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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, depending on the experimental model used. In this study, we provide evidence that both FcγR and complement are obligatory 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.

Antiglomerular 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, which resulted into the development of two distinct experimental models for anti-GBM disease. In the direct model, heterologous Abs against mouse GBM are injected (4, 5). However, to induce disease, large amounts of Abs are required. Therefore, an additional model was used in which mice were first preimmunized with heterologous IgG, followed a week later with injection of heterologous anti-GBM Ab. In this so-called accelerated model, flamboyant inflammation takes place (6, 7). However, because 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

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4 Abbreviations used in this paper: GBM, glomerular basement membrane; PAS, periodic acid-Schiff; WT, wild type; DIG, digitonin.

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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 accelerated model, the complement system even seems to have a role in the activation of FcγR (25), and anti-C1q Ab-induced lupus nephritis (26).

In contrast, the accelerated model is dependent on the presence of activating FcγR (8, 10, 30), which might be explained by the strong immune response elicited by the preimmunization (29). In the accelerated model, the complement system even seems to have a protective function as glomerular injury was more severe in C1q−/− and C3−/− mice (6, 7, 27). The latter might be explained by the absence of C1q-mediated immune complex clearance, and C3-mediated Ag-antibody complex solubilisation (31), resulting in increased glomerular IgG and/or immune-complex deposition, respectively (6, 7). Importantly, the complement system and FcγR were not important for progression of disease in the second, autologous, phase of both models (27, 8, 10, 30), in which potential roles for inflammatory cells other than neutrophils (i.e., T cells) as well as angiotensin II were postulated (26). Thereafter, kidneys were collected, snap frozen and stored at −20°C until further use. A fraction of MsRb 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 αGBM Ab (200 μl in PBS). After 6 days, 1 mg of monoclonal MsRb 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 MsRb IgG (acute model), or one month after MsRb 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 until further use. A fraction of MsRb IgG was coupled to digoxigenin (DIG) as described before (32).

**Histological analysis**

Mouse kidneys were sectioned into 3-μm slides to assess deposition of complement components and IgG. The presence of rabbit IgG (αGBM 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 mAb (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 and
(Molecular Probes, Leiden, The Netherlands). 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 αGBM followed 6 days later by either MsrAb 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 αGBM and MsrAb 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 αGBM Ab and MsrAb IgG as well as complement deposition in mouse kidneys, mice were sacrificed 24 h after injection of the MsrAb IgG, and kidney sections were examined using immunofluorescent stainings (Fig. 2C). Presence of αGBM 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 αGBM Abs, and that this was distributed in a GBM-like pattern. Mice that received both αGBM as well as MsrAb IgG showed a deposition of mouse IgG in a GBM-like pattern. Although glomeruli of mice that received αGBM 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 MsrAb 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 αGBM and MsrAb IgG compared with mice that received αGBM 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 αGBM and MsrAb 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 αGBM 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 MsrAb IgG, significantly more leukocytes were observed in glomeruli of αGBM and MsrAb 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 αGBM and MsrAb 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 αGBM plus MsrAb IgG, compared with mice that had received αGBM alone (Fig. 2D); the average percentage of glomeruli affected in αGBM control mice was 2 ± 3.5% (n = 6) compared with 20.8 ± 7.2% (n = 8) in mice that obtained both αGBM as well as MsrAb IgG. In addition, mice that had received both αGBM plus MsrAb IgG had diffuse PAS-positive deposits, whereas the minor percentage of affected glomeruli of mice that received αGBM 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 αGBM and MsrAb 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 FcRγ-chain−/− mice, which lack all activatory IgG Fc receptors. As shown in Fig. 4, FcRγ-chain−/− mice were completely protected from development of albuminuria (Fig. 4), although all FcRγ-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 FcRγ-chain−/− mice with αGBM followed 6 days later with MsαRb IgG, similar to experiments mentioned above. Furthermore, FcRγ-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 from development of albuminuria (Fig. 4), although all FcRγ-chain−/− mice exhibited glomerular depositions of Ab and complement (data not shown).

**FIGURE 4.** Role of FcγR in development of albuminuria. FcRγ-chain−/− mice and WT mice were injected with rabbit αGBM, followed 6 days later by an injection of MsαRb IgG. Albumin excretion in urine was measured and expressed as mg/24 h. ***, p < 0.001.

**FIGURE 5.** Mice injected with αGBM and MsαRb IgG develop and maintain albuminuria. WT, C3−/−, FcRγ-chain−/−, and FcRγ-chain−/− × C3−/− double-knockout mice were injected with rabbit αGBM and 6 days later with MsαRb IgG (arrow in A). As a control, WT mice were injected with αGBM Ab alone (depicted as “αGBM only”). Urine was collected three times a week and analyzed for albumin content. Mice were sacrificed at day 37. A, Average albumin excretion is shown per group. The percentage of mice with mesangial matrix expansion (B), and glomerular sclerosis (C) at day 37 are shown per group. D, Representative pictures of PAS stained kidney sections of αGBM or αGBM plus MsαRb IgG injected mice (magnification ×250). No major abnormalities were detected in mice injected with αGBM only, whereas mesangial matrix expansion (lower left), segmental matrix accumulation (lower middle) and sclerosis (arrow, lower middle) and tubular casts (white asterisks, lower right) are observed in αGBM plus MsαRb IgG injected mice.
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
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 MsoRb 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 nephrotic 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 MsoRb 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 then 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 I mo, mouse IgG deposition was not increased in glomeruli of control mice which received αGBM only (fluorescent 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 note that C3 deposition was higher in FcR-γ-chain−/− mice, as compared with the WT mice. This observation is compatible with the absence of FcRγ-mediated activation of infiltrating neutrophils. It has been reasoned that secretion of proteolytic enzymes by activated neutrophils can lead to degradation of deposited C3 fragments, which may therefore determine the amount of activated C3 that remains deposited at the site of tissue injury (49). Histological analysis of kidney tissue revealed that ~50% of the WT mice developed sclerotic glomeruli on day 37 of the disease (Fig. 5, C and D). This sclerosis was completely absent in double-knockout mice lacking both C3 and FcγR (FcR-γ-chain−/− × C3−/−), while mice lacking either FcγR (FcR-γ-chain−/− × C3−/−) mice, while mice lacking either FcγR (FcR-γ-chain−/− × C3−/−) mice, or C3 (C3−/−) mice) displayed a reduced degree of sclerosis. Furthermore, mesangial matrix expansion was observed in ~65% of WT mice animals, and was reduced in mice lacking FcγR (Fig. 5, B and D). However, mesangial matrix expansion was increased in C3−/− mice (~80% of mice). This might be explained by the absence of C3-mediated Ag-antibody complex solubilization (31), which can lead to the presence of more immune-complexes in glomeruli, and thus an increased Ab-mediated injury. The latter might also explain the presence of mesangial matrix expansion in mice deficient for both FcγR and C3. These pathological phenomena were not observed in the acute model at 24 h after injection of MsoRb IgG. This may be explained by the time interval of 24 h, which seems to be too short for development of glomerular expansion and sclerosis.

Taken together, our novel well controllable model of anti-GBM disease shows an interplay between humoral mechanisms including Abs and complement (the latter being partially responsible for tissue injury and leukocyte attraction), and cellular mechanisms. Together, these mechanisms lead to acute glomerular injury, which can proceed into a chronic renal injury that remains complement and Fc receptor dependent. These results provide insight into the different mechanisms that play a role in anti-GBM disease, and, furthermore, this novel attenuated model may also provide a useful platform for the evaluation of drugs that may ultimately be beneficial for the treatment of Goodpasture’s syndrome.

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Disclosures
The authors have no financial conflict of interest.

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