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Both Complement and IgG Fc Receptors Are Required for Development of Attenuated Antiglomerular Basement Membrane Nephritis in Mice

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To elucidate the mechanisms of glomerulonephritis, including Goodpasture’s syndrome, mouse models are used that use heterologous Abs against the glomerular basement membrane (GBM) with or without preimmunization with foreign IgG from the same species. These studies have revealed the requirement of either FcγR or complement for a full-blown inflammation in a novel attenuated passive model of anti-GBM disease. We demonstrate that administration of subnephritogenic doses of rabbit anti-GBM Abs followed by a fixed dose of mouse mAbs to rabbit IgG, allowing timing and dosing for the induction of glomerulonephritis, resulted in reproducible complement activation via the classical pathway of complement and albuminuria in wild-type mice. Because albuminuria was absent in FcR-γ-chain−/− mice and reduced in C3−/− mice, a role for both FcγR and complement is postulated. Because C1q−/− and C4−/− mice lacking a functional classical and lectin pathway did develop albuminuria, we suggest involvement of the alternative pathway of complement. Anti-GBM glomerulonephritis occurs acutely following the administration of mouse anti-rabbit IgG, and proceeds in a chronic fashion dependent on both FcγR and complement. This novel attenuated model allows elucidating the relative contribution of different mediator systems of the immune system to the development of renal injury, and also provides a platform for the assessment of different treatment protocols and evaluation of drugs that ultimately may be beneficial for the treatment of anti-GBM mediated glomerulonephritides. The Journal of Immunology, 2009, 183: 3980–3988.

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ntiglomerular basement membrane (GBM)-mediated glomerulonephritis plays an essential role in autoimmune diseases such as Goodpasture’s syndrome. Although the pathological processes that damage the kidney following deposition of anti-GBM Abs are incompletely understood, Goodpasture’s syndrome, a life-threatening renal disease, is characterized by rapidly progressive glomerulonephritis and a linear deposition of Abs along the GBM (1). Binding of these autoantibodies to the GBM (anti-GBM Ab) leads to autoimmune injury characterized by strong complement activation (as evidenced by the deposition of C3), leukocyte infiltration and proteinuria. This can ultimately lead to crescent formation, scarring, and loss of renal function (2). Until now, the only treatment available for Goodpasture’s syndrome is to slow down disease progression using corticosteroids and plasmapheresis (1). However, no curative therapy is available. Therefore, development of controlled experimental animal models is crucial.

To better understand the mechanism of anti-GBM disease, several animal models have been developed. Earlier experimental studies have mainly used rat models to investigate the pathology of anti-GBM disease (3). However, to profit from animals that have genetic deficiencies, a number of investigators switched to mice, genetic deficiencies, a number of investigators switched to mice, and because this response is variable between individual mice, this model is difficult to control. Furthermore, due to the injection of high amounts of Ab in the direct model, the degree of renal inflammation in the latter model is strongly dependent on the initial immune response against the IgG used for immunization, and because this response is variable between individual mice, this model is difficult to control. Furthermore, due to the injection of high amounts of Ab in the direct model, and due to the preimmunization in the accelerated model, both models comprise two phases, a first, heterologous, phase which mimics the Ab-mediated disease, and a second, autologous, phase, in which mice have developed an immune response against the foreign Abs (8–10). As this autologous phase is independent of Ab-mediated effector mechanisms, these models are less adequate to study chronic effects of anti-GBM disease. Furthermore, these direct and
accelerated models differ in their mechanism of Ab-induced renal injury.

Autoantibodies might provoke damage via distinct effector mechanisms. Firstly, Abs might cause injury due to the activation of the complement system (11). The complement system consists of three pathways: the classical, the lectin, and the alternative pathway, which all merge at the level of C3. Activation of the complement system can lead to inflammatory processes, such as production of chemotactic fragments resulting in the attraction and activation of leukocytes, formation of the lytic membrane attack complex (C5b-9), and release of inflammatory mediators (12), which together determine the degree of glomerular injury (13-15).

Classically, IgG Ab have been shown to be able to activate the classical pathway of complement. However, in a growing list of Ab-induced autoimmune diseases, such as Ab-mediated arthritis (16), subepidermal blistering disease (17), anti-phospholipid syndrome (12), ischemia/reperfusion injury (18), and cryoglobulin-induced immune-complex glomerulonephritis (19), alternative pathway involvement has been implicated. Secondly, Abs can also induce injury through activation of IgG Fc receptors (FcγR) (20). IgG binding to FcγR on immune cells can activate immune effector functions including Ab-dependent cellular cytotoxicity, phagocytosis, and release of inflammatory mediators and reactive oxygen species by leukocytes whose capacity to infiltrate the site of inflammation is strongly correlated with the induction of proteinuria in most models of anti-GBM disease. FcγR have been shown to be involved in numerous autoimmune diseases, such as Ab-induced arthritis (21), bullous pemphigoid (22), immune hemolytic anemia (23), and lupus nephritis (24). Furthermore, a potential role for both complement and FcγR has been shown in anti-GPI Ab-induced arthritis (25), and anti-C1q Ab-induced lupus nephritis (26).

For induction of anti-GBM disease, the direct model depends on complement activation, as was shown in C3−/− animals (27, 28). This was, at least partially, elicited via the classical pathway, as C4−/− mice (which lack an intact classical and lectin pathway) showed reduced numbers of inflammatory cells and renal injury compared with WT mice (28, 29). However, a potential role for the alternative pathway of complement was postulated as well (29). In the accelerated model, the complement system even seems to have an alternative contribution of different mediator systems of the immune system to the development of renal injury, and also provides a very useful platform for the assessment of different treatment protocols and evaluation of drugs that ultimately may be beneficial for the treatment of anti-GBM disease.

Materials and Methods

Mice

C57BL/6 wild-type (WT) mice were purchased from Charles River Laboratories. The C1q−/−, C4−/−, and C3−/− mice were provided by Marina Botto (Imperial College, London, U.K.) and Mike Carroll (Harvard Medical School, Boston, MA), respectively, and backcrossed for six generations to the C57BL/6 background. The FcR−γ-chain−/− mice, generated in C57BL/6 background, are a gift of Takashi Saito (Yokohama, Japan). All strains, including the FcR−γ-chain−/− × C3−/− double-knockout strain, which was generated by crossing FcR−γ-chain−/− mice with C3−/− mice, were maintained by the Department of Human Genetics and the Nephrology Department, as described before (26). All experiments were approved by the Leiden University animal ethics committee, and performed according to institutional and national guidelines.

Antibodies

Rabbit anti-mouse GBM Abs (oGBM Abs) were obtained as described before (26). The mouse mAb (IgG2a) anti-rabbit IgG (MsαRb IgG, Fc-specific) was produced from hybridoma CRL-1753 (American Type Culture Collection) in IMDM (Lonza BioWhittaker) supplemented with 5% FCS, 0.05 mM 2-ME, 0.5 mg/ml IL6 (Strathmann Biotech) and antibiotics. MsαRb IgG in the supernatant was concentrated, centrifuged at 10,000 rpm and purified by protein G Sepharose chromatography (Amersham Biosciences). Presence of MsαRb IgG in the elution fractions was confirmed by ELISA. The positive fractions were pooled, concentrated and, after dialyzing against PBS, aliquotted and stored at −20°C until further use. A fraction of MsαRb IgG was coupled to digoxigenin (DIG) as described before (32).

Induction of glomerulonephritis

At day 0, mice were injected i.v. with 0.5 mg oGBM Ab (200 μl in PBS). After 6 days, 1 mg of monoclonal MsαRb IgG was given i.p. Urine samples were collected at day 0, 5, and 6 to measure albumin excretion in urine (albuminuria). Mice were sacrificed 24 h after administration of MsαRb IgG (acute model), or one month after MsαRb IgG injection (chronic model). Thereafter, kidneys were collected, snap frozen and stored at −150°C for immunohistochemical analysis.

Albuminuria and creatinine

To collect urine, mice were placed in metabolic cages for 18 h. Urine was centrifuged and subsequently stored at −20°C. Rocket immuno-electro- phoresis (protocol modified from Ref. 33) was used to quantitate albumin levels in urine. Albuminuria per mouse is plotted as mg/24 h. Urine creatinine levels were determined by a kinetic colorimetric assay using a commercially available kit (Creatinine Jaffé method, Roche diagnostics) and a Cobas Integra 800 analyzer (Roche).

Histological analysis

Mouse kidneys were sectioned into 3-μm slides to assess deposition of complement components and IgG. The presence of rabbit IgG (oGBM Ab) in glomeruli was detected using a goat anti-rabbit IgG FITC Ab (Nordic Immunological Laboratories). Mouse IgG was detected using goat anti-mouse IgG coupled to Oregon Green (Invitrogen). C1q deposition was visualized using a polyclonal rabbit anti-mouse C1q, which was coupled to DIG (26). C4 and C3 were detected using rat anti-mouse C4 and C3 mAb (HyCult Biotechnology) followed by DIG-coupled mouse anti-rat Fab (His 8, provided by Dr. N.A. Bos, Department of Histology and Cell Biology, Groningen University, The Netherlands), which was then detected using a (Fab’), sheep anti-DIG-FITC (Roche Diagnostics). The intensity of staining was scored by at least two independent observers, who were blinded to the code of the sections. A score between 0 (no staining) and 3 (maximal staining) was given. For each mouse, the score was determined by averaging the scores given by the observers.

Leukocytes and granulocytes were visualized using an anti-CD45 Ab (AbD Serotec, Oxford, U.K.) and mAb Gr-1 (a gift from Dr. R. Toes, Department of Rheumatology, LUMC, The Netherlands), respectively, followed by incubation with goat anti-rat IgG, coupled to Alexa fluor 488.
Leukocytes were counted by a pathologist who was blinded to the code of the sections.

For light microscopy, the kidney was fixed in paraformaldehyde, embedded in paraffin, and 3-μm sections were stained with H&E, and periodic acid-Schiff (PAS). Evaluation of histopathologic changes was performed by a pathologist who was blinded to the code of the sections.

Statistics

Statistical differences were determined by one-way ANOVA using Kruskal Wallis statistics, Dunn’s correction for multiple comparison, and Chi-square analysis. Significance was accepted when \( p < 0.05 \).

Results

Set up and evaluation of the attenuated mouse model for anti-GBM glomerulonephritis

To facilitate interactions with mouse effector systems in a natural fashion, mice were injected with a fixed subnephritogenic amount of rabbit αGBM Ab (0.5 mg i.v.) that serves as an anchor on the GBM for the subsequent binding of mouse mAb anti-rabbit IgG (MsoRb IgG), which was given 6 days later. This MsoRb IgG binds well to the αGBM Ab, as shown in Fig. 1. In in vivo MsoRb IgG dose-finding pilot experiments, an injection of 1 mg MsoRb IgG (i.p. at day 6) was chosen to be the best dose to induce glomerulonephritis.

To evaluate the manifestation of disease, as measured by albuminuria, glomerular complement deposition, leukocyte influx, and glomerular pathology, C57BL/6 mice were injected with PBS (as a control) or with αGBM Ab, followed 6 days later with the injection of PBS (as a control) or MsoRb IgG. Albuminuria was determined in all groups 24 h after the second injection. Mice that received PBS or αGBM Ab alone, did not develop albuminuria (Fig. 2A). Only when both αGBM and MsoRb IgG were given, overt albuminuria was detected, indicating that renal damage was specifically elicited by the injection of the mouse Ab (Fig. 2A). Blood urea nitrogen, as well as urine creatinine levels were unaltered between these three groups (data not shown).
FIGURE 3. Role of complement activation in renal injury. A. Groups of WT, C1q−/−, C4−/−, and C3−/− mice were injected with rabbit anti-GBM followed 6 days later by either MsoRb IgG or PBS as control. Urinary albumin excretion was measured by rocket immunoelectrophoresis and expressed as mg/24 h. B–D, Kidneys of WT, C1q−/−, C4−/−, and C3−/− mice injected with both rabbit anti-GBM and MsoRb IgG were stained for the presence of C1q, C4, and C3. Semiquantitative analysis of complement deposition was performed in the different strains. Fluorescence intensity (range 0–3) was scored by at least two blinded observers, and average score for each mouse was calculated. *, p < 0.05 compared with WT; **, p < 0.01 compared with WT.

To determine binding of anti-GBM Ab and MsoRb IgG as well as complement deposition in mouse kidneys, mice were sacrificed 24 h after injection of the MsoRb IgG, and kidney sections were examined using immunofluorescent stainings (Fig. 2C). Presence of anti-GBM Ab was visualized using a rabbit IgG staining, which showed that rabbit IgG was equally present in the glomeruli of all the mice that received anti-GBM Abs, and that this was distributed in a GBM-like pattern. Mice that received both anti-GBM as well as MsoRb IgG showed a deposition of mouse IgG in a GBM-like pattern. Although glomeruli of mice that received anti-GBM alone were positive for mouse IgG, staining was significantly less as compared with mice that received both Abs (a score of 0.5 ± 0.2 vs 2.3 ± 0.4; p < 0.01, respectively). These data indicate that MsoRb IgG colocalizes with rabbit IgG on the GBM in vivo. Glomerular rabbit or mouse IgG deposition was not detectable in mice that were injected with PBS alone.

Ab deposition was associated with complement activation in mouse glomeruli, as C1q, C4, and C3 were also deposited in glomeruli in a GBM-like pattern (Fig. 2C). C1q and C3 deposition were significantly increased in glomeruli of mice that received both anti-GBM and MsoRb IgG compared with mice that received anti-GBM alone (a score of 2.4 ± 0.3 vs 1.4 ± 0.1 for C1q; p < 0.01, and a score of 3.1 ± 0.35 vs 1.9 ± 0.4 for C3; p < 0.01, respectively), whereas differences in C4 deposition were not significant. Control (PBS) mice had no detectable glomerular deposition of C1q, C4, or C3.

At 24 h after the last injection, the group of mice that received both anti-GBM and MsoRb IgG had an average of 2.5 leukocytes per glomerulus, which consisted of granulocytes and some macrophages (Fig. 2B). These leukocyte numbers were not significantly different from anti-GBM control mice (p = 0.09). The influx of neutrophils might have been missed as glomerular neutrophil influx peaks at several hours, and resolves within 24 h in most models (4, 5, 9, 28, 34–36), and this was also shown in a neutrophil-dependent direct model for anti-GBM disease (4, 28). Indeed, in an experiment in which mice were sacrificed within 15 h after injection of MsoRb IgG, significantly more leukocytes were observed in glomeruli of anti-GBM and MsoRb IgG experimental mice, compared with controls (4.3 ± 1.1 vs 1.6 ± 0.4 cells/glomerulus; p < 0.01).

Furthermore, although glomerular sclerosis, endocapillary proliferation, and mesangial matrix expansions were not detected in mice that had received both anti-GBM and MsoRb IgG, the percentage of glomeruli with PAS-positive deposits, as well as the location of these PAS-positive deposits, was altered in mice that received anti-GBM plus MsoRb IgG, compared with mice that had received anti-GBM alone (Fig. 2D); the average percentage of glomeruli affected in anti-GBM control mice was 2 ± 3.5% (n = 6) compared with 20.8 ± 7.2% (n = 8) in mice that obtained both anti-GBM as well as MsoRb IgG. In addition, mice that had received both anti-GBM plus MsoRb IgG had diffuse PAS-positive deposits, whereas the minor percentage of affected glomeruli of mice that received anti-GBM alone showed deposits that were only present in afferent arterioles (Fig. 2D).

The attenuated anti-GBM model is dependent on both complement activation and FcγR

The underlying mechanisms of anti-GBM mediated renal injury were evaluated by using C57Bl/6 mouse strains, deficient for specific complement components. WT, C1q−/−, C4−/−, and C3−/− mice were injected with both anti-GBM and MsoRb IgG and the development of glomerulonephritis was assessed by measuring albuminuria, and evaluation of complement deposition.

Compared with WT mice, development of albuminuria was significantly reduced in C3−/− mice, implying an important role for complement in development of renal injury (Fig. 3A). Both C1q−/− mice (which lack an intact classical complement pathway) as well as C4−/− mice (which lack a functional classical and lectin pathway) developed albuminuria similar to that observed in WT mice, indicating that neither C1q nor C4 are crucial for development of anti-GBM mediated glomerulonephritis (Fig. 3A). However, C1q, the first component of the classical pathway, was present in glomeruli of mice deficient for C3 or C4 (Fig. 3B), whereas C4 deposition, which is downstream of C1q activation, was reduced to background in mice lacking C1q (Fig. 3C), indicating that the lectin pathway most likely does not play a significant role. Furthermore, C3 deposition was present but clearly decreased in glomeruli of C1q−/− and C4−/− mice (Fig. 3D), which indicates that the classical pathway of complement is, at least partially, involved in the glomerular deposition of C3 in this model. All knockout mice lacked significant staining for the complement component that was knocked out, confirming the specificity of the staining (Fig. 3, B–D).
To further unravel the underlying mechanism of anti-GBM mediated glomerulonephritis, the role of FcγR was evaluated in FcR-γ-chain−/− mice, which lack all activatory IgG Fc receptors. As shown in Fig. 4, FcR-γ-chain−/− mice were completely protected from development of albuminuria (Fig. 4), although all FcR−γ-chain−/− mice exhibited glomerular depositions of Ab and complement (data not shown).

The attenuated anti-GBM model results in chronic renal injury

To investigate whether anti-GBM mediated glomerulonephritis could proceed into a chronic fashion, and to evaluate whether albuminuria would remain dependent on the presence on an intact complement system and on the presence of FcγR, we injected WT, C3−/−, and FcR-γ-chain−/− mice with αGBM followed 6 days later with MsαRb IgG, similar to experiments mentioned above. Furthermore, FcR-γ-chain−/− × C3−/− double-knockout mice, which lack both FcγR as well as an intact complement system, were included, as well. However, instead of sacrificing the animals 24 h after injection of MsαRb IgG, mice were followed for 37 days, and urine samples were collected three times a week. WT control mice receiving αGBM Ab alone did not develop any albuminuria during the whole period (Fig. 5A). In contrast, a rapid rise of albuminuria was observed in WT mice in the first two days...
after injection of MsαRb IgG, after which albuminuria declined slowly, but remained significantly present up until day 37 (Fig. 5A). Similar to what was observed in the acute setup, C3−/− mice showed decreased albuminuria, whereas albuminuria was completely absent in FcR-γ-chain−/− mice. In C3−/− mice, albuminuria was slightly above background up until day 37. As expected, albuminuria was absent in FcR-γ-chain−/− × C3−/− double-knockout mice, as well (Fig. 5A).

At day 37, mice were sacrificed and kidneys were examined histologically. No differences were found in leukocyte numbers, which might be due to the late time point on which the mice were sacrificed (data not shown). However, clear histological injury was now observed in WT mice injected with αGBM and MsαRb IgG, as evidenced by significantly increased mesangial matrix expansion (Fig. 5, B and D), and segmental matrix accumulation and glomerular sclerosis (Fig. 5, C and D), as compared with mice injected with αGBM alone. Furthermore, tubular casts, representative for heavy proteinuria, was observed in 50% of WT animals, whereas tubular casts were not observed in αGBM control mice (Fig. 5D).

Mouse kidneys were furthermore stained for rabbit IgG (αGBM), mouse IgG (MsαRb IgG), and complement products. In all mice, αGBM could still be observed in significant amounts in the glomeruli, indicating that rabbit IgG was not cleared from the GBM. Furthermore, no significant differences were observed in rabbit IgG staining between WT (either injected with αGBM alone, or injected with both αGBM and MsαRb IgG) and knockout mice (Fig. 6A, and data not shown). Mouse IgG staining was observed in all mice that received αGBM and MsαRb IgG, whereas hardly any mouse IgG could be observed in mice injected with αGBM alone, indicating that MsαRb IgG remained anchored to the αGBM Ab for over a month (Fig. 6A). At day 37, complement

FIGURE 6. Rabbit IgG, mouse IgG, and complement depositions are present at day 37. A, Groups of mice injected with rabbit αGBM alone (depicted as “αGBM”) or injected with both αGBM and MsαRb IgG were sacrificed after 37 days, and the presence of rabbit IgG, mouse IgG, C1q, C4, and C3 was determined by immunofluorescence (representative stainings, magnification ×200). B–D, Quantitative analysis of C1q, C4, and C3 deposition was performed on kidneys of the different mouse strains. Fluorescence intensity (range 0–3) was scored by at least two blinded observers, and the average score for each mouse was calculated. *, p < 0.05 compared with WT; **, p < 0.01 compared with WT.
deposition, as measured with C1q, C3, and C4, was low in control mice (injected with αGBM alone) whereas all and knockout mice injected with both Abs showed significant complement deposition (Fig. 6, B–D), indicating either low clearance of deposited complement or continuous complement activation. Compared with WT mice, C3 deposition was absent in C3−/− mice, thereby confirming staining specificity, and was significantly increased in FcR-γ-chain−/− mice (Fig. 6D).

Discussion

In this manuscript, we describe a novel model for anti-GBM mediated glomerulonephritis, characterized by albuminuria, complement activation, and involvement of FcγR. The model is based on passive administration of a fixed amount of rabbit αGBM Ab which serves as an anchor for the subsequent binding of a mouse mAb against rabbit IgG. In this way, the present model differs from earlier described models where either high amounts of rabbit or goat anti-GBM Ab were administered to naive mice (4, 5), or the model where mice were first preimmunized with IgG of the anti-GBM Ab species, followed a week later with anti-GBM Ab (6, 7). The advantage of the model described in the present study is that the degree of proteinuria is dependent on the amount of Ab used. In pilot experiments we first determined the required amount of rabbit αGBM Ab and the amount of MsαRb IgG, which resulted in a choice of 0.5 mg polyclonal rabbit Ab and 1.0 mg monoclonal mouse Ab per mouse. Compared with the earlier described models, the present model results in a controllable degree of renal injury, which is not dependent on the immune response of individual mice. Indeed, when higher doses of monoclonal mouse anti-rabbit Abs were used, a more severe disease occurred (data not shown). Therefore, this approach seems to circumvent potential variations in the strength of the immune response against either rabbit immunoglobulins or collagen type IV fragments as required in the accelerated nephrotoxic nephritis model of anti-GBM disease (6, 7) and the model of Goodpasture’s syndrome (37–40), respectively.

The induction of disease was rapid, with an average proteinuria of 5 mg/24 h in WT mice. Furthermore, a clear involvement of classical complement activation was observed with deposition of C1q, C4, and C3 in glomeruli of mice receiving both rabbit αGBM and MσαRb IgG. Interestingly, mice with a deficiency in the classical pathway (C1q−/− mice) or the classical and the lectin pathway (C4−/− mice) displayed almost the same degree of proteinuria as compared with WT mice. This observation is compatible with the finding that C3 deposition in these mice was still present, while C4 and C1q deposition at the tissue level was clearly absent or diminished (Fig. 3). Whereas C3 deposition in C1q−/− and C4−/− mice was present but clearly reduced, C4 deposition in C1q−/− mice was reduced to background, indicating that the classical pathway clearly contributes to complement activation, whereas the lectin pathway most likely does not play a significant role in this model. Although C1q−/− and C4−/− mice displayed a reduced level of C3 deposition, there still remained clear deposition of C3 indicating a diversion of complement activation from the classical to the alternative pathway, and therefore maintenance of the degree of proteinuria in these knockout mice.

This is in contrast with an experimental mouse model for lupus nephritis, a glomerular immune complex disease which was shown to be mediated via the classical pathway of complement (26). However, similar observations have been made in other passive Ab-mediated models of experimental disease in mice, like the collagen arthritis model (16), subepidermal blistering disease (17), anti-phospholipid syndrome (12), ischemia/reperfusion injury (18), and cryoglobulin-induced immune complex glomerulonephritis (19), where the alternative pathway was shown to be both required and sufficient for disease induction.

The question is why a diversion of complement activation from classical to the alternative pathway is observed. For our observation, which is in line with previous studies in another model of anti-GBM mediated disease in mice (28), there are, at least, two explanations. The first explanation is that certain Abs, like rabbit Abs may elicit activation of the alternative pathway as has been reported by several groups (41, 42). Additionally, studies in collagen Ab-induced arthritis have shown that mouse Abs can activate the complement system in mice via the alternative pathway (16). A second explanation may be found in the observation that modified self tissue may lead to the exposure of intracellular Ags which may directly activate the alternative pathway as exemplified by the binding of properdin to, for instance, DNA (43). Binding of Abs to different cells such as endothelial cells and mesangial cells may lead to apoptosis with a flip-flop of the cell membrane. This mechanism is associated with a loss of protection of the tissue involved against complement attack. Various studies have shown that apoptotic cells are more prone to complement-recognition than their intact counterparts (44–46). Additionally, induction of apoptosis is accompanied with direct binding of properdin (43, 47), which then can serve as a focus point for the direct binding of C3b. This will lead to local alternative pathway complement activation, as there is continuous turnover of C3 into C3b and C3d in the circulation (48).

That C3 activation is critical for the development of proteinuria is evidenced by the observation that C3−/− mice display a clearly reduced degree of proteinuria (Fig. 3A). C3 activation and deposition is associated with the further recruitment and engagement of infiltrating cells such as neutrophils and macrophages (Fig. 2B, and data not shown). These cells can than directly interact via their FcγR and complement receptors with deposited IgG and complement fragments, respectively. Involvement of FcγR in the induction of renal injury was investigated in FcR-γ-chain−/− mice, which have a complete absence of all stimulatory FcγR, either on infiltrating inflammatory cells or on resident kidney cells. FcR-γ-chain−/− mice did not show any signs of proteinuria at 24 h of disease. In this respect, the present observations are discrepant from earlier reports in mice preimmunized with IgG from the species of the anti-GBM Abs (6, 7, 27). In these studies, it was demonstrated that there was a protective role for complement in the degree of renal damage as was shown by proteinuria and other parameters of renal injury. We reason that these differences are dependent on the severity of the renal damage i.e., the amount, time span, isotype, and the species-origin of the IgG that is involved in the ongoing process (36). At high amounts of IgG, complement activation is not obligatory, as has been observed by Sheerin et al. (29). Therefore, we feel that the model we describe in the present manuscript allows the further analysis of the contribution of both FcγR and complement in the process of anti-GBM mediated renal damage in mice. These observations suggest that modulation of FcγR engagement or interference with the activation of complement may provide strategies for the control of anti-GBM disease.

In additional experiments summarized in Fig. 5 and 6, we demonstrate that the attenuated model of anti-GBM disease has a chronic course. In WT mice, proteinuria was persistently present for at least 37 days. Sustained proteinuria was dependent both on complement and the presence of activatory FcγR. Although the absence of FcγR is a prerequisite for disease chronicity, the absence of complement leads to a moderate disease over time. Immunofluorescence analysis revealed that Ab deposition (both rabbit IgG and mouse IgG) persisted over the whole period and that
this was accompanied with C1q, C4, and C3 deposition. Importantly, even after 1 mo, mouse IgG deposition was not increased in glomeruli of control mice which received αGBM only (fluorescence intensity was similar to irrelevant control staining), supporting the view that the injected amount of rabbit αGBM did not induce an autologous immune response. It is of interest to porting the view that the injected amount of rabbit

ey tissues.

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