

# Glomerular Activation of the Lectin Pathway of Complement in IgA Nephropathy Is Associated with More Severe Renal Disease

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IgA nephropathy (IgAN) is characterized by glomerular co-deposition of IgA and complement components. Earlier studies showed that IgA activates the alternative pathway of complement, whereas more recent data also indicate activation of the lectin pathway. The lectin pathway can be activated by binding of mannose-binding lectin (MBL) and ficolins to carbohydrate ligands, followed by activation of MBL-associated serine proteases and C4. This study examined the potential role of the lectin pathway in IgAN. Renal biopsies of patients with IgAN ( $n = 60$ ) showed mesangial deposition of IgA1 but not IgA2. Glomerular deposition of MBL was observed in 15 (25%) of 60 cases with IgAN and showed a mesangial pattern. All MBL-positive case, but none of the MBL-negative cases showed glomerular co-deposition of L-ficolin, MBL-associated serine proteases, and C4d. Glomerular deposition of MBL and L-ficolin was associated with more pronounced histologic damage, as evidenced by increased mesangial proliferation, extracapillary proliferation, glomerular sclerosis, and interstitial infiltration, as well as with significantly more proteinuria. Patients who had IgAN with or without glomerular MBL deposition did not show significant differences in serum levels of MBL, L-ficolin, or IgA or in the size distribution of circulating IgA. Furthermore, *in vitro* experiments showed clear binding of MBL to polymeric but not monomeric patient IgA, without a significant difference between both groups. Together, these findings strongly point to a role for the lectin pathway of complement in glomerular complement activation in IgAN and suggest a contribution for both MBL and L-ficolin in the progression of the disease.

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IgA nephropathy (IgAN) is a common renal disease that is characterized primarily by mesangial deposition of IgA (reviewed in references [1,2]). Mesangial IgA deposition induces an inflammatory process that involves mesangial proliferation, interstitial damage, and proteinuria and slowly progresses to sclerosis and end-stage renal failure in approximately one third of cases. Several effector mechanisms are likely to be involved in the induction of renal inflammation and damage, including direct interactions between IgA and mesangial cells (3–5) and IgA-mediated complement activation (1,6,7).

IgA deposition in IgAN is commonly associated with deposition of complement factors, most often C3, the terminal com-

plex C5b-9, and properdin (8,9). Furthermore, increased levels of soluble split products of activated C3 have been observed in the circulation of patients with IgAN (10–12). These findings suggest involvement of the alternative pathway. Indeed, *in vitro* studies with IgA from humans and rodents, as well as *in vivo* studies in rats, have indicated that IgA can activate directly the alternative pathway of complement (6,13–15).

The complement system can be activated *via* three pathways: The classical pathway, the alternative pathway, and the lectin pathway. These pathways converge at the cleavage of C3, followed by activation of the common terminal pathway and formation of C5b-9. The classical pathway involves binding of C1q to immune complexes, for example, leading to generation of the C3 convertase C4b2a. The more recently discovered lectin pathway of complement is activated after an interaction of the plasma lectins mannose-binding lectin (MBL), L-ficolin, or H-ficolin with their carbohydrate ligands (16–19). This leads to activation of MBL-associated serine proteases (MASP), which are present in a proenzymatic complex with these lectins. Ac-

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tivated MASP-2 generates C4b2a, followed by C3 cleavage. In contrast to the classical pathway and the lectin pathway, the alternative pathway leads to activation of C3 in a C4-independent way, involving factors B and D and properdin.

Until now, the mechanism of complement activation in IgAN has been defined incompletely. In addition to the presence of factors that are indicative for alternative pathway activation, several studies have shown glomerular deposition of C4, as well as circulating C4 activation products in a subpopulation of patients with IgAN (10,20). Because *in vitro* studies indicated that IgA cannot activate the classical complement pathway and evidence for C4 activation in IgAN was observed in the absence of C1q deposition, activation of the lectin pathway of complement was hypothesized. Deposition of MBL in association with IgA, as a marker for lectin pathway activation, was reported in a subpopulation of patients with IgAN by several authors (21–23), but these findings were questioned by others (24). Furthermore, the relation of glomerular MBL positivity with parameters of renal damage and complement activation *via* the lectin pathway was inconsistent between the different studies.

Recently, we reported that human polymeric serum IgA can bind to human MBL (25). This interaction involves the lectin domain of MBL and results in activation of C4 and C3. Therefore, we hypothesize that C4 activation in IgAN may result from binding of serum MBL–MASP-2 complexes to IgA that is deposited in the renal mesangium. In this study, we analyzed the possible contribution of the lectin pathway of complement to activation of C4 and induction of renal damage in a well-defined set of renal biopsies of patients with IgAN. In addition to MBL, deposition of the functionally related molecule L-ficolin was studied. The results indicate activation of the lectin pathway, involving both MBL and L-ficolin, in a subpopulation of patients with IgAN, which is strongly associated with markers of disease progression.

## Materials and Methods

### *Patients and Biopsies*

Renal biopsies were selected from patients who had IgAN and from whom a renal biopsy was taken between January 2001 and December 2003. Patients were selected when adequate tissue was obtained for diagnostics (at least eight glomeruli in light microscopy sections and complete immunohistology and electron microscopy examination) and when sufficient frozen material was available for additional staining after immunodiagnosis (at least six glomeruli in at least 15 tissue sections [5  $\mu$ m thick]). Patients with Henoch-Schönlein purpura, systemic lupus erythematosus, liver cirrhosis, or other systemic diseases were excluded. In total, 60 biopsies were selected for evaluation.

Among selected patients, 69% were male and 31% were female. Serum creatinine levels ranged from 44 to 972  $\mu$ mol/L at the time of renal biopsy. Creatinine clearance was calculated according to the Cockcroft formula (range 10 to 160 ml/min). Presentation at time of renal biopsy was as follows: 58% urinary abnormalities, 32% chronic renal failure, and 10% acute renal failure. Chronic renal failure was defined as serum creatinine value  $>124$   $\mu$ mol/L or creatinine clearance  $<80$  ml/min in at least three determinations before renal biopsy and confirmed further during hospitalization. Acute renal failure was defined as an abrupt decline in GFR detected at patient admission, without previous altered examinations and without ultrasonographic signs

of chronic renal injury. Urine samples (24-h collection) and serum samples were obtained at time of renal biopsy.

For additional immune fluorescence studies, a more recent series of renal biopsies was collected from patients with IgAN ( $n = 25$ ) using the same selection criteria as described above. From these patients, detailed clinical and histologic information has not been collected.

### *Serologic Analysis*

Serum IgA concentration was measured by standard nephelometric immunoassay. MBL complex activity was assessed by ELISA using coated mannan as a ligand and detecting the activation of purified C4, as described before (26) with some modifications (27). MBL complex activity was expressed in arbitrary units per milliliter on the basis of the activity of a normal human pool serum. MBL concentrations were assessed by sandwich ELISA as described previously (25). Concentrations of L-ficolin were assessed using a similar protocol. In brief, plates (Maxisorb; Nunc, Roskilde, Denmark) were coated with mAb GN5 (5  $\mu$ g/ml; mouse mAb against human L-ficolin) or mAb 3E7 (5  $\mu$ g/ml; mouse IgG1 anti-human MBL) in coating buffer (100 mM Na<sub>2</sub>CO<sub>3</sub>/NaHCO<sub>3</sub> [pH 9.6]). After each step, plates were washed with PBS that contained 0.05% Tween 20. Residual binding sites were blocked by incubation with PBS that contained 1% BSA. Serum samples and subsequent detection antibodies were diluted in PBS that contained 1% BSA and 0.05% Tween 20 and incubated for 1 h at 37°C. Primary antibodies were mAb 3E7 and mAb GN5, respectively, conjugated to digoxigenin (dig; Roche Diagnostics, Almere, The Netherlands). Binding of mAb was detected using horseradish peroxidase-conjugated sheep anti-dig antibodies (Fab fragments; Roche Diagnostics). Concentrations of MBL and L-ficolin were calculated using serial dilutions of a standard serum with a known concentration of MBL (provided by Dr. P. Garred, Copenhagen, Denmark) or L-ficolin (provided by Dr. D.C. Kilpatrick, Edinburgh, UK), respectively.

### *Purification of Serum IgA and Assessment of MBL Binding to IgA*

IgA was purified starting from 350  $\mu$ l of patient serum ( $n = 12$ ). The serum was applied directly to an IgA affinity column (Sephacrose beads conjugated to mAb HisA43 anti-human IgA; provided by Dr. J. van den Born, Amsterdam, The Netherlands), using Veronal-buffered saline that contained 2 mM EDTA as a running buffer. IgA was eluted using 0.1 M Glycine/0.3 M NaCl (pH 2.8), and fractions were neutralized immediately by 1 M Tris (pH 8.0). Fractions were assessed for IgA content, and the IgA peak was pooled and concentrated until approximately 350  $\mu$ l. These samples were purified further on a Sephadex 200 gel filtration column (25 ml; GE Healthcare, Munich, Germany), using the same running buffer. Fractions of 0.3 ml were collected.

On basis of the protein profile, fractions that contained polymeric, dimeric, and monomeric IgA were selected. IgA concentration in these fractions was quantified by IgA sandwich ELISA. Increasing concentrations of IgA were coated on ELISA plates, and binding of purified human MBL was assessed as described previously (25) using dig-conjugated mAb 3E7 for detection. In parallel, the amount of immobilized IgA was detected using biotinylated goat anti-human IgA (Dako, Glostrup, Denmark), followed by horseradish peroxidase-conjugated streptavidin (Zymed, Invitrogen, Breda, The Netherlands). The relative amounts of polymeric, dimeric, and monomeric serum IgA were quantified on basis of the protein profile that was obtained after gel filtration of purified IgA, using calculation of the area under the curve.

### *Immunohistology and Immunofluorescence*

For immunofluorescence and immunoperoxidase stainings, the unfixed renal tissue was embedded in OCT compound (Sakura Tissue-

Tek; Bayer, Leverkusen, Germany), snap-frozen in a mixture of isopentane and dry ice, and stored at  $-80^{\circ}\text{C}$ . Subsequently,  $5\text{-}\mu\text{m}$  sections were placed on slides and stored at  $-20^{\circ}\text{C}$  until immunostaining.

We used mouse mAb directed against the following molecules: MBL (mAb 3E7 [28], mAb 1C10, and mAb 2A9 [29]), L-ficolin (mAb GN4 and mAb GN5 [30]), MASP-1/3 heavy chain (mAb 1E2 and mAb 4C2 [31,32]), C4 binding protein (mAb from Quidel, San Diego, CA), C5b-9 (aE11; Abcam, Cambridge, UK), IgA1 (mAb NI69-11; Nordic, Tilburg, The Netherlands [33]), and IgA2 (mAb 14-3-26 [Becton Dickinson, Erembodegem, Belgium] and mAb NI512 [Nordic] [33]). Rabbit polyclonal antibodies were applied for detection of C3 (FITC-labeled anti-human C3c; Dako), C4d (Biomedica, Vienna, Austria [34]), and MASP-2 (a MASP-2-specific antibody generated in the laboratory of nephrology using the recombinant protease domain of MASP-2 [35,36]; provided by Dr. P. Gal, Institute of Enzymology, Budapest, Hungary; to be described elsewhere).

For indirect immunofluorescence, after fixation in cold acetone, tissues were incubated sequentially with the primary antibody and the proper fluorescently labeled secondary antibody (Alexa Fluor 488-conjugated goat anti-mouse Ig or goat anti-rabbit Ig; Molecular Probes, Eugene, OR). Slides finally were mounted with an antifading aqueous mounting medium (Fluorsave; Calbiochem, San Diego, CA).

Immunoperoxidase staining of MBL was performed as described previously (37). Briefly, after incubation with 0.5% avidin (Sigma Chimica, Milan, Italy) and 0.01% biotin (Sigma), to suppress endogenous avidin-binding activity, tissue sections were fixed in a methanol- $\text{H}_2\text{O}_2$  solution to block endogenous peroxidase. After washing, sections were incubated with primary antibody, followed by biotinylated anti-mouse antibody (Zymed) and peroxidase-labeled streptavidin (Zymed). Peroxidase activity was detected with 3,5-diaminobenzidine (Sigma), and sections were counterstained with Harry's hematoxylin, dehydrated, and mounted in Permount. In both immunofluorescence and immunoperoxidase methods, specificity of antibody labeling was demonstrated concurrently using proper control immunoglobulins (Zymed).

### Evaluation of Renal Tissue

Evaluation of renal tissue was performed blindly by two independent observers. For immunostaining, tissues were scored as negative (0) or positive (1), according to the detection of staining in the majority of glomeruli, in at least three tubular cross-sections per field, and in vessel endothelium.

For histology, sections were stained using standard periodic-acid Schiff, periodic-acid-silver methenamine, and/or Trichrome techniques. Mesangial proliferation was scored as 1+ when mild to moderate (*i.e.*, between four and six cells per mesangial area) and 2+ when intense (more than six cells per mesangial area). Extracapillary proliferation, global sclerosis, and segmental sclerosis were calculated as percentage of the total number of glomeruli. Interstitial infiltration and fibrosis were scored 0 when absent, 1+ when mild (involving  $<30\%$  of the interstitium), 2+ when moderate (30 to 60% of the interstitium involved), or 3+ when intense (when present in  $>60\%$  of the renal interstitium). Hyalinosis of the vessel wall was indicated when absent (0) or present (1).

### Statistical Analyses

Data were compared between patients who had IgAN and showed positive and negative glomerular staining for MBL, respectively. Frequency analysis was performed using  $\chi^2$  test. Other comparisons were evaluated using the Mann Whitney *U* test. The Spearman rank corre-

lation coefficient was used to analyze correlation. Differences were considered statistically significant at  $P < 0.05$ .

## Results

### Evidence for Glomerular Lectin Pathway Activation in IgAN

The presence of MBL was examined in 60 renal biopsies from patients with IgAN. Glomerular staining for MBL was observed in a predominantly mesangial pattern in 15 biopsies (25%; Figure 1, A and C), whereas glomeruli in 45 IgAN biopsies stained negative for MBL (Figure 1D). A similar positive staining was observed using three different mAb directed against MBL, whereas staining with an isotype control mAb was completely negative (Figure 1B).

Next we examined the presence of other molecules involved in the lectin pathway of complement. All renal biopsies with positive glomerular staining for MBL also showed glomerular staining for L-ficolin, as shown by two different mAb, whereas glomerular L-ficolin staining was negative in all MBL-negative cases (Table 1, Figure 2). Similarly, all cases that were positive for MBL and L-ficolin but none of the negative cases showed positive mesangial staining for the common heavy chain of MASP-1 and MASP-3, using two mAb, and for C4d and C4 binding protein (Table 1, Figure 2). The majority of biopsies in both MBL-negative and MBL-positive groups showed deposition of C3, whereas C1q was negative (Table 1). All IgAN biopsies showed a typical mesangial deposition of IgA1 (Table 1, Figure 2). In contrast, we were unable to show a positive staining for IgA2, using two different IgA2-specific mAb (Table 1, Figure 2), although these antibodies showed clear positive staining on renal biopsies from patients with lupus nephritis (data not shown).

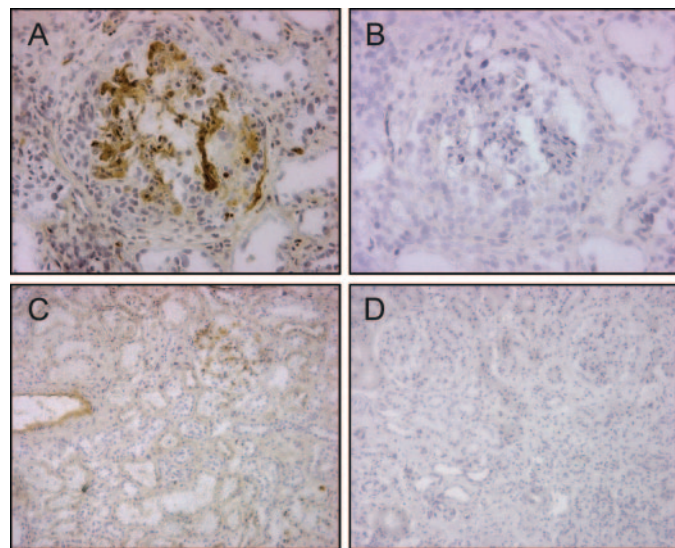


Figure 1. Glomerular mannose-binding lectin (MBL) deposition in patients with IgA nephropathy (IgAN). Biopsies from patients with IgAN were stained with mAb 1C10 (A), mAb 3E7 (C and D), or an isotype control mAb (B). A, C, and D are derived from different patients who showed positive (A and C) or negative (D) staining for MBL. A and B are from the same patient. Note tubular and vascular staining for MBL in C, in addition to glomerular staining.



Table 1. Molecular composition of glomerular deposition in IgAN<sup>a</sup>

MBL Staining	L-Ficolin	MASP-1/3	C4d	C4-Binding Protein	C1q	C3	IgA1	IgA2
Negative (%; n = 45)	0	0	0	0	0	82	100	0
Positive (%; n = 15)	100	100	100	100	0	60	100	0

<sup>a</sup>Data indicate the percentage of biopsies with positive staining for a certain marker. Positive staining means clear positivity in the majority of glomeruli. IgAN, IgA nephropathy; MASP, mannose-binding lectin-associated serine proteases; MBL, mannose-binding lectin.

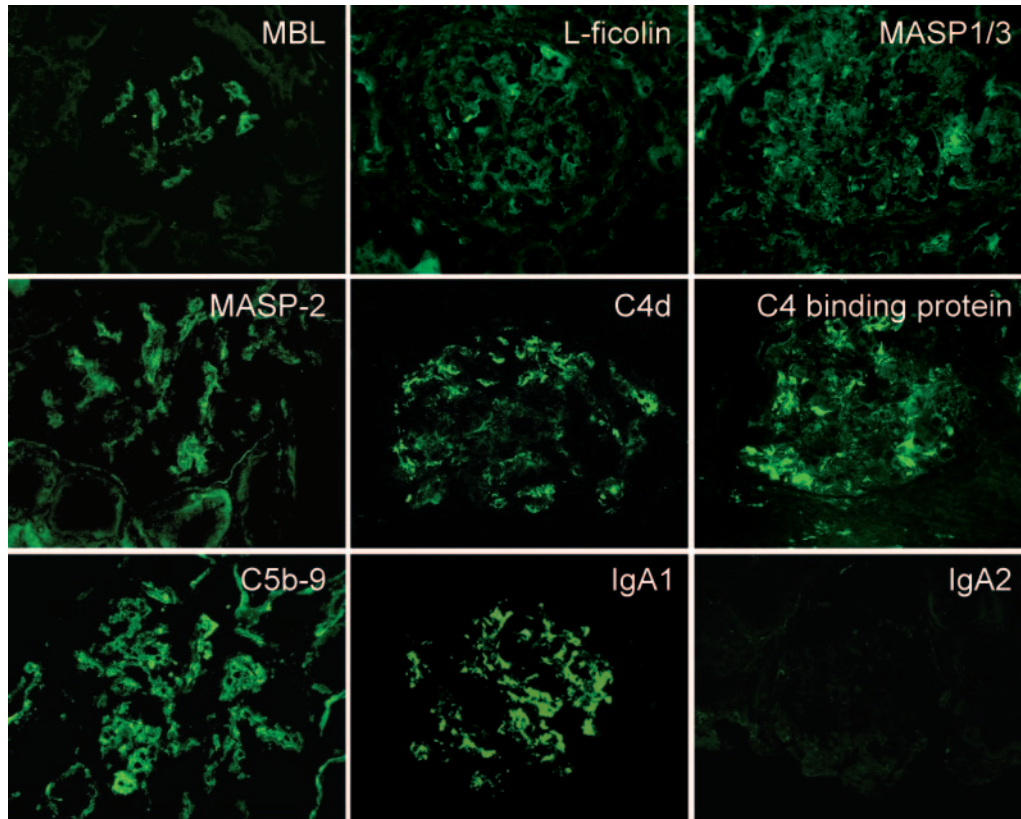


Figure 2. Lectin pathway activation in IgAN. Renal tissue from patients with IgAN was stained for the presence of MBL (mAb 3E7), L-ficolin (mAb GN4), MBL-associated serine proteases 1 and 3 (MASP-1/3; mAb 1E2), MASP-2, C4d, C4-binding protein, C5b-9, IgA1, and IgA2 (mAb NI512), as indicated. Representative images are shown.

To extend further the analysis of complement activation in IgAN, we evaluated deposition of MASP-2, which is the C4-cleaving enzyme of the lectin pathway, and C5b-9 as the final product of the terminal pathway of complement. Because of a shortage of renal tissue, we used an additional series of 25 renal biopsies for these studies. From these biopsies, six (24%) showed positive glomerular staining for MBL, confirming the results presented above in an independent study. All MBL-positive biopsies showed positive glomerular staining for MASP-2 (Figure 2), whereas MASP-2 was negative in all other biopsies. Staining for C5b-9 clearly was positive in a mesangial pattern in all IgAN biopsies. However, in MBL-positive biopsies, staining was observed with a high intensity (Figure 2), whereas intensity showed a marked variation in MBL-negative biopsies (data not shown).

#### Glomerular Lectin Pathway Activation in IgAN Is Associated with Markers of Disease Progression

Data indicated above show that patients with IgAN can be divided in two groups: Those with (25%) and without (75%) evidence of glomerular activation of the lectin pathway of complement. These two patient groups were characterized further.

MBL-positive and -negative cases had a similar male/female distribution and a similar age at the time of the renal biopsy (Table 2). However, patients with glomerular MBL positivity presented twice as often with renal failure at the time of renal biopsy, mostly chronic renal failure ( $P < 0.0001$ ; Table 2). Furthermore, MBL-positive patients showed significantly more proteinuria than MBL-negative patients with IgAN, whereas, in contrast, fewer MBL-positive patients with IgAN experienced

Table 2. Clinical and laboratory data from patients with IgAN<sup>a</sup>

Parameter	MBL-Negative Cases	N	MBL-Positive Cases	N	P
Age at renal biopsy (yr; median)	35	36	27	12	0.28
Female gender (%)	36	36	17	12	0.21
Renal failure (% chronic)	33 (73% chronic)	45	67 (80% chronic)	15	<0.0001
Proteinuria (g/24 h; median)	0.7	45	2.5	15	0.004
Macroscopic hematuria present (%)	46	39	13	15	0.025
Serum creatinine ( $\mu\text{mol/L}$ ; median)	97	45	133	15	0.042
Creatinine clearance (ml/min; median)	92	25	61	10	0.43
Serum MBL (ng/ml; median)	1299	28	873	12	0.80
MBL complex activity (U/ml; median)	738	29	865	12	0.27
Serum L-ficolin ( $\mu\text{g/ml}$ ; median)	2.60	28	1.82	12	0.15
Serum IgA (mg/ml; median)	2.54	36	2.28	15	0.37
Monomeric serum IgA (%; median)	73	6	73	6	0.70
Dimeric serum IgA (%; median)	23	6	20	6	0.39
Polymeric serum IgA (%; median)	4.0	6	5.4	6	0.09

<sup>a</sup>MBL-negative cases and MBL-positive cases are defined on the basis of glomerular staining. The size distribution of purified serum IgA is expressed as percentage of total IgA. All data were obtained at the time of the renal biopsy.

episodes of macroscopic hematuria (Table 2, Figure 3, A and B). In our group of patients with IgAN, macroscopic hematuria seemed to be associated with a more benign disease course, because patients with macroscopic hematuria showed significantly less proteinuria than patients without macroscopic hematuria, also when MBL-negative cases were analyzed only ( $P = 0.04$ , not shown). MBL-positive patients also showed a higher serum creatinine level than MBL-negative patients with IgAN, although the creatinine clearance was not significantly different (Table 2). However, the latter parameter could be analyzed for only some of the patients in our study.

These data suggest that glomerular MBL positivity in IgAN is associated with a more severe disease. This is strongly supported by semiquantitative histologic evaluation of renal damage, demonstrating that biopsies from patients who had IgAN with positive glomerular staining for MBL had more intense mesangial proliferation as well as significantly more extracapillary proliferation, global sclerosis, and interstitial infiltration than MBL-negative patients (Figure 3, C through F; Table 3). Increased glomerular and tubulointerstitial damage in MBL-positive patients, as compared with MBL-negative patients, is illustrated in Figure 4.

No difference between the two groups was found for the presence of vascular lesions. Glomerular staining for MBL was significantly associated with staining for MBL in vessels and tubuli (Table 3, Figure 1C). However, tubular and vascular staining for MBL was only weakly associated with mesangial proliferation ( $P = 0.03$ ), and no association was found with proteinuria or other parameters of disease activity. Furthermore, tubulointerstitial staining for MBL was not clearly associated with markers of complement activation (data not shown).

#### Characterization of Circulating MBL, L-Ficolin, and IgA

We further examined whether the observed dichotomy in patients with IgAN with respect to glomerular lectin pathway

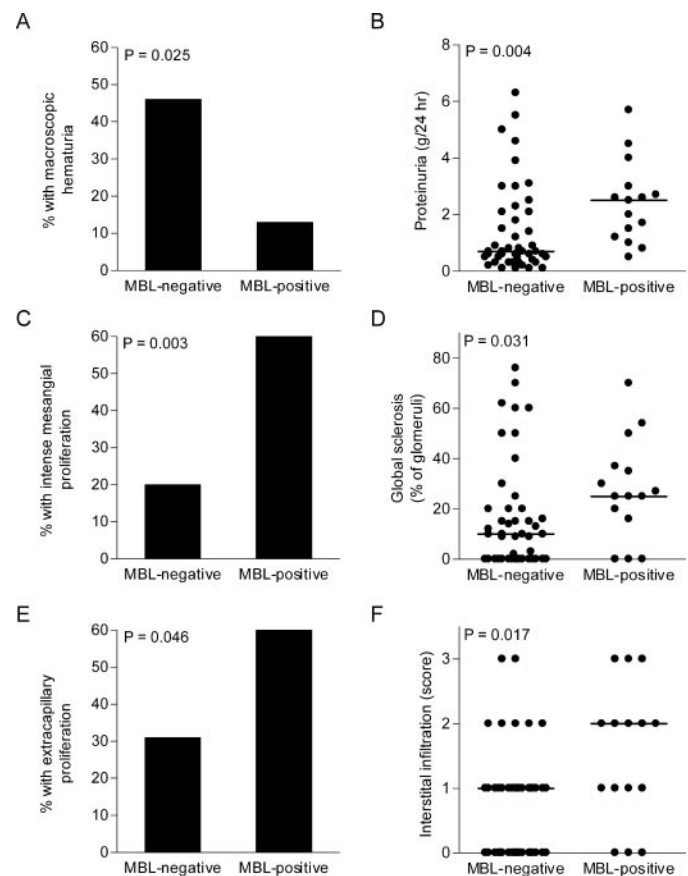


Figure 3. MBL positivity is associated with markers of renal damage. Patients with IgAN were divided in two groups on basis of glomerular positivity for MBL. Clinical (A and B) and histologic data (C through F) presented are scored as explained in Materials and Methods. Horizontal lines in B, D, and F represent the median. Statistical analysis was performed as described in Tables 2 and 3.

Table 3. Histologic data from patients with IgAN<sup>a</sup>

Parameter	MBL-Negative Cases ( <i>n</i> = 45)	MBL-Positive Cases ( <i>n</i> = 15)	<i>P</i>
Intense mesangial proliferation (% of cases)	20	60	0.003
Extracapillary proliferation present (% of cases)	31	60	0.046
Global sclerosis (% of glomeruli; median)	10	25	0.031
Segmental sclerosis (% of glomeruli; median)	0	8	0.069
Interstitial infiltration (0 to 3 scale scoring; median)	1	2	0.017
Interstitial fibrosis (0 to 3 scale scoring; median)	1	2	0.12
Vessel lesions present (% of cases)	33	40	0.64
Vascular MBL staining positive (% of cases)	36	73	0.01
Tubular MBL staining positive (% of cases)	22	80	0.0001

<sup>a</sup>MBL-negative cases and MBL-positive cases are defined on basis of glomerular staining.

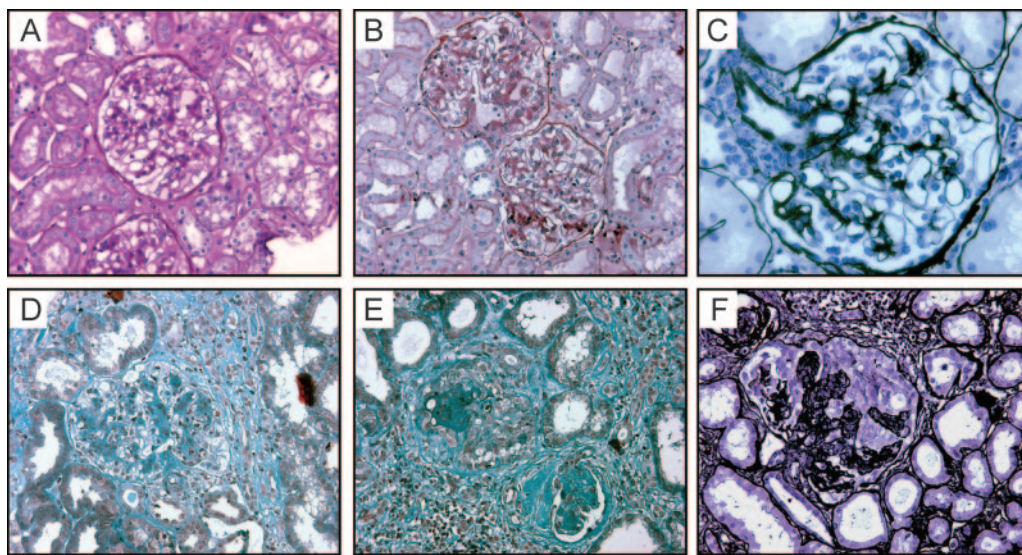


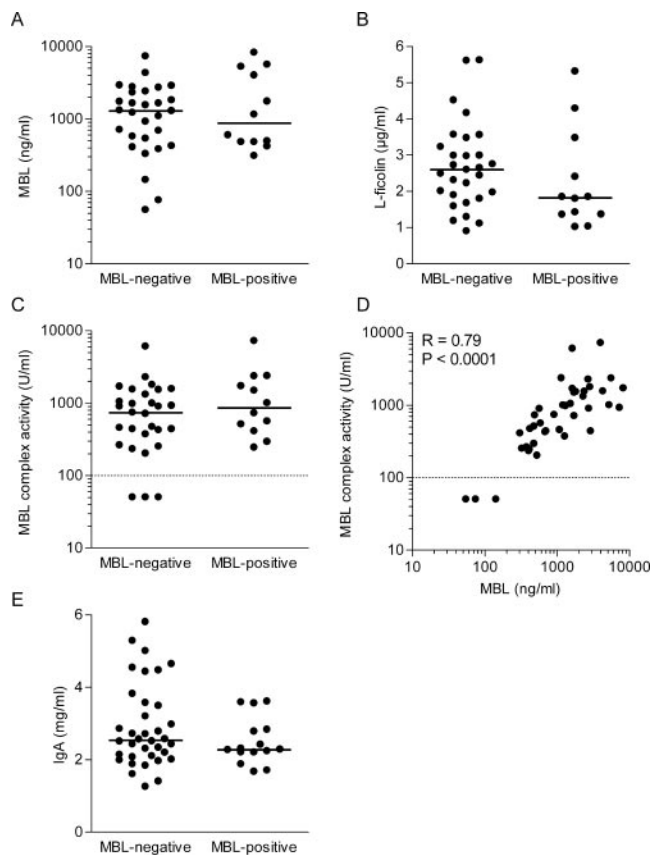
Figure 4. Renal histologic damage in patients with IgAN. Renal histology from three patients without glomerular MBL deposition (A through C) shows glomeruli that were affected by mild mesangial proliferation and mild mesangial matrix expansion. No major tubulointerstitial lesions are visible. (D through F) Patients with glomerular MBL deposition (*n* = 3). Glomerular injury is characterized by intense mesangial proliferation and mesangial matrix expansion (D), segmental and global sclerosis (E), and extracapillary proliferation (F), whereas tubular dilations, interstitial infiltration, and fibrosis are evident in the interstitium (D through F). Sections were stained with periodic acid-Schiff (A and B), periodic-acid-silver methenamine (C and F), or trichrome (D and E) techniques.

activation could be associated with certain properties of the lectin pathway of complement and/or the IgA in the circulation. Serum levels of MBL are genetically determined and highly variable in the human population (27). A similar variability was observed for patients with IgAN in our study, without a significant difference between patients who showed positive and negative staining for MBL (Figure 5A, Table 2). Serum levels of L-ficolin showed less variability and also did not distinguish between both groups (Figure 5B, Table 2). As a functional parameter, we assessed MBL complex activity, which is a measure of the ability of the MBL–MASP-2 complex to activate C4. MBL complex activity showed a similar distribution in both groups (Figure 5C, Table 2) and was strongly correlated to MBL serum concentration (Figure 5D). As ex-

pected, three patients with clear MBL deficiency (Figure 5D) showed negative glomerular staining for MBL.

Because these data exclude the simple explanation that lectin pathway deficiency explains the lack of glomerular lectin pathway activation in most patients with IgAN, we further studied whether properties of serum IgA may explain the observed dichotomy. The concentration of serum IgA was not significantly different between both groups (Figure 5E, Table 2). To address the question more in depth, we purified serum IgA from serum from patients with IgAN. From patients with and without glomerular MBL deposition, we selected six patients in each group. Patients with a possible MBL deficiency were excluded. IgA was purified from serum and subjected to gel filtration. The protein profile clearly reveals the presence of





**Figure 5.** Circulating MBL, L-ficolin, and IgA in patients with IgAN. Concentrations of MBL (A), L-ficolin (B), and IgA (E), as well as MBL complex activity (C) were quantified in serum of patients with IgAN as described in Materials and Methods. Patients with IgAN were divided in two groups on the basis of glomerular positivity for MBL. Horizontal solid lines represent the median, and dashed lines indicate the detection limit. (D) Correlation between MBL concentration and MBL complex activity.

polymeric, dimeric, and monomeric IgA (Figure 6A). The size distribution of serum IgA in patients who had IgAN with and without glomerular MBL deposition showed a low variability without any significant differences (Figure 6B, Table 2).

Subsequently, we studied the binding of purified MBL to patient IgA of various sizes. Increasing concentrations of monomeric, dimeric, and polymeric IgA were coated on ELISA plates, and binding of MBL was assessed. The results showed a high variability in the MBL binding properties of IgA from various donors, although similar amounts of IgA were immobilized on the ELISA plates (Figure 7A). Monomeric IgA did not show MBL binding, whereas strong but donor-dependent MBL binding could be observed to polymeric IgA (Figure 7B). MBL binding to dimeric IgA was significantly less than that to polymeric IgA ( $P < 0.01$ ). Quantification of the binding of MBL per unit of immobilized IgA did not reveal a significant difference between patients with and without glomerular MBL positivity, respectively (Figure 7B). However, two patients whose IgA showed strong MBL binding both showed positive deposition of MBL in the glomerulus.

## Discussion

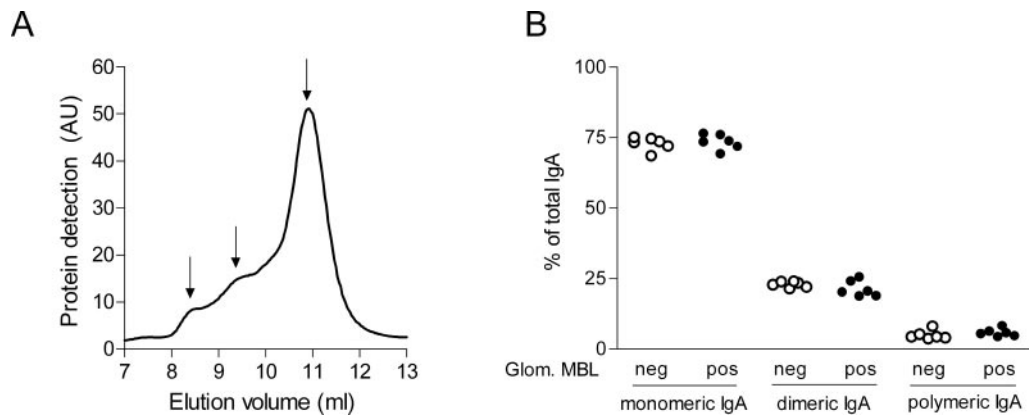
Data from our study indicate that patients with IgAN can be divided in two groups on the basis of the pattern of complement activation. Approximately 75% of patients with IgAN show negative glomerular staining for MBL, L-ficolin, MASP, C4d, and C4-binding protein, indicating that C3 and C5b-9 activation in these patients most likely occurs *via* the alternative pathway. In contrast, 25% of patients with IgAN show glomerular deposition of MBL, L-ficolin, MASP, and C4d but not C1q, which is strongly indicative of activation of complement *via* the lectin pathway of complement. Importantly, the clinical and histologic data clearly indicate that activation of the lectin pathway of complement is associated with more severe renal damage.

Using a well-defined polyclonal antibody to C4d, which now is used frequently in the diagnostic evaluation of renal transplant rejection (34), we observed glomerular deposition of C4d in IgAN exclusively in association with molecules of the lectin pathway. An identical pattern was observed for deposition of C4-binding protein, a regulatory protein that also was reported previously as a sensitive marker for the presence of C4 (20). Activated C4 most likely is generated by MASP-2, the key complement-activating enzyme of the lectin pathway, which was shown to be present in the mesangial area in association with MBL.

In addition to strongly suggesting activation of C4 *via* the lectin pathway in a subpopulation of patients with IgAN, our data indicate glomerular activation of C3 and C5b-9 *via* the alternative pathway in the majority of patients with IgAN, independent of C4. The latter aspect is in agreement with a number of *in vitro* studies that showed alternative pathway activation by IgA from human and rodent species (6,13,15).

We assume that mesangial MBL and L-ficolin are derived from the circulation as preformed complexes with MASP, although local production of molecules of the lectin pathway cannot be excluded (38). Our previous *in vitro* studies showed that MBL is able to interact *via* its lectin domain with purified polymeric serum IgA (25). Further support for an interaction between MBL and IgA is provided by studies in Henoch Schönlein purpura nephritis (39,40). In this study, we show that MBL binds to polymeric IgA from patients with IgAN. It is possible that MBL binds to glycans that are present on the IgA heavy chains. IgA consists of two subclasses, IgA1 and IgA2, the former of which dominates in the circulation (90% IgA1). However, mucosal IgA may contain up to 65% IgA2 (7). It is interesting that circulating IgA1 from patients with IgAN was reported to have aberrant glycosylation of O-linked glycans, which potentially are involved in recognition by lectins (41).

Although it generally is believed that IgA deposits in IgAN consist almost exclusively of IgA1 (1,2,7,33,42,43), biopsies from patients with IgAN also may contain IgA2 (44). A recent study in this field by Hisano *et al.* (23) showed that approximately 50% of a Japanese population of patients with IgAN showed deposition of IgA2 next to IgA1, whereas glomerular MBL deposition was observed exclusively in these IgA2-positive patients. Similar results were reported recently in Henoch-Schönlein purpura nephritis (40). Stimulated by these striking



**Figure 6.** Size distribution of circulating IgA. (A) Typical example of a protein profile obtained after gel filtration of purified IgA. Position of polymeric, dimeric, and monomeric IgA is indicated. (B) Quantification of IgA size distribution of IgA from 12 patients with IgAN showing positive or negative glomerular staining for MBL.

observations, we investigated the glomerular presence of IgA2 in our patient population, using two different IgA2-specific mAb, including mAb 14-3-26 that also was used by Hisano *et al.* (23). However, we were not able to detect any glomerular positivity for IgA2 in IgAN biopsies. This difference might be due to ethnic differences between patient populations. Furthermore, although we tried several amplification methods to acquire a signal for IgA2, it still could be that IgA2 is present in tiny amounts and/or masked by other molecules, thereby hampering detection.

IgA that is deposited in the renal mesangium of patients with IgAN is classically accepted as being polymeric IgA (45). Polymeric IgA contains several other molecular components, which currently are only partially identified (45,46). It cannot be excluded that MBL binds to a glycosylated molecule that is associated with polymeric IgA and present in the mesangial deposits of patients with IgAN. An alternative explanation would be that MBL binds to injured tissue in the glomerulus, which is in line with the binding of MBL to apoptotic and necrotic cells (47).

To our best knowledge, data from our study are the first indication for a role of L-ficolin in disease. *In vitro* data indicate that ligand specificities of MBL and L-ficolin only partially overlap (16,17,48). However, recent experiments in our group indicate that L-ficolin also binds to polymeric IgA (Roos *et al.*, manuscript in preparation). MBL and ficolins have an important role in innate immune defense. However, evidence is increasing that MBL and the lectin pathway of complement also can be harmful for the host as mediators of inflammation. In this respect, MBL has been proposed to be involved in ischemia/reperfusion injury (49), diabetic nephropathy (50), and ulcerative colitis (51). In this study, we observed that patients who had IgAN with glomerular MBL deposition and lectin pathway activation showed significantly more renal damage, as evidenced by increased mesangial proliferation, extracapillary proliferation, glomerular sclerosis, interstitial infiltration, and proteinuria. An association of glomerular MBL positivity with decreased renal function and increased mesangial proliferation and proteinuria was also reported by Matsuda *et al.* (21) but

could not be confirmed by studies from Endo *et al.* (22) and Hisano *et al.* (23). In Henoch-Schönlein purpura nephritis, MBL deposition was associated with increased progression of renal disease (40). Increased disease progression in association with glomerular lectin pathway activation may possibly be ascribed to proinflammatory activation products of the complement system, including C5a and C5b-9, and potentially also to a direct effect of MBL.

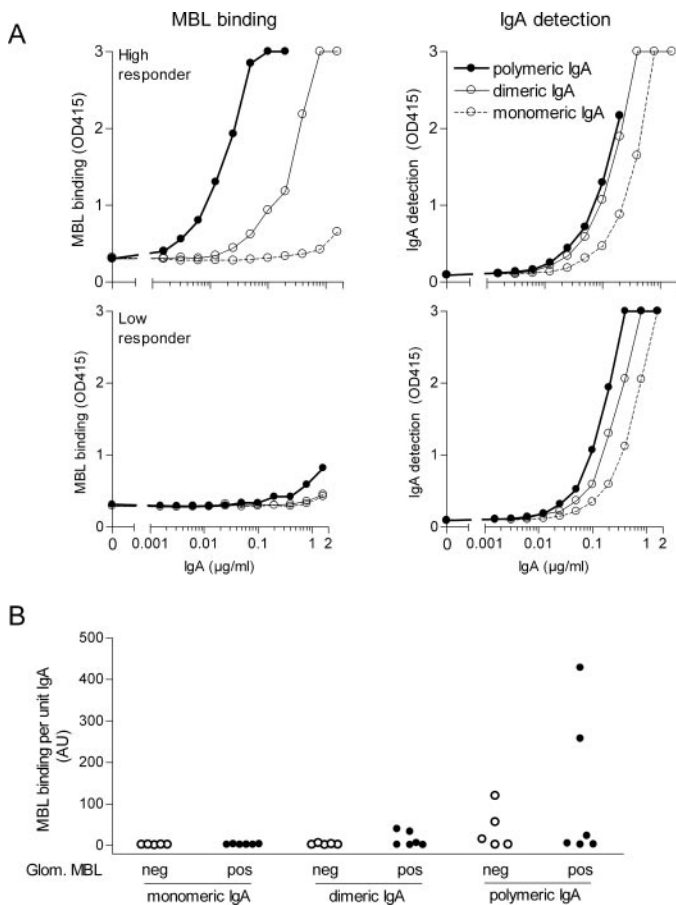
Similar to several other studies (21–23), we observed lectin pathway activation only in a subpopulation of patients with IgAN. The majority of patients with IgAN without mesangial MBL deposition did not show any evidence for deficiency of MBL or L-ficolin. It is interesting that serum concentrations of MBL in this population of patients with IgAN ( $n = 41$ ; median 1213 ng/ml) were significantly higher than those in a group of white healthy control subjects ( $n = 190$ ; median 677 ng/ml;  $P = 0.002$ ; data not shown). This difference could be explained by genetic differences and/or by conditions that are associated with renal disease.

We hypothesize that the difference between patients who have IgAN with and without mesangial MBL deposition is based on differential availability of the ligand, potentially related to differences in IgA glycosylation. To examine this hypothesis further, we investigated the binding of purified MBL to polymeric, dimeric, and monomeric IgA from patients who had IgAN and were either positive or negative for glomerular MBL deposition. This analysis revealed binding of MBL to polymeric serum IgA with a large interindividual variation, but the degree of MBL binding could not be related directly to glomerular lectin pathway activation. However, mesangial IgA is not necessarily similar to serum IgA; furthermore, it could be that glomerular IgA is differentially accessible in patients with IgAN. We assume that the observed dichotomy in patients with IgAN is due to several factors acting in combination.

## Conclusion

Our study shows that MBL and the lectin pathway of complement are involved in complement activation in a subpopulation of patients with IgAN, which is highly likely to play an





**Figure 7.** Binding of MBL to polymeric circulating IgA from patients with IgAN. (A) IgA of various sizes was coated, followed by assessment of MBL binding and, in parallel, detection of immobilized IgA, as indicated. Two representative examples are shown for IgA samples that show a high or low binding of MBL. (B) Quantification of MBL-binding properties of patient IgA of various sizes on the basis of a calibration line and corrected for the amount of immobilized IgA.

adverse role in the disease. *In vitro* data point to a role for polymeric IgA in lectin pathway activation. Precise identification of the ligand for MBL and L-ficolin in the mesangium, which presumably is present and/or accessible in only some of the patients with IgAN, will provide novel insight in the pathogenesis of IgAN and may provide novel therapeutic options to treat disease progression.

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## References

1. Floege J, Feehally J: IgA nephropathy: Recent developments. *J Am Soc Nephrol* 11: 2395–2403, 2000
2. Donadio JV, Grande JP: IgA nephropathy. *N Engl J Med* 347: 738–748, 2002
3. van den Dobbelen MEA, Van der Woude FJ, Schroeijers WEM, van den Wall Bake AWL, Van Es LA, Daha MR: Binding of dimeric and polymeric IgA to rat renal mesangial cells enhances the release of interleukin 6. *Kidney Int* 46: 512–519, 1994
4. Duque N, Gomez-Guerrero C, Egido J: Interaction of IgA with Fcα receptors of human mesangial cells activates transcription factor nuclear factor-κB and induces expression and synthesis of monocyte chemoattractant protein-1, IL-8, and IFN-inducible protein 10. *J Immunol* 159: 3474–3482, 1997
5. Lai KN, Tang SC, Guh JY, Chuang TD, Lam MF, Chan LY, Tsang AW, Leung JC: Polymeric IgA1 from patients with IgA nephropathy upregulates transforming growth factor-β synthesis and signal transduction in human mesangial cells via the renin-angiotensin system. *J Am Soc Nephrol* 14: 3127–3137, 2003
6. Bogers WM, Stad RK, Van Es LA, Daha MR: Immunoglobulin A: Interaction with complement, phagocytic cells and endothelial cells. *Complement Inflamm* 8: 347–358, 1991
7. Van Es LA, de Fijter JW, Daha MR: Pathogenesis of IgA nephropathy. *Nephrology* 3: 3–12, 1997
8. Bene MC, Faure GC: Composition of mesangial deposits in IgA nephropathy: Complement factors. *Nephron* 46: 219, 1987
9. Rauterberg EW, Lieberknecht HM, Wingen AM, Ritz E: Complement membrane attack (MAC) in idiopathic IgA-glomerulonephritis. *Kidney Int* 31: 820–829, 1987
10. Wyatt RJ, Kanayama Y, Julian BA, Negoro N, Sugimoto S, Hudson EC, Curd JG: Complement activation in IgA nephropathy. *Kidney Int* 31: 1019–1023, 1987
11. Abou-Ragheb HHA, Williams AJ, Brown CB, Milford-Ward A: Plasma levels of the anaphylatoxins C3a and C4a in patients with IgA nephropathy/Henoch-Schönlein nephritis. *Nephron* 62: 22–26, 1992
12. Zwirner J, Burg M, Schulze M, Brunkhorst R, Gotze O, Koch KM, Floege J: Activated complement C3: A potentially novel predictor of progressive IgA nephropathy. *Kidney Int* 51: 1257–1264, 1997
13. Hiemstra PS, Gorter A, Stuurman ME, Van Es LA, Daha MR: Activation of the alternative pathway of complement by human serum IgA. *Eur J Immunol* 17: 321–326, 1987
14. Stad RK, van Gijlswijk-Janssen DJ, Van Es LA, Daha MR: Complement depletion abolishes IgA-mediated glomerular inflammation in rats. *Exp Nephrol* 2: 182–189, 1994
15. Janoff EN, Fasching C, Orenstein JM, Rubins JB, Opstad NL, Dalmaso AP: Killing of *Streptococcus pneumoniae* by capsular polysaccharide-specific polymeric IgA, complement, and phagocytes. *J Clin Invest* 104: 1139–1147, 1999
16. Petersen SV, Thiel S, Jensenius JC: The mannan-binding lectin pathway of complement activation: Biology and disease association. *Mol Immunol* 38: 133–149, 2001
17. Holmskov U, Thiel S, Jensenius JC: Collectins and ficolins

- lins: Humoral lectins of the innate immune defense. *Annu Rev Immunol* 21: 547–578, 2003
18. Matsushita M, Endo Y, Fujita T: Complement-activating complex of ficolin and mannose-binding lectin-associated serine protease. *J Immunol* 164: 2281–2284, 2000
  19. Matsushita M, Kuraya M, Hamasaki N, Tsujimura M, Shiraki H, Fujita T: Activation of the lectin complement pathway by H-ficolin (Hakata antigen). *J Immunol* 168: 3502–3506, 2002
  20. 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* 21: 335–340, 1984
  21. Matsuda M, Shikata K, Wada J, Sugimoto H, Shikata Y, Kawasaki T, Makino H: Deposition of mannan binding protein and mannan binding protein-mediated complement activation in the glomeruli of patients with IgA nephropathy. *Nephron* 80: 408–413, 1998
  22. 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* 13: 1984–1990, 1998
  23. Hisano S, Matsushita M, Fujita T, Endo Y, Takebayashi S: Mesangial IgA2 deposits and lectin pathway-mediated complement activation in IgA glomerulonephritis. *Am J Kidney Dis* 38: 1082–1088, 2001
  24. Lhotta K, Wurzner R, Konig P: Glomerular deposition of mannose-binding lectin in human glomerulonephritis. *Nephrol Dial Transplant* 14: 881–886, 1999
  25. Roos A, Bouwman LH, van Gijlswijk-Janssen DJ, Faber-Krol MC, Stahl GL, Daha MR: Human IgA activates the complement system via the mannan-binding lectin pathway. *J Immunol* 167: 2861–2868, 2001
  26. Petersen SV, Thiel S, Jensen L, Steffensen R, Jensenius JC: An assay for the mannan-binding lectin pathway of complement activation. *J Immunol Methods* 257: 107–116, 2001
  27. Roos A, Garred P, Wildenberg ME, Lynch NJ, Munoz J, Zuiverloon T, Bouwman LH, Schlagwein N, Fallaux-van den Houten FC, Faber-Krol MC, Madsen HO, Schwaeble WJ, Matsushita M, Fujita T, Daha MR: Antibody-mediated activation of the classical pathway of complement may compensate for mannose-binding lectin deficiency. *Eur J Immunol* 34: 2589–2598, 2004
  28. Matsushita M, Takahashi A, Hatsuse H, Kawakami M, Fujita T: Human mannose-binding protein is identical to a component of Ra-reactive factor. *Biochem Biophys Res Commun* 183: 645–651, 1992
  29. Collard CD, Vakeva A, Morrissey MA, Agah A, Rollins SA, Reenstra WR, Buras JA, Meri S, Stahl GL: Complement activation after oxidative stress: Role of the lectin complement pathway. *Am J Pathol* 156: 1549–1556, 2000
  30. Kilpatrick DC, Fujita T, Matsushita M: P35, an opsonic lectin of the ficolin family, in human blood from neonates, normal adults, and recurrent miscarriage patients. *Immunol Lett* 67: 109–112, 1999
  31. Terai I, Kobayashi K, Matsushita M, Fujita T: Human serum mannose-binding lectin (MBL)-associated serine protease-1 (MASP-1): Determination of levels in body fluids and identification of two forms in serum. *Clin Exp Immunol* 110: 317–323, 1997
  32. Kuraya M, Matsushita M, Endo Y, Thiel S, Fujita T: Expression of H-ficolin/Hakata antigen, mannose-binding lectin-associated serine protease (MASP)-1 and MASP-3 by human glioma cell line T98G. *Int Immunol* 15: 109–117, 2003
  33. Valentijn RM, Radl J, Haaijman JJ, Vermeer BJ, Weening JJ, Kauffmann RH, Daha MR, Van Es LA: Circulating and mesangial secretory component-binding IgA-1 in primary IgA nephropathy. *Kidney Int* 26: 760–766, 1984
  34. Bohmig GA, Exner M, Habicht A, Schillinger M, Lang U, Kletzmayer J, Saemann MD, Horl WH, Watschinger B, Regele H: Capillary C4d deposition in kidney allografts: A specific marker of alloantibody-dependent graft injury. *J Am Soc Nephrol* 13: 1091–1099, 2002
  35. Harmat V, Gal P, Kardos J, Szilagy K, Ambrus G, Vegh B, Naray-Szabo G, Zavodszky P: The structure of MBL-associated serine protease-2 reveals that identical substrate specificities of C1s and MASP-2 are realized through different sets of enzyme-substrate interactions. *J Mol Biol* 342: 1533–1546, 2004
  36. Ambrus G, Gal P, Kojima M, Szilagy K, Balczer J, Antal J, Graf L, Laich A, Moffatt BE, Schwaeble W, Sim RB, Zavodszky P: Natural substrates and inhibitors of mannan-binding lectin-associated serine protease-1 and -2: A study on recombinant catalytic fragments. *J Immunol* 170: 1374–1382, 2003
  37. Rastaldi MP, Ferrario F, Tunesi S, Yang L, D'Amico G: Intraglomerular and interstitial leukocyte infiltration, adhesion molecules, and interleukin-1 alpha expression in 15 cases of antineutrophil cytoplasmic autoantibody-associated renal vasculitis. *Am J Kidney Dis* 27: 48–57, 1996
  38. Kuncewicz T, Sheta EA, Goldknopf IL, Kone BC: Proteomic analysis of s-nitrosylated proteins in mesangial cells. *Mol Cell Proteomics* 2: 156–163, 2003
  39. Endo M, Ohi H, Ohsawa I, Fujita T, Matsushita M, Fujita T: Complement activation through the lectin pathway in patients with Henoch-Schonlein purpura nephritis. *Am J Kidney Dis* 35: 401–407, 2000
  40. Hisano S, Matsushita M, Fujita T, Iwasaki H: Activation of the lectin complement pathway in Henoch-Schonlein purpura nephritis. *Am J Kidney Dis* 45: 295–302, 2005
  41. Coppo R, Amore A: Aberrant glycosylation in IgA nephropathy (IgAN). *Kidney Int* 65: 1544–1547, 2004
  42. Conley ME, Cooper MD, Michael AF: Selective deposition of immunoglobulin A1 in immunoglobulin A nephropathy, anaphylactoid purpura nephritis, and systemic lupus erythematosus. *J Clin Invest* 66: 1432–1436, 1980
  43. Lomax-Smith JD, Zabrowarny LA, Howarth GS, Seymour AE, Woodroffe AJ: The immunochemical characterization of mesangial IgA deposits. *Am J Pathol* 113: 359–364, 1983
  44. Andre C, Berthoux FC, Andre F, Gillon J, Genin C, Sabatier JC: Prevalence of IgA2 deposits in IgA nephropathies: A clue to their pathogenesis. *N Engl J Med* 303: 1343–1346, 1980
  45. van der Boog PJ, Van Kooten C, de Fijter JW, Daha MR: Role of macromolecular IgA in IgA nephropathy. *Kidney Int* 67: 813–821, 2005
  46. van der Boog PJ, van Zandbergen G, de Fijter JW, Klar-Mohamad N, van Seggelen A, Brandtzaeg P, Daha MR,

- Van Kooten C: Fc alpha RI/CD89 circulates in human serum covalently linked to IgA in a polymeric state. *J Immunol* 168: 1252–1258, 2002
47. Nauta AJ, Raaschou-Jensen N, Roos A, Daha MR, Madsen HO, Borrias-Essers MC, Ryder LP, Koch C, Garred P: Mannose-binding lectin engagement with late apoptotic and necrotic cells. *Eur J Immunol* 33: 2853–2863, 2003
48. Le Y, Lee SH, Kon OL, Lu J: Human L-ficolin: Plasma levels, sugar specificity, and assignment of its lectin activity to the fibrinogen-like (FBG) domain. *FEBS Lett* 425: 367–370, 1998
49. Walsh MC, Bourcier T, Takahashi K, Shi L, Busche MN, Rother RP, Solomon SD, Ezekowitz RA, Stahl GL: Mannose-binding lectin is a regulator of inflammation that accompanies myocardial ischemia and reperfusion injury. *J Immunol* 175: 541–546, 2005
50. Hansen TK, Tarnow L, Thiel S, Steffensen R, Stehouwer CD, Schalkwijk CG, Parving HH, Flyvbjerg A: Association between mannose-binding lectin and vascular complications in type 1 diabetes. *Diabetes* 53: 1570–1576, 2004
51. Rector A, Lemey P, Laffut W, Keyaerts E, Struyf F, Wolants E, Vermeire S, Rutgeerts P, Van Ranst M: Mannan-binding lectin (MBL) gene polymorphisms in ulcerative colitis and Crohn's disease. *Genes Immun* 2: 323–328, 2001

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