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Anti-C1q autoantibodies deposit in glomeruli but are only pathogenic in combination with glomerular C1q-containing immune complexes

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Anti-C1q autoantibodies are present in sera of patients with several autoimmune diseases, including systemic lupus erythematosus (SLE). Strikingly, in SLE the presence of anti-C1q is associated with the occurrence of nephritis. We have generated mouse anti-mouse C1q mAb's and used murine models to investigate whether anti-C1q autoantibodies actually contribute to renal pathology in glomerular immune complex disease. Administration of anti-C1q mAb JL-1, which recognizes the collagen-like region of C1q, resulted in glomerular deposition of C1q and anti-C1q autoantibodies and mild granulocyte influx, but no overt renal damage. However, combination of JL-1 with a subnephritogenic dose of C1q-fixing anti-glomerular basement membrane (anti-GBM) antibodies enhanced renal damage characterized by persistently increased levels of infiltrating granulocytes, major histological changes, and increased albuminuria. This was not observed when a non-C1q-fixing anti-GBM preparation was used. Experiments with different knockout mice showed that renal damage was dependent not only on glomerular C1q and complement activation but also on Fcγ receptors. In conclusion, anti-C1q autoantibodies deposit in glomeruli together with C1q but induce overt renal disease only in the context of glomerular immune complex disease. This provides an explanation why anti-C1q antibodies are especially pathogenic in patients with SLE.

Introduction

Systemic lupus erythematosus (SLE) is a systemic autoimmune disease immunologically characterized by B cell hyperactivity, production of a multitude of different autoantibodies, and immune complex formation (1, 2). It affects 0.04% of the general population of developed countries. Since nearly 80% of the cases occur in women in the childbearing years, it may affect as many as 1 in 1,000 young women (3). The etiology of SLE is largely unknown, but it involves genetic, hormonal, and environmental factors (4). The complement system plays an important role in the onset as well as the effector phase of SLE (5, 6) and may also be the target of an autoantibody response (7). In SLE many organs may be affected, including serosa, joints, CNS, skin, and kidney.

Lupus nephritis (LN), the renal disease that accompanies SLE, is present in 25–50% of the cases (8) and is the major cause of morbidity and mortality (9). Understanding the sequence of events leading to full-blown LN in these patients is of major importance. Anti-C1q autoantibodies have been suggested to be closely associated with LN (10). This association is concluded from the correlation between

anti-C1q autoantibody positivity and renal involvement (11, 12), the predictive value of anti-C1q autoantibody titers for flares of nephritis (13, 14), and the accumulation of anti-C1q autoantibodies in LN kidneys (15, 16). Conversely, in the absence of anti-C1q autoantibodies, no LN develops (17, 18). However, no causal relationship has been established until now. Anti-C1q autoantibodies may be associated with other immune complex renal diseases as well; however, the total number of patients studied limits firm conclusions (10). Interestingly, anti-C1q autoantibodies can be found in several other conditions as well, such as the hypocomplementemic urticarial vasculitis syndrome (HUVS), and even in some healthy individuals (10), but in these instances they are unrelated to renal pathology. Anti-C1q autoantibodies also occur in murine models of SLE (19, 20). In MRL-lpr mice, rising anti-C1q autoantibody titers parallel a rise in LN, and anti-C1q autoantibodies also accumulate in glomeruli in murine SLE (21) as in human SLE.

In the present study we have investigated how anti-C1q autoantibodies contribute to the development of nephritis in mouse models. We show that administration of anti-C1q mAb's to naive mice results in glomerular deposition of C1q and anti-C1q autoantibodies but not in overt renal disease. However, administration of anti-C1q autoantibodies to mice pretreated with C1q-fixing anti-glomerular basement membrane (anti-GBM) antibodies, as a model for glomerular immune complex disease, resulted in strong synergistic enhancement of renal disease. Therefore, anti-C1q autoantibodies can be pathogenic to

Nonstandard abbreviations used: CLR, collagen-like region; DIG, digoxigenin; FcγR, Fcγ receptor; GBM, glomerular basement membrane; gh, globular head; HUVS, hypocomplementemic urticarial vasculitis syndrome; LN, lupus nephritis; SLE, systemic lupus erythematosus.

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the kidney but only in the context of C1q-containing glomerular immune complexes as found in SLE.

Results

Generation and characterization of anti-C1q mAb's. Following immunization of C1q-/- mice with purified mouse C1q, we obtained several mouse anti-mouse C1q mAb's. The stable clones JL-1, JL-2, and JL-3 were of the IgG2b, IgG2a, and IgM isotypes, respectively. Purified Ig's reacted in a dose-dependent fashion with C1q in ELISA (Figure 1A). For all our experiments we used an IgG2b control mAb as negative control. All 3 anti-C1q mAb's recognize different epitopes, since digoxigenin-conjugated (DIG-conjugated) mAb's were only competed by the respective unlabeled mAb but not by any of the other mAb's (Figure 1B). Anti-C1q mAb's were used to stain sections of spleens of WT mice and C1q-/- mice as an additional argument for specificity. Both mAb's JL-1 and JL-2 stain mouse C1q specifically on follicles of spleen of WT mice, as do polyclonal antibodies, but not on spleen of C1q-/- mice (Figure 1C). Both mAb JL-3 (data not shown) and control mAb (Figure 1C) did not stain either the WT or the C1q-/- spleen. Western blot analysis showed that whereas the polyclonal antibody recognized all 3 chains of C1q, mAb JL-1 specifically recognized the A/B chain and JL-2 recognized the C chain. Neither JL-3 nor IgG2b recognized C1q in Western blots (data not shown). In humans, anti-C1q autoantibodies react primarily with the collagen-like region (CLR), or "tail," of the C1q molecule (10, 21). Using recombinant heads or tails (CLRs), we found that JL-1, which was the only mAb showing cross-reactivity with human C1q (data not shown), specifically recognized the CLR only (Figure 1D). Similarly, serum from autoimmune MRL-lpr mice, but not normal mouse serum, reacted dose-dependently with CLRs (Figure 1E). Based on these in vitro data we concluded that mAb JL-1 is the most appropriate anti-C1q mAb to test in vivo.

Anti-C1q mAb's deplete circulating C1q and deposit in glomeruli but do not induce overt renal damage. Administration of mAb JL-1 (1 mg intraperitoneally) resulted in complete depletion of circulating C1q levels at 2 hours, which remained low at least up to 24 hours, whereas injection of IgG2b control mAb did not alter circulating C1q levels (Figure 2A). Administration of mAb JL-1 resulted in an increased glomerular depo-

sition of mouse IgG and especially mouse C1q, whereas injection of control mAb did not result in additional IgG or C1q deposition (Figure 2, B and C). Administration of mAb JL-1 to C1q-/- mice did not result in enhanced glomerular IgG deposition, which excludes the possibility that mAb JL-1 is reacting with glomerular antigens other than C1q (Figure 2B). Administration of mAb JL-1 resulted in a mild but significant glomerular granulocyte influx (Figure 2D). However, none of the mice developed albuminuria (Figure 2E).

Anti-C1q mAb JL-1 deposits on to glomerular C1q in a planted antigen-like fashion. Previous studies (22) have demonstrated that Ig-deficient, Rag2-/- mice, when compared with WT mice, not only lack circulating IgG but also lack IgG in glomeruli. As a consequence these Rag2-/- mice also lack glomerular C1q, since there is no glomerular IgG as a focus for glomerular C1q localization. Administration of JL-1 to Rag2-/- mice, which have comparable levels of circulating C1q, resulted in a similar depletion of circulating C1q (Figure 3A). However, this did not lead to glomerular deposition of C1q and anti-C1q autoantibodies as seen in WT mice (Figure 3, B and C). Reconstitution of circulating IgG led to the presence of both IgG and C1q in glomeruli (Figure 3, B and C). Subsequent injection of these IgG-reconstituted mice with JL-1 induced glomerular deposition of C1q and anti-C1q autoantibodies as seen in WT mice (Figure 3, B and C). These experiments show that JL-1

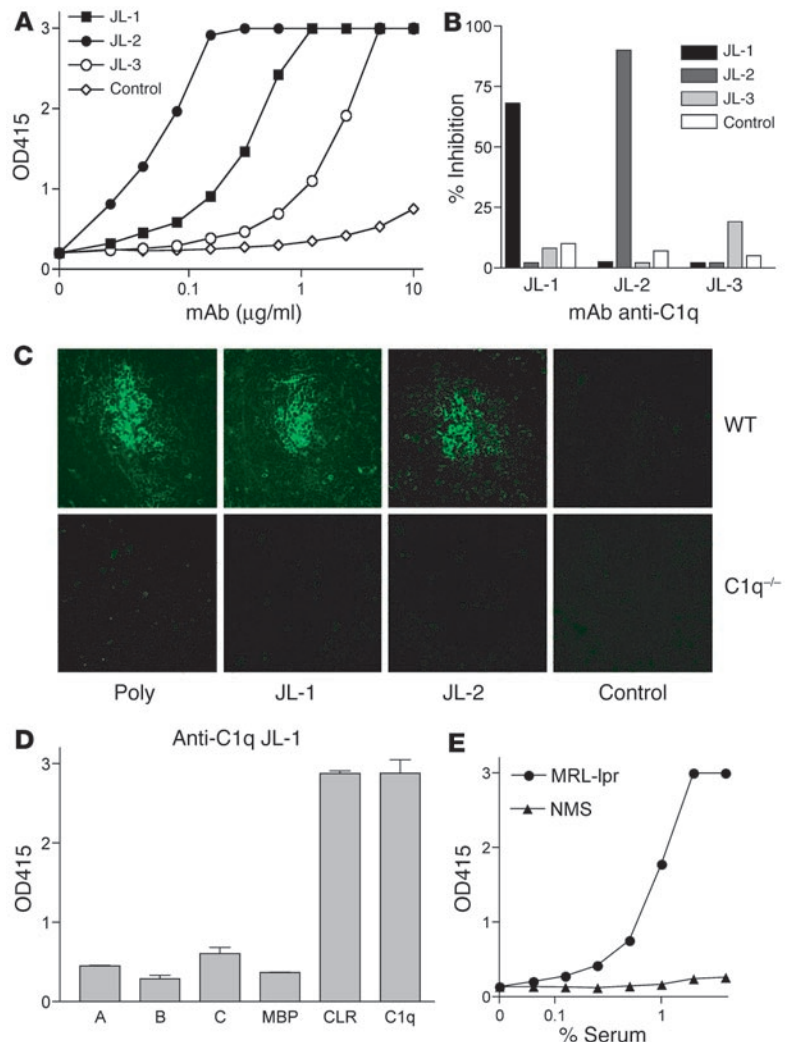
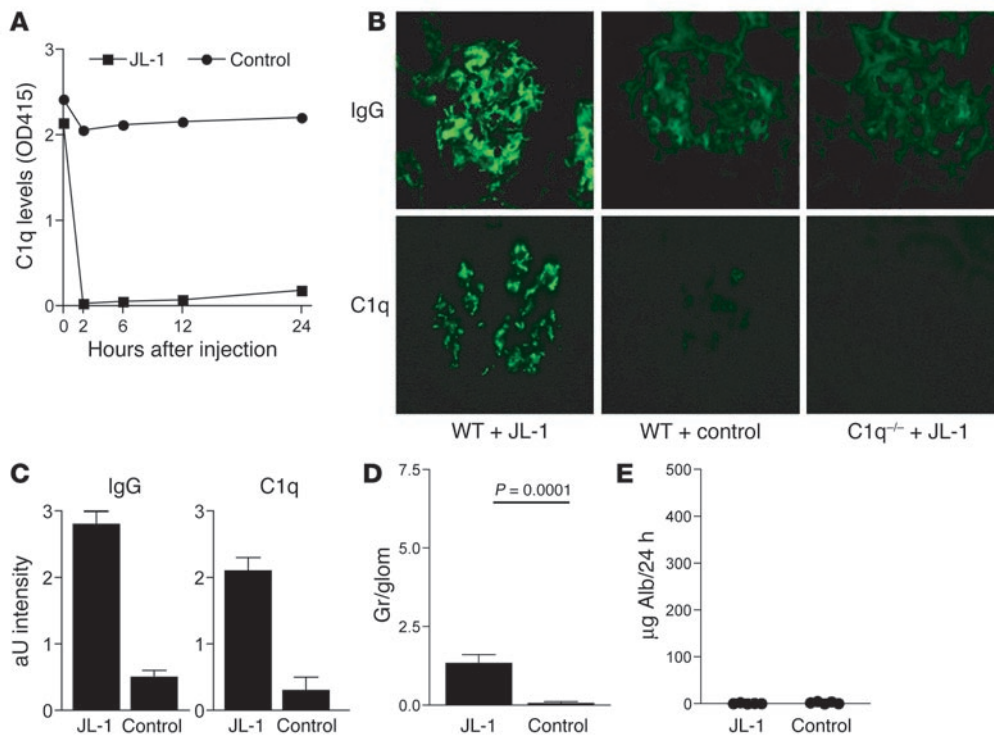


Figure 1 In vitro characterization of mouse anti-mouse C1q mAb. (A) Anti-C1q detection ELISA for anti-C1q mAb's JL-1, JL-2, and JL-3 and control mAb IgG2b under 0.5 M NaCl buffer conditions. OD415, OD at 415 nm. (B) Epitope competition ELISA showing inhibition of binding of DIG-labeled anti-C1q mAb to mouse C1q by unlabeled anti-C1q mAb or control mAb IgG2b. (C) Immunohistochemistry of WT or C1q-/- mouse spleen stained with anti-C1q mAb or controls. Original magnification, x250. Poly, polyclonal antibody. (D) C1q head domains of the human A, B, and C chains or the control maltose-binding protein (MBP), CLRs, and intact human C1q were coated, and mAb JL-1 binding was analyzed. (E) Anti-C1q tail ELISA using human CLRs and serum of autoimmune MRL-lpr mice (MRL-lpr) or nonautoimmune normal mouse serum (NMS) for binding.

**Figure 2**

Effects of administration of anti-C1q mAb to naive mice. (A) C1q sandwich ELISA for the detection of serum C1q levels following administration of anti-C1q mAb and control mAb. (B) Immunofluorescence analysis of mouse kidneys stained for the presence of mouse IgG and mouse C1q following administration of JL-1 or control mAb to WT or C1q^{-/-} mice. Original magnification, $\times 400$. (C) Quantification of immunofluorescence analysis of the deposition of IgG and C1q in WT mice. Data are expressed as arbitrary units (aU), as described in Methods. (D) Quantification of glomerular granulocyte influx. Depicted on the y axis are granulocytes per glomerular cross section (Gr/glom) at 24 hours after injection. (E) Assessment of albuminuria by ELISA. Alb, albumin.

does not capture C1q from the circulation and deposit in the glomerulus but that mAb JL-1 deposits on to C1q already present in the glomerulus in a planted antigen-like fashion.

Administration of anti-C1q mAb JL-1 induces overt renal damage when combined with a subnephritogenic dose of anti-GBM antibodies. Since LN is characterized by the glomerular deposition of C1q-containing immune complexes, we tested the effect of JL-1 in a murine model of C1q-containing solid-phase glomerular immune complexes. Administration of a subnephritogenic dose of C1q-fixing rabbit anti-mouse GBM antibodies was followed by administration of JL-1 or control mAb. Equal administration of anti-GBM antibodies was demonstrated by staining of renal sections for rabbit IgG (Figure 4, A and B). Administration of control mAb IgG2b directly following anti-GBM administration did not result in glomerular localization of additional mouse IgG or C1q and did not increase the mild albuminuria observed with this subnephritogenic dose of anti-GBM (Figure 4, A and D). In sharp contrast, mAb JL-1 gave a strong glomerular deposition of mouse IgG and also of additional C1q (Figure 4, A and B). This increase in IgG and C1q deposition involved all glomeruli (Figure 4A) and was highly consistent between individual mice (Figure 4, A and B). Confocal analysis following double staining for mouse IgG and mouse C1q and for mouse IgG and rabbit IgG revealed that rabbit IgG was present on the GBM and that both mouse C1q and mouse IgG (anti-C1q mAb) followed the same pattern (Figure 4C). Importantly, the glomerular

deposition of mAb JL-1, in this model of immune complex glomerulonephritis, resulted in markedly increased albuminuria ($P = 0.0026$ compared with IgG2b) (Figure 4D).

Mechanism of deposition of anti-C1q mAb JL-1 in combination with anti-GBM antibodies. We used C1q^{-/-} mice to verify that the observed renal damage was dependent on C1q and not caused by contaminating factors like endotoxin. Administration of the rabbit anti-GBM preparation to WT mice induced linear deposition of both rabbit IgG and mouse C1q in glomeruli. Administration of the same preparation to C1q^{-/-} mice led to the linear deposition of only rabbit IgG, not mouse C1q (Figure 5A). In contrast to WT mice, C1q^{-/-} mice did not display any enhancement of albuminuria upon coinjection of JL-1 (see Figure 8).

We next investigated the significance of serum C1q versus glomerular C1q in this model of glomerulonephritis. We compared a C1q-fixing rabbit anti-GBM preparation and a non-C1q-fixing sheep

anti-GBM preparation. Administration of both antibody preparations induced linear deposition of anti-GBM antibodies, but linear deposition of mouse C1q was only observed in the mice injected with C1q-fixing anti-GBM (Figure 5A). Administration of JL-1 to these mice enhanced renal damage only in the mice injected with C1q-fixing anti-GBM (Figure 5B). This points to a dependence on glomerular C1q.

Glomerular histological changes induced by anti-GBM and anti-C1q mAb JL-1. In addition to causing albuminuria, administration of JL-1 in the model of C1q-containing glomerular immune complexes induced major histological changes in glomeruli analyzed 24 hours after injection, characterized by pronounced inflammatory cell influx, focal capillary tuft occlusion by microthrombi, necrotizing lesions, nuclear debris, and wireloop-like lesions (Figure 6A). We did not observe crescents, extracapillary proliferation, or mesangial proliferation. Glomeruli of mice coinjected with control mAb only displayed limited inflammatory cell influx. Quantification of histological changes was performed using the activity index as described by Austin et al. (23). The JL-1-coinjected mice consistently displayed a much higher activity index than the control-coinjected mice (Figure 6B). Electron microscopy revealed capillary tuft occlusion by either granulocytes or fibrinlike material in mice injected with anti-GBM and JL-1, whereas in mice coinjected with control mAb IgG2b the capillaries showed normal contours, without alterations of the endothelium or GBM (Figure 6C). At higher magnifications, some

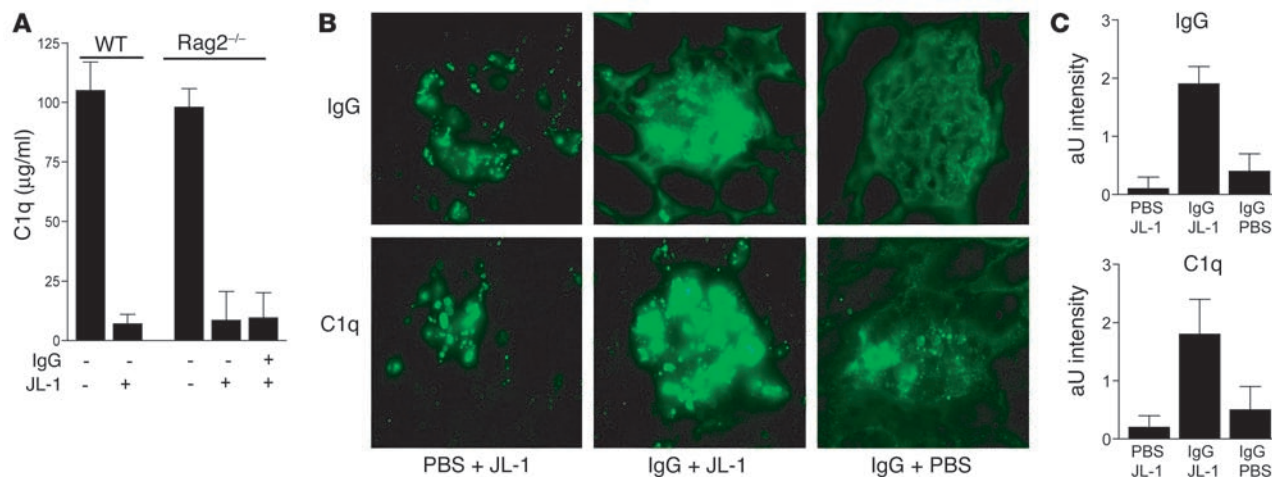


Figure 3 Anti-C1q autoantibodies react with C1q in the glomerulus in a planted antigen-like fashion. (A) C1q sandwich ELISA for serum levels of C1q in naive WT mice, WT mice treated with JL-1, naive Rag2^{-/-} mice, and Rag2^{-/-} mice pretreated with PBS or with mouse IgG and then treated with JL-1. (B) Immunofluorescence analysis of renal sections of Rag2^{-/-} mice, either naive or reconstituted for mouse IgG followed by injection of either JL-1 or PBS. Images show the absence of IgG and C1q in naive Rag2^{-/-} mice and positivity for IgG and C1q, both mesangially and along the GBM, in IgG-reconstituted mice. The positivity for IgG and C1q is highly increased following administration of JL-1. Original magnification, ×400. (C) Quantification of immunofluorescence analysis of the glomerular deposition of IgG and C1q.

subendothelial electron-dense deposits (wireloop-like lesions) were seen in mice injected with anti-GBM and JL-1 but not in mice coinjected with the control mAb IgG2b (Figure 6C).

Glomerular granulocyte influx is pronounced and extended in JL-1-injected mice. Analysis of renal sections of mice injected with anti-GBM in combination with either mAb JL-1 or IgG2b for granulocytes revealed significantly more infiltrating granulocytes at both 2 and 24 hours after injection in the JL-1-injected mice compared with the IgG2b-injected mice (Figure 7A). Granulocyte influx in this model of anti-GBM nephritis is known to peak at 2 hours after injection and then to diminish over time (24). Strikingly, the granulocyte influx at 24 hours was maintained in the JL-1-injected mice and was decreased, as expected, in the IgG2b-injected control mice (Figure 7A). Using confocal microscopy, we confirmed the increased number of infiltrating granulocytes (Figure 7B). Interestingly, the staining for granulocytes occasionally colocalized with the linear deposition of C1q (Figure 7B; colocalization in white; see arrow).

Requirements for JL-1 in combination with anti-GBM to enhance renal disease. To investigate the requirements for the enhanced renal injury and albuminuria, the combination of the C1q-fixing anti-GBM preparation and JL-1 was administered to various mice genetically deficient for complement components or Fcγ receptors. We found that the enhanced albuminuria was dependent on complement activation, based on the observations in C3^{-/-} mice. Potentially, complement may be activated via 3 pathways, the classical, alternative, and lectin pathways of complement activation. Since C4^{-/-} mice were also protected, either the classical or the lectin pathway was activated (Figure 8). In addition to complement activation albuminuria in this model is also dependent on Fcγ receptor (FcγR), since injection of anti-GBM in combination with mAb JL-1 did not result in any albuminuria in FcγR triple (CD64, CD32, CD16) deficient mice (Figure 8).

Discussion

SLE is immunologically characterized by the production of a wide variety of autoantibodies and the formation and deposition of

immune complexes. Renal involvement in this multiorgan disease is a major cause of morbidity and mortality (8, 9). One parameter strongly linked to renal involvement is the presence of anti-C1q autoantibodies (10). In the present study we demonstrate that anti-C1q antibodies are pathogenic to the kidney in conditions of glomerular C1q-containing immune complexes. These data provide essential evidence on how anti-C1q autoantibodies participate in the sequence of events leading to renal inflammation.

Previously, 2 studies have reported the deposition of anti-C1q antibodies in the kidney (25, 26) following the administration of high concentrations of human C1q and anti-human C1q antibodies in mice. Since the heterologous nature of such reagents may have been responsible for either the deposition or the lack of overt renal disease, we have generated mouse anti-mouse C1q autoantibodies by immunizing C1q^{-/-} mice with purified mouse C1q. Since C1q^{-/-} mice are reported to be autoimmune-prone if on a mixed genetic background (27), we used non-autoimmune-prone C1q^{-/-} mice on a full C57BL/6 background (28) to avoid the generation of polyreactive autoantibodies. We determined anti-C1q reactivity using an anti-C1q ELISA as described previously (21), under 0.5 M NaCl buffer conditions to exclude Fc tail interactions with C1q (29). Since IgG2b antibodies can interact with C1q via their Fc tails, we used, in all our in vivo experiments, an isotype-matched IgG2b control mAb that has a similar capacity to interact with C1q via its Fc tail.

Characterization of the mouse anti-mouse C1q mAb revealed reactivity with different epitopes on mouse C1q in ELISA. The mAb's JL-1 and JL-2 displayed reactivity toward C1q in splenic sections of WT but not of C1q^{-/-} mice, confirming their specificity. Since mAb JL-1 also recognized human C1q, we had the unique opportunity to test whether it would be reactive with either the C1q heads or the CLR, since these are only available for human C1q. We found our mAb to be reactive with the CLR only, which is in fact the same region with which the autoantibodies in mice (ref. 19 and current article) and humans (21, 30) are reactive. The

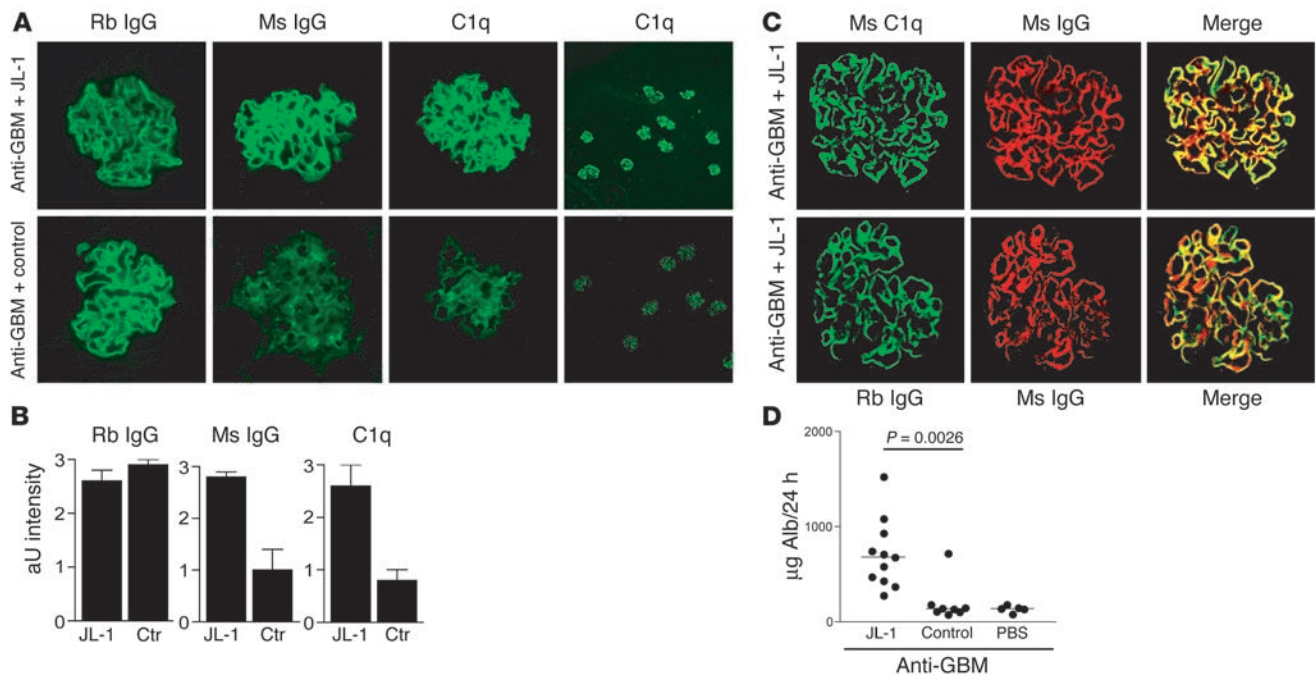


Figure 4 Effects of administration of anti-C1q mAb to mice pretreated with C1q-fixing anti-GBM antibodies. (A) Immunofluorescence of renal sections obtained from mice pretreated with rabbit (Rb) anti-GBM antibodies combined with either mAb JL-1 or IgG2b control mAb stained for the presence of mouse (Ms) IgG, mouse C1q, or rabbit IgG. Images show linear, GBM-like deposition of anti-GBM antibodies, linear fixation of C1q and anti-C1q in the JL-1-coinjected mice, and only mild mesangial positivity for C1q and anti-C1q in control-coinjected mice. Original magnification, $\times 400$. Right panels: Low-power magnifications of renal sections of mice injected with anti-GBM in combination with either JL-1 or control and stained for C1q. Original magnification, $\times 100$. (B) Quantification of immunofluorescence analysis of the glomerular deposition of rabbit IgG, mouse IgG, and C1q. Ctrl, control. (C) Confocal analysis of kidney sections of mice injected with rabbit anti-GBM and JL-1. Representative pictures are shown for the colocalization (yellow) of mouse C1q (green) and mouse IgG (red) and the colocalization (yellow) of rabbit IgG (green) and mouse IgG (red). They indicate that rabbit IgG, mouse C1q, and mouse IgG do colocalize in these mice, in a linear, GBM-like pattern. (D) Albuminuria of mice injected with anti-GBM antibodies followed by JL-1, control mAb, or PBS.

CLR of C1q is most likely exposed and accessible after binding of C1q to a ligand, whereas the heads are occupied. Based on these observations, we have chosen mAb JL-1 as the most appropriate mAb for in vivo testing.

Administration of anti-C1q mAb JL-1 to naive mice resulted in glomerular deposition of both C1q and anti-C1q autoantibodies, but without overt signs of renal damage. Previously we reported similar findings using a rabbit anti-mouse C1q polyclonal antibody (31). These results are consistent with the observation that anti-C1q autoantibodies can be found, unrelated to renal problems, in serum of HUVS patients and healthy individuals. Since no renal biopsies are performed without indications of renal problems, to our knowledge there are no data available on glomerular deposition of C1q and IgG in either HUVS or healthy individuals positive for anti-C1q autoantibody.

Injection of JL-1 in mice that had glomerular C1q-containing immune complexes resulted in enhanced renal damage. Several reports describe that the vast majority of SLE patients have glomerular deposits of both IgG and C1q even in the absence of clinically overt renal disease (32–34). This glomerular C1q could potentially serve as a target for anti-C1q autoantibody. Thus, the occurrence of anti-C1q autoantibodies may be highly relevant to the development of nephritis. Although most SLE patients have glomerular immune complexes containing C1q, only those positive for anti-C1q autoantibodies suffer overt renal disease. Con-

versely, patients who do not have anti-C1q autoantibodies do not develop nephritis (17). We provide causal evidence to suggest that anti-C1q autoantibodies are essential but not sufficient for the development of full-blown renal inflammation (18).

Although anti-C1q autoantibodies are an important determinant for LN, the degree of disease also seems to correlate with the amount of C1q present in the glomerulus. For instance, in glomeruli of Rag2^{-/-} mice, in the absence of glomerular C1q, no deposition takes place and no renal damage occurs. In naive WT mice, trace amounts of C1q are present in glomeruli, which facilitates the glomerular deposition of anti-C1q autoantibodies; however, this is not sufficient for overt renal damage. Only when there is a relatively high concentration of C1q in the glomerulus, as is the case for glomerular C1q-containing immune complexes, will anti-C1q autoantibodies not only deposit but also induce overt renal disease.

We verified that the enhanced renal damage observed was the effect of anti-C1q reactivity by testing the combination of the C1q-fixing anti-GBM preparation and JL-1 in C1q^{-/-} mice. No enhancement of disease was observed in C1q^{-/-} mice, which indicates that it was indeed the interaction between anti-C1q autoantibodies and C1q, and not cross-reactivity of mAb JL-1 with rabbit IgG or contaminating factors like endotoxin, that enhanced renal disease in this model (35).

The enhanced renal damage as observed with the combination of anti-GBM antibodies and anti-C1q mAb JL-1 can be explained

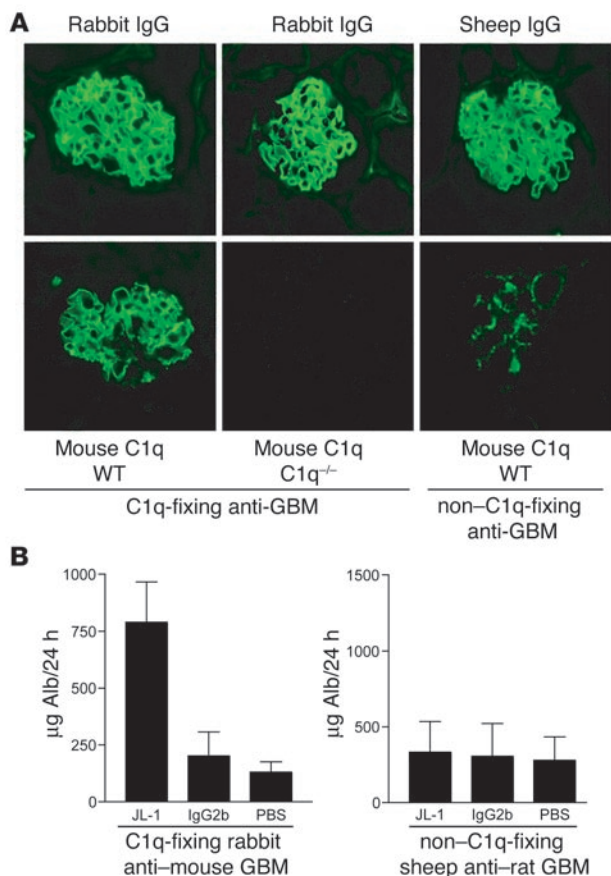


Figure 5

The disease-enhancing effect of JL-1 is dependent on glomerular C1q. (A) Immunofluorescence analysis of anti-GBM antibody deposition and C1q deposition for the C1q-fixing anti-GBM preparation in WT and C1q^{-/-} mice and the non-C1q-fixing anti-GBM preparation in WT mice. The images show the linear IgG fixation for both anti-GBM preparations, but only linear fixation of C1q for the rabbit anti-GBM preparation. Only some mild mesangial C1q positivity can be observed in the mice given sheep anti-GBM. Original magnification, ×400. (B) Groups of WT mice were injected with either the C1q-fixing or the non-C1q-fixing anti-GBM preparation and coinjected with JL-1, control, or PBS, and albuminuria was determined.

by a “2-hit” model. In this model, binding of anti-GBM antibodies to the GBM and the separate deposition of C1q and anti-C1q autoantibodies may trigger pathological effects. In an alternative synergistic model, events such as binding of anti-GBM to the GBM, fixation of C1q by the Fc parts of the anti-GBM, and deposition of anti-C1q autoantibodies on to this immune complex C1q could converge. We tested the 2 possible mechanisms by using a C1q-fixing and a non-C1q-fixing anti-GBM preparation. In the C1q-fixing model, the enhancing effect of JL-1 was observed only in WT, not in C1q^{-/-} mice. It was also absent in the model using a non-C1q-fixing anti-

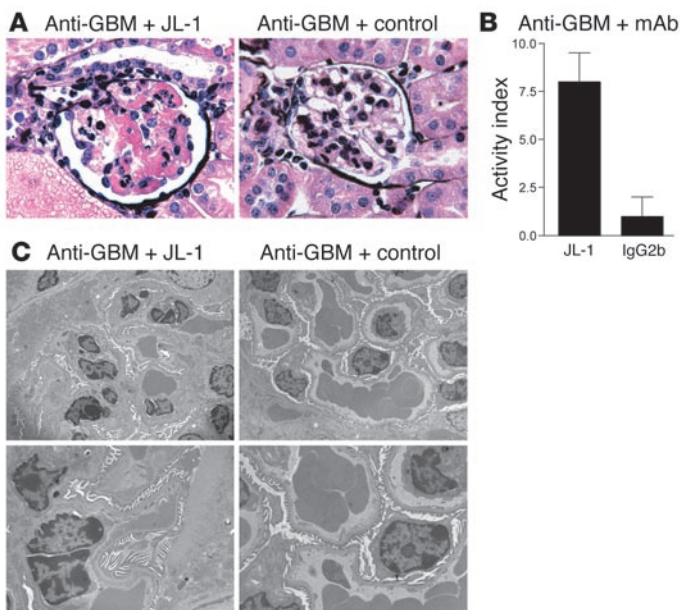
GBM preparation. This indicates that, in our model, anti-C1q autoantibodies, in the short term, enhances renal disease only in the context of renal C1q-containing immune complexes and, therefore, has a “synergistic” effect.

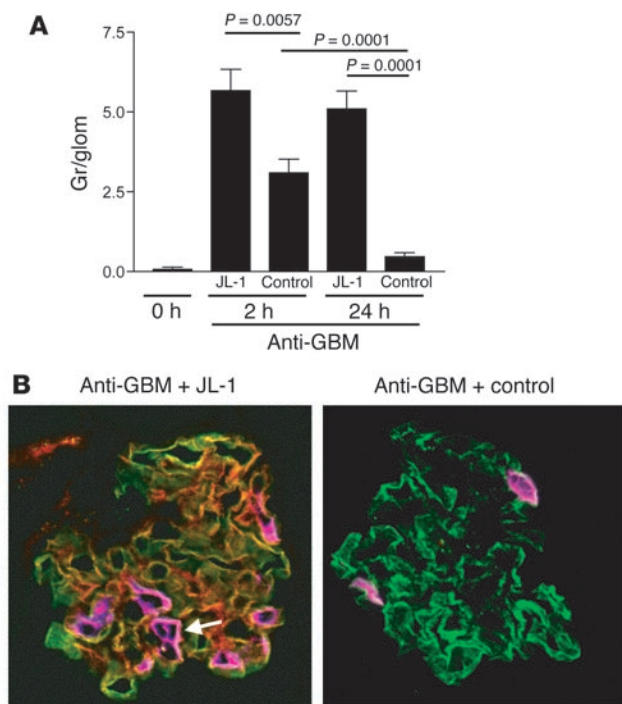
The damage induced by anti-C1q autoantibodies, in combination with anti-GBM antibodies, involves increased numbers of glomerular granulocytes, capillary tuft occlusion, microthrombi, and wireloop-like lesions. LN is categorized in several WHO classifications, depending on histological abnormalities (8). Many histological criteria of LN are present in the lesions induced by our anti-C1q mAb, such as IgG and C1q deposition, formation of sub-endothelial immune deposits, influx of leukocytes, and glomerular tuft occlusion. The fact that our experiments lasted only 24 hours may explain the lack of proliferative lesions or crescent formation.

The mechanism by which these anti-C1q autoantibodies enhance immune complex renal disease involves both complement activation and Fc receptors. Several reports have described the anti-GBM model as complement-independent (35–37), although others did report an effect of complement (24, 38, 39). Most likely, the dose of antibody given will determine the dependence on complement. Low concentrations, as used in the present study, are still dependent on complement, whereas high doses of antibody damage the kidney in a complement-independent way (38). In contrast, both concentrations of antibody work Fc receptor–dependently (37, 40). We have shown our

Figure 6

Histological changes induced by JL-1 at both the light microscopic and the electron microscopic levels. (A) Histological analysis of Silver-stained renal sections of mice injected with rabbit anti-mouse GBM and coinjected with either mAb JL-1 or IgG2b control mAb, obtained at 24 hours after injection. For JL-1–coinjected mice, images show pronounced inflammatory cell influx, focal capillary tuft occlusion by microthrombi, necrotizing lesions, nuclear debris, and wireloop-like lesions. Control-coinjected mice only display marginal inflammatory cell influx. Original magnification, ×400. (B) Quantification of histological changes using the activity index as described in Methods. (C) Electron microscopic analysis of glomerular lesions of mice injected with rabbit anti-mouse GBM and coinjected with either anti-C1q mAb JL-1 or control mAb IgG2b. At higher magnification, we observed several wireloop-like lesions in the JL-1–coinjected mice, whereas the IgG2b-coinjected mice did not display any abnormalities. Original magnifications, ×2,000 (left) and ×4,000 (right).





model to be indeed dependent on the C1q, the antigen for anti-C1q autoantibodies, by using C1q^{-/-} mice. Using C3^{-/-} mice, we also found that complement activation was essential. Our experiments using C4^{-/-} mice demonstrate that our model was dependent on C4 activation, suggesting involvement of either the classical or the lectin pathway of complement activation. Complement activation itself may damage the kidney or it may attract and activate leukocytes, which, in turn, may cause damage via Fcγ receptor-dependent mechanisms. Therefore, we have analyzed our model in Fcγ receptor-deficient mice and observed normal deposition of C1q and anti-C1q but no enhanced albuminuria, which indicates that Fcγ receptor triggering is also important in this model. Based on our data and the literature, we postulate that the sequence of events leading to severe immune complex renal disease is deposition of immune complexes on the GBM (Figure 9A), fixation of C1q from the circulation (Figure 9B), binding of anti-C1q autoantibodies (Figure 9C), and massive complement activation and attraction of inflammatory cells (Figure 9D). In conclusion, anti-C1q antibodies induce glomerular deposition of C1q and anti-C1q in naive mice but do not cause overt renal disease. However, anti-C1q autoantibodies strongly enhance renal disease when C1q-containing immune complexes are present in the glomerulus, which may explain the relationship between these antibodies and nephritis in SLE patients. These data provide essential insight into the mechanisms involved in immune complex-mediated renal inflammation.

Methods

Animals

Male C57BL/6 mice (Harlan), C1q^{-/-} C57BL/6 mice (27), C3^{-/-} C57BL/6 mice (41), C4^{-/-} C57BL/6 mice (41), FcγR triple ^{-/-} (CD64, CD32, CD16) mice (42), and Rag2^{-/-} C57BL/6 mice (43) (Taconic) were maintained at our facility. Mice were 8–12 weeks of age and had free access to water and standard chow. Animal care and experimental procedures were performed in accordance with the NIH guidelines for the care and use of laboratory animals.

Figure 7

Quantification of glomerular granulocyte influx. (A) Mice were injected with anti-GBM antibodies in combination with either JL-1 or control mAb. Granulocytes per glomerular cross section were scored either at 2 hours or at 24 hours after injection. (B) Confocal analysis of sections stained for mouse C1q (green), mouse IgG (red), and mouse granulocytes (purple). The pictures are merged and show, in yellow, colocalization of green and red, and, in white, colocalization of green and purple. The anti-GBM antibodies induced linear fixation of C1q in both groups, but only in the JL-1-coinjected mice is there colocalization between C1q and IgG and a pronounced influx of granulocytes. Original magnification, ×400. The white arrow indicates the white colocalization between C1q and granulocytes.

Purification and detection of mouse C1q

Mouse C1q was purified as described previously (31). Briefly, mouse serum (Harlan) was adjusted to 10 mM EDTA and applied to a rabbit IgG Sepharose column. After extensive washing with PBS containing 10 mM EDTA, bound C1q was eluted using 1 M NaCl containing 10 mM EDTA. The C1q-enriched fractions were identified by ELISA, pooled and dialyzed against PBS, concentrated to 0.5 mg/ml, and stored in aliquots at -80°C. Levels of mouse C1q were detected by sandwich ELISA as described previously (31).

Generation of mouse anti-mouse C1q mAb

Anti-C1q mAb's were generated by immunization of C1q^{-/-} C57BL/6 mice with purified mouse C1q. Mice were injected subcutaneously with 20 μg C1q in 50 μl CFA (Difco), followed by 3 boosts with 20 μg mouse C1q in 50 μl incomplete Freund's adjuvant (Difco) at 2-week intervals. Splenocytes of immunized animals were fused with SP2.0 cells using standard procedures. Cells were grown in IMDM (BioWhittaker Europe) supplemented with 2% PenStrep (Invitrogen Corp.), 0.5 ng/ml human IL-6 (PreproTech Inc.), 0.05 mM β-mercaptoethanol, and 10% heat-inactivated FCS (Invitrogen Corp.). Clones were screened for anti-C1q reactivity using an anti-C1q detection ELISA as previously described (21). Briefly, ELISA plates were coated with C1q-binding peptide 2J (44) at 12.5 μM in coating buffer for 2 hours at 37°C. Plates were washed and incubated with Rag2^{-/-}, Ig-deficient serum as a source of C1q at 1:20 in PBS containing 0.05% Tween 20 and 1% BSA (PBS-T-BSA) for 1 hour at 37°C. Serial dilutions of samples were incubated in PBS-T-BSA containing 0.5 M NaCl for 1 hour at 37°C. Bound mouse IgG was detected using goat anti-mouse IgG conjugated to HRP (DAKO A/S). Data are expressed as OD at 415 nm. Isotypes were determined using an Isotype kit (BD Biosciences – Pharmingen).

Positive clones were subjected to 2 rounds of limiting-dilution selection. Large quantities of mAb were generated using in vitro culture. Antibody

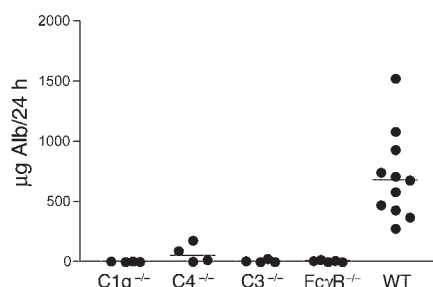


Figure 8

Anti-GBM and JL-1 in various genetically deficient mice. Rabbit anti-mouse GBM in combination with JL-1 was administered to either WT C57BL/6 mice or C1q^{-/-}, C4^{-/-}, C3^{-/-}, or FcγR^{-/-} mice, and albuminuria was assessed.

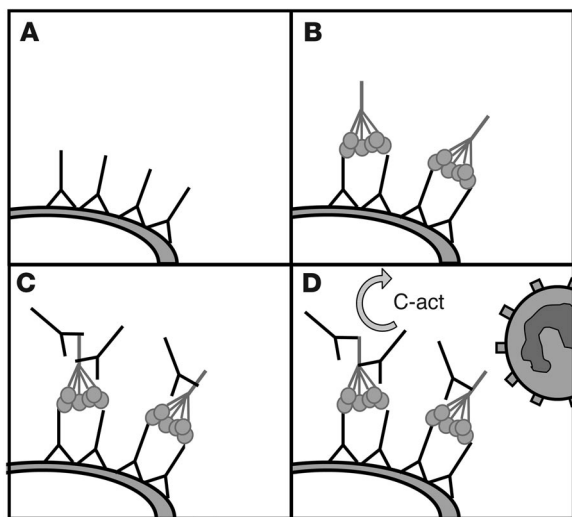


Figure 9
Schematic representation of the pathogenic role of anti-C1q antibodies in immune complex-mediated renal disease. (A) Deposition of immune complexes on the GBM. (B) Fixation of C1q from the circulation by the immune complexes. (C) Binding of anti-C1q autoantibodies to C1q present on the immune complexes. (D) Activation of complement (C-Act) and attraction of inflammatory cells.

purification was performed using Protein A-Sepharose HT columns (Pharmacia Corp.) for IgG2a and IgG2b antibodies and by precipitation and gel filtration for IgM antibody. Purity of the mAb preparations was verified by SDS-PAGE and found to be consistently more than 90%. As a control we used IgG2b mAb anti-TNP (kindly provided by L. Aarden, Sanquin Research, Amsterdam, The Netherlands). Three stable clones of mouse anti-mouse C1q were obtained, designated JL-1, JL-2, and JL-3.

Characterization of anti-C1q mAb’s

Epitope competition. Fixed amounts of DIG-conjugated anti-C1q mAb or control mAb IgG2b were coincubated with an excess of nonconjugated anti-C1q mAb or IgG2b in the anti-C1q detection ELISA. Binding of DIG-conjugated mAb was detected by anti-DIG HRP (Boehringer Mannheim). Data are expressed as percent inhibition compared with noninhibited mAb binding.

Immunohistochemistry. Spleen sections of either WT or C1q^{-/-} mice were stained with DIG-conjugated anti-C1q mAb and DIG-conjugated control antibodies at 5 µg/ml in PBS containing 1% BSA for 1 hour at room temperature followed by FITC-conjugated anti-DIG (Boehringer Mannheim) at 5 µg/ml.

Anti-globular head or –collagen-like tail reactivity of mAb’s

Recombinant forms of the globular regions of human C1q A, B, and C chains (globular head A [ghA], ghB, and ghC, respectively) were generated as described recently (45). Human CLR’s were generated from purified human C1q as described previously (46, 47). The recombinant globular regions, CLR’s, or human C1q were coated in ELISA, and anti-C1q mAb and control mAb were incubated at 2.5 µg/ml in PBS-T-BSA and analyzed for binding. Maltose-binding protein (MBP) was used as a negative control protein, since ghA, ghB, and ghC were expressed in bacteria as fusions to *Escherichia coli* MBP. Anti-CLR reactivity was also analyzed in serum from an autoimmune MRL-lpr mouse of 5 months of age and an age-matched nonautoimmune BALB/c mouse, by coating of CLR and incubation of serially diluted serum.

Generation of anti-GBM antibodies

A C1q-fixing anti-GBM polyclonal antibody was generated by immunization of male New Zealand white rabbits (Harlan) with purified mouse GBM as described previously (48). Subcutaneous injection of 50 µl GBM mixed with 100 µl CFA (Difco) was followed by 3 boosts with 50 µl mouse GBM in 100 µl incomplete Freund’s adjuvant (Difco) at 2-week intervals. Rabbit immune serum was heat-inactivated at 56°C for 30 minutes and centrifuged for 20 minutes at 2,000 g. γ-Globulin precipitation was performed using (NH₄)₂SO₄ at 40% wt/vol saturation, and the precipitate was further purified by anion exchange chromatography using DEAE-Sepharcel (Pharmacia Corp.). Rabbit IgG-containing fractions were pooled, concentrated to 20 mg/ml, dialyzed against 0.15 M NaCl, and stored in aliquots at -20°C.

Alternatively, we have used the γ-1 fraction of a sheep anti-rat GBM polyclonal antiserum that cross-reacts with mouse GBM (49, 50). Although this fraction is known to activate mouse complement (51), we previously observed that this preparation does not fix C1q in vivo.

Experimental procedure

Groups of 2 C57BL/6 mice were injected intraperitoneally with 1 mg of purified anti-C1q mAb or isotype control in 1 ml of sterile PBS. Blood was collected by tail cut at 0, 2, 6, 12, and 24 hours after injection. Serum was prepared and stored at -80°C. Groups of 5 C57BL/6 mice were injected with 1 mg of purified anti-C1q mAb intraperitoneally in 1 ml of sterile PBS. Overnight urine was collected by placement of the mice in metabolic cages. Mice were anesthetized with urethane (Sigma-Aldrich) and sacrificed by heart puncture. To exclude cross-reactivity, an additional group of 3 C1q^{-/-} mice was injected with 1 mg of mAb JL-1.

Three naive Rag2^{-/-} mice were injected with JL-1. Six Rag2^{-/-} mice were reconstituted for mouse IgG by means of mouse IgG injection as described previously (22). One group of reconstituted mice (n = 3) was injected with PBS and the other group (n = 3) with JL-1. Both groups were analyzed for the renal deposition of mouse IgG and mouse C1q by immunofluorescence.

Groups of at least 5 mice were injected intravenously with a subnephritogenic dose of either 2 mg rabbit anti-mouse GBM or 0.5 mg sheep anti-rat GBM polyclonal antibodies in a total volume of 200 µl sterile PBS via the tail vein, and then injected intraperitoneally 10 minutes later with 1 mg purified mAb JL-1 or IgG2b in 1 ml sterile PBS or by 1 ml PBS. Overnight urine was collected by placement of the mice in metabolic cages. Mice were anesthetized with urethane (Sigma-Aldrich) and sacrificed by heart puncture at 24 hours. Similarly, an additional 2 groups of 4 mice were injected with the C1q-fixing rabbit anti-GBM antibody in combination with JL-1, IgG2b control antibody, or PBS only, sacrificed at 2 hours, and analyzed for glomerular granulocyte influx as a measure of glomerular inflammation.

The C1q-fixing anti-GBM preparation was administered in combination with JL-1 to groups of C1q^{-/-}, C4^{-/-}, C3^{-/-}, and FcγR triple -/- (CD64, CD32, CD16) mice (n = 4 in each group), and urine was collected and analyzed for albuminuria.

Histological analysis

Immediately after sacrifice, 1 kidney from each mouse was frozen in pre-cooled 2-methylbutane for immunohistochemistry, and the other was fixed in methyl Carnoy’s solution for light microscopy.

For immunofluorescence, cryostat sections of 3 µm were fixed using acetone, all antibodies were diluted in PBS containing 1% BSA, and sections were incubated in a humid incubator for 1 hour at room temperature. Sections were washed 3 times for 5 minutes using PBS. Sections were stained for the presence of mouse IgG, rabbit IgG, and mouse C1q using goat anti-mouse IgG Oregon Green (Invitrogen Corp.), goat anti-rabbit IgG conjugated to FITC (Nordic Immunological Laboratories), and rabbit anti-mouse



C1q DIG, followed by sheep anti-DIG conjugated to HRP, where appropriate. Evaluation of the extent and intensity of immunofluorescence was performed by scoring of the intensity of staining for individual glomeruli as 0 (negative), 1 (positive above background), 2 (positive), and 3 (brightly positive) for at least 20 glomeruli per section. Means and SDs for at least 3 mice per group are shown. For double staining, goat anti-mouse IgG Alexa 546 (Invitrogen Corp.) was used in combination with anti-C1q and anti-rabbit IgG stainings, as described above. For triple staining, the antibodies described above were combined with rat anti-mouse granulocyte mAb GR-1 (a kind gift of G. Kraal, Vrije Universiteit Medisch Centrum, Amsterdam, The Netherlands), and rat IgG was detected using goat anti-rat IgG Alexa 594 (Invitrogen Corp.). The double- and triple-stained sections were mounted in Mowiol 40-88 (Sigma-Aldrich Chemie) and examined using an LSM 510 confocal microscope (Carl Zeiss AG).

For immunohistochemistry, sections were stained for mouse granulocytes using rat anti-mouse granulocyte mAb GR-1, and rat IgG was detected using goat anti-rat IgG conjugated to HRP (DAKO), preincubated with 10% normal mouse serum to absorb anti-mouse IgG cross-reactivity. Nova-RED (Vector Laboratories Inc.) was used as a substrate. Granulocyte influx was scored by counting of the number of granulocytes per glomerular cross section. Granulocytes were counted by 2 observers on coded sections.

For light microscopy, the other kidney was fixed and embedded in paraffin, and 3- μ m sections were stained with H&E, periodic acid-Schiff, and silver. Evaluation of histopathological changes was performed by a pathologist who was blinded to the code of the sections, using the activity index as described by Austin et al. (23). This index scores several parameters of active renal inflammation, such as glomerular proliferation, leukocyte exudation, karyorrhexis/fibrinoid necrosis, and hyaline deposits.

For electron microscopy, small pieces of cortex were fixed in 1% paraformaldehyde/1.5% glutaraldehyde overnight and washed in 0.1 M cacodylate buffer. Pieces of kidney were postfixed in 1% OsO₄, dehydrated, embedded in Epon 812, and cut into ultrathin sections using a Leica UCT

ultramicrotome (Leica Microsystems Inc.). On the grids, sections were poststained with uranyl acetate and lead citrate and examined with a JEM-1011 electron microscope (JEOL USA Inc.).

Renal damage

Renal damage was determined by analysis of urinary albumin excretion using an autoanalyzer (Hitachi 911; Roche). Data are expressed as micrograms albumin excreted in urine per 24 hours.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 3.03 software (GraphPad Software Inc.). Differences between parameters were analyzed using unpaired Student's *t* tests. The *P* values were considered statistically significant at *P* less than 0.05.

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