of patient death (HR 3.6; 95% CI 1.22-10.6, p = 0.02). MBL was also significantly associated with an increased risk of patient death when analyzed as a continuous parameter (p = 0.013). MBL remained significantly associated with patient death when entered into a multivariate model adjusted for recipient age, sex and baseline CRP using the Cox regression method (Table 2).

The reasons for patient death in the high and low MBL groups are shown in table 3. The excess mortality in patients with an MBL level above 400 ng/ml was explained to a large extent by a higher cardiovascular mortality in this group. No significant difference in infection-related deaths between the low and high MBL-groups was observed.

Table 2. Risk factors for patient death[a].

	univariate			multivariate[b]		
	HR	95% CI	P-value	HR	95% CI	P-value
MBL> 400 ng/ml	6.28	1.89-20.87	0.003	4.44	1.3-15.1	0.017
Log MBL ng/ml	2.75	1.24-6.11	0.013	2.56	1.04-6.3	0.04
MBL genotype A/A	3.6	1.22-10.06	0.02			
Significant coronary stenosis at baseline	2.00	0.92-4.30	0.077			
Male recipient	0.61	0.29-1.26	0.18			
Recipient age > 40 yr	1.52	0.73-3.19	0.27			
Smoking	0.96	0.49-2.01	0.96			
Baseline cholesterol > 5 mmol/l	0.99	0.47-2.09	0.99			
Years diabetes	0.99	0.93-1.06	0.8			
CRP	1.03	0.98-1.07	0.25			
MMF vs. Aza	0.53	0.22-1.31	0.17			
Pre-emptive transplantation	1.25	0.58-2.73	0.57			

[a] HR, hazard ratio; MBL, mannose-binding lectin; CI, confidence interval; CRP, c-reactive proteine, MMF, mycophenolate mofetil; Aza, Azathioprine [b]For the multivariate analysis MBL was adjusted for recipient sex, age and CRP

Table 3. Reason for death according to MBL levels[a]

	Recipient MBL-level				
	MBL ≤ 400 ng/ml		MBL > 400 ng/ml		
	n	%	n	%	P-value
	34	-	65	-	-
All causes	3	8.8	26	40	0.001
Cardiovascular	0	0	11	16.9	0.014
Malignancy	1	2.9	4	6.2	0.66
Infection	0	0	3	4.6	0.55
Other	1	2.9	4	6.2	0.66
Undetermined	1	2.9	4	6.2	0.66

[a] MBL, mannose-binding lectin

Discussion

Our study demonstrates superior graft and patient survival after SPKT in recipients with low MBL levels. A high MBL level was associated with an increased incidence of death-censored loss of both the renal and the pancreatic allograft. Furthermore, a high-MBL status was associated with markedly increased mortality, and we demonstrate that this high MBL-status is genetically determined.

These findings corroborate our recent report demonstrating an association of MBL levels above 400 ng/ml with poorer graft survival after deceased donor kidney transplantation [18]. Our earlier study on the role of MBL in kidney transplantation showed a non-significant trend towards poorer patient survival in renal allograft recipients with a high MBL level. This difference between the two studies may be explained by the higher risk profile in the type 1 diabetic population receiving SPKT compared with the general population of kidney allograft recipients. In addition the harmful effect of MBL in cardiovascular mortality may be enhanced in the diabetic population.

Reports on the cardiovascular effects of MBL deficiency in the general population have been inconclusive. The predictive value of MBL levels for myocardial infarction was studied in the population-based Reykjavik study [21]. In this population MBL levels

above 1000 ng/ml were associated with a lower odds ratio for myocardial infarction. Interestingly no data on mortality were reported. In the Strong Heart Study cohort, native Americans with coronary heart disease had an increased frequency of variant MBL genotypes when compared with a matched cohort without coronary heart disease [22]. Contrary to these findings, but in agreement with our current data, a recent study in 964 apparently healthy men did show an association of elevated MBL levels with coronary heart disease [23].

So how can we explain the adverse effect of high MBL levels on graft and patient survival in our study? The finding of superior graft survival after SKPT confirms our earlier report demonstrating superior allograft survival in recipients with low MBL levels after deceased donor renal transplantation [18]. Like in the current SPKT cohort the incidence of acute rejection was similar in the high and low MBL groups, but graft loss due to rejection occurred much more frequently in recipients with high MBL levels. We hypothesize that MBL contributes to tissue damage in various inflammatory settings including graft rejection. Models of ischemia/reperfusion damage in heart, intestine and kidney have shown that MBL A & C-deficient mice are protected from ischemia/reperfusion injury as compared to wild type animals [13;24;25]. In line with these findings MBL deposition has been detected in human kidneys with ischemia/ reperfusion damage [26], indicating that wildtype MBL may contribute to local complement activation and enhanced inflammation in tissue damage. Next to the interaction of MBL with apoptotic and necrotic cells [27], MBL-mediated damage may also be related to its antibody-binding capacities [28-30]. A recent study has failed to show an association between MBL levels and patient or graft survival after kidney transplantation [31]. In comparison with our studies it has to be noted that the analysis was performed using the median MBL level or the third quartile as cut-off values which may not be ideal for detecting MBL-mediated effects.

In addition to the effect of MBL on graft survival, we also observed a strong association of high MBL levels with inferior patient survival which was independent of graft survival. Earlier studies have pointed towards a detrimental role of MBL in patients with diabetes. High levels of MBL have been associated with an increased frequency of cardiovascular disease and proteinuria in patients with type 1 diabetes mellitus [15;16]. Similarly, high MBL levels have also been related to increased mortality in type 2 diabetics [17]. It may well be that MBL exerts a specific harmful effect in the diabetic milieu and the increased mortality in high-MBL subjects may be related to microvascular damage obtained prior to the pancreas transplantation. Additionally, the unfavorable effect of MBL observed in the context of ischemia/

reperfusion damage may also contribute to tissue damage and mortality following cardiovascular events.

Since intraindividual MBL-levels are highly stable over time [21] we are convinced that serum MBL levels measured prior to transplantation adequately represent the exposition to MBL. Moreover, our MBL assay strongly correlates with both the functional activity of the lectin pathway [19] and the presence of SNPs of the MBL gene. In fact, measurement of MBL levels in serum may be a more powerful and convenient method of detecting MBL-mediated effects than genotyping since not all intraindividual variations in MBL levels are explained by the known polymorphisms of exon 1 and other parts of the MBL gene.

Recently low MBL levels have been related to an increased incidence of clinically important infections after liver transplantation [12]. However, no association between MBL-deficiency and infection-related mortality was detected in our cohort. Low infection-related mortality after SPKT has been reported before [32]. Thus, although we cannot exclude that MBL deficiency is associated with an increased incidence of infections after SPKT this did not contribute to graft survival or patient mortality in our cohort.

We conclude that MBL levels are a powerful predictor of graft and patient survival after SPKT. If these findings can be confirmed in other study populations, determination of MBL levels and/or MBL genotyping may aid risk stratification prior to SPKT. Whether these findings eventually lead to new therapeutic approaches will depend on the elucidation of the underlying pathophysiological mechanisms.

Acknowledgements

This study was supported in part by the Dutch Kidney Foundation (grant number: CO3-694). We thank Prof. Teizo Fujita (Fuhushima, Japan) for providing the anti-MBL antibody (3E7). We also acknowledge the excellent technical assistance by Nicole Schlagwein and Reinier van der Geest and thank Rolf H. Vossen (Dept. of Genetics, LUMC) for his help with the genotyping.

Reference List

1. Israni AK, Feldman HI, Propert KJ, Leonard M, Mange KC. Impact of simultaneous kidney-pancreas transplant and timing of transplant on kidney allograft survival. *Am J Transplant* 2005; 5(2):374-382.
2. Ojo AO, Meier-Kriesche HU, Hanson JA, Leichtman A, Magee JC, Cibrik D et al. The impact of simultaneous pancreas-kidney transplantation on long-term patient survival. *Transplantation* 2001; 71(1):82-90.
3. Smets YF, Westendorp RG, van der Pijl JW, de Charro FT, Ringers J, de Fijter JW et al. Effect of simultaneous pancreas-kidney transplantation on mortality of patients with type-1 diabetes mellitus and end-stage renal failure. *Lancet* 1999; 353(9168):1915-1919.
4. Pruijm MT, de Fijter HJ, Doxiadis II, Vandenbroucke JP. Preemptive versus Non-preemptive simultaneous pancreas-kidney transplantation: a single-center, long-term, follow-up study. *Transplantation* 2006; 81(8):1119-1124.
5. Zhou W, Farrar CA, Abe K, Pratt JR, Marsh JE, Wang Y et al. Predominant role for C5b-9 in renal ischemia/reperfusion injury. *J Clin Invest* 2000; 105(10):1363-1371.
6. Pratt JR, Basheer SA, Sacks SH. Local synthesis of complement component C3 regulates acute renal transplant rejection. *Nat Med* 2002; 8(6):582-587.
7. Brown KM, Kondeatis E, Vaughan RW, Kon SP, Farmer CK, Taylor JD et al. Influence of donor C3 allotype on late renal-transplantation outcome. N Engl J Med 2006; 354(19):2014-2023.
8. Garred P, Larsen F, Madsen HO, Koch C. Mannose-binding lectin deficiency--revisited. *Mol Immunol* 2003; 40(2-4):73-84.
9. Garred P, Pressler T, Lanng S, Madsen HO, Moser C, Laursen I et al. Mannose-binding lectin (MBL) therapy in an MBL-deficient patient with severe cystic fibrosis lung disease. *Pediatr Pulmonol* 2002; 33(3):201-207.
10. Mullighan CG, Heatley S, Doherty K, Szabo F, Grigg A, Hughes TP et al. Mannose-binding lectin gene polymorphisms are associated with major infection following allogeneic hemopoietic stem cell transplantation. *Blood* 2002; 99(10):3524-3529.
11. Koch A, Melbye M, Sorensen P, Homoe P, Madsen HO, Molbak K et al. Acute respiratory tract infections and mannose-binding lectin insufficiency during early childhood. *JAMA* 2001; 285(10):1316-1321.
12. Bouwman LH, Roos A, Terpstra OT, de Knijff P, van Hoek B, Verspaget HW et al. Mannose binding lectin gene polymorphisms confer a major risk for severe infections after liver transplantation. *Gastroenterology* 2005; 129(2):408-414.
13. Moller-Kristensen M, Wang W, Ruseva M, Thiel S, Nielsen S, Takahashi K et al. Mannan-binding lectin recognizes structures on ischaemic reperfused mouse kidneys and is implicated in tissue injury. *Scand J Immunol* 2005; 61(5):426-434.
14. Walsh MC, Bourcier T, Takahashi K, Shi L, Busche MN, Rother RP et al. Mannose-binding lectin is a regulator of inflammation that accompanies myocardial ischemia and reperfusion injury. *J Immunol* 2005; 175(1):541-546.
15. Hansen TK, Tarnow L, Thiel S, Steffensen R, Stehouwer CD, Schalkwijk CG et al. Association between mannose-binding lectin and vascular complications in type 1 diabetes. *Diabetes* 2004; 53(6):1570-1576.

16. Hovind P, Hansen TK, Tarnow L, Thiel S, Steffensen R, Flyvbjerg A et al. Mannose-binding lectin as a predictor of microalbuminuria in type 1 diabetes: an inception cohort study. *Diabetes* 2005; 54(5):1523-1527.
17. Hansen TK, Gall MA, Tarnow L, Thiel S, Stehouwer CD, Schalkwijk CG et al. Mannose-binding lectin and mortality in type 2 diabetes. *Arch Intern Med* 2006; 166(18):2007-2013.
18. Berger SP, Roos A, Mallat MJ, Fujita T, de Fijter JW, Daha MR. Association between mannose-binding lectin levels and graft survival in kidney transplantation. *Am J Transplant* 2005; 5(6):1361-1366.
19. Roos A, Garred P, Wildenberg ME, Lynch NJ, Munoz JR, Zuiverloon TC et al. Antibody-mediated activation of the classical pathway of complement may compensate for mannose-binding lectin deficiency. *Eur J Immunol 2*004; 34(9):2589-2598.
20. Zhou L, Wang L, Palais R, Pryor R, Wittwer CT. High-resolution DNA melting analysis for simultaneous mutation scanning and genotyping in solution. *Clin Chem* 2005; 51(10):1770-1777.
21. Saevarsdottir S, Oskarsson OO, Aspelund T, Eiriksdottir G, Vikingsdottir T, Gudnason V et al. Mannan binding lectin as an adjunct to risk assessment for myocardial infarction in individuals with enhanced risk. *J Exp Med* 2005; 201(1):117-125.
22. Best LG, Davidson M, North KE, MacCluer JW, Zhang Y, Lee ET et al. Prospective analysis of mannose-binding lectin genotypes and coronary artery disease in American Indians: the Strong Heart Study. *Circulation* 2004; 109(4):471-475.
23. Keller TT, van Leuven SI, Meuwese MC, Wareham NJ, Luben R, Stroes ES et al. Serum levels of mannose-binding lectin and the risk of future coronary artery disease in apparently healthy men and women. *Arterioscler Thromb Vasc Biol* 2006; 26(10):2345-2350.
24. Hart ML, Ceonzo KA, Shaffer LA, Takahashi K, Rother RP, Reenstra WR et al. Gastrointestinal ischemia-reperfusion injury is lectin complement pathway dependent without involving C1q. *J Immunol* 2005; 174(10):6373-6380.
25. Jordan JE, Montalto MC, Stahl GL. Inhibition of mannose-binding lectin reduces postischemic myocardial reperfusion injury. *Circulation* 2001; 104(12):1413-1418.
26. de Vries B, Walter SJ, Peutz-Kootstra CJ, Wolfs TG, van Heurn LW, Buurman WA. The mannose-binding lectin-pathway is involved in complement activation in the course of renal ischemia-reperfusion injury. *Am J Pathol* 2004; 165(5):1677-1688.
27. Nauta AJ, Raaschou-Jensen N, Roos A, Daha MR, Madsen HO, Borrias-Essers MC et al. Mannose-binding lectin engagement with late apoptotic and necrotic cells. *Eur J Immunol* 2003; 33(10):2853-2863.
28. Roos A, Bouwman LH, Gijlswijk-Janssen DJ, Faber-Krol MC, Stahl GL, Daha MR. Human IgA activates the complement system via the mannan-binding lectin pathway. *J Immunol* 2001; 167(5):2861-2868.
29. Roos A, Rastaldi MP, Calvaresi N, Oortwijn BD, Schlagwein N, Gijlswijk-Janssen DJ et al. Glomerular activation of the lectin pathway of complement in IgA nephropathy is associated with more severe renal disease. *J Am Soc Nephrol* 2006; 17(6):1724-1734.
30. Arnold JN, Wormald MR, Suter DM, Radcliffe CM, Harvey DJ, Dwek RA et al. Human serum IgM glycosylation: identification of glycoforms that can bind to mannan-binding lectin. *J Biol Chem* 2005; 280(32):29080-29087.

31. Hjelmesaeth J, Ueland T, Flyvbjerg A, Bollerslev J, Leivestad T, Jenssen T et al. Early Posttransplant Serum Osteoprotegerin Levels Predict Long-Term (8-Year) Patient Survival and Cardiovascular Death in Renal Transplant Patients. *J Am Soc Nephrol* 2006; 17(6):1746-1754.
32. Smets YF, van der Pijl JW, van Dissel JT, Ringers J, de Fijter JW, Lemkes HH. Infectious disease complications of simultaneous pancreas kidney transplantation. Nephrol Dial Transplant 1997; 12(4):764-771.

Chapter 5

Infectious complications after simultaneous pancreas-kidney transplantation: A role for the lectin pathway of complement activation

Jeffrey J.W. Verschuren[1],
Anja Roos[1,2],
Alexander F.M. Schaapherder[3],
Marko J.K. Mallat[1],
Mohamed R. Daha[1],
Johan W. de Fijter[1] and
Stefan P. Berger[1]

Departments of [1]Nephrology, [2]Clinical Chemistry, and [3]Surgery,
Leiden University Medical Center, Leiden, The Netherlands

Transplantation 2008; 85:75-80

Summary

Mannose-binding lectin (MBL) is a recognition molecule of the lectin pathway of complement activation and its serum levels are largely determined by frequently occurring polymorphisms of the MBL gene. We questioned whether MBL deficiency influences infectious complications after simultaneous pancreas-kidney transplantation (SPKT).

Infectious complications in the first year after transplantation were scored retrospectively in 152 consecutive SPKT patients who received their transplant at our center between 1990 and 2005. Pre-transplant serum MBL levels were determined by ELISA. Every 500 ng/mL increase in baseline MBL was associated with an odds ratio of 0.83 (P = 0.045) for urinary tract infections and an odds ratio of 0.68 (P = 0.029) for urosepsis. Urosepsis was significantly more common in patients with low baseline MBL (< 400 ng/mL) compared to higher MBL levels (22.7% vs. 8.3%, P = 0.015). No significant influence of MBL on the occurrence of wound infections and cytomegalovirus disease could be demonstrated.

With the current study we show that high levels of serum MBL are associated with protection against urinary tract infections and more specifically against urosepsis after SPKT. These data indicate an important role for the lectin pathway of complement activation in antimicrobial defense in these transplant recipients.

Introduction

Simultaneous pancreas-kidney transplantation (SPKT) offers the opportunity to correct both renal failure and the underlying metabolic disease in type 1 diabetics with severe diabetic nephropathy. Technical success rates have improved and SPKT has become a routine procedure in many transplant centers. Nevertheless, this procedure is still characterized by a high rate of rejection [1] and infectious complications [2] when compared to kidney transplantation alone.

The complement system plays an important role in solid organ transplantation in both ischemia/reperfusion injury and rejection-mediated damage [3;4]. Mannose-binding lectin (MBL) is a major initiation molecule of the lectin pathway of complement activation and its levels are largely determined by polymorphisms within exon 1 and the promoter region of the *MBL-2* gene. MBL binds to carbohydrate moieties of pathogens and damaged host tissue which leads to opsonisation and activation of the complement cascade. We recently reported a strong association of high MBL levels with mortality and graft loss in SPKT recipients [5], a finding possibly explained by a contribution of the lectin pathway to tissue damage occurring during ischemia/reperfusion injury. Experimental data have demonstrated an important role of the lectin pathway in ischemia/reperfusion damage of the heart and kidney [6;7]. On the other hand, the lectin pathway may be of particular importance in host defense in the immune compromised host. Various studies have linked low serum levels of MBL and MBL SNPs to increased infectious complications in various settings of impaired immunity including cystic fibrosis and early childhood [8;9]. Medical interventions associated with states of impaired adaptive immunity such as stem cell transplantation and chemotherapy have also been associated with increased infection rates in the presence of low MBL levels [10;11]. We recently showed that the transplantation of livers with variant MBL genotypes results in low MBL levels in the recipient and an significantly increased rate of severe infections [12].

Taken together, MBL deficiency can have dual effects, dependent on the clinical situation. In our recent publication on the role of MBL in SPKT [5], we showed that a low-MBL status is clinically beneficial, since it is associated with lower mortality and less graft loss. In line with the well-established role of MBL in host defense, we now report that a low-MBL status of the recipient is strongly associated with an increased susceptibility to infections in this highly infection-prone cohort of immunosuppressed patients. In particular, results indicate an association of low MBL levels with an increased risk for urinary tract infections after SPKT.

Materials and Methods

From January 1990 until July 2005, 183 simultaneous pancreas/kidney transplantations were performed in the Leiden University Medical Center. Ten recipients were excluded because they lost their pancreas allograft within one week after transplantation due to arterial thrombosis. A pre-transplant serum sample was available from 152 of the 173 remaining patients. In 144 of these patients exocrine pancreatic juices were drained via the bladder and primary enteric drainage was used in 8 patients.

Data analysis was performed using the Leiden Transplant Database containing donor variables (gender, date of birth and death, cytomegalovirus (CMV) status, transplantation date), recipient variables (gender, age at time of transplantation, CMV status) and post-transplantation features (immune suppression, rejection history, rejection treatment and graft loss). This information was collected routinely for all transplant patients. After transplantation, patients were followed until death or July 2006.

The study was performed according to the guidelines of the ethics committee of the Leiden University Medical Center and patient anonymity was maintained.

Antibiotic prophylaxis

As viral prophylaxis high dose acyclovir (57 patients), valaciclovir (12 patients) or ganciclovir (44 patients) was given during the first year after transplantation. CMV prophylaxis was given indiscriminately to all patients irrespective of their CMV status until December 2002. From 2003 onwards CMV prophylaxis was discontinued and a pre-emptive treatment protocol was introduced. From 1995 onwards, cotrimoxazol was given as a prophylaxis for both urinary tract infections and Pneumocystis pneumonia (PCP) (100 patients). As prophylaxis for *Candida spp.* stomatitis and vaginitis, amfotericine B fluid and miconazol cream were given up to 1 year after transplantation, respectively.

Immunosuppression

All patients transplanted before August 1996 received an immunosuppressive regimen consisting of triple therapy including prednisone, cyclosporin and azathioprine (32.2%). From August 1996 on, azathioprine was replaced by mycophenolate mofetil (MMF, 67.8%). Eighteen patients received induction treatment with OKT-3 between 1991 and 1994. From 1999 onwards induction treatment was reinitiated and consisted of either polyclonal antithymocyte globulin (ATG-Fresenius) or daclizumab.

Infections
All clinically significant infections (site, pathogen, antibiotic treatment) were scored retrospectively with a follow-up of one year after transplantation using Center for Disease Control and Prevention (CDC) criteria [13]. Infection was defined as a positive culture from the site expected to be the focus of infection followed by subsequent appropriate antibiotic therapy.

Criteria for the most frequent infections are briefly described. Urinary tract infections (UTI) were divided into cystitis, pyelonephritis and urosepsis. Cystitis was defined as a positive urine culture (> 10^4 cfu/ml) and pyuria with or without other symptoms. Pyelonephritis was defined as a positive urine culture, pyuria and fever (>38.5°C). If blood culture was also positive, the infection was scored as urosepsis. Wound infections (surgical site infections) were divided in superficial and deep (i.e., intra-abdominal) infections and defined as positive tissue or drain cultures. Furthermore, superficial wound infection was defined as involvement of skin or subcutaneous tissue around the incision and deep wound infection by involvement of intra-abdominal tissue with or without fever. The diagnosis CMV disease was made after laboratory documentation of CMV replication (positive pp65) in the presence of clinical symptoms (fever >38.5° with respiratory, hepatic, hematological, gastro-intestinal, central nervous system, renal, or musculoskeletal findings that could not be attributed to another cause) followed by starting or adjustment of antiviral treatment [2].

ELISA
Serum MBL concentrations were assessed by sandwich ELISA as described previously [14]. In short, 96-well ELISA plates were coated with 3E7 (mouse IgG1 anti-MBL at 5 μg/mL). After blocking residual binding sites with PBS containing 1% BSA, serum samples were diluted 1:50 and 1:500 in PBT (PBS containing 1% BSA and 0.05% Tween 20) and incubated. Dig-conjugated 3E7 was added as second antibody. Detection of MBL binding was performed by adding Fab anti-Dig-HRP (Fab fragments, Roche, Mannheim, Germany) followed by enzyme activity detection with ABTS (Sigma Chemical Co., St. Louis, MO). Optical density was measured at 415 nm using a micro plate biokinetics reader (EL312e, Biotek Instruments, Winooski, VT). A calibration line was obtained using a serial dilution of human serum with a known concentration of MBL.

Statistical analysis

Logistic regression analysis was used to test the influence of MBL on the different infectious parameters. MBL concentrations < 400 ng/mL were classified as low MBL, whereas MBL > 400 ng/mL were considered normal/high MBL levels. Categorical characteristics among the different MBL groups were compared using cross tables with calculations of exact p-values using the Pearson Chi-Square test or with the Fisher's Exact test. Continuous variables were analyzed using the Student's t-test, when test assumptions were met, and otherwise with the Mann-Whitney test. To identify independent risk factors for infectious complications multivariate binary logistic regression analysis was performed. The forced entry method was applied.

P-values of < 0.05 were considered to be statistically significant. All analyses were performed with SPSS Statistical Software Package (version 12.01, SPSS Inc.).

Results

Patient characteristics of the 152 selected patients are summarized in table 1. Their mean age was 40.8 years and 62% were male. One year patient survival was 95.3% and death censored pancreas and kidney allograft survival were 92.8% and 96.1%, respectively.

Infectious complications after SPKT

We recorded 529 clinically relevant infections during the first year after transplantation and 138 SPKT recipients (90.8%) experienced at least one infection. The majority, 118 patients (78%) were treated for cystitis, 34 (22%) had at least one episode of pyelonephritis and 20 episodes of urosepsis were recorded in 19 patients (13%). Wound infections were registered in 48 patients (32%) and 46 patients were treated for CMV infection (30%). The distribution of infectious episodes during the first year after SPKT is shown in table 2. Women experienced significantly more urinary tract infections than men (4.4 vs 2.9 episodes per patient, P = 0.001). *Escherichia coli* was the most frequent cause of urinary tract infections (33%) followed by *Enterococcus faecalis* (20%) and *coagulase negative staphylococci* (15%). In 7 cases of bacteremia no definite focus could be identified.

Table 1. Patient characteristics

	Mean / N	Range / %
Sex		
Male	94	61.8
Female	58	38.2
Age (years)	40.8[a]	24-58
MBL level pre-Tx (ng/mL)†	904†	28-5153
CMV status*		
D+/R+	30	19.7
D+/R-	36	23.7
D-/R+	29	19.1
D-/R-	57	37.5
Prophylaxis		
Cotrimoxazol	98	64.5
Viral	113	74.3
Immunosuppression▪		
Azathioprine	49	32.2
Mycophenolate Mofetil	103	67.8
Enteric pancreas drainage	8	5.1
Rejections per patient	1.39[a]	0-4
Pancreas failure#,°	11	7.2
Kidney failure#,°	9	5.9
Patient survival°	145	95.3

† Non-Gaussian distribution, median is given, * IgG serology, ▪ Immunosuppression consisted of triple therapy with prednison, cyclosporin A and either azathioprine or mycophenolate mofetil # Death censored, ° Organ faillure and patient survival in 1st year after transplantation, N, number; MBL, mannose-binding lectin; D, donor; R, recipient; +, positive; -, negative

Of the 8 patients with primary enteric drainage, 2 suffered from urinary tract infections, of which one developed urosepsis. Two patients developed CMV disease and 3 had a wound infection. Four of these 8 patients had no infections in the first year after transplantation.

Table 2. Localization of infections

	Infections	%	Patients	%
Urinary tract infections	362	68	129	85
Cystitis	306	58	118	78
Pyelonefritis	36	7	34	22
Urosepsis	20	4	19	13
Wound infection	64	12	48	32
CMV	55	10	46	30
Other	47	9	43	28
Total	529	100	139	91

CMV, cytomegalovirus disease

MBL and Infections

The median MBL level in the study population was 904 ng/ml (range 24-5153). We first examined the influence of MBL on infectious complications in steps of 500 ng/ml (table 3). For every 500 ng/mL increase in baseline MBL an odds ratio of 0.84 (P = 0.045) for urinary tract infections, of 0.68 (P= 0.029) for urosepsis and of 0.70 (P = 0.016) for all episodes of bacteremia was detected. With this analysis no significant influence of increasing MBL levels could be detected for the development of cystitis, pyelonephritis, wound infection, or CMV disease.

Figure 1 shows the pre-transplantation MBL concentrations of the patients without UTI (median 1634 ng/mL), with cystitis (median 810 ng/mL), and with urosepsis (median 373 ng/mL). Differences in median baseline MBL were significant between groups (no UTI vs. cystitis, P = 0.013, no UTI vs. urosepsis, P = 0.001).

A cut-off of 400 ng/ml was used to differentiate between high and low MBL levels. This cut-off level had been established in earlier studies and strongly correlates with the presence of MBL polymorphisms [15;16]. Furthermore, in a receiver operator characteristics analysis a cut-off level around 400 ng/ml was determined to have the best possible significance and specificity for predicting infectious complications. Twenty-nine % of the SPKT cohort had MBL levels below this cut-off. Table 3 shows the characteristics and infectious complications in the high and low MBL recipients. The sex and age distribution was similar in both groups. The high and low MBL groups had the same incidence of acute rejection (68.5% vs. 68.2%., P = 0.556). UTI (95.5%

vs. 80.6%, P = 0.023), cystitis (88.6% vs. 73.1%, P = 0.038) and urosepsis (22.7% vs. 8.3%, P = 0.015) were significantly more common in patients with low baseline MBL compared to the high MBL group. Episodes of pyelonephritis were also more frequent in the low MBL group, but this difference did not reach statistical significance (P = 0.074). The total number of UTI per patient was significantly higher in the low MBL group (2.93 vs. 2.16, P = 0.036). Although infectious complications were more common in patients with low baseline MBL, MBL was not associated with increased infection-related mortality. Only 4 patients in the entire cohort died of infections during the first year after transplantation.

Table 3. Change in Risk of Infectious Complications with increasing baseline MBL levels

	P-value	Odds ratio	95% Confidence interval	
			lower	upper
UTI	0.045	0.84	0.70	1.00
Cystitis	0.243	0.91	0.78	1.07
Pyelonephritis	0.507	0.94	0.79	1.13
Urosepsis	0.029	0.68	0.49	0.96
Bacteraemia	0.016	0.70	0.52	0.94
Wound infection	0.204	0.90	0.76	1.06
CMV	0.517	0.95	0.81	1.11
All infections	0.044	0.81	0.66	0.99

Significant p-values are bold and underlined. Odds ratio = change in Odds ratio every 500 ng/mL MBL.
UTI, urinary tract infection; CMV, cytomegalovirus disease

Multivariate analysis of risk factors for development of urosepsis, including sex, age, immunosuppression, PCP prophylaxiswith co-trimoxazol, initial method of exocrine pancreatic drainage and MBL level, indicated that MBL was the only significant risk factor (table 5a). A baseline MBL level below 400 ng/mL was associated with an odds ratio of 3.58 for developing urosepsis (P = 0.016). Interestingly the introduction of cotrimoxazol prophylaxis did not reduce the risk of urosepsis in our cohort. When multivariate analysis was performed primary enteric drainage was associated with an odds ratio of 0.11 for cystitis (P = 0.014). Male recipients had an odds ratio of 0.4 (P = 0.059) for experiencing cystitis and baseline MBL levels below 400 ug/ml were associated with an odds ratio of 2.78 (P = 0.064) (table 5b). Cotrimoxazol prophylaxis did not have a beneficial effect on the prevention of cystitis.

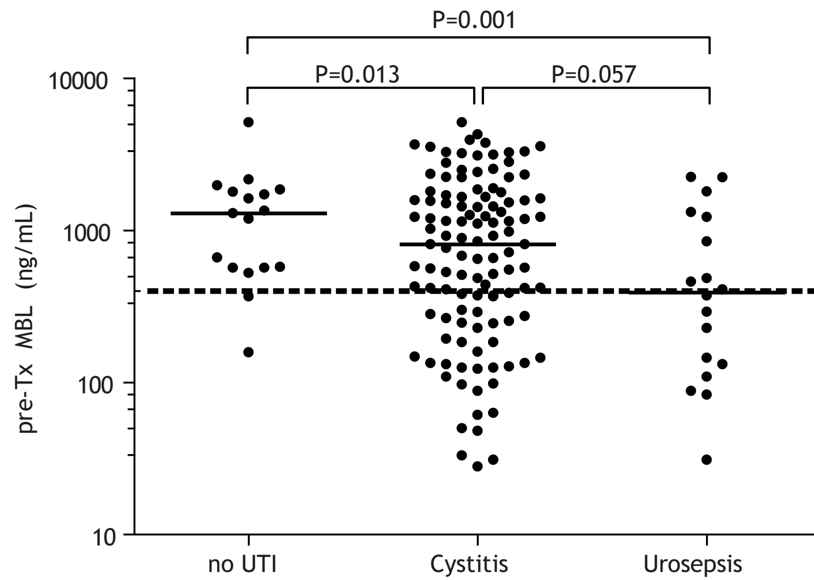


Figure 1. MBL levels in patients without urinary tract infections, with cystitis and with urosepsis. The dotted line indicates the cut-off level of 400 ng/mL and the solid lines indicate the median. MBL levels are represented in a log scale. Pre-Tx MBL, pre-transplantation mannose-binding lectin concentration; UTI, urinary tract infection.

Table 4. Patient characteristics according to MBL level

	Pre-tx MBL (ng/mL)		
	<400	>400	p-value
N	44	108	
Male (%)	63.6	61.1	0.771
Recipient age (years)	41.2	40.6	0.630
Donor age (years)	32.1	30.0	0.269
UTI (%)	95.5	80.6	0.023
Cystitis (%)	88.6	73.1	0.038
Pyelonephritis (%)	31.8	18.5	0.074
Urosepsis (%)	22.7	8.3	0.015
N of UTI per patient	2.93	2.16	0.036
Bacteraemia (%)	25.0	13.0	0.069
CMV (%)	36.4	27.8	0.296
Wound infection (%)	29.5	32.4	0.731
Deep(%)	20.5	23.1	0.718
Superficial(%)	9.1	7.9	0.713
Primair enteric drainage (%)	2.3	6.5	0.439

Significant p-values are bold and underlined. N, number; Pre-tx MBL, pre-transplantation mannose-binding lectin concentration; UTI, urinary tract infection; CMV, cytomegalovirus disease

Table 5. Multivariate analysis of risk factors for experiencing urosepsis (A) and cystitis (B)

	P-value	Odds ratio	95% Confidence interval	
			lower	upper
A) Urosepsis				
Male	0.398	1.63	0.53	5.04
Patient age	0.605	0.98	0.92	1.05
Azathioprine	0.910	1.09	0.26	4.46
Cotrimoxazol prophylaxis	0.636	0.72	0.18	2.82
Enteric pancreas drainage	0.829	1.30	0.12	14.46
MBL <400 ng/mL	0.016	3.58	1.27	10.04
B) Cystitis				
Male	0.059	0.40	0.16	1.03
Patient age	0.487	1.02	0.96	1.08
Azathioprine	0.289	1.88	0.59	6.00
Cotrimoxazol prophylaxis	0.750	1.19	0.40	3.54
Enteric pancreas drainage	0.014	0.11	0.02	0.64
MBL <400 ng/mL	0.064	2.78	0.94	8.18

Significant p-values are bold and underlined. MBL, mannose-binding lectin

Discussion

Our previous study on the role of the lectin pathway in SPKT showed that low MBL levels were associated with superior organ and graft survival [5]. We wondered whether this survival advantage was associated with the disadvantage of more infectious complications. In the current study, we now show that low MBL levels indeed are associated with an increased risk of infections after SPKT. Transplant recipients with MBL levels below 400 ng/ml had a higher risk of bacterial cystitis and more episodes of urosepsis as compared to patients with MBL levels above 400 ng/ml. In the multivariate analysis MBL deficiency was the only identifiable risk factor for urosepsis.

The innate immune system is of particular importance in patients with a suppressed adaptive immune system. MBL binds to a broad range of microorganisms [17], including S*taphylococcus aureus*, certain *E. coli* strains and *Candida* species. Binding of MBL may lead to activation of the lectin pathway of complement activation and enhanced phagocytosis [18). Although MBL deficiency does not seem to be a major risk factor for infections in the general population [19], immunosuppressed patients depend more on the lectin pathway for antimicrobial defense as has been shown in patients treated with chemotherapy [10] or hematopoietic stem cell transplantation [11].

Solid organ transplantation is associated with an especially high risk for infectious complications. Liver transplantation is an example of this high risk of infections, resulting in significant morbidity and mortality. We recently reported that MBL deficiency after liver transplantation is associated with a high risk of clinically significant infections consisting of sepsis, peritonitis and pneumonia [12]. Similar to liver transplantation, SPKT is also associated with a high rate of infectious complications [2]. In our study population 138 patients (90.8%) suffered from at least one clinical significant infection in their first year after transplantation, in spite of the given antibiotic prophylaxis. Especially UTI are a major problem, since 85% of the patients experienced at least one episode and 59% experienced recurrent UTI. This high rate of UTI is probably related to pre-existing bladder dysfunction, the use of catheters and in particular the metabolic and anatomic consequences of the exocrine drainage of the pancreas to the bladder next to the intense immunosuppression. Although only 8 patients in our cohort had primary enteric drainage of the pancreas, a significant protective effect against cystitis was detected when compared with primary bladder drainage.

Interestingly, low MBL levels were not only associated with urosepsis but also with an increased occurrence of uncomplicated cystitis. We are not aware of any studies examining the role of MBL in cystitis. At this point we do not know whether MBL enters the urinary tract via the kidney or whether it is possibly produced by urothelial cells. In a preliminary study we were able to detect low levels of MBL in urine during urinary tract infections in transplant recipients (unpublished observations).

Different cut-off values for serum MBL levels have been used in several studies to define MBL-deficiency. However it is likely that the physiologically relevant MBL level resulting in clinical manifestations differs in different diseases. Genotyping has also been used. The drawback of this classification is that individuals with identical genotypes for all known variants may differ up to 10-fold in MBL levels [20]. From analysis with different cut-off levels of MBL, it appeared that 400 ng/

mL was the most optimal cut-off level in our patient group of SPKT patients. This cut-off was found earlier to clearly distinguish between wildtype MBL individuals and those with a polymorphism [16]. On the other hand we did also find a continuous effect when MBL was studied in steps of 500 ng/ml. This indicates that higher MBL levels are associated with increasing antibacterial protection, most likely also in a range of MBL concentrations that are subject to regulation of expression by promoter polymorphisms, rather than associated with genetically-based deficiency.

A recent study demonstrated that MBL levels determined under baseline conditions are highly predictive of MBL levels during the acute phase response after surgery [21], justifying the use of pre-transplant sera in our study. Due to lack of international standardization it must be noted that the used cut-off level of 400 ng/ml refers to our well established in house ELISA [16] and the cut-off levels may be somewhat different in other test systems using other MBL-detecting antibodies.

Our study demonstrates increased susceptibility to urinary tract infections after SPKT in patients with low MBL levels (<400 ng/mL) compared to patients with high MBL levels. A recently published study investigated MBL polymorphisms in non-transplanted females with pyelonephritis due to *Escherichia coli* [22]. MBL polymorphisms associated with low levels of MBL were not more frequent in women with pyelonephritis when compared with a control population and were not associated with an increased risk of bacteremia. The discrepancies between our study and these findings are possibly explained by the specific importance of MBL in the setting of immunosuppression after SPKT and by the lack of sensitivity when using genotyping instead of determination of MBL concentrations to identify low MBL individuals. Cereva et al. studied *MBL2*, MBL-associated serine protease-2 (*MASP2*) and Toll-like receptor 4 (*TLR4*) gene mutations in a cohort of 33 SPKT recipients and 203 recipients of kidney transplants alone [23]. The presence of gene mutations was not associated with increased bacterial infections in this cohort. Again, the discrepancy with our findings may be explained by the use of genotyping instead of phenotypic characterization. Additionally, only small proportion of the patients in this study had a combined pancreas-kidney transplantation. MBL related effects may have been missed in this patient group with its specific risk of infectious complications.

We did not detect an effect of MBL on other types of infections, including wound infections and CMV (re)activation. Manuel et al. reported an increased risk for the development of CMV infections in a small group of high risk renal transplant recipients with MBL levels below 500 ng/ml [22]. The difference between the two studies may be explained by the patient selection, different prophylactic and immunosuppressive protocols and different methods of CMV detection (e.g. pp65 versus PCR).

Conclusion

Although MBL deficiency is favorable for patient and graft survival following SPKT, we now show that MBL deficiency is associated with urinary tract infections and more specifically with an increased incidence of urosepsis after SPKT. These data indicate an important role of the innate immune system in antimicrobial defense in immune compromised transplant recipients. If confirmed, pre-transplant MBL levels may support risk stratification prior to SPKT and guide decisions concerning the method of exocrine pancreatic drainage and antimicrobial prophylaxis.

Acknowledgements

We thank Prof. Teizo Fujita (Fuhushima, Japan) for providing the anti-MBL antibody (3E7). We also acknowledge the excellent technical assistance by Nicole Schlagwein.

Reference List

1. Demartines N, Schiesser M, Clavien PA. An evidence-based analysis of simultaneous pancreas-kidney and pancreas transplantation alone. *Am J Transplant* 2005;5: 2688-2697.
2. Smets YF, van der Pijl JW, van Dissel JT, Ringers J, de Fijter JW, Lemkes HH. Infectious disease complications of simultaneous pancreas kidney transplantation. *Nephrol Dial Transplant* 1997;12: 764-771.
3. Pratt JR, Basheer SA, Sacks SH. Local synthesis of complement component C3 regulates acute renal transplant rejection. *Nat Med* 2002;8: 582-587.
4. Brown KM, Kondeatis E, Vaughan RW et al. Influence of donor C3 allotype on late renal-transplantation outcome. *N Engl J Med* 2006;354: 2014-2023.
5. Berger SP, Roos A, Mallat MJ et al. Low Pretransplantation Mannose-Binding Lectin Levels Predict Superior Patient and Graft Survival after Simultaneous Pancreas-Kidney Transplantation. *J Am Soc Nephrol* 2007;18: 2416-2422.
6. Walsh MC, Bourcier T, Takahashi K et al. Mannose-binding lectin is a regulator of inflammation that accompanies myocardial ischemia and reperfusion injury. *J Immunol* 2005;175: 541-546.
7. Moller-Kristensen M, Wang W, Ruseva M et al. Mannan-binding lectin recognizes structures on ischaemic reperfused mouse kidneys and is implicated in tissue injury. *Scand J Immunol* 2005;61: 426-434.
8. Garred P, Pressler T, Madsen HO et al. Association of mannose-binding lectin gene heterogeneity with severity of lung disease and survival in cystic fibrosis. *J Clin Invest* 1999;104: 431-437.
9. Koch A, Melbye M, Sorensen P et al. Acute respiratory tract infections and mannose-binding lectin insufficiency during early childhood. *JAMA* 2001;285: 1316-1321.
10. Peterslund NA, Koch C, Jensenius JC, Thiel S. Association between deficiency of mannose-binding lectin and severe infections after chemotherapy. *Lancet* 2001;358: 637-638.
11. Mullighan CG, Heatley S, Doherty K et al. Mannose-binding lectin gene polymorphisms are associated with major infection following allogeneic hemopoietic stem cell transplantation. *Blood* 2002;99: 3524-3529.
12. Bouwman LH, Roos A, Terpstra OT et al. Mannose binding lectin gene polymorphisms confer a major risk for severe infections after liver transplantation. *Gastroenterology* 2005;129: 408-414.
13. Garner JS, Jarvis WR, Emori TG, Horan TC, Hughes JM. CDC definitions for nosocomial infections, 1988. *Am J Infect Control* 1988;16: 128-140.
14. Roos A, Bouwman LH, Gijlswijk-Janssen DJ, Faber-Krol MC, Stahl GL, Daha MR. Human IgA activates the complement system via the mannan-binding lectin pathway. *J Immunol* 2001;167: 2861-2868.
15. Berger SP, Roos A, Mallat MJ, Fujita T, de Fijter JW, Daha MR. Association between mannose-binding lectin levels and graft survival in kidney transplantation. *Am J Transplant* 2005;5: 1361-1366.
16. Roos A, Garred P, Wildenberg ME et al. Antibody-mediated activation of the classical pathway of complement may compensate for mannose-binding lectin deficiency. *Eur J Immunol* 2004;34: 2589-2598.

17. Neth O, Jack DL, Dodds AW, Holzel H, Klein NJ, Turner MW. Mannose-binding lectin binds to a range of clinically relevant microorganisms and promotes complement deposition. *Infect Immun* 2000;68: 688-693.
18. Kuhlman M, Joiner K, Ezekowitz RA. The human mannose-binding protein functions as an opsonin. *J Exp Med* 1989;169: 1733-1745.
19. Dahl M, Tybjaerg-Hansen A, Schnohr P, Nordestgaard BG. A Population-based Study of Morbidity and Mortality in Mannose-binding Lectin Deficiency. *J Exp Med* 2004;199: 1391-1399.
20. Steffensen R, Thiel S, Varming K, Jersild C, Jensenius JC. Detection of structural gene mutations and promoter polymorphisms in the mannan-binding lectin (MBL) gene by polymerase chain reaction with sequence-specific primers. *J Immunol Methods* 2000;241: 33-42.
21. Van Till JW, Boermeester MA, Modderman PW et al. Variable mannose-binding lectin expression during postoperative acute-phase response. *Surg Infect (Larchmt)* 2006;7: 443-452.
22. Manuel O, Pascual M, Trendelenburg M, Meylan PR. Association between mannose-binding lectin deficiency and cytomegalovirus infection after kidney transplantation. *Transplantation* 2007;83: 359-362.
23. Cervera C, Lozano F, Saval N et al. The influence of innate immunity gene receptors polymorphisms in renal transplant infections. *Transplantation* 2007;83: 1493-1500.

Chapter 6

Properdin binds to late apoptotic and necrotic cells independently of C3b, and regulates alternative pathway complement activation

Wei Xu[1],
Stefan P. Berger[1],
Leendert A. Trouw [2],
Hetty C. de Boer[1,3],
Nicole Schlagwein[1],
Chantal Mutsaers[1],
Mohamed R. Daha[1] and
Cees van Kooten[1]

[1]Department of Nephrology, [2]Department of Rheumatology,
[3]Einthoven Laboratory for Experimental Vascular Medicine,
Leiden University Medical Center, Leiden, the Netherlands

J Immunol 2008; 180(11):7613-7621

Summary

Cells that undergo apoptosis or necrosis are promptly removed by phagocytes. Soluble opsonins such as complement can opsonize dying cells, thereby promoting their removal by phagocytes and modulating the immune response. The pivotal role of the complement system in the handling of dying cell has been demonstrated for the classical pathway (via C1q) and lectin pathway (via MBL and ficolin). Here we report that the only known naturally occurring positive regulator of complement, properdin, binds predominantly to late apoptotic and necrotic cells, but not to early apoptotic cells. This binding occurs independent of C3b, which is additional to the standard model wherein properdin binds to pre-existing clusters of C3b on targets and stabilizes the convertase C3bBb. By binding to late apoptotic or necrotic cells, properdin serves as a focal point for local amplification of alternative pathway complement activation. Furthermore, properdin exhibits a strong interaction with DNA that is exposed on late stage of dying cells. Our data indicate that direct recognition of dying cells by properdin is essential to drive alternative pathway complement activation.

Introduction

Under steady-state conditions, cells that undergo apoptosis and necrosis can be safely and silently eliminated by professional phagocytes, i.e. immature dendritic cells (DCs) and macrophages (Mφ) [1-3]. Apoptotic cells are a rich source of autoantigens, which are involved in the induction of self-tolerance and autoimmunity [4]. Compelling evidence has emerged that abnormal clearance of apoptotic cells is associated with development of the autoimmune disease systemic lupus erythematosus (SLE) [5;6].

Soluble factors from the innate immune system such as complement or pentraxins can opsonize apoptotic cells, thereby promoting their removal by phagocytes [6-8]. In humans, homozygous deficiency of any of the early components of the classical pathway of complement activation (C1q, C1r, C1s, C4, and C2) predisposes to the development of SLE [9], suggesting that complement is involved in removal of dying cells and the immune regulation associated with this process. Complement-mediated clearance of apoptotic cells has been well documented both *in vitro* [10] and *in vivo* [11]. Nevertheless, the role of the complement system in the handling of dying cells has been mostly linked to the classical pathway (via C1q) and lectin pathway (via MBL and ficolin) [10-14]. It was suggested that the main product of complement activation, iC3b, facilitates the removal of dead material and mediates peripheral tolerance [10;15;16].

The alternative pathway of complement is thought to be activated following hydrolysis of C3, generation of C3b and formation of a positive feedback loop to mount a rapid local response [17]. The alternative pathway was initially recognized to amplify complement activation triggered by classical pathway. Properdin, discovered in 1954 [18], is the only known naturally occurring positive regulator of complement activation [19]. It was originally shown that properdin binds to C3b and increases the stability of the alternative pathway convertases at least 10-fold on target surfaces and immune complexes [20]. It has been recently suggested that properdin could bind directly to microbial targets [21], which is consistent with a proposal made more than 50 years ago [18].

In the present study, we investigated whether properdin, like C1q and MBL, contributes to the recognition and opsonization of dying cells. We found that properdin binds predominantly to late apoptotic and necrotic cells independent of C3b, but not to early apoptotic cells, leading to alternative pathway-mediated complement activation. DNA was identified as one of the ligands on dying cells to which properdin binds. This accounts for a C3b independent mechanism of properdin-initiated complement activation on dying cells.

Material and methods

Induction of apoptosis and necrosis

Jurkat cells were cultured in RPMI culture medium. Early or late apoptosis was induced in Jurkat cells by exposure to ultra violet (UV)-C light (Philips TUV lamp, predominantly 254 nm) at a dose of 50 J/m^2, followed by 3 or 30 hours culture in RPMI serum-free medium, respectively. Necrosis was induced by treating cells at 56°C for 0.5 h or 5 cycles of freeze-thaw from -80°C to 36°C. Both apoptosis and necrosis were confirmed by double staining with fluorescein isothiocyanate (FITC)-labeled annexin V and propidium iodide (PI, VPS Diagnostics, Hoeven, the Netherlands) according to established methods [22]. In addition, light microscopy and fluorescent microscopy (Leica DC300F, Leica, Rijswijk, the Netherlands) were performed to detect the morphology and Hoechst nuclear staining of these cells, respectively. Alternatively, human umbilical cord endothelial cells (HUVEC), U937 cell (monocytic cell lines), and Epstein-Barr virus-transformed B lymphoblastoid cell lines (EBV-LCLs) were used for the induction of necrosis.

In some experiments, splenocytes were obtained from C3 knockout (C3$^{-/-}$) [23] or C57BL/6 wild type (WT) mice (Harlan). Splenocytes was rendered necrotic by incubating them at 56°C, as described above.

Phagocytosis assay

Phagocytosis of early apoptotic, late apoptotic and necrotic cells was assessed by using a protocol described previously [24]. Briefly, a subset of macrophages (Mφ2) were generated from CD14$^+$ monocytes in RPMI culture medium (RPMI 1640 containing 10% heat-inactivated FCS, 90 U/ml penicillin and 90 µg/ml streptomycin) (all from Gibco/Life technologies, Breda, the Netherlands) in the presence of 5 ng/ml M-CSF (R&D systems / ITK Diagnostics, Uithoorn, the Netherlands) for 6 days. Jurkat cells were used as the target for phagocytosis. Prior to the induction of apoptosis or necrosis, Jurkat cells were fluorescently labeled with carboxyfluorescein diacetate succinamidyl ester (CFSE, Molecular Probes, Leiden, the Netherlands). Labeled early, late apoptotic or necrotic cells (1×10^5) were investigated with or without opsonization using normal human serum (NHS). Dying cells were co-cultured with Mφ2 in 1:1 ratio at 37°C for 0.5 h in 100 µl RPMI culture medium in round-bottom glass tubes. As a control, co-culture was performed at 4°C to detect the binding of dying cells to phagocytes. *M*φ2 were stained with a PE-conjugated mAb against CD11b (BD Biosciences, San Jose, CA) and uptake was analyzed by a two-color flow cytometry.

The percentage of CD11b-positive cells that stained positive for CFSE was used as a measure for the percentage of Mφ2 that ingested and/or bound apoptotic cells.

Isolation of properdin, C1q and MBL, C3 and C3b

Properdin was isolated from pooled human serum from volunteer donors. Serum was first precipitated by dialysis against 5 mM EDTA (pH 6.0). The precipitate was dissolved in Veronal-buffered saline (2×VBS, 1.8 mM Na-5,5-diethylbarbital, 0.2 mM 5,5-diethylbarbituric acid, 145 mM NaCl), and then dialyzed against 0.01 M NaAc containing 2 mM EDTA (pH 6.0) and applied to a Sulphopropyl C50 column. Properdin was eluted with a linear salt gradient. Properdin-containing fractions, as determined by ELISA, were pooled, concentrated, and subsequently applied to a Sephacryl S-300 gel filtration column (Pharmacia Biotech, Uppsala, Sweden). Fractions containing properdin were dialyzed against PBS, 2 mM EDTA and further purified using human IgG coupled to a Biogel A5 (Bio-Rad, Hercules, CA) to remove contaminating C1q. Purity of the properdin preparation was determined by analysis on 10% SDS-PAGE gel. A single 220-kD band was observed. C1q and MBL were purified from pooled human plasma obtained from healthy donors as described previously [25;26]. C3 was purified from serum using different steps of chromatography, whereas C3b was generated by brief trypsin cleavage (60 seconds) of purified C3 followed by direct inactivation. The purity of C3 and C3b was determined by SDS-PAGE gel.

Serum

C4-depleted serum (C4ds) was used as a complement source lacking both classical and lectin pathway activity, and was prepared as following: Blood was obtained by venapuncture, allowed to clot at room temperature for 1 hour and then centrifuged. The serum was brought to a NaCl concentration of 0.3M by addition of NaCl and then mixed with an immunoabsorbent of rabbit IgG anti-human C4. Coupling of rabbit IgG anti-C4 to Sepharose was performed according to manufacturers' instructions (Amarsham Biosciences, Roosendaal, the Netherlands). Following absorption by gentle mixing for 30 minutes at 4ºC, the mixture was centrifuged at 1000g and the supernatant aliquotted and frozen at -80ºC. The C4 depleted serum had no detectable complement activity at a dilution of 1/5 in a hemolytic test using sheep erythrocytes sensitized with rabbit anti-SRBC, while the starting serum induced 1 unit of C-activity at a dilution of 1/240. Additionally, C4 hemolytic activity could be restored in 1/25 diluted C4-deficient serum with 10 ug/ml purified C4.

Properdin-depleted serum (Pds) was obtained by immune absorption using Biogel-coupled monoclonal Ab against human properdin (a gift of Statens Serum Institut, Copenhagen, Denmark). Pds showed normal classical and lectin pathway activities in hemolytic assays.

C3 deficient serum was (C3-def) obtained from a patient who was deficient for C3 while containing normal properdin concentration (17.5 ug/ml). In C3-def, the C3 level was below detection limit as measured by ELISA (data not shown).

Normal human sera from 9 healthy donors were used as C3 full sera as confirmed by C3 ELISA. The properdin concentration in those C3 full sera was ranging from 10.4 to 25.1 ug/ml.

Binding assay for properdin, C1q and MBL

Binding of properdin to viable, early apoptotic, late apoptotic or necrotic cells was investigated by incubating cells with up to 40 μg/ml human purified properdin at 37^oC for 1 h in serum-free RPMI culture medium. We used serum-free medium as a standard buffer to exclude a possible contribution of serum constituents, unless specifically indicated. Then cells were extensively washed and incubated with a rabbit-anti-human properdin polyclonal Ab (generated by immunizing rabbit with purified properdin), and detected with phycoerythrin (PE)-conjugated goat $F(ab)_2$ anti-rabbit Ig (Southern Biotechnology Associates, Birmingham, US). The cells were analyzed by flow cytometry. Data from 10^4 events were acquired. Alternatively, C3 deficient serum or C3 full serum was used as a source of properdin to detect binding of properdin. These sera were diluted in serum-free RPMI medium as 40%. In some experiments, purified C3 or C3b was used to detect its binding to properdin which has been pre-bound on necrotic cells.

Binding of C1q (30 μg/ml) and MBL (10 μg/ml) were performed in the same way as properdin binding and detected with a monoclonal antibody (mAb) directed against C1q (mAb 2204) or MBL (clone 3E7), respectively. Binding was visualized with phycoerythrin (PE)-conjugated goat $F(ab)_2$ anti-mouse Ig (DAKO, Glossstrup, Denmark).

In some experiments, cells were pre-incubated with C1q or7 MBL, followed by incubation of properdin and *vice versa*.

To detect the binding of properdin to DNA, double stranded DNA (dsDNA) from calf thymus (Sigma-Aldrich), single stranded DNA (ssDNA, Isogen, Maarssen, the Netherlands) or human albumin (Sigma-Aldrich) were coated in PBS on microtiter plates overnight, and then blocked with 2%BSA before adding purified properdin.

After washing, bound properdin was detected with Dig-labelled rabbit-anti-human properdin. Bound antibody was developed with anti-Dig-HRP (Roche Diagnostics GmbH, Mannheim, Germany), and measured for absorbance at OD 451 nm.

To confirm that DNA is exposed on late apoptotic cells and necrotic cells, a monoclonal mouse anti-human dsDNA Ab (ImmunoTools, Friesoythe, Germany) was used to detect DNA and its binding assessed with a PE-conjugated goat $F(ab)_2$ anti-mouse Ig. For double staining, late apoptotic cells and necrotic cells were opsonized with properdin at 37ºC for 1 h in serum-free RPMI culture medium. Cells were then incubated with a rabbit-anti-human properdin Ab and a mouse anti-human dsDNA Ab, and developed by PE-conjugated goat $F(ab)_2$ anti-rabbit Ig and FITC- conjugated goat $F(ab)_2$ anti-mouse Ig (BD Biosciences). For confocal microscopy analysis, primary Abs were visualized by Alexa 568 or Alexa 488-labelled secondary Abs. Above cells were followed by a Hoechst nuclear staining prior to fixation by 1% paraformaldyde, and then mounted onto the slides for analysis by a confocal laser scanning microscope LSM 510 (Carl Zeiss AG), as described previously [24]. Images were visualized using a 63 × /1.40 numeric aperture oil objective, and were processed using Zeiss LSM Image Examiner software.

Complement activation by dying cells

Activation of complement by dying cells was assessed as follows: early, late apoptotic or necrotic cells were pre-incubated with or without properdin (20 ug/ml) at 37ºC for 1 h in serum-free RPMI culture medium, washed extensively and then exposed to different dilutions of Pds, C4ds or NHS for 0.5 h at 37ºC. Deposition of C3, C4 and C5b-9 on the cell surfaces were detected by flow cytometry using mAbs against C3 (RFK22, [27]), C4 (anti-C4-4 [28]) and C5b-9 (AE11, kindly provided by Dr. T.E. Mollnes, Nordland Central Hospital, Bodø, Norway), respectively.

Statistical analysis

Statistical analysis was performed by one sample *t* test using GraphPad Prism (GraphPad software, San Diego, CA). Differences were considered statistically significant when p values were less than 0.05.

Results

Complement-mediated phagocytosis of late apoptotic and necrotic cells

Relatively pure populations of viable (90-98%), early apoptotic (40-70%), late apoptotic (90-100%) and necrotic cells (100%) were obtained, based on the annexin V and PI staining (Figure 1A). The difference between late apoptotic cells and necrotic cells was confirmed by light microscopy and Hoechst nuclear staining (Figure 1B), demonstrating that late apoptotic cells showed blebbing on the cell surfaces and nuclear segmentation, whereas necrotic cells showed condensed nuclei.

In agreement with our earlier findings, M-CSF-driven anti-inflammatory Mφ2 preferentially recognized and ingested early apoptotic cells, as compared to the ingestion of late apoptotic and necrotic cells [24] (Figure 1C). However, opsonization of early apoptotic cells with normal human serum (NHS) did not enhance their uptake by Mφ2 (Figure 1C, D), while opsonization of late apoptotic and necrotic cells with NHS significantly increased their uptake by Mφ2 ($p < 0.01$) (Figure 1C, D). Enhancement of phagocytosis by NHS was also restricted to late apoptotic and necrotic cells when using monocyte-derived dendritic cells and GM-CSF-driven Mφ1 (data not shown). We next questioned whether the observed enhanced phagocytosis is associated with complement deposition on the dying cells. Indeed, NHS-exposed late apoptotic and necrotic cells, but not early apoptotic cells, displayed strong deposition of C3 by flow cytometry (Figure 1E). Therefore we assessed the pathways involved in the activation of complement on these cells.

Properdin binds to late apoptotic and necrotic cells

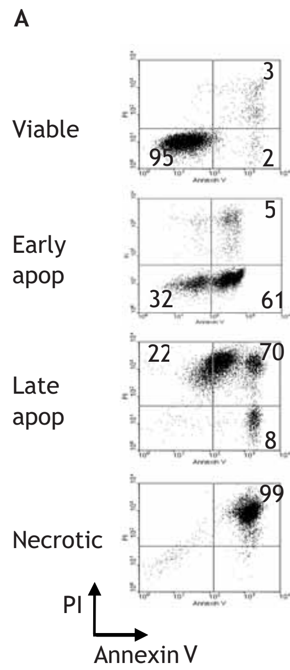
We investigated whether properdin, the only naturally occurring positive complement regulator, can bind directly to dying cells that are at different stages of cell death. Similar to C1q, and MBL, properdin showed a predominant interaction with late apoptotic cells and necrotic cells over early apoptotic or viable cells (Figure 2A, B). Properdin was shown to bind to both late apoptotic and necrotic cells in a dose-dependent manner (Figure 2C). To rule out the possibility that the observed binding of properdin is cell type or method specific, different cell lines and methods for induction of necrosis were used. Properdin was shown to bind to necrotic HUVEC, U937, HK-2 and EBV-LCL cells, and also to Jurkat cells that were rendered necrotic by 5 cycles of freeze-thawing (data not shown), suggesting that binding of properdin to necrotic cells is a universal phenomenon and irrespective of specific cell types.

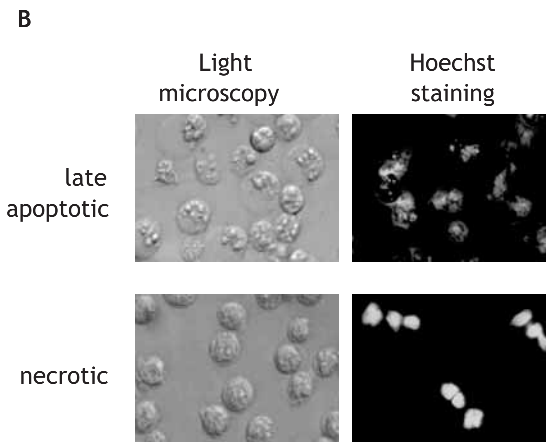
Binding of properdin to dying cells can occur independent of C3

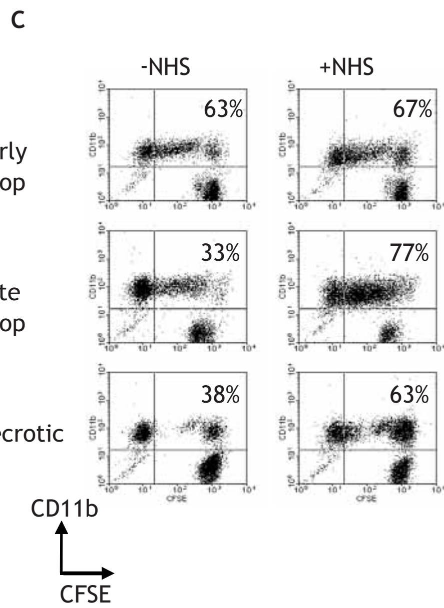
It has been established for a long time that properdin can bind to pre-existing clusters of surface-bound C3b [29], thereby stabilizing the C3b-dependent C3 convertase C3bBb [20]. However, the experiments presented above were performed in the absence of serum, suggesting that binding of properdin to dying cells occurs independent of C3b. This seems to be consistent with a recent publication showing that properdin binds directly to bacterial targets [30]. To exclude the possibility for endogenous generation of C3b by dying Jurkat cells, we investigated whether properdin could bind to necrotic splenocytes derived from C3 knockout ($C3^{-/-}$) mice. Properdin was shown to bind to necrotic splenocytes of $C3^{-/-}$mice to a similar extent as binding to necrotic cells from WT mice (Figure 3A). Properdin did not bind to viable splenocytes derived from either $C3^{-/-}$ or WT mice (Figure 3A).

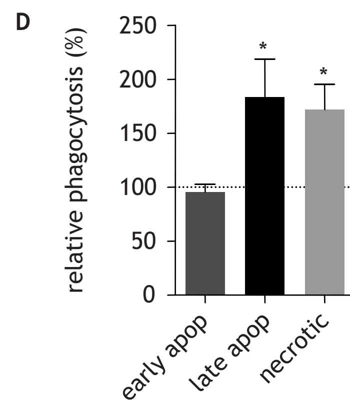
In addition, to confirm C3 independence, we also used C3 deficient serum in EDTA containing medium (obtained from a C3 deficient patients with a normal concentration of properdin) as a source of properdin to opsonize necrotic cells. We found similar binding of properdin to necrotic cells in C3 deficient serum as compared to C3 full serum (NHS) and purified properdin (Figure 3B), although the extent of the binding is low since the concentration of properdin in these sera was around 7ug/ml. In the absence of EDTA, C3 full sera showed much higher binding of properdin, indicating that complement activation amplifies the properdin binding. Together these data suggest that binding of properdin to dying cells can occur independently of C3.

We next investigated whether purified C3 or C3b can bind to properdin bound on the surface of dying cells. We first opsonized necrotic cells with properdin to allow sufficient binding of properdin on the surface of necrotic cells. Next increasing concentrations of C3 and C3b were added, and binding was detected with a monoclonal antibody recognizing both C3 and C3b. Under these conditions, C3 or C3b did not bind to non-opsonized necrotic cells, showing specificity for the interaction with properdin (data not shown). Binding to properdin was almost exclusive for C3b, and only minor interaction with intact C3 was seen at the highest concentration (Figure 3C). Although preparations were pure as based on SDS-PAGE analysis (data not shown), small contamination with C3b cannot be excluded. Together, these data show that properdin binds C3b instead of intact C3 and suggest that local generation of C3b is a prerequisite for the focal properdin-driven complement activation / amplification.


A
Viable
3
95
2
Early apop
5
32
61
Late apop
22
70
8
Necrotic
99
PI
Annexin V


B
Light microscopy
Hoechst staining
late apoptotic
necrotic


C
-NHS
+NHS
Early apop
63%
67%
Late apop
33%
77%
Necrotic
38%
63%
CD11b
CFSE


D
relative phagocytosis (%)
250
200
150
100
50
0
early apop
late apop
necrotic

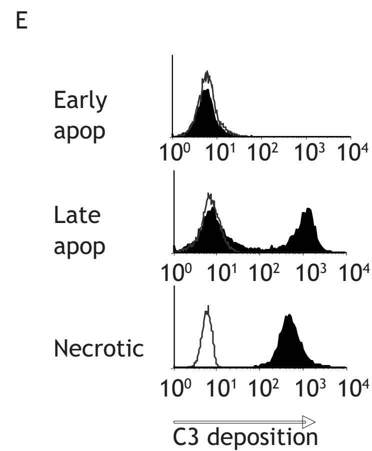

E
Early apop
Late apop
Necrotic
C3 deposition

Figure 1. Complement-mediated phagocytosis of dying cells. (**A**) Early or late apoptosis was induced in Jurkat cells by exposure to ultra violet (UV)-C light at a dose of 50 J/m^2, followed by 3 or 30 hours culture in RPMI serum-free medium, respectively. Necrosis was induced by treating cells at 56°C for 0.5 h. Cells were stained with annexin V and PI by flow cytometry. (**B**) Late apoptotic cells and necrotic cells are scored by light microscopy or fluorescent microscopy for hoechst staining on cytospins of these cells. Magnification, 200×. (**C**) CFSE-labeled early apoptotic, late apoptotic or necrotic cells (1×10^5) were first opsonized with or without normal human serum (NHS), then co-cultured with Mφ2 in 1:1 ratio at 37°C for 0.5 h. *M*φ2 were stained with a PE-conjugated mAb against CD11b and uptake was analyzed by a two-color flow cytometry. $CD11b^+CFSE^+$ cells were used as a measure for the percentage of *M*φ2 that ingested apoptotic cells. (**D**) Relative phagocytosis was calculated as uptake of NHS-opsonized dying cells versus non-opsonized cells. Data are mean ±SEM of 3 independent experiments. *, $p < 0.01$, one sample paired *t* test. (**E**) C3 deposition (filled histogram) after NHS opsonization on early, late apoptotic and necrotic cells was detected by flow cytometry. Open histograms are the matched isotype controls (see page 154 for color image B).

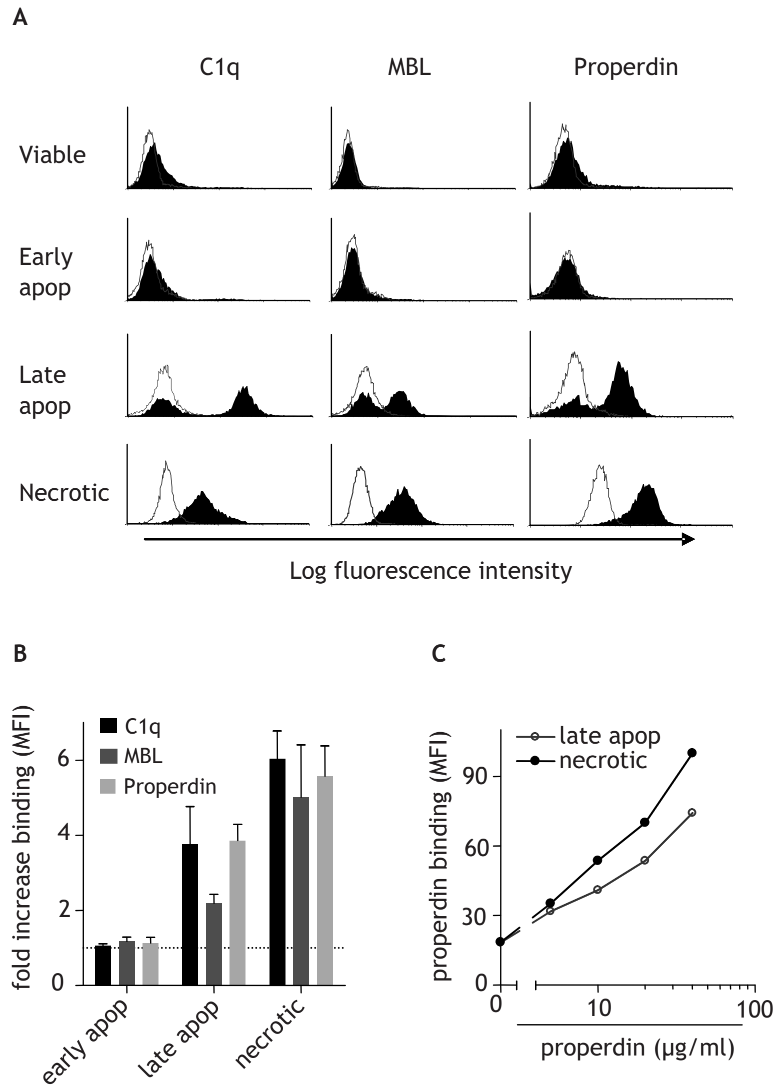


Figure 2. Properdin binds to late apoptotic and necrotic cells. (**A**) Viable, early apoptotic, late apoptotic and necrotic cells were collected and incubated with C1q, MBL or properdin. Specific binding (filled histogram) of C1q, MBL and properdin were detected by mAb against C1q (clone 2204), MBL (clone 3E7) and rabbit-anti-human properdin. Open histograms are the matched isotype controls. (**B**) Fold increase of binding was calculated as the mean fluorescence intensity (MFI) of C1q, MBL, or properdin divided by the MFI of matched isotype controls. Data shown are mean ±SEM of at least 4 independent experiments. (**C**) Late apoptotic and necrotic cells were incubated with increasing concentration of properdin (up to 40 μg/ml), and detected for properdin binding. Data shown are MFI.

To test the physiological relevance of properdin binding to dying cells, we used sera from nine healthy donors (properdin concentration ranges from 10.4 to 25.1 ug/ml). All sera tested showed predominant binding to late apoptotic cells and necrotic cells, but not to early apoptotic or viable Jurkat cells (Figure 3D).

Properdin is a focal point for amplification of alternative pathway complement on dying cells

To investigate whether binding of properdin to dying cells might act as a focal point for local amplification of the complement system, we analyzed complement activation on necrotic cells using properdin-depleted serum (Pds). Cells pre-incubated with purified properdin alone, as expected, did not show C3 and C5b-9 deposition (Figure 4A). In Pds, a reduced C3 deposition was observed, which is accompanied with a lack of deposition of the membrane attack complex C5b-9 (Figure 4A). Necrotic cells that had been pre-exposed to properdin, washed extensively, and subsequently incubated with Pds, displayed significantly increased C3 and C5b-9 deposition (Figure 4A), suggesting that properdin is essential for local amplification of the complement cascade on necrotic cells.

To prove that cell-bound properdin can activate complement independently of the classical and lectin pathways, we used C4-depleted serum (C4ds) as a source of complement, since C4 is a crucial factor for both pathways. Exposure of both late apoptotic cells (Figure 4B) and necrotic cells (Figure 4C) that had been pre-incubated with properdin to C4ds significantly induced the deposition of C5b-9 in a dose-dependent manner, as compared with those without properdin. There was no C4 deposition neither on late apoptotic (Figure 4B) nor on necrotic cells (Figure 4C) after opsonization of C4ds, confirming that C4 had been effectively depleted in our C4ds preparation. When a fixed amount (30%) of C4ds was used, the increase of C5b-9 on the cell surface was dose dependently affected by the amount of properdin (Figure 4D). Overall, this shows that properdin is a rate limiting factor that mediates complement activation at the surface of late apoptotic and necrotic cells via alternative pathway activation.

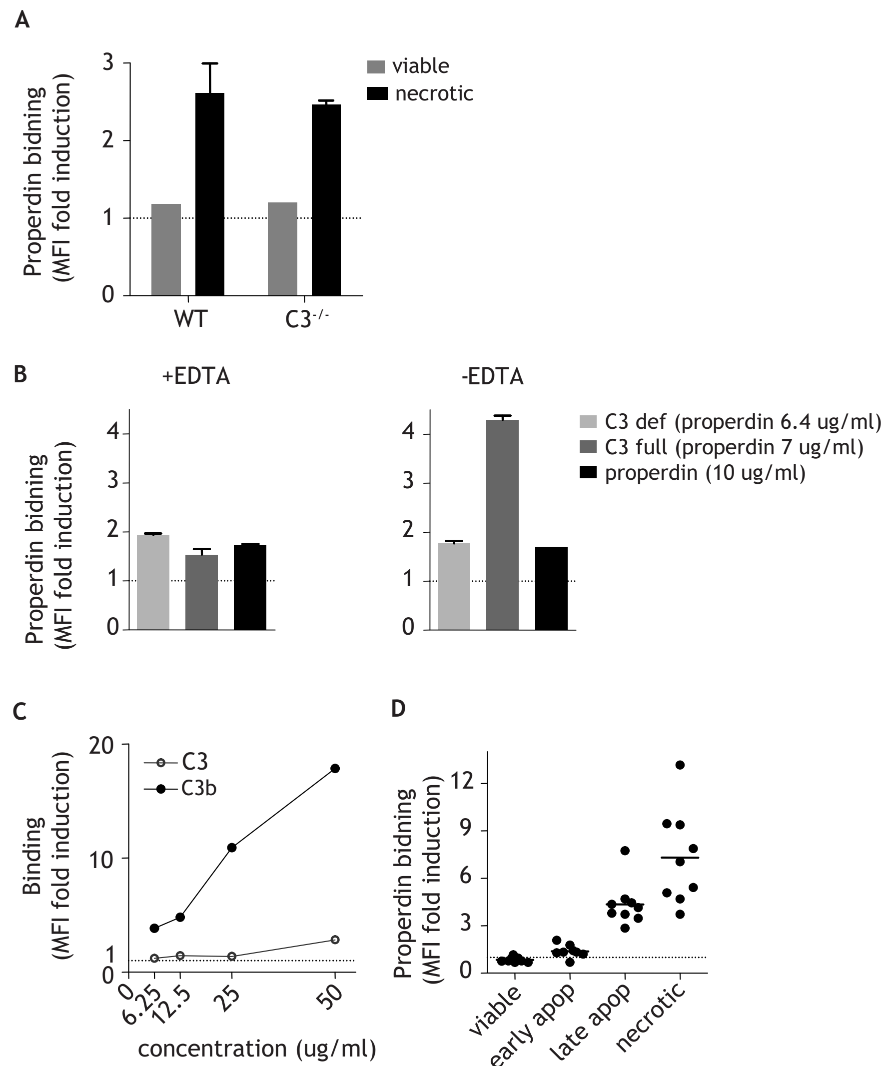


Figure 3. Binding of properdin to dying cells is independent of C3b. (**A**) Splenocytes from $C3^{-/-}$ or WT mice were first rendered necrotic by heating or kept viable, and measured for properdin binding. Fold increase of MFI was shown. Data shown are mean ±SEM of 4 independent experiments. (**B**) Necrotic cells were opsonized with C3 full serum (normal human serum from a healthy donor, properdin concentration was 16 ug/ml), or C3 deficient serum (obtained from a patient who was deficient for C3, properdin concentration was 17.5 ug/ml) for 1 hour at 37°C in the presence or absence of 10 mM EDTA. 40% of the serum (diluted in serum-free RPMI medium) was used, thus the final concentration of

properdin presented in the opsonization was 6.4 ug/ml (C3-full serum) and 7 ug/ml (C3-def serum), respectively. As a control, purified properdin (10 ug/ml) was used for the binding assay. Binding of properdin was detected with a polyclonal rabbit anti-human properdin antibody and developed by a goat anti-rabbit Ab. Fold induction of MFI in flow cytometry is shown. Data are mean ±SD of two experiments. (**C**) Necrotic cells were first opsonized with properdin (30ug/ml) for 1 hour at 37°C to allow sufficient binding of properdin on the surface of necrotic cells. Next increasing concentrations of C3 and C3b were added (up to 50 ug/ml), and detected with a monoclonal antibody (RFK22) recognizing both C3 and C3b. Fold induction of MFI in flow cytometry is shown. Data are representative of three independent experiments. (**D**) Sera from 9 healthy donors (properdin concentration ranges from 10.4 to 25.1 ug/ml), diluted as 40% in RPMI serum-free medium, were used to opsonize viable, early apoptotic, late apoptotic or necrotic cells. Properdin binding (MFI fold induction) is shown.

Properdin does not compete with binding of C1q and MBL to necrotic cells

We showed previously that C1q and MBL share binding ligands on apoptotic cells [31]. Since properdin was shown to bind to late apoptotic and necrotic cells in a similar pattern as C1q and MBL (Figure 1A), we hypothesized that properdin may bind to a similar structure on dying cells. Necrotic cells were pre-incubated with properdin, followed by incubation with increasing concentrations of C1q. A dose-dependent binding of C1q was observed, but pre-incubation with properdin did not inhibit the binding of C1q to the cells (Figure 5A). In a reverse way, pre-incubation of necrotic cells with C1q did not decrease properdin binding either (Figure 5B). Similarly, pre-incubation of necrotic cells with properdin did not interfere with MBL binding and *vice versa* (Figure 5C). Therefore, our data suggest that properdin binds to a yet unknown ligand, which is different from the one to which C1q and MBL bind.

Properdin binds to DNA

One of the autoantigens exposed on apoptotic cells and necrotic cells is DNA [4;32]. Based on the finding that properdin specifically binds to late apoptotic and necrotic cells, we hypothesized that properdin might bind to DNA exposed on the surface of dying cells. Properdin showed a strong binding to both dsDNA and ssDNA at concentrations of 1 μg/ml and higher on microtiter plates (Figure 6A). Furthermore, pre-incubation of properdin with calf thymus dsDNA dose-dependently inhibited binding of properdin to necrotic cells (Figure 6B), suggesting a strong interaction between DNA and properdin.

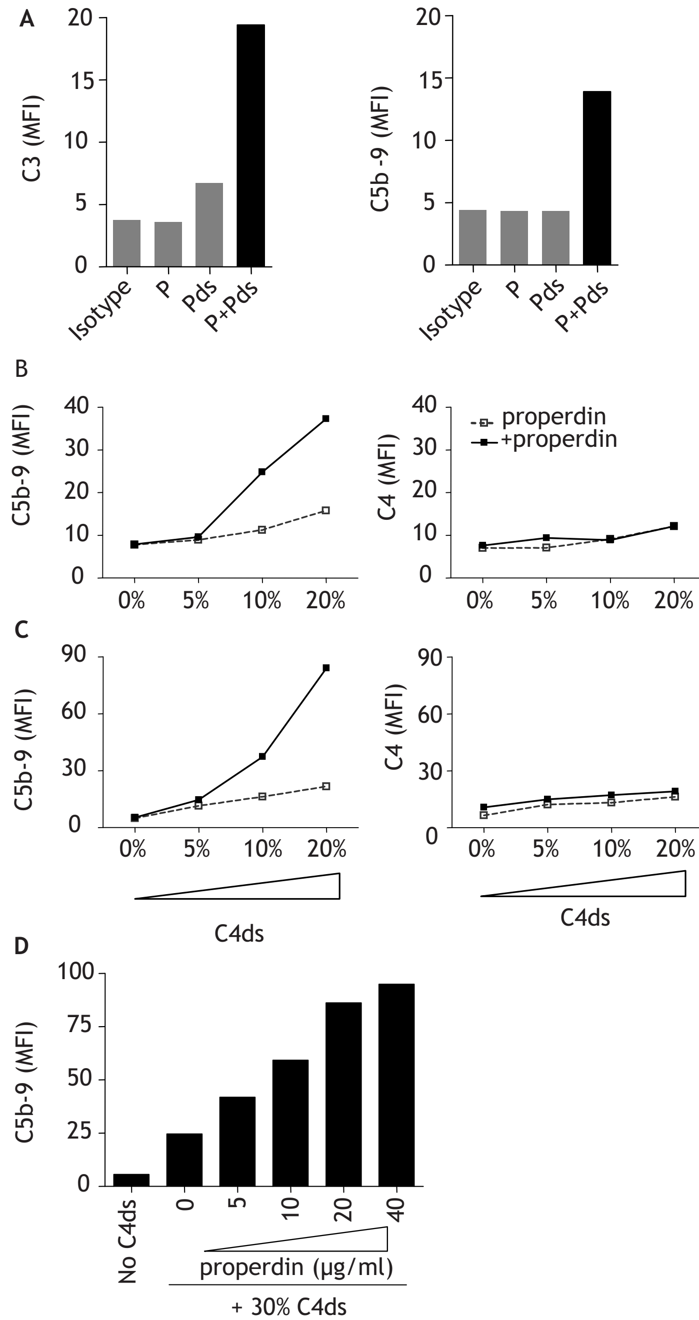


Figure 4. Properdin activates complement via alternative pathway. (**A**) Necrotic Jurkat cells were pre-incubated with or without properdin (20 µg/ml), and then washed extensively before adding 30% properdin-depleted serum (Pds). Data shown are C3 and C5b-9 deposition

on the cells. (**B**) Late apoptotic and (**C**) necrotic cells were pre-incubated with or without properdin, and then washed extensively before adding increasing concentration of C4-depleted serum (C4ds). C5b-9 and C4 deposition on the cells were measured. (**D**) Necrotic cells were pre-incubated with increasing concentration of properdin, and then washed extensively before adding 30% C4ds. Cells were measured for C5b-9 formation. Data are representative of 2 independent experiments.

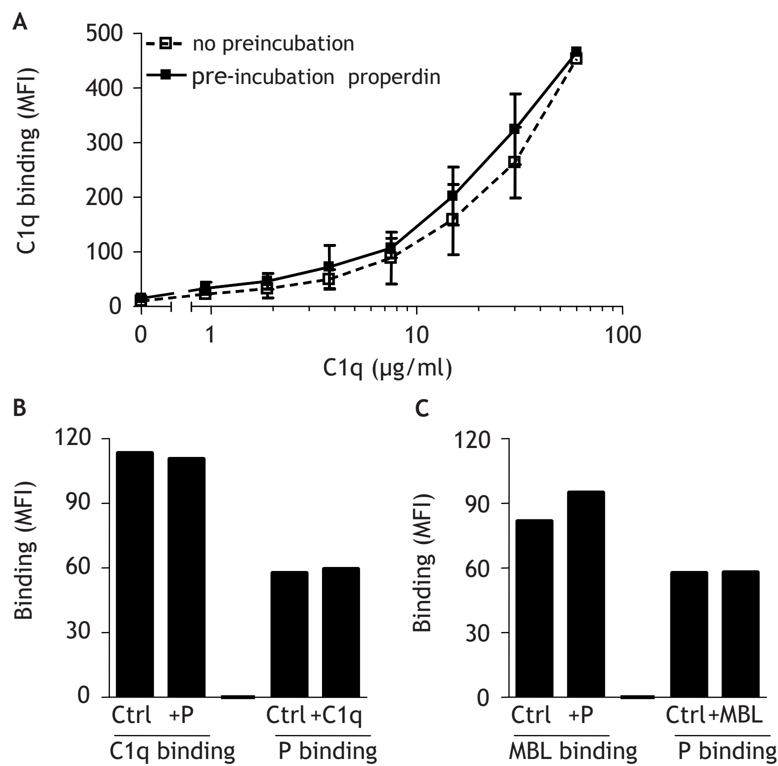


Figure 5. Properdin does not compete for binding with C1q and MBL. (**A**) Necrotic cells were pre-incubated with properdin (40 µg/ml) and followed by incubation with increasing concentrations of C1q (up to 60 µg/ml). C1q binding was measured. Data shown are mean ±SEM of 2 independent experiments. (**B**) Necrotic cells were pre-incubated with properdin (40 µg/ml) or C1q (30 µg/ml), then followed by incubation with C1q (10 µg/ml), or properdin (20 µg/ml), respectively. C1q and properdin binding were measured by flow cytometry. (**C**) Competition between properdin and MBL (10 µg/ml) was investigated as described in (B).

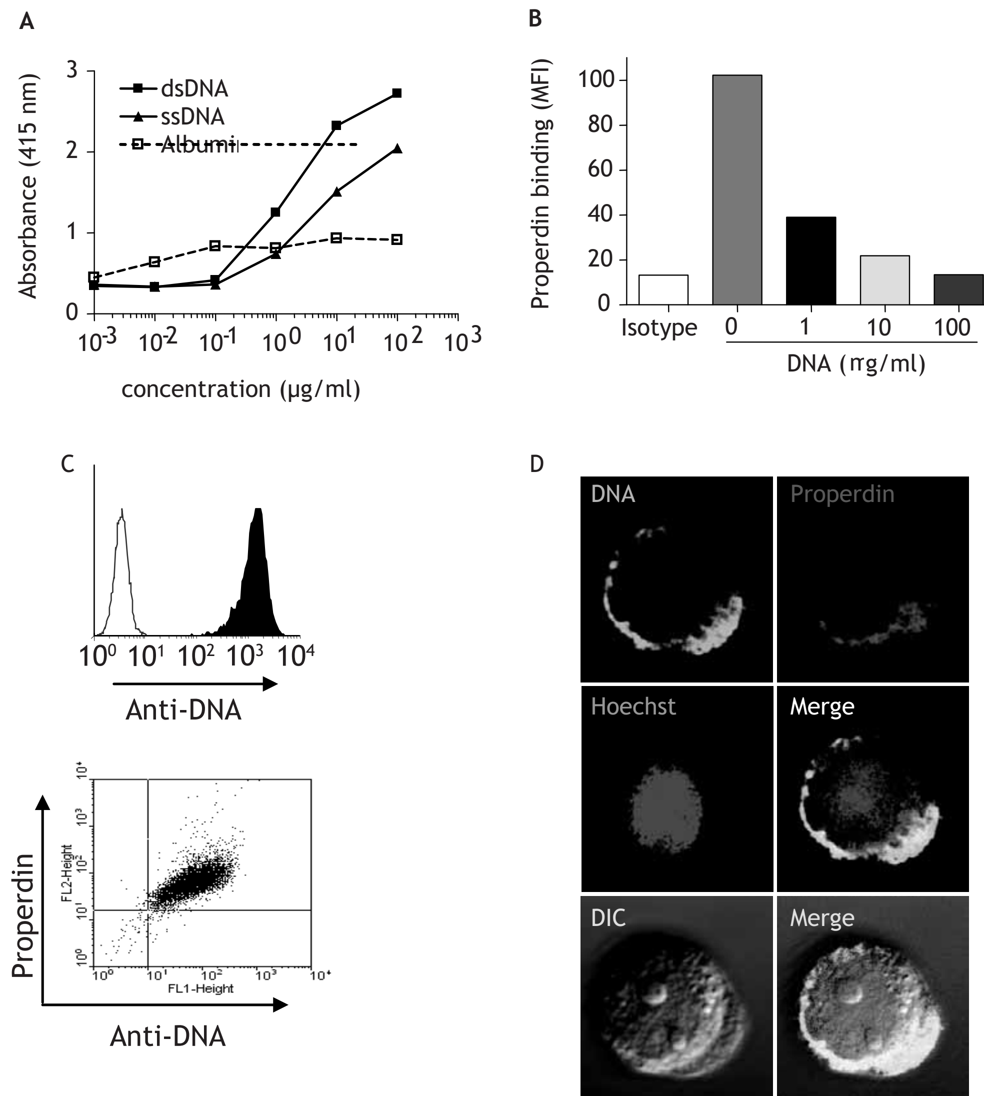


Figure 6. Properdin binds to DNA. (**A**) Different concentration of dsDNA and single strand DNA (ssDNA) or human albumin were coated on microtiter plates overnight, and then blocked with 2%BSA before adding properdin. After washing, plates were incubated with Dig-labelled rabbit-anti-human properdin. Signal was developed by anti-Dig-HRP, and measured for absorbance at OD 451 nm. (**B**) Properdin was pre-incubated with increasing concentrations of calf thymus double strand DNA (dsDNA), and then incubated with necrotic cells. Data shown are properdin binding to the cells (MFI). (**C**) Necrotic cells were incubated with a mouse anti-human dsDNA Ab, and developed by PE-conjugated goat

$F(ab)_2$ anti-mouse Ig. For double staining, cells were first opsonized with properdin (40 ug/ml) at 37ºC for 1 h in serum-free RPMI culture medium, followed by incubation with a rabbit-anti-human properdin Ab and a mouse anti-human dsDNA Ab, and developed by PE-conjugated goat $F(ab)_2$ anti-rabbit Ig and FITC-conjugated goat $F(ab)_2$ anti-mouse Ig (BD Biosciences). (D) Confocal laser scanning microscopy (LSM 510, Carl Zeiss AG), was performed on properdin-opsonized necrotic cells that were stained for properdin, DNA and Hoechst. Green: DNA; Red: properdin; Blue: Hoechst; Yellow: DNA co-localizes with properdin; DIC: differential interference contrast. Magnification, 400× (see page 155 for color image D).

We confirmed that DNA is indeed exposed on late apoptotic cells and necrotic cells using a monoclonal anti-dsDNA antibody as detected by flow cytometry (Figure 6C, 7A). Necrotic cells that were pre-incubated with properdin showed double positivity for both properdin binding and anti-DNA (Figure 6C). The binding of properdin to DNA on necrotic cells was further confirmed by confocal microscopy showing that properdin and DNA are co-localized on necrotic cells (Figure 6D). As a control, properdin-opsonized viable cells were negative for either properdin binding or DNA (date not shown).

Interestingly, different from necrotic cells, cells made late apoptotic were not all recognized by properdin (Figure 2A), which prompted us to further dissect these cell populations in detail. To better analyze the data, we divided these cells into two populations based on the forward and side scatter characteristics, namely R1 and R2 (Figure 7A). Cells in R2 are Annexin V^+PI^-, characteristics of early apoptotic cells (Figure 7A)., and as expected these cells did not bind properdin and did not expose DNA (Figure 7B). Cells in R1 are all Annexin V^+PI^+, indicative for late apoptotic cells (Figure 7A). Within the R1 population, part of the cells were both negative for properdin binding and DNA, and binding of properdin is related to the degree that DNA is exposed on these dying cells (Figure 7B). This suggests that during reorganization of dying cells, including blebbing, ligands for properdin are not equally distributed over the cellular fragments. Such unequal distribution of late apoptotic cells was not only applied to binding of properdin, but also to complement activation after NHS opsonization as measured for C3 and C5b-9 deposition on these cells (Figure 7C). Confocal microscopy confirmed that properdin was co-localized exclusively with fragmented DNA exposed on these cells (Figure 7D).

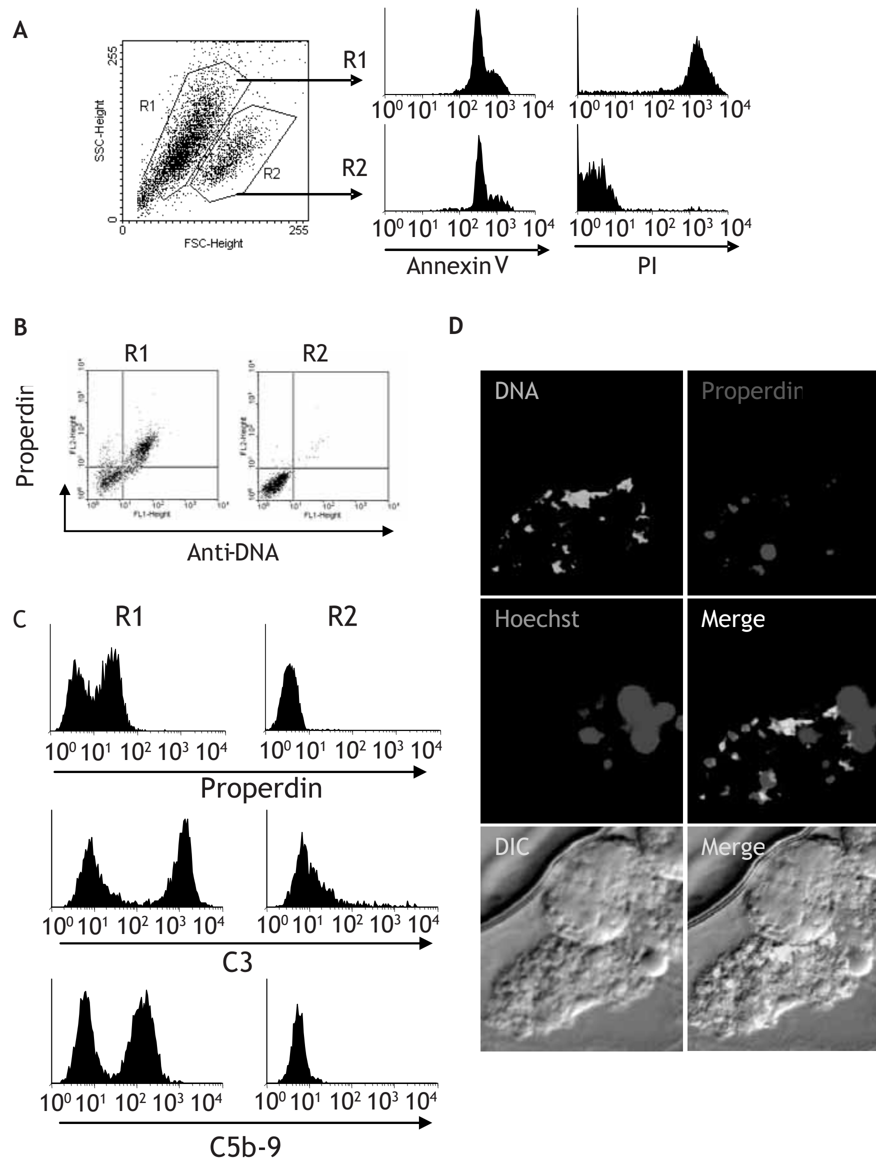


Figure 7. Properdin binds to a subpopulation of late apoptotic cells. (**A**) Jurkat cells made late apoptotic by culturing cells for 30 h after UV irradiation were stained with Annexin V and PI by flow cytometry. Cells were divided into R1 (late apoptotic cells) and R2 (early apoptotic cells). (**B**) Double staining of properdin and DNA to late apoptotic cells that were pre-opsonized with properdin was shown based on different gate on R1 or R2 region. (**C**) Histogram of properdin binding to late apoptotic cells was shown based on gate R1 and R2. C3 and C5b-9 depositions are shown on these cells after opsonization with NHS. (**D**) Confocal microscopy was performed on properdin-opsonized late apoptotic cells

that were stained for properdin, DNA and Hoechst. Green: DNA; Red: properdin; Blue: Hoechst; Yellow: DNA co-localizes with properdin; DIC: differential interference contrast. Magnification, 400× (see page 158 for color image D).

Discussion

We describe here that properdin specifically binds to late apoptotic or necrotic cells, but not to early apoptotic cells. Furthermore, DNA exposed on dying cells is one of the ligands to which properdin binds. We provide evidence that binding of properdin to late apoptotic cells and necrotic cells can occur independent of C3b, and serves as a focal point for the local amplification of the alternative pathway of complement.

In the past, studies on complement-mediated clearance of dying cells have mainly focused on the classical pathway [10-12]. Properdin is a positive regulator of the alternative pathway, which has been shown to bind to C3b and to stabilize the labile C3b-dependent C3 convertase C3bBb [19;20]. Two models have been proposed for the role of properdin in alternative pathway activation of complement. The first model suggests that properdin binds to a pre-formed C3bBb resulting in stabilization of the alternative pathway of C3 convertase [20]. The other model suggests that properdin first binds to a surface ligand via one of its subunits and then promotes the assembly C3bBb at the ligand-binding sites of its adjoining subunits [33]. Very recently, it has been shown that properdin can bind directly to bacterial surfaces (34). Our data showed that properdin binds to late apoptotic and necrotic cells prior to C3 deposition on the cell surface. Evidence that binding of properdin to dying cells can occur independent of C3b was further supported by experiments showing that properdin binds strongly to necrotic splenocytes derived from $C3^{-/-}$ mice (Figure 3A), and by experiments using C3 deficient serum (Figure 3B). We further showed that properdin pre-bound on necrotic cells binds to purified C3b instead of intact C3. Thus, we suggest that properdin binds to dying cells first in the absence of C3, and following the generation of C3b serves as a focal point for local amplification and boost of the properdin-driven complement activation cascade.

Our data suggest that DNA is one of the targets for properdin on dying cells. During apoptosis, DNAse cleaves DNA into nucleosomal units [35]. Indeed, DNA has been shown to be one of the major autoantigens exposed on apoptotic cell surfaces [4;36]. Using a monoclonal anti-DNA antibody, we demonstrate that both late apoptotic cells and necrotic cells expose DNA. Confocal images indicate that properdin and DNA are

co-localized on late apoptotic cells or necrotic cells. Interestingly, it seems that only the DNA recognized by anti-DNA Abs was accessible for properdin. It is likely that during apoptosis, DNAse digested small fragments of DNA, which are targeted by properdin, while properdin does not bind to the complete nucleosomal units of DNA. In pathological situations, DNA is considered as one of the immunologically active autoantigens [37], that can stimulate immune cells via Toll-like receptors [38;39]. In autoimmune lupus, DNA is one of the major immunogens to trigger autoantibody production [40]. Our finding that properdin binds to DNA opens the possibility that properdin may interfere with unwanted immune activation when DNA is exposed on dying cells during a large scale cell death.

Several other serum components have been suggested to interact with DNA, including C1q [41], MBL [42], serum amyloid-P component (SAP)[43], and C4b-binding protein (C4BP) [44]. We showed that properdin does not compete with the binding of C1q and MBL to dying cells, suggesting that C1q and MBL interacts with DNA structures different from the ones that properdin recognizes. It has been suggested that properdin binds to sulfatide (sulfated glycosphingolipids) and weakly to phosphatidylserine (PS) [45]. However, it is not likely that PS exposed on the surface of dying cells is a major ligand, since properdin does not bind to early apoptotic cells although these cells do express PS. Whether sulfatide or other phospholipids are one of the additional ligands on dying cells for properdin recognition is currently under investigation.

Involvement of properdin in the handling of dying cells was initially suggested by Kemper *et al.* reporting that properdin binds to early and late apoptotic cells [46]. Here we demonstrate that properdin binds predominantly to late apoptotic and necrotic cells, but not to early apoptotic cells. A similar restriction has been demonstrated for the binding of C1q, MBL (reviewed in [8]), Ficolin [14], natural IgM [47] and pentraxin family members SAP [48) and PTX3 [49]. Therefore, soluble opsonins especially seem to contribute to a safe clearance of late apoptotic and necrotic material. Together with our previous findings that early apoptotic cells are preferentially cleared by anti-inflammatory macrophages [24], we suggest that a hierarchy exists in the clearance mechanism of dying cells. Uptake of early apoptotic cells by local macrophages with anti-inflammatory properties is an initial step; whereas complement-mediated processes via all three pathways are a rather late event [9], most likely ensuring a safe clearance when an overload of apoptosis or defects in phagocytic capacity occur, thereby preventing a break of tolerance [50-52].

In humans, individuals deficient for properdin are prone to lethal pyonenic (particular neisserial) infections [53;54]. Mice deficient for properdin provided evidence that properdin is essential in driving LPS-mediated alternative complement activation [55]. No reports have shown that deficiency for properdin predisposes to the development of SLE, whereas in humans homozygous deficiency of any of the early components of the classical pathway of complement activation (C1q, C1r, C1s, C4, and C2) predisposes to the development of SLE [9]. Although many of other opsonins such as MBL, CRP and PTX3 also bind to dying cells and help their clearance, deficiency of these opsonins do not lead to the development of autoimmunity]8]. This might indicate a differential role for opsonins in the handling of dying cells, including augmentation of phagocytosis and /or a role in immune regulation. Among those opsonins, C1q is the strongest genetic factor that is linked to the development of SLE. Next to promoting clearance [13], C1q has been suggested to modulate dendritic cell function by imprinting these cells with tolerogenic properties [56]. Based on this, it is attempting to speculate that properdin might also have a dual function: 1.) amplifying complement activation on dying cells to promote complement-mediated clearance; 2.) immunomodulating properties, which deserve to be studied in detail.

In conclusion, we provide evidence that properdin binds specifically to late apoptotic and necrotic cells via ligands such as DNA, and acts as a focal point for the local amplification of alternative pathway complement activation. This process occurs independently of C3b. We propose here that properdin is a rate limiting factor and focal point for local alternative pathway complement activation on late stages of dying cells, thereby supporting a safe clearance.

Acknowledgements

We thank Ngaisah Klar-Mohamad and Roelof Flierman for technical assistance. We thank Frans Prins (Pathology, LUMC) for the excellent assistance with the confocal microscopy. We are grateful to Anja Roos for helpful discussion and ongoing collaboration.

This study is supported in part by a grant (C02.2015) from the Dutch Kidney Foundation.

Reference List

1. Savill, J., I. Dransfield, C. Gregory, and C. Haslett. 2002. A blast from the past: clearance of apoptotic cells regulates immune responses. *Nat. Rev. Immunol.* 2: 965-975.
2. Henson, P. M., D. L. Bratton, and V. A. Fadok. 2001. Apoptotic cell removal. *Curr. Biol.* 11: R795-R805.
3. Xu, W., A. Roos, M. R. Daha, and C. van Kooten. 2006. Dendritic cell and macrophage subsets in the handling of dying cells. *Immunobiology* 211: 567-575.
4. Casciola-Rosen, L. A., G. Anhalt, and A. Rosen. 1994. Autoantigens targeted in systemic lupus erythematosus are clustered in two populations of surface structures on apoptotic keratinocytes. *J. Exp. Med.* 179: 1317-1330.
5. Baumann, I., W. Kolowos, R. E. Voll, B. Manger, U. Gaipl, W. L. Neuhuber, T. Kirchner, J. R. Kalden, and M. Herrmann. 2002. Impaired uptake of apoptotic cells into tingible body macrophages in germinal centers of patients with systemic lupus erythematosus. *Arthritis Rheum.* 46: 191-201.
6. Carroll, M. C. 2004. A protective role for innate immunity in systemic lupus erythematosus. *Nat. Rev. Immunol.* 4: 825-831.
7. Nauta, A. J., M. R. Daha, C. Kooten, and A. Roos. 2003. Recognition and clearance of apoptotic cells: a role for complement and pentraxins. *Trends Immunol.* 24: 148-154.
8. Roos, A., W. Xu, G. Castellano, A. J. Nauta, P. Garred, M. R. Daha, and C. van Kooten. 2004. Mini-review: A pivotal role for innate immunity in the clearance of apoptotic cells. *Eur. J. Immunol.* 34: 921-929.
9. Manderson, A. P., M. Botto, and M. J. Walport. 2004. The role of complement in the development of systemic lupus erythematosus. *Annu. Rev. Immunol.* 22: 431-456.
10. Mevorach, D., J. O. Mascarenhas, D. Gershov, and K. B. Elkon. 1998. Complement-dependent clearance of apoptotic cells by human macrophages. *J Exp Med* 188: 2313-2320.
11. Taylor, P. R., A. Carugati, V. A. Fadok, H. T. Cook, M. Andrews, M. C. Carroll, J. S. Savill, P. M. Henson, M. Botto, and M. J. Walport. 2000. A hierarchical role for classical pathway complement proteins in the clearance of apoptotic cells in vivo. *J. Exp. Med.* 192: 359-366.
12. Ogden, C. A., A. deCathelineau, P. R. Hoffmann, D. Bratton, B. Ghebrehiwet, V. A. Fadok, and P. M. Henson. 2001. C1q and mannose binding lectin engagement of cell surface calreticulin and CD91 initiates macropinocytosis and uptake of apoptotic cells. *J. Exp. Med.* 194: 781-795.
13. Nauta, A. J., G. Castellano, W. Xu, A. M. Woltman, M. C. Borrias, M. R. Daha, C. van Kooten, and A. Roos. 2004. Opsonization with C1q and mannose-binding lectin targets apoptotic cells to dendritic cells. *J. Immunol.* 173: 3044-3050.
14. Jensen, M. L., C. Honore, T. Hummelshoj, B. E. Hansen, H. O. Madsen, and P. Garred. 2007. Ficolin-2 recognizes DNA and participates in the clearance of dying host cells. *Mol. Immunol.* 44: 856-865.
15. Verbovetski, I., H. Bychkov, U. Trahtemberg, I. Shapira, M. Hareuveni, O. Ben Tal, I. Kutikov, O. Gill, and D. Mevorach. 2002. Opsonization of apoptotic cells by autologous iC3b facilitates clearance by immature dendritic cells, down-regulates DR and CD86, and up-regulates CC chemokine receptor 7. *J. Exp. Med.* 196: 1553-1561.

16. Sohn, J. H., P. S. Bora, H. J. Suk, H. Molina, H. J. Kaplan, and N. S. Bora. 2003. Tolerance is dependent on complement C3 fragment iC3b binding to antigen-presenting cells. *Nat. Med.* 9: 206-212.
17. Walport, M. J. 2001. Complement. First of two parts. *N. Engl. J. Med.* 344: 1058-1066.
18. PILLEMER, L., L. BLUM, I. H. LEPOW, O. A. ROSS, E. W. TODD, and A. C. WARDLAW. 1954. The properdin system and immunity: I. Demonstration and isolation of a new serum protein, properdin, and its role in immune phenomena. *Science* 120: 279-285.
19. Schwaeble, W. J. and K. B. Reid. 1999. Does properdin crosslink the cellular and the humoral immune response? *Immunol. Today* 20: 17-21.
20. Fearon, D. T. and K. F. Austen. 1975. Properdin: binding to C3b and stabilization of the C3b-dependent C3 convertase. *J. Exp. Med.* 142: 856-863.
21. Spitzer, D., L. M. Mitchell, J. P. Atkinson, and D. E. Hourcade. 2007. Properdin can initiate complement activation by binding specific target surfaces and providing a platform for de novo convertase assembly. *J. Immunol.* 179: 2600-2608.
22. Koopman, G., C. P. Reutelingsperger, G. A. Kuijten, R. M. Keehnen, S. T. Pals, and M. H. van Oers. 1994. Annexin V for flow cytometric detection of phosphatidylserine expression on B cells undergoing apoptosis. *Blood* 84: 1415-1420.
23. Wessels, M. R., P. Butko, M. Ma, H. B. Warren, A. L. Lage, and M. C. Carroll. 1995. Studies of group B streptococcal infection in mice deficient in complement component C3 or C4 demonstrate an essential role for complement in both innate and acquired immunity. *Proc. Natl. Acad. Sci. U. S. A* 92: 11490-11494.
24. Xu, W., A. Roos, N. Schlagwein, A. M. Woltman, M. R. Daha, and C. van Kooten. 2006. IL-10-producing macrophages preferentially clear early apoptotic cells. *Blood,* 107: 4930-4937.
25. van den Berg, R. H., M. Faber-Krol, L. A. Van Es, and M. R. Daha. 1995. Regulation of the function of the first component of complement by human C1q receptor. *Eur. J. Immunol.* 25: 2206-2210.
26. Roos, A., L. H. Bouwman, D. J. Gijlswijk-Janssen, M. C. Faber-Krol, G. L. Stahl, and M. R. Daha. 2001. Human IgA activates the complement system via the mannan-binding lectin pathway. *J. Immunol.* 167: 2861-2868.
27. Nauta, A. J., L. A. Trouw, M. R. Daha, O. Tijsma, R. Nieuwland, W. J. Schwaeble, A. R. Gingras, A. Mantovani, E. C. Hack, and A. Roos. 2002. Direct binding of C1q to apoptotic cells and cell blebs induces complement activation. *Eur. J. Immunol.* 32: 1726-1736.
28. Wolbink, G. J., J. Bollen, J. W. Baars, R. J. ten Berge, A. J. Swaak, J. Paardekooper, and C. E. Hack. 1993. Application of a monoclonal antibody against a neoepitope on activated C4 in an ELISA for the quantification of complement activation via the classical pathway. *J. Immunol. Methods* 163: 67-76.
29. Farries, T. C., P. J. Lachmann, and R. A. Harrison. 1988. Analysis of the interaction between properdin and factor B, components of the alternative-pathway C3 convertase of complement. *Biochem. J.* 253: 667-675.
30. Spitzer, D., L. M. Mitchell, J. P. Atkinson, and D. E. Hourcade. 2007. Properdin can initiate complement activation by binding specific target surfaces and providing a platform for de novo convertase assembly. *J. Immunol.* 179: 2600-2608.

31. Nauta, A. J., N. Raaschou-Jensen, A. Roos, M. R. Daha, H. O. Madsen, M. C. Borrias-Essers, L. P. Ryder, C. Koch, and P. Garred. 2003. Mannose-binding lectin engagement with late apoptotic and necrotic cells. *Eur. J. Immunol.* 33: 2853-2863.
32. Radic, M., T. Marion, and M. Monestier. 2004. Nucleosomes are exposed at the cell surface in apoptosis. *J. Immunol.* 172: 6692-6700.
33. Hourcade, D. E. 2006. The role of properdin in the assembly of the alternative pathway C3 convertases of complement. *J. Biol. Chem.* 281: 2128-2132.
34. Spitzer, D., L. M. Mitchell, J. P. Atkinson, and D. E. Hourcade. 2007. Properdin can initiate complement activation by binding specific target surfaces and providing a platform for de novo convertase assembly. *J. Immunol.* 179: 2600-2608.
35. Nagata, S. 2005. DNA degradation in development and programmed cell death. *Annu. Rev. Immunol.* 23: 853-875.
36. Radic, M., T. Marion, and M. Monestier. 2004. Nucleosomes are exposed at the cell surface in apoptosis. *J. Immunol.* 172: 6692-6700.
37. Marshak-Rothstein, A. and I. R. Rifkin. 2007. Immunologically active autoantigens: the role of toll-like receptors in the development of chronic inflammatory disease. *Annu. Rev. Immunol.* 25: 419-441.
38. Barrat, F. J., T. Meeker, J. Gregorio, J. H. Chan, S. Uematsu, S. Akira, B. Chang, O. Duramad, and R. L. Coffman. 2005. Nucleic acids of mammalian origin can act as endogenous ligands for Toll-like receptors and may promote systemic lupus erythematosus. *J. Exp. Med.* 202: 1131-1139.
39. Viglianti, G. A., C. M. Lau, T. M. Hanley, B. A. Miko, M. J. Shlomchik, and A. Marshak-Rothstein. 2003. Activation of autoreactive B cells by CpG dsDNA. *Immunity.* 19: 837-847.
40. Mohan, C., S. Adams, V. Stanik, and S. K. Datta. 1993. Nucleosome: a major immunogen for pathogenic autoantibody-inducing T cells of lupus. *J. Exp. Med.* 177: 1367-1381.
41. Van Schravendijk, M. R. and R. A. Dwek. 1982. Interaction of C1q with DNA. *Mol. Immunol.* 19: 1179-1187.
42. Palaniyar, N., J. Nadesalingam, H. Clark, M. J. Shih, A. W. Dodds, and K. B. Reid. 2004. Nucleic acid is a novel ligand for innate, immune pattern recognition collectins surfactant proteins A and D and mannose-binding lectin. *J. Biol. Chem.* 279: 32728-32736.
43. Pepys, M. B. and P. J. Butler. 1987. Serum amyloid P component is the major calcium-dependent specific DNA binding protein of the serum. *Biochem. Biophys. Res. Commun.* 148: 308-313.
44. Trouw, L. A., S. C. Nilsson, I. Goncalves, G. Landberg, and A. M. Blom. 2005. C4b-binding protein binds to necrotic cells and DNA, limiting DNA release and inhibiting complement activation. *J. Exp. Med.* 201: 1937-1948.
45. Holt, G. D., M. K. Pangburn, and V. Ginsburg. 1990. Properdin binds to sulfatide [Gal(3-SO4)beta 1-1 Cer] and has a sequence homology with other proteins that bind sulfated glycoconjugates. *J. Biol. Chem.* 265: 2852-2855.
46. Kemper C., Mitchell L.M., and Hourcade D.E. 2007. Properdin: Cell death's little helper? *Mol. Immunol.* 107: Abstract.
47. Fu, M., P. S. Fan, W. Li, C. X. Li, Y. Xing, J. G. An, G. Wang, X. L. Fan, T. W. Gao, Y. F. Liu, and S. Ikeda. 2007. Identification of poly-reactive natural IgM antibody that recognizes late apoptotic cells and promotes phagocytosis of the cells. *Apoptosis.* 12: 355-362.

48. Bijl, M., G. Horst, J. Bijzet, H. Bootsma, P. C. Limburg, and C. G. Kallenberg. 2003. Serum amyloid P component binds to late apoptotic cells and mediates their uptake by monocyte-derived macrophages. *Arthritis Rheum.* 48: 248-254.
49. Rovere, P., G. Peri, F. Fazzini, B. Bottazzi, A. Doni, A. Bondanza, V. S. Zimmermann, C. Garlanda, U. Fascio, M. G. Sabbadini, C. Rugarli, A. Mantovani, and A. A. Manfredi. 2000. The long pentraxin PTX3 binds to apoptotic cells and regulates their clearance by antigen-presenting dendritic cells [In Process Citation]. *Blood* 96: 4300-4306.
50. Savill, J. and V. Fadok. 2000. Corpse clearance defines the meaning of cell death. *Nature* 407: 784-788.
51. Sauter, B., M. L. Albert, L. Francisco, M. Larsson, S. Somersan, and N. Bhardwaj. 2000. Consequences of cell death: exposure to necrotic tumor cells, but not primary tissue cells or apoptotic cells, induces the maturation of immunostimulatory dendritic cells. *J. Exp. Med.* 191: 423-434.
52. Ip, W. K. and Y. L. Lau. 2004. Distinct maturation of, but not migration between, human monocyte-derived dendritic cells upon ingestion of apoptotic cells of early or late phases. *J. Immunol.* 173: 189-196.
53. Densen, P., J. M. Weiler, J. M. Griffiss, and L. G. Hoffmann. 1987. Familial properdin deficiency and fatal meningococcemia. Correction of the bactericidal defect by vaccination. *N. Engl. J. Med.* 316: 922-926.
54. Kolble, K. and K. B. Reid. 1993. Genetic deficiencies of the complement system and association with disease--early components. *Int. Rev. Immunol.* 10: 17-36.
55. Kimura, Y., T. Miwa, L. Zhou, and W. C. Song. 2007. Activator-specific requirement of properdin in the initiation and amplification of the alternative pathway complement. *Blood.*
56. Castellano, G., A. M. Woltman, N. Schlagwein, W. Xu, F. P. Schena, M. R. Daha, and C. van Kooten. 2007. Immune modulation of human dendritic cells by complement. *Eur. J. Immunol.* 37: 2803-2811.

Chapter 7

Complement activation by tubular cells is mediated by properdin binding

Hilde Gaarkeuken,
Machiel A. Siezenga,
Kim Zuidwijk,
Cees van Kooten,
Ton J. Rabelink,
Mohamed R. Daha and
Stefan P. Berger

Department of Nephrology, Leiden University Medical Center,
Leiden, the Netherlands

Am J Physiol Renal Physiol 2008; 295(5):F1397-403

Summary

Activation of filtered complement products on the brush border of the tubular epithelium is thought to be a key factor underlying proteinuria-induced tubulointerstitial injury. However, the mechanism of tubular complement activation is still unclear. Recent studies on mechanisms of complement activation indicate a key role for properdin in the initiation of alternative pathway. We hypothesized that properdin serves as a focal point for complement activation on the tubulus.

We observed a strong staining for properdin on the luminal surface of the tubules in kidney biopsies from patients with proteinuric renal disease. In vitro experiments revealed dose-dependent binding of properdin to PTEC whereas no significant binding to endothelial cells was detected. Exposure of PTEC with normal human serum as a source of complement resulted in complement activation with deposition of C3 and generation of C5b-9. These effects were virtually absent with properdin deficient serum. Pre-incubation of PTEC with properdin before addition of properdin-depleted serum fully restored complement activation on the cells, strongly suggesting a key role for properdin in the activation of complement at the tubular surface.

In proteinuric renal disease, filtered properdin may bind to PTEC and act as a focal point for alternative pathway activation. We propose that this contribution of properdin is pivotal in tubular complement activation and subsequent damage. Interference with properdin binding to tubular cells may provide an option for the treatment of proteinuric renal disease.

Introduction

Worldwide, the number of patients suffering from chronic kidney disease (CKD) is increasing dramatically [1]. The two most important factors contributing to the global rise in CKD are ageing of the population and the epidemic of type 2 diabetes mellitus [2]. It has been well established now that in chronic kidney disease, regardless of the aetiology, proteinuria is a strong and independent predictor for the progression of chronic renal failure to end-stage renal disease (ESRD) [3;4]. Anti-proteinuric treatment is associated with preservation of renal function [5;6].

Several pathophysiologic mechanisms have been proposed to account for proteinuria-induced tubulointerstitial injury. These include lysosomal rupture due to reabsorbed proteins, oxidative damage induced by transferrin reabsorption, and the stimulatory effects of various plasma proteins on the expression of proinflammatory and profibrotic mediators in renal tubular epithelial cells [7-9]. There is accumulating evidence for complement activation as a powerful mechanism underlying the progression of proteinuric renal disease.

In the setting of proteinuria, plasma complement components may enter the tubular lumen [10]. If these complement components are then locally activated this would lead to cell activation and resulting tubular damage and interstitial fibrosis [11;12]. Indeed, proximal tubular epithelial cells (PTEC) activate serum complement *in vitro* via the alternative pathway [13-15]. Also *in vivo*, both in human chronic proteinuric disease and in experimental models, evidence of complement activation can be detected on the apical surface of the renal tubules [14;16;17]. The protective effect of C6 deficiency in the puromycin model of nephrotic syndrome, as well as in the remnant kidney model, provides further evidence for the role of complement in mediating tubulointerstitial injury [16;18]. Targeting complement inhibitory molecules to the proximal tubules in a rat model of proteinuric kidney disease protects against renal dysfunction [19].

However, the exact mechanism of the unique complement activating property of the proximal tubules has not yet been elucidated. Previous studies reported a role for local ammonium (NH_4) in initiating alternative complement pathway activity [20;21]. We hypothesize that besides ammonium, other mechanisms might be involved in triggering tubular complement activation.

The alternative pathway of complement is triggered by spontaneous hydrolysis of C3, which generates C3a and C3b. Cleavage of C3 results in the formation of a positive feedback loop to produce a rapid local response [22]. Properdin, discovered in 1954

by Pillemer *et al.* [23], is the only known positive regulator of the complement system and consists of dimers, trimers and tetramers arranged in a head-to-tail orientation [24;25]. Properdin binds to C3b and enhances complement activation by stabilizing the alternative pathway C3 convertase [26]. Lately, there has been renewed interest in properdin. It was shown that target-bound properdin may serve as a focal point for amplification of C3 activation. Each subunit in the oligomer provides a ligand-binding site and the unoccupied ligand-binding sites can assemble the alternative pathway convertase on target surfaces [27;28]. It has recently been re-emphasized that properdin may act as a focal point in the activation of the alternative pathway of complement [27-30]. It was suggested already in 1954 that properdin might interact directly with cell surfaces [23;31].
In this study, we show that properdin binds to viable tubular epithelial cells and via this mechanism initiates complement activation.

Materials and Methods

Immunohistochemical staining

Frozen 4 μm tissue sections were used to determine the presence of properdin in cortical tissue of human kidneys. After the sections were fixed with acetone, endogenous peroxidase activity was blocked with 0.1% H_2O_2 and 0.1% NaN_3 for 30 min at room temperature (RT). Then the slides were washed and subsequently blocked with phosphate-buffered saline (PBS), 1% bovine serum albumin (BSA) and 5% heat-inactivated normal human serum for 45 min at RT. Next, sections were incubated with a polyclonal rabbit anti-human properdin antibody (Laboratory of Nephrology, Leiden, the Netherlands) in PBS, 1% BSA and 1% normal human serum in a humid atmosphere overnight at RT. After washing with PBS, antibody binding was detected with horseradish peroxidase (HRP)-labeled goat anti-rabbit Ig (DAKO, Glostrup, Denmark) in PBS, 1% BSA and 1% normal human serum (60 min RT) followed by washing with PBS, incubation with Tyramide-fluorescein isothiocyanate in tyramide buffer (NEN™ Life Science Products, Boston, MA, USA; 20 min RT), washing with PBS, incubation with HRP-conjugated rabbit anti-fluorescein isothiocyanate (DAKO) for 60 min at RT, washing with PBS and development with DAB (Sigma, St Louis, MO, USA). Sections were counterstained with hematoxylin (Merck, Darmstadt, Germany) and mounted with imsol (Klinipath, Duiven, The Netherlands).

Cell culture

The immortalized renal proximal tubular epithelial cell-line HK-2 was kindly provided by M. Ryan, University College Dublin, Ireland [32]. Cells were grown in serum-free DMEM/HAMF12 (Bio-Whittaker, Walkersville, MD) supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin (Invitrogen, Breda, the Netherlands), insulin (5 µg/ml), transferrin (5 µg/ml), selenium (5 ng/ml), tri-iodothyronine (40 pg/ml), epidermal growth factor (10 ng/ml), hydrocortisone (36 ng/ml, all purchased from Sigma). Primary human proximal tubular epithelial cells (PTEC) were isolated from pre-transplant biopsies or from kidneys not suitable for transplantation and cultured as described earlier [33]. HUVEC were isolated from umbilical cords as described previously [34]. Cells were cultured on a matrix of fibronectin in M199 medium containing 20% heat-inactivated FCS, 100 U/ml penicillin, 100 ug/ml streptomycin, 50 ug/ml Bovine Pituitary Extract (all from Invitrogen) and 10 U/ml heparin (LEO Pharma B.V., Breda, the Netherlands). The cell lines ECRF-24, Jurkat, HL-60 and U937 were cultured as described earlier [35;36].

Isolation of properdin

Properdin was isolated from pooled human donor serum. First, a precipitation step was performed by dialyzing the serum against water containing 5 mM EDTA, pH 6.0. The resulting precipitate was dissolved in Veronal-buffered saline (2x VBS, 1.8 mM Na-5,5-diethylbarbital, 0.2 mM 5,5-diethylbarbituric acid, 145 mM NaCl), dialyzed against 0.01 M NaAc containing 2mM EDTA, pH 6.0 and applied to a Sulphopropyl Sephadex C50 cation exchange colomn (Pharmacia Biotech, Uppsala, Sweden). Properdin was eluted from the column with a linear salt gradient. Properdin-containing fractions, as determined by enzyme-linked immunosorbent assay (ELISA), were pooled, concentrated, and subsequently applied to a Sephacryl S-300 gel filtration column (Pharmacia), after which properdin-containing fractions were pooled. In order to remove contaminating C1q from the preparation, the properdin-pool was dialyzed against PBS, 2 mM EDTA and further purified using human IgG coupled to a Biogel A5 column (Bio-Rad, Hercules, CA). Purity of the properdin preparation was confirmed by analysis on 10% non-reducing SDS-PAGE gel. A single band of 220 kDa was observed.

Serum preparation

Normal human serum was depleted of properdin by immune adsorption using Biogel-coupled anti-human properdin monoclonal antibodies (a gift of State Serum Institute, Copenhagen, Denmark). The properdin-depleted serum showed normal classical and

lectin pathway activity in hemolytic assay. C4-depleted serum, which lacks both classical and lectin pathway activity, was prepared by affinity adsorption using goat anti-human C4 IgG coupled to CNBr-activated Sepharose 4 Fast Flow (Amersham Bioscience Europe, Roosendaal, the Netherlands). After C4 depletion, the serum was free of C4 antigen and classical pathway hemolytic activity could be restored fully by purified hemolytically active C4.

FACS analysis

Deposition of complement on cells was determined by flow cytometry. Properdin binding to the cells was visualized using a polyclonal rabbit anti-human properdin antibody followed by RPE-conjugated goat anti-rabbit IgG (Southern Biotechnology Associates, Birmingham, US). Deposition of C3, C5b-9, C1q, and MBL on the cells was detected using a mouse monoclonal anti-human C3 antibody (RFK22, Laboratory of Nephrology, Leiden, the Netherlands), anti-human C5b-9 (mAb AE11, kindly provided by Dr. T.E. Mollnes, Nordland Central Hospital, Bodo, Norway), anti-human C1q (mAb 2204, kindly provided by Dr. C.E. Hack, Sanquin Research, Amsterdam) and anti-human MBL (mAb 3E7, kindly provided by Dr. T. Fujita, Medical University School of Medicine, Fukushima, Japan) respectively, followed by RPE-conjugated polyclonal goat anti-mouse Ig (DAKO). All antibody incubations were performed on ice for 30 min. Cell surface staining was assessed using a FACScalibur flow cytometer (Becton Dickinson, Mountain View, CA). Propidium iodide (1µg/ml, Molecular Probes, Leiden, the Netherlands) was used for exclusion of dead cells.

Properdin binding and complement activation assays

For FACS experiments, cells were grown to confluence in 48-well tissue culture plates. HK-2 cells and HUVEC were exposed to 20% normal human serum diluted in serum-free DMEM/HAMF12 for 2 h at 37°C. C3, C5b-9, C1q, MBL and properdin were assessed on the cell surface by FACS analysis. Alternative pathway mediated complement activation by HK-2 was tested by incubating the cells with 20% normal human serum in the presence of 5 mM Mg EGTA. Properdin binding to HK-2, primary PTEC, HUVEC, ECRF-24, U937, HL-60 and Jurkat was assessed by incubating the cells with purified human properdin (20 µg/ml) diluted in serum-free DMEM/HAMF12 for 1 h at 37°C. Dose-dependent properdin binding to HK-2 and HUVEC was tested by incubating the cells with increasing concentrations of human properdin (10 to 40 µg/ml). The functional consequences of properdin binding were determined by incubating the cells with 5% properdin-depleted, normal human serum or C4-depleted

human serum as a complement source, diluted in serum-free DMEM/HAMF12 culture medium, for 2 h at 37°C after pre-incubation with properdin. Following properdin and/or serum incubation, the cells were washed twice in PBS, harvested by scraping and resuspended in FACS-buffer (1% BSA and 0.02% sodium azide in PBS) for FACS staining.

Results

Properdin is present on the tubular brush border in proteinuric kidneys

The presence of properdin on the brush border of the proximal tubules was determined in renal biopsies of three patients with membranous nephropathy and in pretransplant renal biopsies of three living related kidney donors. Properdin could be detected along the brush border of the tubules in diseased kidneys, whereas properdin was absent in the tubules of healthy kidney tissue (Figure 1). Since the presence of properdin on the tubular brush border of proteinuric kidneys does not distinguish where in the cascade of complement activation properdin comes in, we proceeded to *in vitro* studies to determine whether properdin is an initiating factor in tubular complement activation.

Complement activation by HK-2 cells

Incubation of Human Kidney-2 (HK-2) cells with normal human serum resulted in fixation of complement products on the cell surface. C3, C5b-9 and properdin, but not C1q and mannan-binding lectin (MBL) could be detected (Figure 2a). The complement system was activated on the cell surface via the alternative pathway since deposition of C3 and C5b-9 was unaffected by Mg EGTA, which interferes with the classical and lectin pathway of complement by chelating calcium (Figure 2b). In contrast, complement fixation was completely blocked by EDTA, which inhibits all three pathways of complement activation. C3 and C5b-9 deposition was also detected on HK-2 cells after exposure to C4-depleted human serum, which excludes involvement of the classical or lectin pathway (Figure 2c). To assure that complement activation was localized to the apical surface, serum incubations were performed on cells that were grown to confluence in a tissue culture plate. Human umbilical vein endothelial cells (HUVEC) were used as a control. No complement deposition was observed on these cells after treatment with normal human serum.

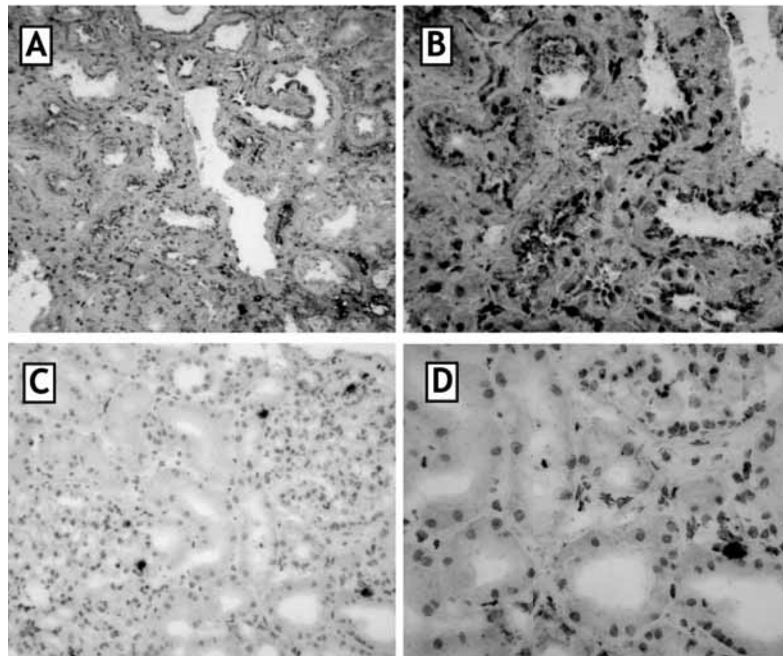


Figure 1. **Properdin staining on the tubular brush border in proteinuric kidneys.** Cryosections of (**A** and **B**) a renal biopsy of a patient with membranous nephropathy and (**C** and **D**) a pretransplant biopsy of a healthy donor were stained immunohistochemically for properdin. (**A** and **C**) Original magnifications were either × 100 or (**B** and **D**) × 250. Pictures are representative for three patients with membranous nephropathy and three healthy kidneys donors (see page 159 for color image).

Binding of properdin to HK-2 cells

We then questioned whether properdin could bind to tubular cells prior to the activation of complement and the deposition of its known ligand C3b. In order to study binding of properdin to the cell surface, confluent cells in a tissue culture plate were incubated with purified human properdin at a concentration of 20 µg/ml. Properdin binding was analysed by flow cytometry. Only cells which were negative for propidium iodide staining were analysed in order to exclude properdin binding to dead cells. As shown in Figure 3a, strong binding of properdin to viable HK-2 cells was detected, whereas no significant binding was shown on HUVEC. As a negative control, the fluorescence intensity of cells incubated with detection antibody only, i.e., without pre-incubation with properdin, is shown. Properdin binds to viable HK-2 cells in a dose-dependent manner (Figure 3b). The cell lines HL-60, U937 (monocytes),

Jurkat (T-cell leukaemia) and ECRF-24 (immortalized HUVEC) were all negative for properdin binding (Figure 3c).

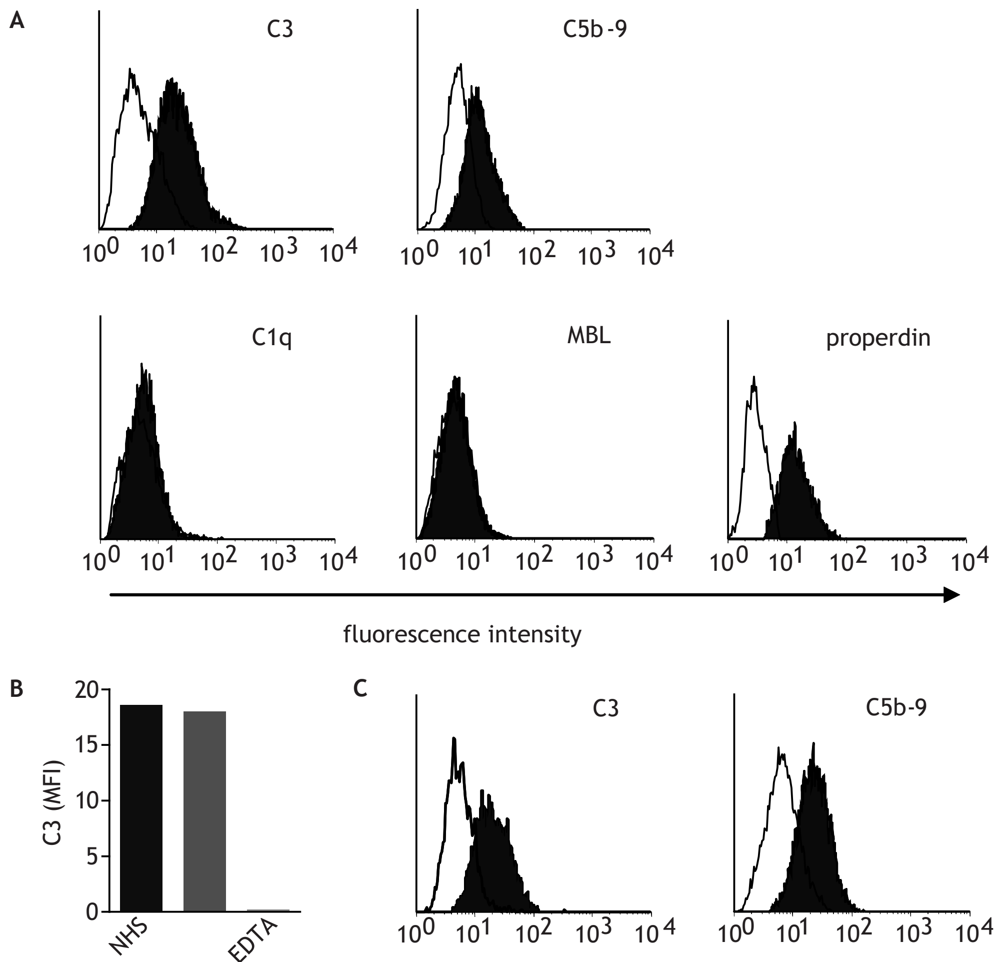


Figure 2. Complement activation by HK-2 cells. (**A**) HK-2 cells were incubated with 20% normal human serum (NHS). C3, C5b-9, C1q, mannan-binding lectin (MBL) and properdin binding (filled histograms) were assessed on the cells using the mAbs RFK22, AE11, 2204, 3E7 and a polyclonal rabbit anti-properdin antibody, respectively. Open histograms show staining on cells that were not exposed to serum. (**B**) C3 deposition was assessed on HK-2 cells after incubation with 20% human serum in the presence or absence of 5mM Mg EGTA or 10 mM EDTA. Results are expressed as the mean fluorescence intensity, MFI. (**C**) C3 and C5b-9 deposition on HK-2 cells after exposure to 20% C4-depleted human serum.

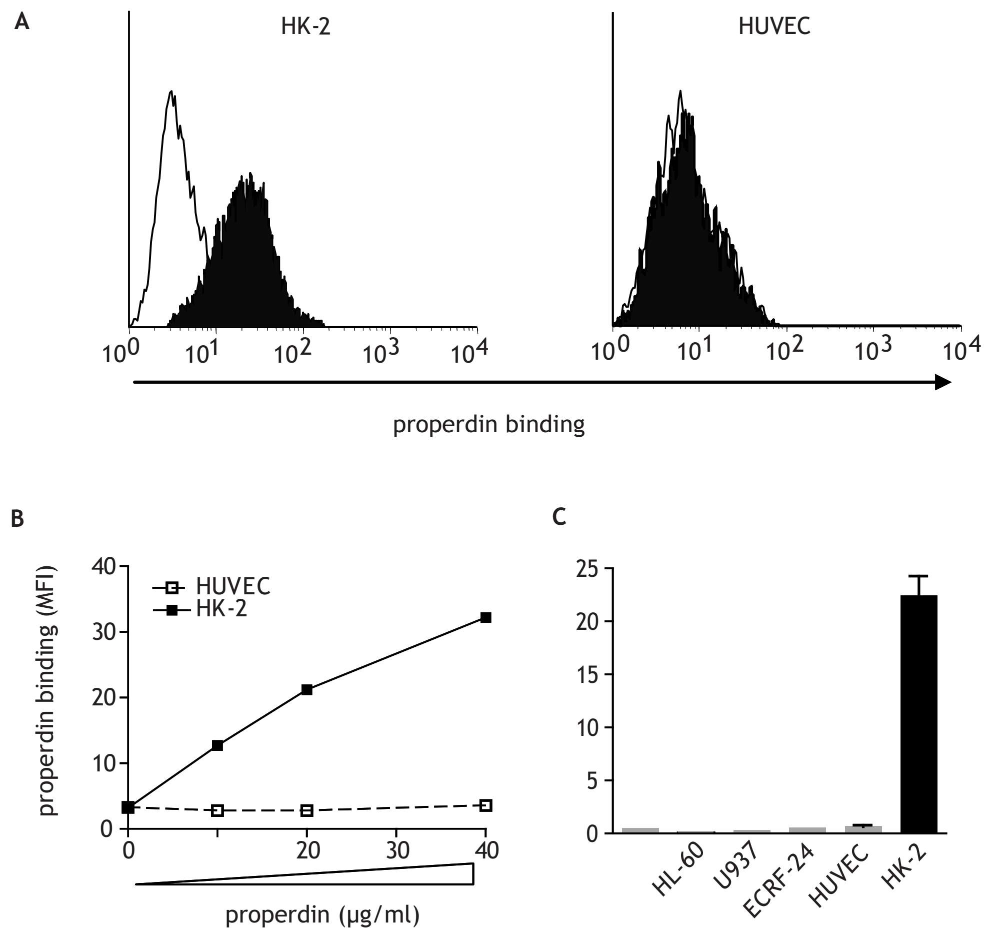


Figure 3. Properdin binding to HK-2 cells. (A) HK-2 cells and human umbilical vein endothelial cells (HUVEC) were incubated with 20 µg/ml purified human properdin. Binding of properdin (filled histograms) was detected with a polyclonal rabbit anti-human properdin antibody followed by goat anti-rabbit conjugated with PE. As a negative control, staining with both primary and secondary antibody was performed on cells that were not exposed to properdin (open histograms). (B) Dose-dependent binding of properdin to HK-2 and HUVEC is shown as the mean fluorescence intensity (MFI). Data are representative for two individual experiments. (C) Binding of properdin (shown as the mean fluorescence intensity, MFI) to the cell lines Jurkat, HL-60, U937, ECRF-24, HUVEC and HK-2. Data are expressed as the mean ± SD of three independent experiments.

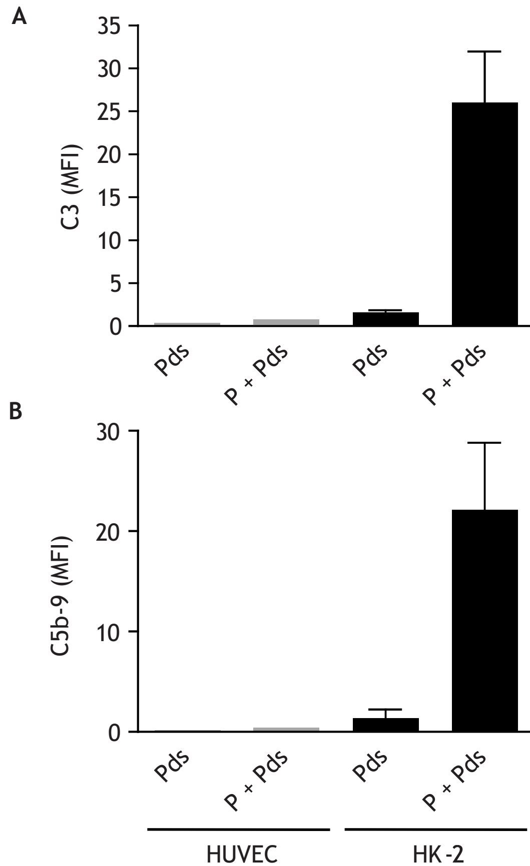


Figure 4. Properdin-dependent complement activation. HK-2 cells and human umbilical vein endothelial cells (HUVEC) were pre-incubated with properdin (P, 20 µg/ml), washed and subsequently exposed to 5% properdin-depleted human serum (Pds). (**a**) C3 and (**b**) C5b-9 deposition (shown as the mean fluorescence intensity, MFI) was detected on the cells using the mAbs RFK22 and AE11, respectively. The results are expressed as the mean ± SD of three independent experiments.

Properdin binding is a focal point for alternative pathway activation on HK-2 cells

Next, we investigated whether properdin, after binding to the tubular surface, acts as a focal point for local amplification of the alternative pathway of complement. To demonstrate properdin-dependent complement activation, deposition of C3 and C5b-9 was assessed on HK-2 cells and HUVEC incubated with properdin-deficient normal human serum, with and without pre-incubation of the cells with purified properdin. HK-2 cells incubated with properdin-depleted serum show a strongly reduced C3 deposition compared to cells exposed to normal human serum. This is accompanied by a strong reduction of C5b-9 deposition. Complement activation was restored completely on cells that had been pre-incubated with properdin, prior to exposure to properdin-deficient serum (Figure 4a and b). HUVEC showed no significant

complement activation, both with and without pre-incubation with purified properdin. This indicates that properdin, bound to the cellular surface of HK-2, initiates and targets the amplification of the complement cascade to the surface of tubular cells.

To confirm that complement activation on HK-2 is properdin-dependent, cells were pre-exposed to different concentrations of purified properdin, ranging from 2,5 to 40 μg/ml, before incubation with 5% normal human serum. Properdin was shown to increase the deposition of both C3 and C5b-9 on HK-2 in a dose-dependent way (Figure 5a). Similar dose-dependent effects were detected when increasing concentrations of properdin were added prior to incubation with C4-depleted human serum (Figure 5b).

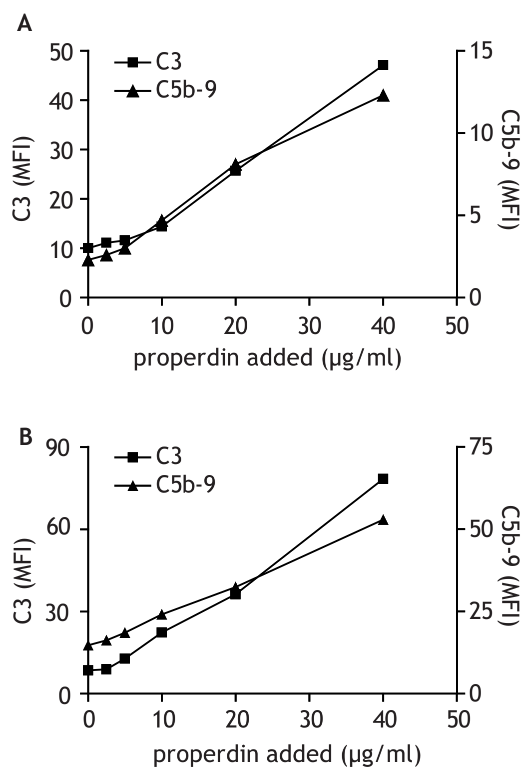


Figure 5. Dose-dependent effect of properdin on complement deposition. HK-2 cells were pre-incubated with increasing concentrations of human properdin. After extensive washing, cells were exposed to (**A**) 5% normal human serum or (**B**) 5% C4-depleted human serum. Complement deposition (expressed as the mean fluorescence intensity, MFI) was assessed by flow cytometry using mAbs RFK22 and AE11 for staining of C3 and C5b-9, respectively. Results represent one out of two experiments.

Properdin binding and complement activation on PTEC

In order to test whether the PTEC cell line HK-2 is representative for primary PTEC lines, properdin binding and properdin-dependent complement activation was assessed on primary PTEC cultures. As shown in Figure 6a, properdin binding on PTEC is comparable to HK-2 (Figure 3a). The six tested PTEC cell lines showed variability in properdin binding (Figure 6b). None of the three HUVEC cell lines showed significant binding of properdin. However, the extent of properdin binding to PTEC was strongly correlated with the level of C3 deposition on these cells, r = 0.96 / p = 0.002 (Figure 6c).

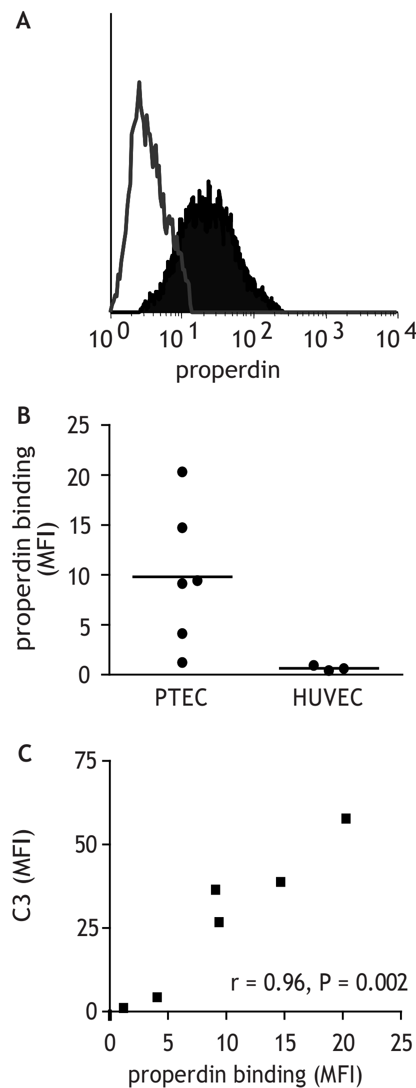


Figure 6. Properdin-mediated complement fixation on primary PTEC. (A) Primary proximal tubular epithelial cell (PTEC) lines were analysed for properdin binding (filled histogram) by flow cytometry after incubation with 20 µg/ml purified human properdin. The open histogram shows staining on cells that were not incubated with properdin. (B) Binding of properdin to different PTEC and human umbilical vein endothelial cell (HUVEC) lines. Properdin binding is expressed as the mean fluorescence intensity (MFI). The background fluorescence (primary and secondary antibody without properdin pre-incubation) is subtracted for each cell line individually. (C) Properdin binding and resulting properdin-dependent complement activation was tested by incubating the cells with 5% properdin-depleted human serum (Pds) after pre-exposure to 20 µg/ml purified properdin. Properdin binding and C3 deposition is shown as the mean fluorescence intensity (MFI). The association between properdin binding and the level of C3 deposition was analysed by calculating the Pearson correlation coefficient.

Discussion

In the present study, we show that properdin binds to the surface of viable PTEC. Properdin binding serves as a focal point for local amplification of the alternative pathway of complement on PTEC and explains the complement activating capacity of these cells.

It has been known for a long time that the apical surface of human proximal tubular epithelial cells activates the complement system *in vitro* and *in vivo* via the alternative pathway [13;14]. In patients suffering from chronic proteinuric renal disease, deposition of complement along the tubular brush border is accompanied by tubulointerstitial injury and progressive loss of renal function. Experimental models of non-selective proteinuria provide further evidence for the role of tubular complement activation in mediating tubulointerstitial injury [17;19]. C6 deficiency protects kidney function in the remnant kidney model as well as in the puromycin-induced model of nephrotic syndrome [16;18].

Although in physiological conditions complement components are not filtered through the glomerular barrier, several studies demonstrated the presence of complement activation products (CAP) in the urine of patients with nephrotic syndrome due to a variety of causes [10;37-39]. These studies showed a positive correlation between tubular C3 fixation and the excretion of complement components as well as complement activation products (including iC3b, Bb and C5b-9) in the urine. Interestingly, the level of urinary CAP excretion was significantly decreased after two weeks of oral sodiumbicarbonate administration [38;40]. The protective effect of bicarbonate was suggested to be due to lowering of the tubular ammonium concentration but may also be explained by a direct effect of increasing the urinary pH [41].

Despite extensive research, the mechanism of complement activation on the tubular brush border has not yet been fully elucidated. It was suggested that local ammonium reacts biochemically with the thioester of C3 and thereby acts as a C3 activator [20;21]. However, the addition of ammonium to serum only resulted in 15% increase in lysis of rabbit erythrocytes. This weak effect of ammonium on complement activation was only present in the lower concentration range. At higher concentrations, ammonium inhibited the alternative pathway. Recently, the activation of complement in proximal tubule cells was studied using proteinuric urine [41]. Increasing concentrations of ammonium resulted in an inhibition of complement activation. Ammonium excretion obviously does not fully explain the propensity of the renal tubule cells to activate the complement system.

Others have suggested that the lack of complement regulatory molecules on the apical surface of PTEC may explain the capacity of these cells to activate complement. Indeed, CD46 (membrane cofactor protein, MCP) only seems to be expressed on the basolateral surface of PTEC and CD55 (decay accelerating factor, DAF) could not be detected at all [42;43]. On the other hand CD59 is expressed abundantly on PTEC and surface expression of both CD46 and CD55 were detected on a PTEC cell line [44].

We suggest that the unique properdin binding capacity of PTEC critically controls the tubular complement activation in proteinuric states. In 1974, Sato *et al.* described that the damaging effect of intraluminally perfused normal rat serum on the rat kidney proximal tubule could be abolished by pre-incubating the serum with a brush border membrane fraction [45]. Possibly the effect of pre-inbcubation with the brush border membrane fraction is explained by its capacity to absorb properdin.

It was recently re-emphasized that properdin, the only known naturally occurring positive regulator of complement, can act as a focal point for alternative pathway amplification [27;28], thereby directing complement activation to the cell surface of apoptotic and necrotic cells [29;46]. Several decades before, Pillemer *et al.* suggested that properdin might also interact directly with target surfaces [23;31]. Likewise, we hypothesized that properdin might be the activator of the alternative pathway on the tubular brush border by interacting with molecules present on the cell membrane.

At the moment, the ligand on PTEC that mediates the interaction with properdin has not yet been identified. Properdin has been shown to bind to surface-bound C3b via one of its subunits followed by the assembly of the alternative pathway convertase at the ligand-binding sites of the adjoining subunits [27]. At the moment we can not fully exclude that properdin binds to PTEC via cell-bound C3b that is derived from endogenously produced and activated C3. Although C3b is undetectable by flow cytometry on PTEC, it might be present below the detection limit. On the other hand, it seems unlikely that significant amounts of C3b are present on quiescent cells. Recent data suggest that properdin also binds to the glycosphingolipid sulfatide [47]. The presence of sulfatide on the brush border of the tubules has been demonstrated in the rat kidney [48]. It is likely that these molecules are also expressed on the tubules in the human kidney, where they may mediate properdin binding to PTEC.

The mechanism by which a sublytic dose of C5b-9 on PTEC leads to tubular damage and subsequent tubulointerstitial fibrosis is thought to be via activation of proinflammatory and fibrogenic pathways [4]. Insertion of C5b-9 into the cell membrane of PTEC results in the production of proinflammatory cytokines and collagen synthesis. Interestingly, PTEC have been shown to synthesize a functional

alternative pathway of complement, which is capable of activating the cells [49]. This intratubular complement activation is tightly regulated and probably plays a role in protecting the kidney from urinary tract infections. Since the apical tubular surface does not come into contact with high concentrations of plasma proteins in normal physiology, protection against circulating complement is of less importance compared to circulating cells and the endothelium. However, in proteinuric renal disease, the tubules are exposed to filtered complement components. In these circumstances, the complement activating capacity of PTEC is harmful, especially since the apical surface has virtually no protection against complement attack [42].

Our data show that properdin binding to the brush border is the rate-limiting step in tubular complement activation. Targeting the interaction between properdin and the tubular brush border might be a therapeutic approach for controlling tubulointerstitial injury, thereby preventing progressive loss of kidney function in patients with chronic proteinuric renal disease.

Acknowledgements

We thank dr. Anja Roos for helpful discussions.

Reference List

1. Lysaght MJ. Maintenance dialysis population dynamics: current trends and long-term implications. *J Am Soc Nephrol* 2002; 13 Suppl 1:S37-S40.
2. Meguid EN, Bello AK. Chronic kidney disease: the global challenge. *Lancet* 2005; 365(9456):331-340.
3. Burton C, Harris KP. The role of proteinuria in the progression of chronic renal failure. *Am J Kidney Dis* 1996; 27(6):765-775.
4. Remuzzi G, Bertani T. Pathophysiology of progressive nephropathies. *N Engl J Med* 1998; 339(20):1448-1456.
5. Lewis EJ, Hunsicker LG, Bain RP, Rohde RD. The effect of angiotensin-converting-enzyme inhibition on diabetic nephropathy. The Collaborative Study Group. *N Engl J Med* 1993; 329(20):1456-1462.
6. Ruggenenti P, Perna A, Benini R, Bertani T, Zoccali C, Maggiore Q et al. In chronic nephropathies prolonged ACE inhibition can induce remission: dynamics of time-dependent changes in GFR. Investigators of the GISEN Group. Gruppo Italiano Studi Epidemiologici in Nefrologia. *J Am Soc Nephrol* 1999; 10(5):997-1006.
7. Wang Y, Chen J, Chen L, Tay YC, Rangan GK, Harris DC. Induction of monocyte chemoattractant protein-1 in proximal tubule cells by urinary protein. *J Am Soc Nephrol* 1997; 8(10):1537-1545.
8. Tang S, Leung JC, Tsang AW, Lan HY, Chan TM, Lai KN. Transferrin up-regulates chemokine synthesis by human proximal tubular epithelial cells: implication on mechanism of tubuloglomerular communication in glomerulopathic proteinura. *Kidney Int* 2002; 61(5):1655-1665.
9. Wohlfarth V, Drumm K, Mildenberger S, Freudinger R, Gekle M. Protein uptake disturbs collagen homeostasis in proximal tubule-derived cells. *Kidney Int Suppl* 2003;(84):S103-S109.
10. Klajman A, Avital A, Myers BD. Renal handling of the third (C3) and fourth (C4) components of the complement system in the nephrotic syndrome. Nephron 1976; 16(5):333-343.
11. Tang S, Lai KN, Sacks SH. Role of complement in tubulointerstitial injury from proteinuria. *Kidney Blood Press Res* 2002; 25(2):120-126.
12. Abe K, Li K, Sacks SH, Sheerin NS. The membrane attack complex, C5b-9, up regulates collagen gene expression in renal tubular epithelial cells. *Clin Exp Immunol* 2004; 136(1):60-66.
13. Biancone L, David S, Della P, V, Montrucchio G, Cambi V, Camussi G. Alternative pathway activation of complement by cultured human proximal tubular epithelial cells. *Kidney Int* 1994; 45(2):451-460.
14. Camussi G, Stratta P, Mazzucco G, Gaido M, Tetta C, Castello R et al. In vivo localization of C3 on the brush border of proximal tubules of kidneys from nephrotic patients. *Clin Nephrol* 1985; 23(3):134-141.
15. Camussi G, Rotunno M, Segoloni G, Brentjens JR, Andres GA. In vitro alternative pathway activation of complement by the brush border of proximal tubules of normal rat kidney. *J Immunol* 1982; 128(4):1659-1663.
16. Nangaku M, Pippin J, Couser WG. C6 mediates chronic progression of tubulointerstitial damage in rats with remnant kidneys. *J Am Soc Nephrol* 2002; 13(4):928-936.

17. Morita Y, Nomura A, Yuzawa Y, Nishikawa K, Hotta N, Shimizu F et al. The role of complement in the pathogenesis of tubulointerstitial lesions in rat mesangial proliferative glomerulonephritis. *J Am Soc Nephrol* 1997; 8(9):1363-1372.
18. Nangaku M, Pippin J, Couser WG. Complement membrane attack complex (C5b-9) mediates interstitial disease in experimental nephrotic syndrome. *J Am Soc Nephrol* 1999; 10(11):2323-2331.
19. He C, Imai M, Song H, Quigg RJ, Tomlinson S. Complement inhibitors targeted to the proximal tubule prevent injury in experimental nephrotic syndrome and demonstrate a key role for C5b-9. *J Immunol* 2005; 174(9):5750-5757.
20. Nath KA, Hostetter MK, Hostetter TH. Pathophysiology of chronic tubulo-interstitial disease in rats. Interactions of dietary acid load, ammonia, and complement component C3. *J Clin Invest* 1985; 76(2):667-675.
21. Nath KA, Hostetter MK, Hostetter TH. Ammonia-complement interaction in the pathogenesis of progressive renal injury. *Kidney Int Suppl* 1989; 27:S52-S54.
22. Walport MJ. Complement. First of two parts. *N Engl J Med* 2001; 344(14):1058-1066.
23. Pillemer L, Blum L, Lepow IH, Ross OA, Todd EW, Wardlaw AC. The properdin system and immunity. I. Demonstration and isolation of a new serum protein, properdin, and its role in immune phenomena. *Science* 1954; 120(3112):279-285.
24. Schwaeble WJ, Reid KB. Does properdin crosslink the cellular and the humoral immune response? *Immunol Today* 1999; 20(1):17-21.
25. Smith CA, Pangburn MK, Vogel CW, Muller-Eberhard HJ. Molecular architecture of human properdin, a positive regulator of the alternative pathway of complement. *J Biol Chem* 1984; 259(7):4582-4588.
26. Fearon DT, Austen KF. Properdin: binding to C3b and stabilization of the C3b-dependent C3 convertase. *J Exp Med* 1975; 142(4):856-863.
27. Hourcade DE. The role of properdin in the assembly of the alternative pathway C3 convertases of complement. *J Biol Chem* 2006; 281(4):2128-2132.
28. Spitzer D, Mitchell LM, Atkinson JP, Hourcade DE. Properdin can initiate complement activation by binding specific target surfaces and providing a platform for de novo convertase assembly. *J Immunol* 2007; 179(4):2600-2608.
29. Kemper C, Mitchell LM, Hourcade DE. Properdin: Cell death's little helper? *Mol Immunol* 2007; 107(Abstract):Abstract.
30. Kimura Y, Miwa T, Zhou L, Song WC. Activator-specific requirement of properdin in the initiation and amplification of the alternative pathway complement. *Blood* 2007.
31. WARDLAW AC, PILLEMER L. Inhibition of the bactericidal activity of the properdin system by zymosan and other properdin binding agents. *J Immunol* 1959; 82(4):313-318.
32. Ryan MJ, Johnson G, Kirk J, Fuerstenberg SM, Zager RA, Torok-Storb B. HK-2: an immortalized proximal tubule epithelial cell line from normal adult human kidney. *Kidney Int* 1994; 45(1):48-57.
33. van Kooten C, Gerritsma JS, Paape ME, van Es LA, Banchereau J, Daha MR. Possible role for CD40-CD40L in the regulation of interstitial infiltration in the kidney. *Kidney Int* 1997; 51(3):711-721.

34. Taekema-Roelvink ME, van Kooten C, Heemskerk E, Schroeijers W, Daha MR. Proteinase 3 interacts with a 111-kD membrane molecule of human umbilical vein endothelial cells. *J Am Soc Nephrol* 2000; 11(4):640-648.
35. Fontijn R, Hop C, Brinkman HJ, Slater R, Westerveld A, van Mourik JA et al. Maintenance of vascular endothelial cell-specific properties after immortalization with an amphotrophic replication-deficient retrovirus containing human papilloma virus 16 E6/E7 DNA. *Exp Cell Res* 1995; 216(1):199-207.
36. Nauta AJ, Trouw LA, Daha MR, Tijsma O, Nieuwland R, Schwaeble WJ et al. Direct binding of C1q to apoptotic cells and cell blebs induces complement activation. *Eur J Immunol* 2002; 32(6):1726-1736.
37. Lange K, Wenk EJ. Complement components in the sera and urines of patients with severe proteinurias. *Am J Med Sci* 1954; 228(4):448-453.
38. Morita Y, Ikeguchi H, Nakamura J, Hotta N, Yuzawa Y, Matsuo S. Complement activation products in the urine from proteinuric patients. *J Am Soc Nephrol* 2000; 11(4):700-707.
39. Ogrodowski JL, Hebert LA, Sedmak D, Cosio FG, Tamerius J, Kolb W. Measurement of SC5b-9 in urine in patients with the nephrotic syndrome. *Kidney Int* 1991; 40(6):1141-1147.
40. Rustom R, Grime JS, Costigan M, Maltby P, Hughes A, Taylor W et al. Oral sodium bicarbonate reduces proximal renal tubular peptide catabolism, ammoniogenesis, and tubular damage in renal patients. *Ren Fail* 1998; 20(2):371-382.
41. Peake PW, Pussell BA, Mackinnon B, Charlesworth JA. The effect of pH and nucleophiles on complement activation by human proximal tubular epithelial cells. *Nephrol Dial Transplant* 2002; 17(5):745-752.
42. Ichida S, Yuzawa Y, Okada H, Yoshioka K, Matsuo S. Localization of the complement regulatory proteins in the normal human kidney. *Kidney Int* 1994; 46(1):89-96.
43. Blok VT, Daha MR, Tijsma OM, Weissglas MG, van den Broek LJ, Gorter A. A possible role of CD46 for the protection in vivo of human renal tumor cells from complement-mediated damage. *Lab Invest* 2000; 80(3):335-344.
44. Li K, Feito MJ, Sacks SH, Sheerin NS. CD46 (membrane cofactor protein) acts as a human epithelial cell receptor for internalization of opsonized uropathogenic Escherichia coli. *J Immunol* 2006; 177(4):2543-2551.
45. Sato K, Ullrich KJ. Serum-induced inhibition of isotonic fluid absorption by the kidney proximal tubule. II. Evidence that complement is involved. *Biochim Biophys Acta* 1974; 354(2):182-187.
46. Xu W, Berger SP, Trouw LA, de Boer HC, Schlagwein N, Mutsaers C et al. Properdin binds to late apoptotic and necrotic cells independently of c3b and regulates alternative pathway complement activation. *J Immunol* 2008; 180(11):7613-7621.
47. Holt GD, Pangburn MK, Ginsburg V. Properdin binds to sulfatide [Gal(3-SO4)beta 1-1 Cer] and has a sequence homology with other proteins that bind sulfated glycoconjugates. *J Biol Chem* 1990; 265(5):2852-2855.
48. Trick D, Decker J, Groene HJ, Schulze M, Wiegandt H. Regional expression of sulfatides in rat kidney: immunohistochemical staining by use of monospecific polyclonal antibodies. *Histochem Cell Biol* 1999; 111(2):143-151.
49. Peake PW, O'Grady S, Pussell BA, Charlesworth JA. C3a is made by proximal tubular HK-2 cells and activates them via the C3a receptor. *Kidney Int* 1999; 56(5):1729-1736.

Chapter 8

General discussion

Multiple threats to the transplanted kidney

The renal transplant is exposed to numerous potentially harmful insults throughout its life. To start with, a transplanted kidney carries the disease burden of its donor which may limit the potential performance of the organ even before the process of transplantation has been initiated. Donor age, pre-existing cardiovascular disease and the acute disease prior to donation in the case of deceased donor kidney may all lead to structural damage impairing the future performance of the kidney. The transplant procedure itself is characterized by ischemia/reperfusion damage. Depending on the duration of the ischemia and the general condition of the organ, this insult may result in delayed graft function, an increased risk of rejection and poorer long term allograft survival. Next to the direct biochemical consequences of hypoxia and the generation of free radicals during the reperfusion period, organ damage during ischemia-reperfusion is also caused by inflammatory processes with the influx of immune cells, cytokine release and complement activation.

The next threat to the allograft is rejection due to allorecognition. Both cellular and humoral rejection remain important threats to the allograft and take their toll in terms of graft survival. Once the transplanted organ has survived the early phase after transplantation, acute rejection becomes less of a threat and drug toxicity will become an important issue. The use of calcineurin inhibitors has dramatically improved organ survival in the first year after transplantation but long term survival has improved less. This is partially explained by the harmful effect of this class of drugs but the increasing use of marginal donors with poorer quality of the transplanted organs may also play a role. The increasing interstitial and vascular damage now coined as chronic allograft dysfunction has multiple immune and non-immune mediated causes including chronic cellular and humoral rejection and drug toxicity [1].

Other threats to organ survival are infectious complications that have evolved as a consequence of the highly efficient prevention of acute rejection. BK-virus nephropathy is now a major problem after renal transplantation [2].

A further important issue after transplantation is recurrence of the original renal disease. Recurrence of the atypical hemolytic uremic syndrome, focal segmental glomerulosclerosis and membranoproliferative glomerulonephritis is associated with poor allograft survival. IgA nephropathy also recurs frequently but usually does not lead to loss of the allograft. In the setting of chronic allograft dysfunction or the recurrence of the underlying renal disease proteinuria may be present and contribute to progressive loss of function.

Death of the recipient is a further major cause for the loss of renal allografts. Post transplant malignancy and cardiovascular disease are the major causes of mortality after renal transplantation [3].

Potentially, the complement system may contribute to the damage to the transplanted kidney during all the processes described above. In fact considerable evidence has accumulated that complement plays a role at various stages of the transplantation process. In the following the general evidence for a role of complement in transplantation will be briefly reviewed followed by a summary and discussion of the evidence provided by the present thesis.

The role of complement in damage processes in renal transplantation

A considerable body of evidence points towards the role of complement in ischemia-reperfusion damage. In the case of the kidney, activation of the alternative pathway seems to be pivotal for the contribution of the complement system [4]. However, in myocardial and intestinal ischemia a clear role for the lectin pathway has been established [5;6]. Both in vitro [7] and in vivo [8] studies provide evidence for a role of natural IgM in the activation of the lectin pathway in ischemia-reperfusion damage. Preliminary data have also linked the lectin pathway to ischemia-reperfusion in the kidney. MBL can be detected in mouse and human kidneys exposed to ischemia-reperfusion [9] and MBL A and C deficient mice are partially protected against renal damage in a model of renal ischemia-reperfusion[10]. Various complement-inhibitory interventions have been used in animal models of renal ischemia-reperfusion. Both the administration of a C5 blocking antibody in mice and the renal perfusion with a complement regulator derived from CR1 in rats lead to decreased inflammation and clearly improved function in kidneys exposed to ischemia-reperfusion damage [11;12].

The paper by Pratt et al. reporting that mouse kidneys deficient for C3 are protected against rejection in a transplantation model has lead to a whole new area of research investigating the role of complement in regulating the adaptive immune response [13]. It is now clear that complement produced locally by antigen presenting cells, the renal epithelium and T-cells contributes to the generation of the adaptive immune response. Triggering of both the C3a and C5a receptor seems to be essential for this complement mediated costimulatory signal [14;15]. First, epidemiological data now support the concept that C3 contributes to damage in human kidney transplantation. In humans the C3 gene exists as two allotypes, F (fast) and S (slow). If recipients with the S/S allotype receive a kidney with the F allotype, either homo- or heterozygous,

allograft survival is significantly better than in recipients receiving kidneys which are homozygous for the S allotype [16]. At the moment the C3 polymorphisms have not been linked to clear functional consequences making a comprehensive explanation of these findings difficult.

Role of MBL

Allograft survival

The lectin pathway of complement activation may contribute to the fate of the transplanted organs at various stages of the transplant process. In view of the known interactions of MBL with apoptotic cells and immunoglobulins we questioned whether MBL would have an impact on organ survival after kidney transplantation. We hypothesized that MBL would contribute to ischemia/reperfusion damage and rejection-mediated damage after transplantation. **Chapter 3** reports on the role of MBL in allograft survival after deceased donor kidney transplantation [17]. Serum MBL levels were determined in pre-transplant serum samples obtained from 266 consecutive patients who received a renal transplant between 1990 and 1994 at our center. Patients were stratified according to their MBL level. We chose an MBL level of 400 ng/ml since this cutoff correlated best with the presence of a variant MBL genotype in a healthy control population. Recipients with low MBL levels had a significantly superior graft survival compared to those with an MBL level above 400 ng/ml. We did not find an effect of MBL levels on the occurrence of delayed graft function or acute rejection. However, the excess graft loss was explained by more severe and treatment-resistant rejection. From this epidemiological study we concluded that MBL does not contribute to the initiation of rejection but possibly contributes to the damage caused by the rejection. MBL may bind to damaged tissue in the context of rejection by interacting with either apoptotic cells or immunoglobulins. As mentioned above IgM may be specifically interesting in this context. At this point of time we do not have stainings with convincing deposition of MBL in biopsies showing rejection of human kidneys. However, we did find strong interstitial and glomerular deposition of MBL in the Fisher to Lewis rat model of chronic rejection (Figure 1). Next to MBL, co-deposition of IgG, IgM, C4 and C3 was detected, indicating that MBL deposition in this model might be triggered by immunoglobulins. In this context a recent paper studying C4 deposition in a model of humoral rejection of the heart is highly interesting [18]. C4 deposition was found to be related to the presence of both complement activating IgG2 and non-complement activating IgG1 antibodies. The marked reduction of complement deposition in an MBL-free system indicated

a role for the lectin pathway in C4 deposition during humoral allograft rejection. Since neither immunoglobulins, C1q or MBL is detected in human transplant biopsies showing C4 deposition the precise pathways contributing to the cleavage of C4 remain to be elucidated. In our opinion the lectin pathway of complement activation has to be taken into account.

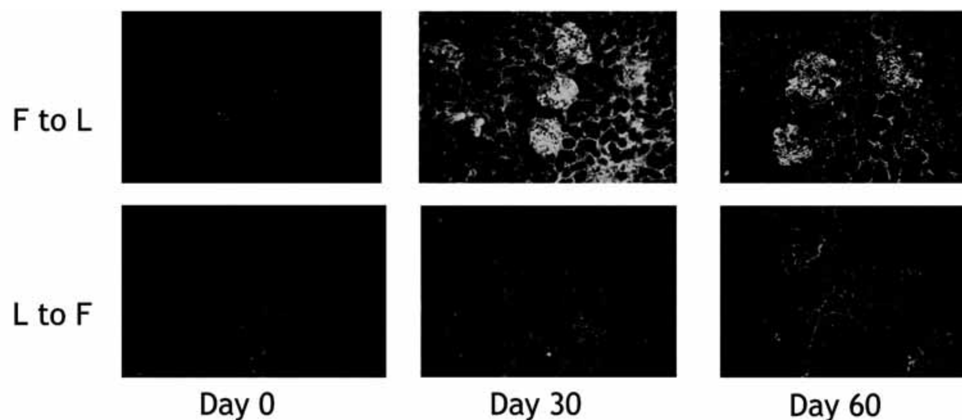


Figure 1. MBL A staining in the Fisher to Lewis model of chronic rejection. Kidneys obtained from Fischer rats were transplanted into Lewis rats (chronic rejection group, top row) and Lewis kidneys were transplanted into Fisher rats (control group, bottom row). Kidneys harvested at time points zero and after 30 and 60 days were stained for the presence of MBL A (see page 159 for color image).

Chapter 4 questioned which role MBL would have in simultaneous pancreas-kidney transplantation (SPKT). We thought it would be interesting to study this group next to the recipients of kidney transplants alone for the following reasons. 1. The type 1 diabetic population receiving a SPKT is characterized by a high burden of cardiovascular morbidity. A number of studies now point towards a role of the lectin pathway in cardiovascular morbidity and mortality. Specifically in the case of diabetes MBL seems to have a detrimental role in cardiovascular outcome. Studies performed in Danish type 1 diabetics demonstrated an association of high MBL levels with an increased incidence of cardiovascular disease and proteinuria [19;20]. In a cohort of subjects with type 2 diabetes followed for over 15 years MBL levels above 1000 were associated with increased mortality.

In our cohort of 99 patients who received a SPKT between 1990 and 2000 we were able to confirm our earlier studies demonstrating an association of MBL levels below 400 ng/ml with superior survival of the transplanted kidney. The first new result in this study was the finding that pancreas allograft survival was also better in the group with low MBL levels. However, the most striking result was the much higher risk of death in the group with MBL levels above 400 ng/ml when compared with the patients with lower MBL levels. We also were able to confirm this association with MBL by genotyping the recipients for the MBL gene polymorphisms associated with low levels. Kidney recipients with an MBL genotype associated with low levels had a relative risk for death of 3.6 compared to the recipients with variant MBL genotypes. As expected, the difference in survival was largely explained by a higher cardiovascular mortality in the group with a high MBL.

As mentioned above, various earlier studies have pointed towards a deleterious role of MBL in diabetes mellitus. However the exact mechanism of a contribution of MBL to cardiovascular damage remains unclear. One possibility is that MBL may not actually cause atherosclerotic disease but once an ischemic event occurs MBL may lead to enhanced damage to the affected organ. In line with this concept we recently described that patients with very low MBL levels undergoing cardiac surgery with the use of the heart/lung machine were protected against the development of multi-organ failure [21].

However, in an Icelandic cohort low MBL levels were associated with an increased risk of myocardial infarction [22]. Interestingly no data in the mortality of these patients was presented. So, possibly MBL does not lead to an increase in the frequency of cardiovascular events but high levels may be associated with more tissue damage and result in higher mortality. Again, the recently described interaction of MBL with IgM may be of interest in an attempt to explain our finding of reduced survival of high MBL recipients. Natural antibodies may interact with apoptotic cells in the setting of ischemia-reperfusion and may thus lead to MBL binding. However, natural antibodies have also been shown to bind to oxidized lipoproteins pointing towards a role of these antibodies in the pathogenesis of atherosclerosis [23]. We speculate that MBL may interact with these antibodies in the atherosclerotic plaque, leading to complement activation and inflammation.

A further interesting option may be the interaction of MBL with glycosylated proteins in diabetes. Enzymatic glycosylation of proteins via the hexosamine pathway is aberrant in diabetes mellitus [24]. Possibly these changed glycosylation patterns in the diabetic milieu allow the recognition of cell surface molecules by MBL and

subsequent activation of the lectin pathway. At this point of time no data on the interaction of MBL with aberrantly glycosylated proteins in diabetes are available.

Infectious complications after transplantation

After having described the role of MBL in allograft and patient survival after transplantation the question about the role of MBL in the protection against infectious complications after transplantation arose. Since the immunosuppressive regimes after transplantation are largely directed against adaptive immunity it would seem logical to expect an important role for the adaptive immune system in the protection against infections. As described in chapter one a role for the lectin pathway in the protection against infections has specifically been found in situations in which the adaptive immune system is impaired [25-27]. Bouwman et al. showed that MBL is protective against infectious complications after liver transplantation [28]. In our study on the role of MBL in infectious complications after simultaneous pancreas-kidney transplantation the transplantations performed between 1990 and 2005 at our center were scored for clinically significant infections in the first year after transplantation. In **chapter 5** we show that patients with high MBL levels at baseline experience less episodes of cystitis and urosepsis compared with patients with MBL levels above 400 ng/ml. Low MBL levels were the only identifiable risk factor for urosepsis in this cohort. Interestingly we did not find an association of MBL with wound infections or CMV infections. We had originally expected a role for MBL in wound infections since MBL has been shown to interact with *Staphylococcus aureus*, the major organism causing post-operative wound infections.

The urinary tract infections in our cohort consisted of infections with *Escherichia coli*, *Entrococcus faecalis* and *Klebsiella* species. We were not able to link the protective role of MBL to a specific organism. Possibly the subgroups were too small to detect specific organisms associated with MBL deficiency. Although infections and specifically urinary tract infections were very frequent, infection related mortality was very low in our cohort. So even if low MBL levels are associated with more infections after SPKT it seems clear that a low MBL status is preferable in view of the markedly better allograft and patient survival. To date we can not explain how MBL protects against urinary tract infections. From numerous studies it is clear that the kidney is a major site of complement synthesis [29;30]. It makes sense that the kidney should be able to generate protective complement molecules since urinary tract infections are one of the most frequent bacterial infections in humans and the renal epithelium comes into contact with ascending bacteria at an early phase of the

infection. However, it has been suggested that uropathogenic bacteria evade killing by the complement system and in fact make use of opsonisation by complement to invade renal cells via CD46 [31]. Currently no data exists that demonstrate local MBL production by the kidney in humans. Of interest is our observation of detectable levels of MBL in the urine during proteinuria and urinary tract infections (unpublished data).

A role for the alternative pathway

Chapters 6 and 7 in the present thesis investigate the rediscovered role of properdin as a pattern recognition molecule. Properdin is classically associated with its capacity to stabilize preformed C3 convertases. Recent papers have pointed towards the capacity of properdin to bind to pathogenic surfaces. The ligand for C3 on these surfaces may be pre-formed C3b but properdin also interacts with poorly defined non-complement ligands on e.g. bacteria [32-34]. In view of the important role of MBL and C1q in the recognition and clearance of apoptotic cells [35], we first questioned whether properdin could bind to apoptotic cells and whether this binding would lead to activation of the alternative pathway. In **chapter 6** we show that properdin binds to late apoptotic and necrotic Jurkat cells leading to activation of the alternative pathway [36]. By using splenocytes obtained from C3-deficient mice we were able to show that properdin can bind to a cellular surface independently of prior C3 activation and deposition of C3b. We also demonstrated that DNA which is expressed on the surface of apoptotic blebs is one of the ligands for properdin. These findings further establish a role for properdin as a pattern recognition molecule that contributes to the clearance of apoptotic cells. The exact quantitative importance of properdin-mediated clearance of apoptotic cells is not clear at the moment. C1q deficiency is strongly linked to the development of systemic lupus erythematosus [37]. No such link has been described for individuals with properdin deficiency who are susceptible for meningococcal infections. We speculate that properdin recognizes apoptotic renal cells in the setting of ischemia/reperfusion or rejection. Whether this interaction results in safe clearance of these damaged cells or contributes to the amplification of the inflammatory process is currently not clear.

In **chapter 7** the role of properdin in the activation of complement in the setting of tubular injury is described. Proteinuria is thought to contribute to progressive renal damage in numerous forms of proteinuric renal disease. Similarly to diseases

of the native kidneys proteinuria has also been established as a powerful predictor of graft loss in the setting of kidney transplantation [38]. The strong association between proteinuria and outcome suggests a causal relationship and activation of complement in the tubules may explain the harmful effect of proteinuria [39]. We show that properdin binds to tubular cells in the absence of other complement molecules and acts as a focal point for the subsequent activation of the alternative pathway. This binding of properdin is specific for the tubulus and was not detected on endothelial or circulating cells. We hypothesize that properdin filtered together with other complement molecules in proteinuric states binds to the proximal tubulus and then leads to activation of the alternative pathway with subsequent activation of tubular cells by sublytic membrane attack complex and the anaphylatoxins C3a and C5a. These findings may have important implications for renoprotective strategies in a large array of proteinuric renal diseases including proteinuria in the transplanted allograft.

Open questions and future plans

The studies in **chapter 3 and 4** show an association between low MBL levels and superior allograft survival after kidney transplantation alone and combined pancreas kidney transplantation. In **chapter 4** we were also able to confirm these results with genotyping excluding a role of an acute phase reaction in our findings. Like in all epidemiological studies it will be essential to confirm these findings in other populations before MBL levels can be used as a prognostic tool in clinical practice. We certainly hope that other groups will find our studies interesting enough and attempt to repeat them in other populations.

In studies investigating the association of MBL with outcome the method of MBL determination is a continuous matter of debate and a number of authors prefer genotyping since the genotype is not influenced by acute phase reactions of storage of the sample. However, currently we are only partially able to predict MBL levels by genotyping and the inter-individual variation of MBL concentrations within a group of individuals with the same genotype is substantial. Relying in the determination of the genotype only may result in missing some MBL-mediated effects since an important part of the variation is not recognized. Furthermore, it has been found that intra-individual MBL levels are highly stable over time [22] and that baseline levels strongly correlate with the levels induced by an acute phase reaction [40].

The used cutoff levels for MBL vary strongly between studies. This may partially be caused by differences in the biological effects that have been studied. Differences in the employed assays may also explain this lack of consensus. Greater standardization of MBL assays is highly desirable.

At this point of time we can only speculate about the mechanisms of MBL-mediated damage in the context of renal transplantation. Further pathophysiological studies are necessary to explain our epidemiological findings. Interesting approaches include studies on the interaction of MBL with IgM and aberrantly glycoslylated proteins.

As discussed in chapter two mutations of the complement regulators factor H and I play an important role in the hemolytic uremic syndrome and age-related macular degeneration. It will be interesting to study the role of these complement regulators in kidney transplantation.

Further research on the role of complement in proteinuria-mediated damage to the kidney may be very important in finding new strategies in the prevention of renal failure. It would be helpful if studies using urine from patients with proteinuria could establish a link between the urinary concentration of complement activation products and renal prognosis.

Next steps are to define the role of properdin in animal models of proteinuric renal disease. Identification of the ligand for MBL on the tubulus cell may allow the development of strategies to specifically inhibit tubular complement activation without blocking the entire alternative pathway.

Reference List

1. Joosten SA, Sijpkens YW, van Kooten C, Paul LC. Chronic renal allograft rejection: pathophysiologic considerations. *Kidney Int* 2005; 68(1):1-13.
2. Bohl DL, Brennan DC. BK virus nephropathy and kidney transplantation. *Clin J Am Soc Nephrol* 2007; 2 Suppl 1:S36-S46.
3. de Mattos AM, Prather J, Olyaei AJ, Shibagaki Y, Keith DS, Mori M et al. Cardiovascular events following renal transplantation: role of traditional and transplant-specific risk factors. *Kidney Int* 2006; 70(4):757-764.
4. Zhou W, Farrar CA, Abe K, Pratt JR, Marsh JE, Wang Y et al. Predominant role for C5b-9 in renal ischemia/reperfusion injury. *J Clin Invest* 2000; 105(10):1363-1371.
5. Hart ML, Ceonzo KA, Shaffer LA, Takahashi K, Rother RP, Reenstra WR et al. Gastrointestinal ischemia-reperfusion injury is lectin complement pathway dependent without involving C1q. *J Immunol* 2005; 174(10):6373-6380.
6. Walsh MC, Bourcier T, Takahashi K, Shi L, Busche MN, Rother RP et al. Mannose-Binding Lectin Is a Regulator of Inflammation That Accompanies Myocardial Ischemia and Reperfusion Injury. *J Immunol* 2005; 175(1):541-546.
7. McMullen ME, Hart ML, Walsh MC, Buras J, Takahashi K, Stahl GL. Mannose-binding lectin binds IgM to activate the lectin complement pathway in vitro and in vivo. *Immunobiology* 2006; 211(10):759-766.
8. Zhang M, Takahashi K, Alicot EM, Vorup-Jensen T, Kessler B, Thiel S et al. Activation of the lectin pathway by natural IgM in a model of ischemia/reperfusion injury. *J Immunol* 2006; 177(7):4727-4734.
9. de Vries B, Walter SJ, Peutz-Kootstra CJ, Wolfs TG, van Heurn LW, Buurman WA. The mannose-binding lectin-pathway is involved in complement activation in the course of renal ischemia-reperfusion injury. *Am J Pathol* 2004; 165(5):1677-1688.
10. Moller-Kristensen M, Wang W, Ruseva M, Thiel S, Nielsen S, Takahashi K et al. Mannan-binding lectin recognizes structures on ischaemic reperfused mouse kidneys and is implicated in tissue injury. *Scand J Immunol* 2005; 61(5):426-434.
11. de Vries B, Matthijsen RA, Wolfs TG, van Bijnen AA, Heeringa P, Buurman WA. Inhibition of complement factor C5 protects against renal ischemia-reperfusion injury: inhibition of late apoptosis and inflammation. *Transplantation* 2003; 75(3):375-382.
12. Patel H, Smith RA, Sacks SH, Zhou W. Therapeutic strategy with a membrane-localizing complement regulator to increase the number of usable donor organs after prolonged cold storage. *J Am Soc Nephrol* 2006; 17(4):1102-1111.
13. Pratt JR, Basheer SA, Sacks SH. Local synthesis of complement component C3 regulates acute renal transplant rejection. *Nat Med* 2002; 8(6):582-587.
14. Liu J, Lin F, Strainic MG, An F, Miller RH, Altuntas CZ et al. IFN-{gamma} and IL-17 Production in Experimental Autoimmune Encephalomyelitis Depends on Local APC-T Cell Complement Production. *J Immunol* 2008; 180(9):5882-5889.
15. Strainic MG, Liu J, Huang D, An F, Lalli PN, Muqim N et al. Locally produced complement fragments C5a and C3a provide both costimulatory and survival signals to naive CD4+ T cells. *Immunity* 2008; 28(3):425-435.

16. Brown KM, Kondeatis E, Vaughan RW, Kon SP, Farmer CK, Taylor JD et al. Influence of donor C3 allotype on late renal-transplantation outcome. *N Engl J Med* 2006; 354(19):2014-2023.
17. Berger SP, Roos A, Mallat MJ, Fujita T, de Fijter JW, Daha MR. Association between mannose-binding lectin levels and graft survival in kidney transplantation. *Am J Transplant* 2005; 5(6):1361-1366.
18. Murata K, Fox-Talbot K, Qian Z, Takahashi K, Stahl GL, Baldwin WM, III et al. Synergistic deposition of C4d by complement-activating and non-activating antibodies in cardiac transplants. *Am J Transplant* 2007; 7(11):2605-2614.
19. Hansen TK, Tarnow L, Thiel S, Steffensen R, Stehouwer CD, Schalkwijk CG et al. Association between mannose-binding lectin and vascular complications in type 1 diabetes. *Diabetes* 2004; 53(6):1570-1576.
20. Hovind P, Hansen TK, Tarnow L, Thiel S, Steffensen R, Flyvbjerg A et al. Mannose-binding lectin as a predictor of microalbuminuria in type 1 diabetes: an inception cohort study. *Diabetes* 2005; 54(5):1523-1527.
21. Bilgin YM, Brand A, Berger SP, Daha MR, Roos A. Mannose-binding lectin is involved in multiple organ dysfunction syndrome after cardiac surgery: effects of blood transfusions. *Transfusion* 2008; 48(4):601-608.
22. Saevarsdottir S, Oskarsson OO, Aspelund T, Eiriksdottir G, Vikingsdottir T, Gudnason V et al. Mannan binding lectin as an adjunct to risk assessment for myocardial infarction in individuals with enhanced risk. *J Exp Med* 2005; 201(1):117-125.
23. Shaw PX, Goodyear CS, Chang MK, Witztum JL, Silverman GJ. The autoreactivity of anti-phosphorylcholine antibodies for atherosclerosis-associated neo-antigens and apoptotic cells. *J Immunol* 2003; 170(12):6151-6157.
24. Buse MG. Hexosamines, insulin resistance, and the complications of diabetes: current status. *Am J Physiol Endocrinol Metab* 2006; 290(1):E1-E8.
25. Mullighan CG, Heatley S, Doherty K, Szabo F, Grigg A, Hughes TP et al. Mannose-binding lectin gene polymorphisms are associated with major infection following allogeneic hemopoietic stem cell transplantation. *Blood* 2002; 99(10):3524-3529.
26. Peterslund NA, Koch C, Jensenius JC, Thiel S. Association between deficiency of mannose-binding lectin and severe infections after chemotherapy. *Lancet* 2001; 358(9282):637-638.
27. Koch A, Melbye M, Sorensen P, Homoe P, Madsen HO, Molbak K et al. Acute respiratory tract infections and mannose-binding lectin insufficiency during early childhood. *JAMA* 2001; 285(10):1316-1321.
28. Bouwman LH, Roos A, Terpstra OT, de Knijff P, van Hoek B, Verspaget HW et al. Mannose binding lectin gene polymorphisms confer a major risk for severe infections after liver transplantation. *Gastroenterology* 2005; 129(2):408-414.
29. Seelen MA, Brooimans RA, van der Woude FJ, van Es LA, Daha MR. IFN-gamma mediates stimulation of complement C4 biosynthesis in human proximal tubular epithelial cells. *Kidney Int* 1993; 44(1):50-57.
30. van den Dobbelsteen ME, Verhasselt V, Kaashoek JG, Timmerman JJ, Schroeijers WE, Verweij CL et al. Regulation of C3 and factor H synthesis of human glomerular mesangial cells by IL-1 and interferon-gamma. *Clin Exp Immunol* 1994; 95(1):173-180.

31. Springall T, Sheerin NS, Abe K, Holers VM, Wan H, Sacks SH. Epithelial secretion of C3 promotes colonization of the upper urinary tract by Escherichia coli. *Nat Med* 2001; 7(7):801-806.
32. Hourcade DE. The role of properdin in the assembly of the alternative pathway C3 convertases of complement. *J Biol Chem* 2006; 281(4):2128-2132.
33. Spitzer D, Mitchell LM, Atkinson JP, Hourcade DE. Properdin can initiate complement activation by binding specific target surfaces and providing a platform for de novo convertase assembly. *J Immunol* 2007; 179(4):2600-2608.
34. Kimura Y, Miwa T, Zhou L, Song WC. Activator-specific requirement of properdin in the initiation and amplification of the alternative pathway complement. *Blood* 2007.
35. Nauta AJ, Castellano G, Xu W, Woltman AM, Borrias MC, Daha MR et al. Opsonization with C1q and mannose-binding lectin targets apoptotic cells to dendritic cells. *J Immunol* 2004; 173(5):3044-3050.
36. Xu W, Berger SP, Trouw LA, de Boer HC, Schlagwein N, Mutsaers C et al. Properdin binds to late apoptotic and necrotic cells independently of c3b and regulates alternative pathway complement activation. *J Immunol* 2008; 180(11):7613-7621.
37. Cook HT, Botto M. Mechanisms of Disease: the complement system and the pathogenesis of systemic lupus erythematosus. *Nat Clin Pract Rheumatol* 2006; 2(6):330-337.
38. Halimi JM, Buchler M, Al Najjar A, Laouad I, Chatelet V, Marliere JF et al. Urinary albumin excretion and the risk of graft loss and death in proteinuric and non-proteinuric renal transplant recipients. *Am J Transplant* 2007; 7(3):618-625.
39. Abbate M, Zoja C, Remuzzi G. How does proteinuria cause progressive renal damage? *J Am Soc Nephrol* 2006; 17(11):2974-2984.
40. Van Till JW, Boermeester MA, Modderman PW, Van Sandick JW, Hart MH, Gisbertz SS et al. Variable mannose-binding lectin expression during postoperative acute-phase response. *Surg Infect* (Larchmt) 2006; 7(5):443-452.

Nederlandse Samenvatting

Het afweersysteem bestaat uit een aangeboren en een verworven deel. Traditioneel wordt in het transplantatieveld veel aandacht aan het verworven deel van het immuunsysteem besteed. Hierbij wordt gedacht dat B- en T-cellen het getransplanteerde weefsel als vreemd herkennen en een specifieke afweerreactie tegen dit als vreemd herkende weefsel veroorzaken. De geneesmiddelen die gebruikt worden om afstoting te voorkomen en te behandelen richten zich dan ook voornamelijk op het verworven afweersysteem.

In de afgelopen jaren is er een toenemende belangstelling voor de rol van het aangeboren afweersysteem in de transplantatiegeneeskunde ontstaan. Hierbij is duidelijk geworden dat het aangeboren afweersysteem een belangrijke rol in de aansturing van het verworven afweersysteem speelt. Het aangeboren afweersysteem speelt niet alleen een rol bij de herkenning van vreemde organismen maar kan ook schade herkennen. Het aangeboren afweersysteem bestaat uit cellen en eiwitten die in staat zijn patronen op micro-organismen en beschadigde cellen te herkennen en deze structuren snel op te ruimen. In de context van niertransplantatie speelt het aangeboren afweersysteem een rol bij de initiële schade door zuurstoftekort (ischemie en reperfusie), de initiatie en controle van afstotingsreacties, de chronische schade bij een chronische allograft nefropathie en bij de terugkeer van de oorspronkelijke ziekte. Reeds voor de transplantatie kan het aangeboren afweersysteem een rol spelen bij het ontstaan van chronische schade in de ontvanger en het te transplanteren orgaan. Naast deze beschadigende rol heeft het aangeboren afweersysteem een belangrijke rol in de bescherming tegen infecties in een situatie waar het verworven deel van het afweersysteem onderdrukt wordt.

Het complementsysteem is een belangrijk onderdeel van het aangeboren afweersysteem. Het bestaat uit ongeveer 30 moleculen die middels drie verschillende routes geactiveerd worden. De drie routes worden elk door de interactie van eigen herkenningsmoleculen met oppervlaktepatronen geactiveerd. Deze patronen kunnen bijvoorbeeld op de oppervlakte van micro-organismen, op immuuncomplexen of op dode (≈ apoptotische) cellen aanwezig zijn. Hoofdstuk twee van dit proefschrift geeft een overzicht over de drie activeringsroutes en hun rol in de nefrologie.

In dit proefschrift concentreren wij ons op de betekenis van de lectine en alternatieve route bij niertransplantatie.

Activering van de lectineroute wordt door binding van de herkenningsmoleculen mannosebindend lectine (MBL) en ficoline aan suikergroepen op het oppervlak

van micro-organismen en beschadigde cellen veroorzaakt. Een gebrek aan het herkenningsmolecuul MBL door frequent voorkomende polymorfismen van het MBL gen resulteert in een verhoogde gevoeligheid voor infecties in situaties waarin het verworven afweersysteem gestoord is. Typische voorbeelden zijn patiënten die chemotherapie of een stamceltransplantatie ondergaan. Uit verschillende lijnen van onderzoek is gebleken dat MBL niet alleen een beschermende rol speelt maar ook aan weefselschade kan bijdragen. De lectineroute speelt bijvoorbeeld een rol bij de weefselschade door ischemie en reperfusie. In epidemiologische studies is gebleken dat patiënten met diabetes mellitus type 1 en een hoog MBL meer nierschade en cardiovasculaire ziekte hebben dan patiënten met een laag MBL.

Gezien deze schadelijke rol van MBL vroegen wij ons af of MBL aan de beschadiging van een getransplanteerde nier bijdraagt. In hoofdstuk drie wordt beschreven dat hoge MBL-spiegels bij ontvangers van een niertransplantatie geassocieerd zijn met een slechtere overleving van het getransplanteerde orgaan. In deze studie hebben wij in een groep van getransplanteerde patiënten de MBL spiegels voor transplantatie bepaald en de overleving van het getransplanteerde orgaan in ontvangers met een laag of hoog MBL vergeleken. Ontvangers met een hoog MBL hadden een duidelijk verhoogd risico om de getransplanteerde nier te verliezen vergeleken met de ontvangers met een laag MBL. Het bleek dat het orgaanverlies bij patiënten met een hoog MBL door moeilijk behandelbare afstoting veroorzaakt werd.

In hoofdstuk 4 wordt de rol van MBL bij patiënten met een gecombineerde nier-pancreas transplantatie beschreven. Deze patiëntengroep wordt door bijzonder veel cardiovasculaire complicaties, afstoting en infectieuze problemen gekenmerkt. Ook in deze groep was een laag MBL met een betere overleving van de getransplanteerde nier geassocieerd. Nieuw was dat ook de overleving van de getransplanteerde pancreas in patiënten met een laag MBL beter was dan bij patiënten met een hoog MBL. Bijzonder indrukwekkend was de bevinding dat ontvangers met een hoog MBL een duidelijk hogere sterfte hadden. Deze verhoogde sterfte werd ook bij patiënten met een MBL-genotype gevonden dat met een hoog MBL geassocieerd is. Omdat wij onze bevindingen middels een genotypering voor de belangrijkste MBL genvarianten konden bevestigen, kunnen wij een artefact door een acute fase response of door bewaren van het serum uitsluiten. De verhoogde sterfte in de groep met een hoog MBL werd vooral door een verhoogde cardiovasculaire sterfte verklaard.

Het lijkt dat MBL niet zozeer een verhoogde incidentie van schadelijke gebeurtenissen veroorzaakt maar eerder de schade en de gevolgen verhoogt.

Nadat wij de betere overleving bij ontvangers met een laag MBL gevonden hadden vroegen wij ons af in hoeverre een laag MBL met een toegenomen risico op infecties gepaard gaat. Tenslotte is in de literatuur overtuigend aangetoond dat MBL een beschermende rol speelt tegen infecties bij patiënten met een gestoorde verworven immuniteit. Om dit te onderzoeken hebben wij bij patiënten die tussen 1990 en 2005 een nier-pancreas transplantatie hebben ondergaan alle relevante infecties in het eerste jaar na de transplantatie gescoord en de MBL waardes in het serum in een direct voor de transplantatie afgenomen monster bepaald. De patiënten met een MBL-spiegel onder 400 ng/ml maakten meer episodes van blaasontsteking door vergeleken met de patiënten met een MBL spiegel boven 400 ng/ml. Ook was een lage MBL spiegel bij de ontvanger geassocieerd met een verhoogde incidentie van door urineweginfectie veroorzaakte sepsis. Er werd geen samenhang tussen MBL en het voorkomen van wondinfecties en cytomegalievirusinfecties gevonden.

Het blijkt dus dat lage MBL spiegels gunstig zijn voor de overleving van het getransplanteerde orgaan en in het geval van een gecombineerde nier-pancreastransplantatie ook voor de overleving van de patiënt. Dit voordeel van een lage MBL spiegel gaat wel gepaard met een verhoogd risico op infecties. Gelukkig zijn de meeste na een transplantatie voorkomende infecties goed te behandelen en overlijden maar weinig patiënten hieraan.

De klaring van apoptotische cellen speelt een belangrijke rol in de initiatie van de afweerreactie bij transplantatie en autoimmuunziekten. De complementmoleculen MBL en C1q herkennen apoptotische cellen en dragen aan de klaring van deze cellen bij. Pas recent werd de rol van properdine als patroonherkenningsmolecuul herontdekt. In hoofdstuk 6 beschrijft dit proefschrift dat properdine aan laat- apoptotische cellen en necrotische cellen bindt. De binding van properdine vertoont een patroon dat vergelijkbaar is met dat van MBL en C1q en is onafhankelijk van de aanwezigheid van geactiveerd C3 (C3b). DNA dat op de oppervlakte van apoptotische en necrotische cellen aanwezig is, is een potentiële ligand voor properdine op deze cellen.

In hoofdstuk 7 beschrijven wij de interactie van properdine met niertubuluscellen. Complementactivatie ter hoogte van de niertubuli wordt gezien als belangrijke oorzaak voor chronische schade bij nierziekten die gepaard gaan met een verhoogde eiwituitscheiding in de urine. Eerdere studies hebben aangetoond dat complement op de oppervlakte van tubuluscellen geactiveerd wordt. Tot nu was het mechanisme van deze complementactivatie onduidelijk. Wij laten zien dat properdine aan levende gekweekte tubuluscellen bindt. Deze binding is essentieel voor de complementactivatie

op de oppervlakte van het tubulus. Indien het lukt het bindende molecuul voor properdine op de tubuluscellen te identificeren is het misschien mogelijk op een specifieke manier proteïnurie-gemedieerde nierschade te voorkomen.

In het onderzoek beschreven in dit proefschrift wordt met epidemiologische en experimentele methodes een belangrijke rol voor het complementsysteem in de context van niertransplantatie beschreven. De bevindingen betreffende de rol van MBL kunnen een rol spelen bij de risicostratificatie en beslisvorming rondom het transplantatieproces. Ook wijzen onze gegevens op mogelijke risico's van MBL suppletie. De interactie van properdine met tubuluscellen biedt een mogelijke nieuwe toegang tot de ontwikkeling van progressie remmende therapieën voor proteïnurische nierziekten.

Curriculum Vitae

De schrijver van dit proefschrift werd geboren op 19 augustus 1969 in Johannesburg, Zuid Afrika. Na schoolbezoek in Engeland, Nederland en Duitsland deed hij 1988 eindexamen (Abitur) aan het Gustav Streseman Gymnasium in Bad Wildungen, Duitsland. Na alternatieve dienst te hebben verricht in een centrum voor ruggenmergletsels begon hij in 1990 met de studie geneeskunde aan de Universiteit Witten-Herdecke, Duitsland. Tijdens zijn studie werkte hij gedurende 10 maanden in het lab nierziekten aan het LUMC aan een project over immuunstimulerende effecten van proteinase 3 onder supervisie van professor M.R. Daha.

Na het behalen van zijn artsexamen in october 1996 is hij in november 1996 met de opleiding interne geneeskunde aan het Universiteitsziekenhuis Mannheim begonnen (opleider Prof. Dr. F.J. van der Woude). Tijdens de periode in Mannheim deed hij onderzoek over orgaanpreconditionering. Van 2000 tot 2003 werd de opleiding tot internist in het LUMC voltooid (opleider Prof. Dr. A.E. Meinders). In 2003 begon hij met de opleiding tot nefroloog in het LUMC (opleiders Prof. Dr. L.C. Paul, Prof. Dr. J.W. de Fijter en Prof. Dr. A.J. Rabelink) en werd het onderzoek over complement en transplantatie begonnen. In 2005 werd de auteur hoofd van de poli nierziekten en transplantatie in het LUMC. Sinds juni 2008 is hij werkzaam als internist-nefroloog in het HagaZiekenhuis te Den Haag.

List of Publications

1. **Berger SP**, Seelen MA, Hiemstra PS, Gerritsma JS, Heemskerk E, Van Der Woude FJ, Daha MR. Proteinase 3, the major autoantigen of Wegener's granulomatosis, enhances IL-8 production by endothelial cells in vitro. *J Am Soc Nephrol* 7: 694-701, 1996.
2. **Berger SP**, Hunger M, Yard BA, Schnuelle P, Van Der Woude FJ. Dopamine induces the expression of heme oxygenase-1 by human endothelial cells in vitro. *Kidney Int* 58: 2314-9, 2000.
3. Schnuelle P, **Berger S**, de Boer J, Persijn G, Van Der Woude FJ. Donor employment of vasopressors and its impact on allograft survival after transplantation. *Transplant Proc* 33: 1282-3, 2001.
4. Schnuelle P, **Berger S**, de Boer J, Persijn G, Van Der Woude FJ. Effects of catecholamine application to brain-dead donors on graft survival in solid organ transplantation. *Transplantation* 72: 455-63, 2001.
5. **Berger SP**, Roos A, Mallat MJ, Fujita T, de Fijter JW, Daha MR. Association between mannose-binding lectin levels and graft survival in kidney transplantation. *Am J Transplant* 5: 1361-6, 2005.
6. **Berger SP**, Roos A, Daha MR. Complement and the kidney: what the nephrologist needs to know in 2006? *Nephrol Dial Transplant* 20: 2613-9, 2005.
7. **Berger SP**, Roos A, Mallat MJ, Fujita T, de Fijter JW, Daha MR. Association between mannose-binding lectin levels and graft survival in kidney transplantation. *Am J Transplant* 5: 1361-6, 2005.
8. **Berger SP**, Roos A, Daha MR. Complement and the kidney: what the nephrologist needs to know in 2006? *Nephrol Dial Transplant* 20: 2613-9, 2005.
9. Bouwman LH, Roos A, Terpstra OT, de Knijff P, van Hoek B, Verspaget HW, **Berger SP**, Daha MR, Frolich M, van der Slik AR, Doxiadis II, Roep BO, Schaapherder AF. Mannose binding lectin gene polymorphisms confer a major risk for severe infections after liver transplantation. *Gastroenterology* 129: 408-14, 2005.
10. **Berger SP**, Groeneveld TWL, Roos A, Daha MR. C1q and the glomerulopathies: therapeutic approaches for the treatment of complement-mediated diseases. In: Zipfel PF, ed. *Complement and Kidney Disease*. Basel: Birkhäuser, p. 37-47, 2006.
11. **Berger SP**. Azathioprin und Mycophenolat bei Lupusnephritis. In: Aringer M, ed. Aktuelle Therapieoptionen beim systemischen Lupus erythematodes. Bremen: Uni-Med Verlag AG, p. 40-45, 2007.

12. **Berger SP**, Daha MR. Complement in glomerular injury. *Semin Immunopathol* 29: 375-84, 2007.
13. Chandie Shaw PK, **Berger SP**, Mallat M, Frolich M, Dekker FW, Rabelink TJ. Central obesity is an independent risk factor for albuminuria in nondiabetic South Asian subjects. *Diabetes Care* 30: 1840-4, 2007.
14. de Boer MG, Bruijnesteijn van Coppenraet LE, Gaasbeek A, **Berger SP**, Gelinck LB, van Houwelingen HC, van den BP, Kuijper EJ, Kroon FP, Vandenbroucke JP. An outbreak of Pneumocystis jiroveci pneumonia with 1 predominant genotype among renal transplant recipients: interhuman transmission or a common environmental source? *Clin Infect Dis* 44: 1143-9, 2007.
15. Roos A, Daha MR, van Pelt J, **Berger SP**. Mannose-binding lectin and the kidney. *Nephrol Dial Transplant* 22: 3370-7, 2007.
16. Bilgin YM, Brand A, **Berger SP**, Daha MR, Roos A. Mannose-binding lectin is involved in multiple organ dysfunction syndrome after cardiac surgery: effects of blood transfusions. *Transfusion* 48: 601-8, 2008.
17. Cohen D, Koopmans M, Kremer H, I, **Berger SP**, van Groningen MR, Steup-Beekman GM, de HE, Bruijn JA, Bajema IM. Potential for glomerular C4d as an indicator of thrombotic microangiopathy in lupus nephritis. *Arthritis Rheum* 58: 2460-9, 2008.
18. Gaarkeuken H, Siezenga MA, Zuidwijk K, van KC, Rabelink TJ, Daha MR, **Berger SP**. Complement activation by tubular cells is mediated by properdin binding. *Am J Physiol Renal Physiol 2008: 295(5), F1397-403*.
19. Verschuren JJ, Roos A, Schaapherder AF, Mallat MJ, Daha MR, de Fijter JW, **Berger SP**. Infectious Complications After Simultaneous Pancreas-Kidney Transplantation: A Role for the Lectin Pathway of Complement Activation. *Transplantation* 85: 75-80, 2008.
20. Xu W, **Berger SP**, Trouw LA, de Boer HC, Schlagwein N, Mutsaers C, Daha MR, van Kooten C. Properdin binds to late apoptotic and necrotic cells independently of c3b and regulates alternative pathway complement activation. *J Immunol* 180: 7613-21, 2008.

Nawoord

In tegenstelling tot velen heb ik het promotietraject niet als zwaar of belastend ervaren. Ik voel ook geen opluchting maar misschien eerder verlies nu ik deze woorden kan schrijven. De onderzoeksprojecten in dit proefschrift hebben mij veel plezier gegeven en ook zonder promotietraject had ik deze studies zo willen doen.

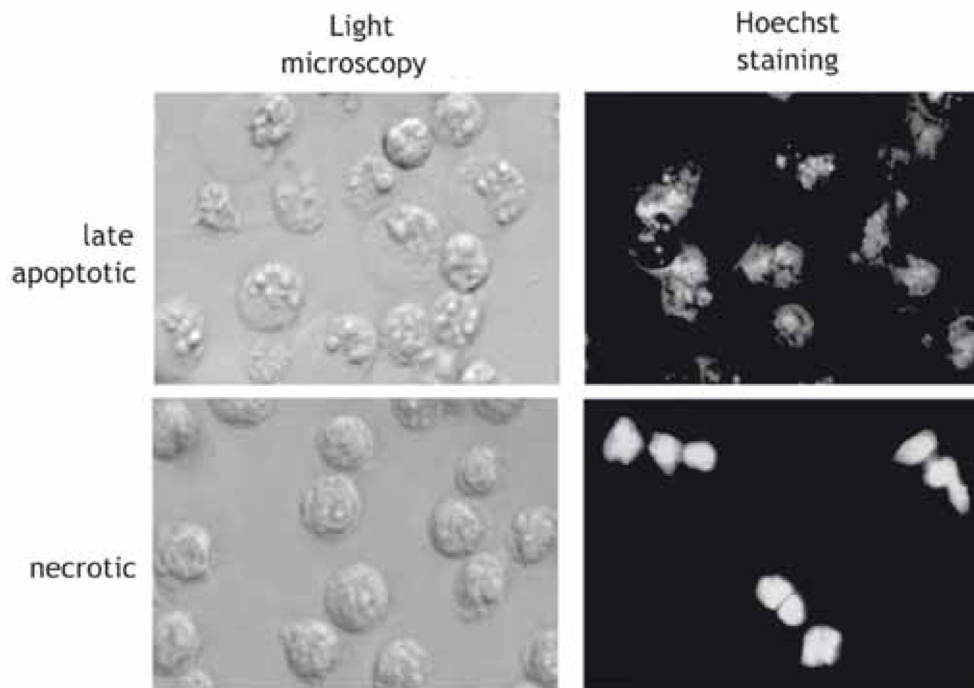
Vanzelfsprekend ben ik velen die mij welwillend geholpen hebben tot dank verschuldigd. Allereerst wil ik de talrijke medewerkers van het lab nierziekten bedanken die mij de afgelopen jaren geholpen hebben. De gedrevenheid en expertise op het lab maken het mogelijk voor (vrijwel) elke vraagstelling een partner te vinden om naar een oplossing te zoeken. Mijn bijzondere dank gaat uit naar Reinier van der Geest en Maria Essers.

Marko! Buurman en stille held. Jij bent mijn epidemiologieleraar en jouw deur stond voor mij altijd open. Juist omdat je mij geholpen hebt om mijn analyses zelf te doen is jouw rol in dit proefschrift en voor mijn wetenschappelijke ontwikkeling bijzonder groot.
Jeffrey, het was een genoegen jou te begeleiden. Met veel trots kijk ik naar het resultaat van onze samenwerking en hoop op wetenschappelijk gebied nog veel van je te horen.
Hilde, de tijd op het lab was voor jou niet altijd makkelijk en niet alle editors deelden mijn enthousiasme over ons werk. Maar onze samenwerking heeft wel iets achtergelaten en ik hoop dat je tevreden met het resultaat bent.

Ik dank alle stafleden en de fellows van de afdeling nierziekten voor de samenwerking en de secretaresses voor alle ondersteuning.

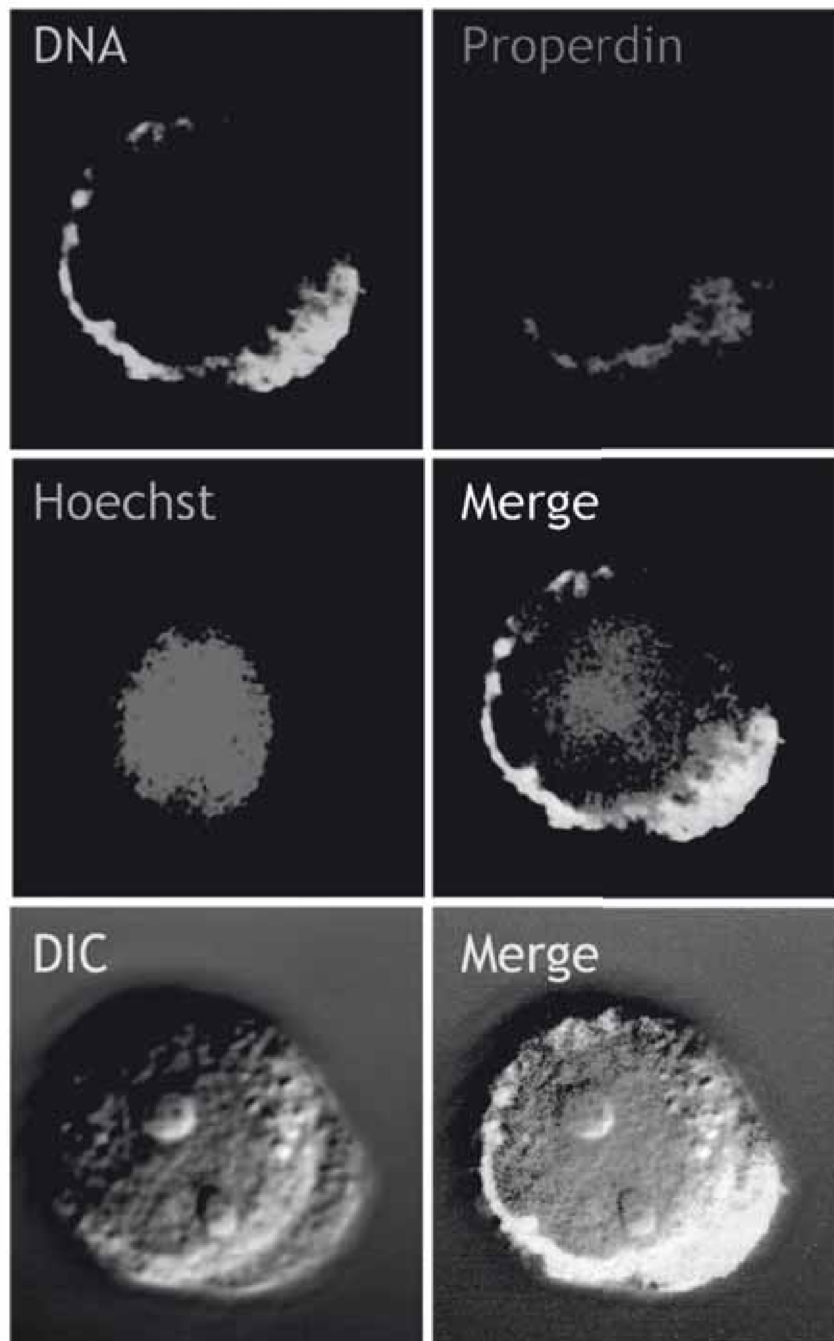
Tanja, jouw directe betrokkenheid bij dit proefschrift was beperkt. Jouw betrokkenheid bij mijn leven is onmeetbaar. Jij bent mijn kompas, mijn vuurtoren, mijn landvast en de moeder van mijn fantastische kinderen.

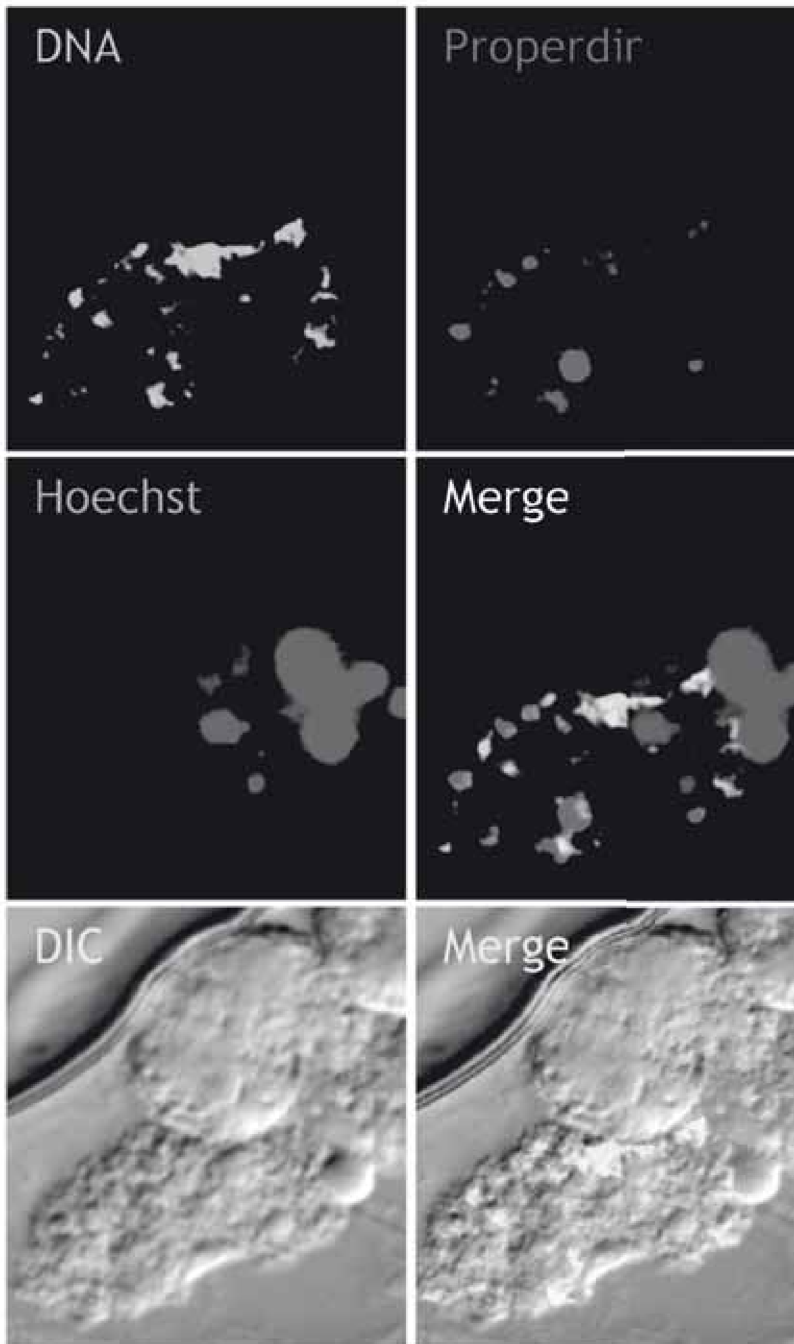
Color figures



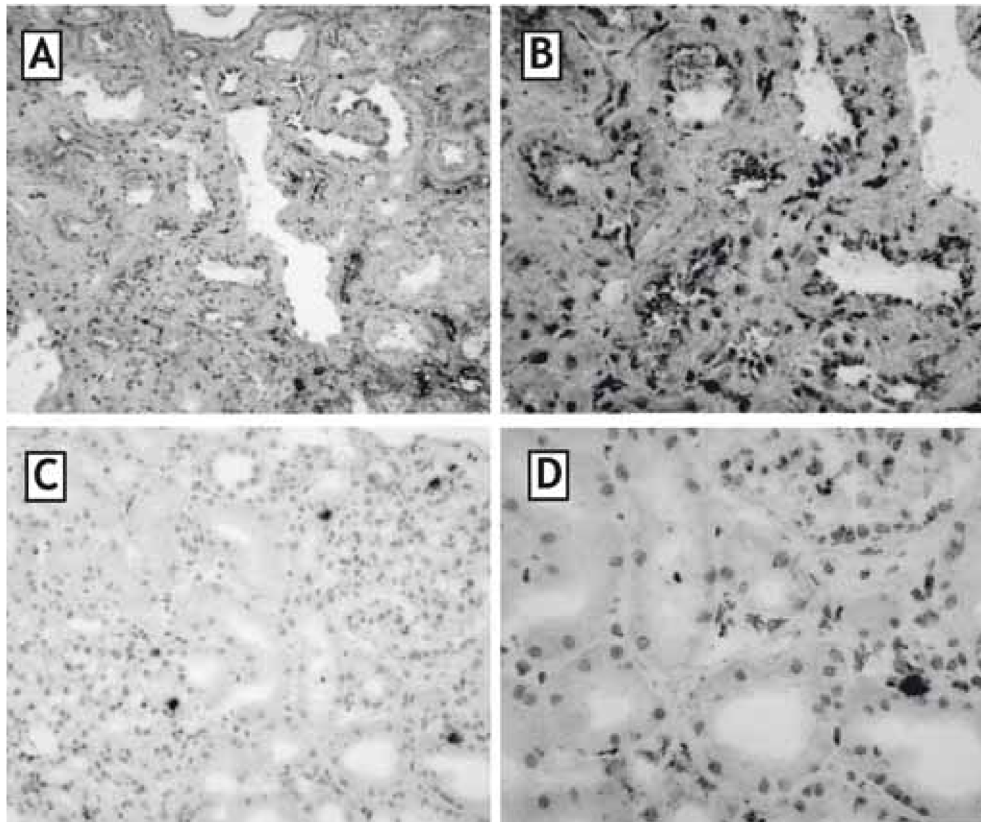
Chapter 6, figure 1. **Complement-mediated phagocytosis of dying cells**. Late apoptotic cells and necrotic cells are scored by light microscopy or fluorescent microscopy for hoechst staining on cytospins of these cells. Magnification, 200× (see page 92 for complete image).

Chapter 6, figure 6. **Properdin binds to DNA**. Confocal laser scanning microscopy (LSM 510, Carl Zeiss AG), was performed on properdin-opsonized necrotic cells that were stained for properdin, DNA and Hoechst. Green: DNA; Red: properdin; Blue: Hoechst; Yellow: DNA co-localizes with properdin; DIC: differential interference contrast. Magnification, 400× (see page 100 for complete image).

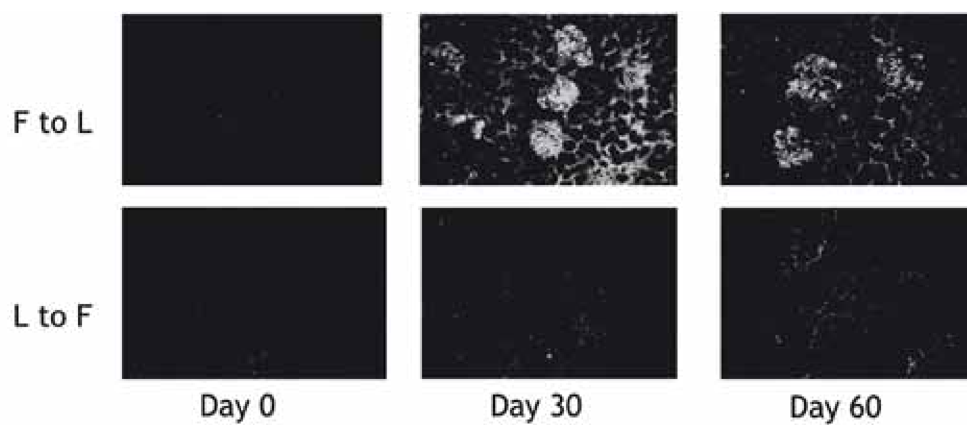

DNA
Properdin
Hoechst
Merge
DIC
Merge



Chapter 6, figure 7. Properdin binds to a subpopulation of late apoptotic cells. Confocal microscopy was performed on properdin-opsonized late apoptotic cells that were stained for properdin, DNA and Hoechst.
Green: DNA;
Red: properdin;
Blue: Hoechst;
Yellow: DNA co-localizes with properdin;
DIC: differential interference contrast.
Magnification, 400× (see page 102 for complete image).



Chapter 7, figure 1. Properdin staining on the tubular brush border in proteinuric kidneys. See page 160 for explanation.



Chapter 8, figure 1. MBL A staining in the Fisher to Lewis model of chronic rejection. See page 160 for explanation.

Chapter 7, figure 1. **Properdin staining on the tubular brush border in proteinuric kidneys**. Cryosections of (A and B) a renal biopsy of a patient with membranous nephropathy and (C and D) a pretransplant biopsy of a healthy donor were stained immunohistochemically for properdin. (A and C) Original magnifications were either × 100 or (B and D) × 250. Pictures are representative for three patients with membranous nephropathy and three healthy kidneys donors (see page 118 for gray image).

Chapter 8, figure 1. **MBL A staining in the Fisher to Lewis model of chronic rejection**. Kidneys obtained from Fischer rats were transplanted into Lewis rats (chronic rejection group, top row) and Lewis kidneys were transplanted into Fisher rats (control group, bottom row). Kidneys harvested at time points zero and after 30 and 60 days were stained for the presence of MBL A (see page 118 for gray image).