Alport Alloantibodies but Not Goodpasture Autoantibodies Induce Murine Glomerulonephritis: Protection by Quinary Crosslinks Locking Cryptic $\alpha$3(IV) Collagen Autoepitopes In Vivo

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Alport Alloantibodies but Not Goodpasture Autoantibodies Induce Murine Glomerulonephritis: Protection by Quinary Crosslinks Locking Cryptic α3(IV) Collagen Autoepitopes In Vivo

Wentian Luo,* † Xu-Ping Wang,* † Clifford E. Kashtan, † and Dorin-Bogdan Borza* ‡

The noncollagenous (NC1) domains of α3α4α5(IV) collagen in the glomerular basement membrane (GBM) are targets of Goodpasture autoantibodies or Alport posttransplant nephritis alloantibodies mediating rapidly progressive glomerulonephritis. Because the autoepitopes but not the alloepitopes become cryptic upon assembly of α3α4α5NC1 hexamers, we investigated how the accessibility of B cell epitopes in vivo influences the development of glomerulonephritis in mice passively immunized with human anti-GBM Abs. Alport alloantibodies, which bind to native murine α3α4α5NC1 hexamers in vitro, deposited linearly along the mouse GBM in vivo, eliciting crescentic glomerulonephritis in FcgR2b−/− mice susceptible to Ab-mediated inflammation. Goodpasture autoantibodies, which bound to murine α3NC1 monomer and dimer subunits but not to native α3α4α5NC1 hexamers in vitro, neither bound to the mouse GBM in vivo nor induced experimental glomerulonephritis. This was due to quinary NC1 crosslinks, recently identified as sulfilimine bonds, which comprehensively locked the cryptic Goodpasture autoepitopes in the mouse GBM. In contrast, non-crosslinked α3NC1 subunits were identified as a native target of Goodpasture autoantibodies in the GBM of squirrel monkeys, a species susceptible to Goodpasture autoantibody-mediated nephritis. Thus, crypticity of B cell autoepitopes in tissues uncouples potentially pathogenic autoantibodies from autoimmune disease. Crosslinking of α3α4α5NC1 hexamers represents a novel mechanism averting autoantibody binding and subsequent tissue injury by posttranslational modifications of an autoantigen. The Journal of Immunology, 2010, 185: 3520–3528.

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Abbreviations used in this paper: APTN, Alport posttransplant nephritis; C.I.C., circulating immune complexes; D, dimer; G, glomeruli; GBM, glomerular basement membrane; GN, glomerulonephritis; GP, Goodpasture; hIgG, human IgG; M, monomer; MAHA, mouse anti-human IgG Abs; mIgG, mouse IgG; NC1, noncollagenous domain 1; NHS, normal human serum; NTS, nephotoxic serum.

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The α3εα5CN1 hexamers are also the target of anti-GBM alloantibodies mediating Alport posttransplant nephritis (APTN), a serious complication affecting ~3–5% of Alport patients receiving a kidney transplant (15–18). APTN is the result of an alloimmune reaction to "foreign" α3εα5(IV) collagen present in the allograft GBM but absent from the Alport patient’s tissues. APTN is most prevalent in patients with X-linked Alport syndrome, who develop alloantibodies against several alloepitopes within the α5CN1 domain (17). Upon binding to the allograft GBM, APTN alloantibodies cause aggressive glomerulonephritis with similar clinical presentation and pathology findings as in autoimmune anti-GBM disease (19). However, the APTN alloepitopes are accessible in α3εα5CN1 hexamers of the human GBM, unlike the GP alloepitopes (4, 17).

Whether differences in the epitope specificity between GP autoantibodies and APTN alloantibodies are pathogenically relevant is not known. We postulated that APTN alloantibodies are more nephrinergic than GP autoantibodies because they bind to all isoforms of α3εα5CN1 hexamers from the GBM. Testing this hypothesis requires a suitable animal model. A landmark study has demonstrated that GP autoantibodies injected into squirrel monkeys bind to the GBM of the recipient host, causing severe glomerulonephritis (20). However, the nephrinergicity of APTN alloantibodies has not been evaluated by passive transfer into animal models.

The purpose of the present study was to determine whether the relative inaccessibility of B cell autoepitopes in the GBM limits the severity of autoantibody-mediated glomerulonephritis. Because rodent models are preferable to nonhuman primates on human grounds, we developed a mouse model of anti-GBM glomerulonephritis by passive immunization with human anti-GBM Abs. We show that APTN alloantibodies but not GP autoantibodies bound to mouse GBM in vivo, causing crescentic glomerulonephritis in susceptible mouse strains. Although cross-reactive with murine α3NC1 subunits in vitro, GP autoantibodies did not bind to native α3εα5CN1 hexamers of the mouse GBM in vivo, nor did they induce disease. Resistance against GP autoantibody-mediated nephritis was due to quinary \(^2\) (intermolecular) NC1 crosslinks locking the cryptic GP autoepitopes in the mouse GBM. In contrast, the GBM of squirrel monkeys contained non-crosslinked α3NC1 subunits reactive with GP autoantibodies under native conditions. These findings indicate that quinary crosslinks of α3εα5CN1 hexamers, recently identified as sulfilamine bonds (22), lock the cryptic GP autoepitopes, conferring protection against nephritis mediated by GP autoantibodies, but not against APTN alloantibody-mediated disease. Thus, potentially pathogenic autoantibodies can be uncoupled from autoimmune disease by posttranslational modifications of an autoantigen that render B cell autoepitopes structurally inaccessible in tissues.

Materials and Methods

**Anti-GBM Abs**

Previously characterized serum or kidney-eluted anti-GBM alloantibodies from four X-linked Alport syndrome patients who developed APTN (17), as well as sera or plasma exchange fluid from six patients with GP disease (18), were used for in vitro binding studies. Only kidney-eluted alloantibodies from one patient with APTN were available in sufficient amounts for in vivo studies. Purified GP IgG autoantibodies used for in vivo experiments were isolated from the plasma exchange fluid of two patients by affinity chromatography on immobilized human α3NC1 (4). Normal human IgG (hIgG) was purchased from Sigma-Aldrich (St. Louis, MO) or purified from healthy donor sera by affinity chromatography on immobilized protein A. Nephrotic nephritis serum from sheep immunized with mouse GBM was a gift from Dr. A. Richard Kitching (Monash University, Clayton, Victoria, Australia).

**Preparation of NC1 hexamers**

Mouse kidneys were purchased from Pel-Freez Biologicals (Rogers, AR). Frozen kidneys from adult squirrel monkeys (Saimiri sp.) were obtained from the Squirrel Monkey Breeding and Research Resource (University of Texas MD Anderson Cancer Center, Houston, TX). Renal basement membranes including the GBM were isolated from homogenized kidney cortex and digested with bacterial collagenase (Worthington, Lakewood, NJ). The NC1 hexamers thus solubilized were purified by passage through a DE-52 ion-exchange column and gel-filtration chromatography (23).

**Immunoaassays**

Indirect ELISA was performed as described (17). To assay the binding of human anti-GBM Abs to mouse Ags, Nunc MaxiSorp 96-well plates were coated overnight with NC1 hexamers from mouse GBM (300 ng/well) in PBS (pH 7.4). Prior to coating, some NC1 hexamers were treated with 6 M guanidine hydrochloride for 15 min at 60°C for dissociation into subunits. Sera diluted 1:100 or purified hIgG Abs (100 ng/ml) were used as primary Abs. The hIgG bound to the immobilized Ads was detected with alkaline phosphatase-conjugated goat anti-human IgG (Rockland Immunochemicals, Gilbertsville, PA). To assay the circulating mouse anti-human IgG Abs, wells coated with normal human IgG (300 ng/well) were incubated with mouse sera diluted 1:200, followed by detection with HRP-conjugated goat Abs specific for mouse IgGl, IgGl2b, or IgGl2c subclasses (Bethyl Laboratories, Montgomery, TX). The levels of human IgG in mouse sera were assayed by capture ELISA in plates coated with goat anti-human IgG(Fc-specific (300 ng/well).

**Western blots and immunoprecipitation**

Western blots were performed as described (17). NC1 hexamers from the mouse or human GBM (1 μg/lane) were separated by SDS-PAGE and transferred onto Immobilon-P for blotting with mAbs specific for NC1 domains. Strips were incubated with purified hIgG Abs (1 μg/ml) or anti-α3NC1 mAb 8D1, which were detected with alkaline phosphatase-conjugated goat anti-κ light chain (BD Pharmingen, San Jose, CA) or FITC-conjugated goat anti-κ heavy chain (Jackson ImmunoResearch Laboratories, West Grove, PA). Stained sections were visualized using an Axioplan 2 fluorescence microscope (Carl Zeiss MicroImaging, Thornwood, NY) and images were captured with the AxioVision 4.8 software.

**Mouse studies**

C57BL/6J (B6) and B6.C57BSγc−/− mice were purchased from The Jackson Laboratory (Bar Harbor, ME), and B6.FcγRIIB−/− mice were from Taconic Farms (Germantown, NY). The mice were maintained in a specific pathogen-free facility with access to water and food. All mouse studies were performed in accordance with the principles for humane treatment of laboratory animals and were approved by the Institutional Animal Care and Use Committee at Vanderbilt University. Each mouse was injected once in the tail vein with 0.1 mg of purified hIgG in 100 μl of PBS, divided in four...
groups: kidney-eluted APTN alloantibodies, affinity-purified GP-1 or GP-2 autoantibodies, and normal human serum IgG. Blood was collected at the indicated time points by saphenous vein puncture, along with spot urine samples. Blood urea nitrogen was measured by a kinetic urease and glutamate dehydrogenase assay, and urinary creatinine was measured by alkaline picrate (Jaffe reaction), using kits from Thermo Electron (Louisville, CO). Urinary albumin excretion was assayed using a mouse albumin ELISA quantitation kit (Bethyl Laboratory, Montgomery, TX). Portions of kidneys and lungs collected from mice sacrificed at the end of experiments were embedded in paraffin or OCT for further evaluation. For light microscopic assessment of the kidney histopathology, 5-μm paraffin sections stained with H&E or with periodic acid–Schiff reagent were observed with an Axioskop 40 microscope. Images were captured with MRGrab software (Carl Zeiss MicroImaging). For direct immunofluorescence, frozen sections of OCT-embedded kidneys were fixed in acetone and stained with FITC-conjugated goat anti-mouse Ig (BD Pharmingen), Alexa Fluor 488 goat anti-human IgG (H+L), or FITC-conjugated goat anti-mouse C3c (Nordic Immunological Laboratory, Tilburg, The Netherlands).

Statistical analyses

Data were analyzed using GraphPad Prism (GraphPad Software, San Diego, CA), by two-tailed t test for two groups or ANOVA followed by Dunnett’s post hoc test for three or more groups. Statistical significance was inferred for p values <0.05.

Results

Patient GP autoantibodies and APTN alloantibodies bind to the NC1 domains of α3α4α5(IV) collagen from the mouse GBM

The lack of cross-reactivity between human autoantibodies and homologous Ags from other species is a potential roadblock preventing the transfer of autoimmune disease to animal models by passive immunization with patient autoantibodies (25). To determine whether mice are a suitable model for this study, we first verified that human anti-GBM Abs cross-react with mouse collagen IV. By Western blot, NC1 domains from mouse GBM bound hIgG from patients with GP disease or APTN, but not normal hIgG (Fig. 1A). Next, we assessed the accessibility of the epitopes in the mouse GBM. By indirect ELISA (Fig. 1B), only APTN alloantibodies exhibited significant reactivity toward native NC1 hexamers from the mouse GBM, whereas GP autoantibodies bound preferentially to dissociated hexamers, revealing the crypticity of GP autoepitopes. This finding was corroborated by indirect immunofluorescence staining (Fig. 1C). Under native conditions, only APTN alloantibodies stained the GBM and tubular basement membranes of normal mouse kidneys. GP autoantibodies produced the same staining pattern on kidney sections treated with acid urea for epitope unmasking, but did not bind to mouse basement membranes under native conditions. The same results were found using kidneys from C57BL/6, 129/Sv, DBA/1, and SJL/J mice (Supplemental Fig. 1). Additionally, the alveolar basement membranes of mouse lungs were stained by APTN alloantibodies under native conditions, but by GP autoantibodies only after acid urea treatment (Supplemental Fig. 2). Finally, we verified that both APTN alloantibodies and GP autoantibodies bind specifically to mouse α3α4α5(IV) collagen because neither stained kidney basement membranes from Col4α5-null Alport mice (Fig. 1D), in contrast to sheep anti-mouse GBM Abs used to elicit murine nephrotic syndrome nephritis, which appear to target other Ags in the mouse GBM. We have thus shown that NC1 hexamers of mouse α3α4α5(IV) collagen harbor cryptic autoepitopes recognized by GP autoantibodies and accessible alloepitopes targeted by APTN alloantibodies.

APTN alloantibodies but not GP autoantibodies bind in vivo to mouse GBM

In vivo binding of human anti-GBM Abs to target Ags was investigated in C57BL/6J mice injected with APTN alloantibodies, GP autoantibodies from two patients, and hIgG from normal human sera. Renal function was monitored for 8 wk postinjection to assess the development of kidney injury. At all time points, urinary albumin excretion (Fig. 2A) and blood urea nitrogen levels (Fig. 2B) were found to be normal in all mice. Light microscopic examination of the kidneys revealed no histological abnormalities in any group (Fig. 2Cf–j). Direct immunofluorescence revealed bright linear staining for hIgG along the GBM in all mice injected with APTN alloantibodies (Fig. 2Cg), but no hIgG deposition was detected in the kidneys of mice injected with either GP autoantibodies (Fig. 2Ci) or normal hIgG (Fig. 2Ce). Linear GBM deposition of mouse IgG (mlgG) (Fig. 2Cc) but not of mC3 (Fig. 2Cm) was also observed in APTN-injected mice, indicating that these mice produced Abs to the foreign globulin. APTN alloantibodies stained in vitro but did
not bind in vivo to other murine basement membranes containing $\alpha 3\alpha 4\alpha 5(IV)$ collagen, such as renal tubular basement membranes or lung alveolar basement membranes (Supplemental Fig. 2). These results show that APTN alloantibodies, unlike GP autoantibodies, bind specifically to the mouse GBM in vivo, but do not induce glomerulonephritis in C57BL/6J mice.

Binding of APTN alloantibodies to the mouse GBM induces severe glomerulonephritis in B6.Fcgr2b$^{-/-}$ mice

The development of experimental glomerulonephritis after passive immunization with human anti-GBM IgG Abs was next investigated using B6.Fcgr2b$^{-/-}$ mice, because the absence of the inhibitory IgG Fc receptor FcγRIIB exacerbates susceptibility to Ab-mediated inflammation and autoimmune disease (26, 27). Injection of APTN alloantibodies (but not of GP-1 and GP-2 autoantibodies, nor normal hIgG) into Fcgr2b$^{-/-}$ mice caused increased albuminuria after 2 wk (Fig. 3A) and abnormally high blood urea nitrogen levels after 4 wk (Fig. 3B). By 8 wk, 80% (four out of five) of mice injected with APTN alloantibodies died or were sacrificed because of end-stage...
renal disease, compared with zero of eight mice injected with GP-1 or GP-2 autoantibodies. Light microscopic examination of the kidneys revealed crescentic necrotizing glomerulonephritis in APTN-treated mice, but not in those receiving GP autoantibodies or normal hIgG (Fig. 3Ca-f). Direct immunofluorescence staining of kidneys from \( Fcgr2b^{-/-} \) mice injected with APTN alloantibodies revealed linear GBM binding of hIgG, along with mIgG and mC3 deposition along the capillary loops (Fig. 3Ca, j, m). However, APTN alloantibodies did not bind in vivo to mouse renal tubular or alveolar basement membranes containing \( \alpha3\alpha4\gamma5(IV) \) collagen (Supplemental Fig. 2). Weak staining for hIgG, mIgG, and mC3 in a mesangial pattern was found in \( Fcgr2b^{-/-} \) mice injected with GP autoantibodies (Fig. 3Ck, n, o) or normal hIgG (Fig. 3Ci, l, o). This pattern of hIgG deposition, judged to be nonspecific because it did not colocalize with mouse \( \alpha3\alpha4\gamma5(IV) \) collagen (Fig. 3Cp, q, r), may be caused by immune complexes between hIgG and mouse anti-human IgG Abs (MAHA). MAHA were detected in mouse sera by 2 wk after passive immunization (Fig. 4A), and rising titers of MAHA paralleled a decrease in the serum levels of hIgG (Fig. 4B). In mice sacrificed at 3 d after the injection of human Abs, prior to the formation of MAHA, nonspecific mesangial deposits of hIgG were not observed (Fig. 4C). Taken together, these results demonstrate that only APTN alloantibodies bind specifically to the GBM of B6. FcγRIIB−/− mice, causing crescentic glomerulonephritis, whereas GP autoantibodies and normal hIgG are significantly less nephritogenic because neither binds specifically to the mouse GBM in vivo.

Susceptibility of B6.\( Fcgr2b^{-/-} \) mice to APTN Ab-induced glomerulonephritis is due to enhanced production of mIgG2b and mIgG2c against hIgG

We next inquired why APTN alloantibodies elicit severe glomerulonephritis in \( Fcgr2b^{-/-} \) but not in wild-type mice. Because the injection of APTN alloantibodies led to C3 deposition along the capillary loops in \( Fcgr2b^{-/-} \) but not in wild-type mice, we hypothesized that the absence of the inhibitory IgG Fc receptor may augment production of complement-fixing mIgG subclasses, linking the GBM-bound hIgG to inflammatory effectors. For all mIgG subclasses, the titers of MAHA were consistently higher in \( Fcgr2b^{-/-} \) mice than in wild-type mice, but they were less influenced by the nature of hIgG injected into mice (Fig. 5). In wild-type mice, MAHA were almost exclusively mIgG1, whereas \( Fcgr2b^{-/-} \) mice also produced significant amounts of mIgG2b and mIgG2c MAHA. These results suggest that mIgG2b and/or mIgG2c Abs to hIgG may exacerbate glomerulonephritis in \( Fcgr2b^{-/-} \) mice injected with APTN alloantibodies.

NC1 crosslinks lock cryptic \( \alpha3\alpha1 \) autoepitopes in the mouse GBM

We finally addressed the molecular basis of the inaccessibility of GP autoepitopes in the mouse GBM. Because GP autoantibodies mediate anti-GBM disease in human patients and squirrel monkeys (20) but not in mice, we hypothesized that species-specific structural features of GBM collagen IV may affect its immunoreactivity toward GP autoantibodies, thus determining the host’s susceptibility to GP autoantibody-mediated glomerulonephritis. We therefore analyzed how human anti-GBM Abs bind to NC1 domains from the GBM of mice and squirrel monkeys using immunoprecipitation (Fig. 6A). As reference, Fig. 6B depicts the reactivity of NC1 hexamers from human GBM toward GP autoantibodies (14) and APTN alloantibodies (17). In both monkeys and mice, APTN alloantibodies coprecipitated \( \alpha3\alpha1 \), \( \alpha4\alpha1 \), and \( \alpha5\alpha1 \) monomers and dimers (Fig. 6A, lanes c, g). This pattern of reactivity, also seen with human GBM NC1 domains (17), indicates that APTN alloantibodies bind to native \( \alpha3\alpha4\alpha5\alpha1 \) hexamers from all species (Fig. 7A, 7C, 7D). Under native conditions, GP autoantibodies mainly targeted \( \alpha3\alpha1 \) and \( \alpha4\alpha1 \) monomers from the monkey GBM (Fig. 6A, lane b). This pattern of reactivity, previously found for human GBM NC1 domains (14), suggests that the GBM of squirrel monkeys also contains non-crosslinked hexamers (Fig. 6B). In contrast,

![FIGURE 4. Time course for hIgG clearance from mouse sera, MAHA production, and glomerular IgG deposition at 3 d after passive immunization. A. Serum levels of MAHA in the \( Fcgr2b^{-/-} \) mice injected with hIgG were measured by indirect ELISA. B. The levels of hIgG in mouse sera were measured by capture ELISA. C. Indirect immunofluorescence analysis of hIgG or mIgG deposition in kidneys from \( Fcgr2b^{-/-} \) mice at 3 d after injection of APTN alloantibodies, GP autoantibodies, or normal hIgG. At this time point, production of MAHA, kidney histology appeared normal by light microscopy (not shown), and nonspecific glomerular deposits of hIgG were not observed. Original magnification \( \times400 \).](http://www.jimmunol.org/content/152/6/3524/F4.large.jpg)

![FIGURE 5. \( Fcgr2b^{-/-} \) mice have enhanced production of mIgG2b and mIgG2c anti-hIgG Abs (MAHA). Titers of circulating MAHA of mIgG (A), mIgG1 (B), mIgG2b (C), and mIgG2c (D) subclasses were measured by indirect ELISA. Sera collected from wild-type (filled bars) and \( Fcgr2b^{-/-} \) mice (open bars) at 4 wk after the injection of patient alloantibodies (APTN), autoantibodies (GP-1, GP-2), or normal hIgG were diluted 1/500. Binding of mIgG to plates coated with normal hIgG was assayed using subclass-specific secondary Abs. The graphs depict the means and SEM of ELISA absorbance values for four or five mice in each group. The statistical significance of differences in MAHA titers between wild-type and \( Fcgr2b^{-/-} \) mice was analyzed by two-way ANOVA followed by Bonferroni posttests (\( *p < 0.05; **p < 0.01; ***p < 0.0001 \)). NHS, normal human serum.](http://www.jimmunol.org/content/152/6/3524/F5.large.jpg)
GP autoantibodies did not bind to native NC1 hexamers from murine GBM (Fig 6A, lane f), indicating that non-crosslinked hexamers do not occur in the mouse kidneys. Nevertheless, GP autoantibodies bound to α3NC1 monomer and α3–α5NC1 dimer subunits produced by in vitro dissociation of mouse GBM NC1 hexamers (Fig. 6A, lane i). Therefore, GP autoepitopes occur in the mouse GBM only within isoforms of autoantigen impenetrable by GP autoantibodies, namely crosslinked NC1 hexamers containing NC1 dimer subunits. Consequently, mice are resistant to GP autoantibody-mediated nephritis (Fig. 7E).

**Discussion**

Anti-GBM Abs binding to the NC1 domains of α3e4α5(IV) collagen in the human GBM mediate the most aggressive forms of rapid progressive glomerulonephritis in patients with APTN (Fig. 7A) or GP disease (Fig. 7B). Because GP autoantibodies and APTN alloantibodies bind to cryptic and accessible GBM epitopes, respectively, we investigated the role of the accessibility of B cell epitopes in vivo in the pathogenesis of these diseases. Crypticity of GP autoepitopes led us to conjecture that APTN alloantibodies would cause more severe experimental nephritis than would GP autoantibodies in a passive transfer model of anti-GBM disease. We found that only APTN alloantibodies bound in vivo to the mouse GBM, inducing crescentic glomerulonephritis in FcγR2b−/− mice susceptible to Ab-mediated inflammation (Fig. 7C, 7D). We have thus demonstrated the nephritogenic potential of APTN alloantibodies and established the first murine model of glomerulonephritis induced by a patient’s anti-GBM Abs that reproduces the severity of human disease. In contrast, GP autoantibodies neither bound to mouse α3e4α5(IV) collagen in vivo nor induced experimental nephritis (Fig. 7E), owing to quinary NC1 crosslinks locking the GP autoepitopes in the mouse GBM. These results show that the crypticity of B cell epitopes in tissues is sufficient to uncouple potentially pathogenic autoantibodies from autoimmune disease.

*The significance of differences in the epitope specificity of GP autoantibodies and APTN alloantibodies*

Our study illustrates how seemingly small differences in the location of epitopes targeted by anti-GBM Abs impact the development of glomerulonephritis. The different accessibility of the collagen IV autoepitopes and alloepitopes in the GBM is thought to reflect the
distinct immune mechanisms triggering GP disease and APTN (28–30). The crypticity of GP autoepitopes in α3ζ4α5NC1 hexamers implies that a perturbation of the native structure of the autoantigen may be instrumental in breaching immune self-tolerance toward α3 (IV) collagen in GP disease. In contrast, APTN alloantibodies target accessible alloepitopes in the α3ζ4α5NC1 hexamers—antigenic determinants encountered in the normal renal allograft but not in the basement membranes of the affected patients, who presumably lack immune tolerance toward missing collagen IV chains. In this study, both GP autoantibodies and APTN alloantibodies were shown to cross-react with NC1 subunits of murine α3ζ4α5(IV) collagen in vitro, yet only the GP autoepitopes were completely inaccessible within the collagen IV network of mouse GBM in vivo. As a result, mice were protected against anti-GBM glomerulonephritis induced by GP autoantibodies, but not by APTN alloantibodies.

Because relatively large amounts of Ag-specific hlgG were used for passive transfer experiments, only anti-GBM Abs from a small number of patients could be tested in vivo. Nevertheless, these samples were judged to be representative based on their in vitro binding to mouse GBM Ags, which are essentially identical to additional samples of GP autoantibodies and APTN alloantibodies tested only in vitro. We also note that APTN alloantibodies injected in mice were eluted from an allograft kidney, and hence they were highly enriched in the nephrotropic hlgG species. In all in vitro immunoassays, kidney-eluted and serum APTN alloantibodies bound to mouse GBM Ags similar to each other but different from GP autoantibodies (Fig. 1 and Supplemental Fig. 1). However, kidney-eluted APTN alloantibodies have higher affinity for α3ζ4α5NC1 hexamers than do serum APTN alloantibodies (17), which may translate into greater nephritogenic potential. To emulate the enrichment in autoantigen-specific hlgG species upon kidney binding and the subsequent elution, GP autoantibodies used for passive immunizations were affinity-purified from plasma exchange fluid using immobi-

ized α3NC1 monomers.

In vivo binding of APTN alloantibodies to basement membranes containing α3ζ4α5(IV) collagen

The proximal step initiating crescentic glomerulonephritis in mice injected with APTN alloantibodies is the GBM deposition of hlgG. Prerequisites for this binding are the ability of human APTN alloantibodies to cross-react with mouse α3ζ4α5(IV) collagen, as well as the structural accessibility of the alloepitopes within the α3ζ4α5NC1 hexamers (Fig. 1). However, other murine basement membranes containing α3ζ4α5(IV) collagen, such as those in renal tubules and lung alveoli, bind APTN alloantibodies in vitro (by indirect immunofluorescence staining) but not in vivo. APTN alloantibodies readily bind to the GBM because fenestrations in glomerular endothelial cells allow the passage of large macromolecules, which is an adaptation to the blood filtration function. In most capillary beds, endothelial cells have tight junctions impermeable to molecules of IgG size, which prevent the access of circulating Abs to the underlying basement membranes under normal physiological conditions. Thus, cellular and anatomic barriers can modulate the accessibility of (auto)antigens in vivo, influenc-

ing which tissues become targets of Ab-mediated inflammation.

Murine anti-GBM Ab-mediated glomerulonephritis is exacerbated by the absence of the inhibitory IgG Fc receptor FcyRIIB

In vivo binding of APTN alloantibodies to the mouse GBM was necessary but not sufficient for induction of glomerulonephritis, because only FcgR2b−/− mice were susceptible to disease. This echoes the finding that hlgG autoantibodies from patients with rheumatoid arthritis are arthritogenic in FcgR2b−/− but not in wild-
type mice (27). Several mechanisms may contribute to the susceptibility of FcgR2b−/− mice to Ab-mediated glomerulonephritis. Because the expression of the inhibitory receptor FcyRIIB on myeloid cells counterbalances signaling through activating IgG Fc receptors (FcyRI, -III, and -IV), neutrophils and macrophages from FcgR2b−/− mice would have a lower threshold of activation by immune complexes (3). FcyRIIB is also a negative regulator of B cells, implicated in the feedback inhibition of Ab production or apoptosis of plasma cells (31). Consequently, FcgR2b−/− mice produce larger amounts of Abs following antigenic stimulation, as observed for MAHA titers in our study. Finally, it has not escaped our attention that significant amounts of mlgG2b/c MAHA are elicited by injection with hlgG only in FcgR2b−/− but not in wild-type mice. Unlike mlgG1, mlgG2b and mlgG2c, an allotype of mlgG2a in mice with the Igh-1b allele (32), are proinflammatory because they can activate complement and have higher affinity for activating IgG Fc receptors (33). Fixation of mlgG2a and mlgG2b (but not mlgG1) to the mouse GBM induces acute glomerular injury due to an FcyRIII- and FcyRIV-dependent influx of neutrophils (34). In contrast, even long-term persistence of mlgG1 in the GBM of mice injected with an anti-α3NC1 mAb does not induce any glomerular pathology (35). Autoimmune pathology in FcgR2b−/− mice immunized with bovine collagen type IV is asso-

associated with enhanced production of mlgG2a/b autoantibodies (36), suggesting that a propensity toward production of proinflammatory mlgG subclasses may contribute to the susceptibility of FcgR2b−/− mice to Ab-mediated glomerulonephritis.

The cryptic GP autoepitopes are locked by quinary NC1 crosslinks, characteristic posttranslational modification of collagen IV

In contrast to APTN alloantibodies, affinity-purified GP IgG autoantibodies did not induce crescentic glomerulonephritis into FcgR2b−/− mice. Even very large amounts of affinity-purified GP autoantibodies (5 mg/mouse, 50 times more than in this study) injected in XenoMouse II only cause mild nephritis with patchy mesangial expansion and glomerular hlgG deposition in a punctate rather than a linear pattern (37). The inability of GP autoantibodies to bind in vivo to the mouse GBM in significant amounts explains the in-

conspicuous glomerular pathology after passive immunization. In this study, we showed that quinary NC1 crosslinks of α3ζ4α5(IV) collagen prevent the binding of GP autoantibodies to mouse GBM both in vitro and in vivo. Previous studies of α3ζ4α5NC1 hexamers from human GBM have shown that crosslinks yield α3–α5NC1 heterodimers and α4–α4NC1 homodimers (9), which reinforce the hexamers and prevent their dissociation by GP autoantibodies in vitro (14), as also demonstrated for α3ζ4α5NC1 hexamers isolated from bovine testes (12). In this study, GP autoantibodies likewise bound to murine α3–α5NC1 dimers and α3NC1 monomers, after the NC1 hexamers from the mouse GBM had been dissociated (Fig. 6A, lane i). Because NC1 crosslinks do not prevent the binding of GP autoantibodies to α3–α5NC1 dimer subunits, the impenetrability of native murine α3ζ4α5NC1 hexamers by GP autoantibodies must be the combined result of the crypticity of GP autoepitopes together with the structural stabilization of NC1 hexamers by quin-

ary crosslinks. Collectively, these findings imply that the NC1 crosslinks “lock” the cryptic GP autoepitopes within the α3ζ4α5 (IV) collagen suprastructure.

The chemical nature of the collagen IV bonds crosslinking NC1 dimers has been elusive. Long presumed to be crosslinked by disulfide bonds (38), NC1 dimers have been shown to contain intermolecular covalent bonds between conserved Met33 and Lys211 residues, initially assigned as thioether bonds (12, 39, 40). Most re-

cently, this unusual posttranslational modification has been identified
as a sulfilimine bond, uniquely found within collagen IV networks in tissues (22). Sulfilimine bonds have been proposed to occur in most metazoa, based on the evolutionary conservation of the Met and Lys in the NC1 domains of collagen IV from vertebrates and most invertebrate phyla, except in hydra, flatworms, sponges, and Phacozoa (22). This implies that the cryptic GP autoepitopes are locked in the mouse GBM by sulfilimine bonds crosslinking the NC1 domains of adjoining α3ε4ε5(IV) collagen molecules. Thus, posttranslational modifications of autoantigens, often implicated in autoimmune etiology (41–43), can also avert autoimmune disease, as showcased by NC1 crosslinks of α3ε4ε5(IV) collagen. 

Species differences in the susceptibility to GP autoantibodies Unlike mice in this study or XenoMouse II (37), human patients or squirrel monkeys are susceptible to anti-GBM nephritis mediated by GP autoantibodies (20). However, biochemical analyses of NC1 domains from the GBM of mice (24), humans (9), and monkeys (44) have revealed a similar architecture, with α3ε4ε5(IV) collagen molecules forming supramolecular networks. We found that the reactivity toward GP autoantibodies is inversely correlated with the extent to which the NC1 hexamers of the GBM undergo posttranslational modifications forming quinary crosslinks. In the murine GBM, α3ε4ε5NC1 hexamers undergo comprehensive crosslinking, thereby locking all GP autoepitopes. In contrast, a small proportion of α3ε4ε5NC1 hexamers without NC1 crosslinks that react with GP autoantibodies under native conditions has been found to occur in the GBM of humans (14) and squirrel monkeys (this study). Why the crosslinking of GP autoantigen varies among species is unknown. As mouse kidneys contained a smaller proportion of NC1 monomers than did human or monkey kidneys (Figs. 1A, 6A), we speculate that a putative enzyme producing the NC1 crosslink may be more active and/or expressed at higher levels in rodent kidneys. It is perhaps not coincidental that NC1 hexamers from rat kidneys have the smallest proportion of NC1 monomers and the lowest reactivity with GP autoantibodies among these species of mammals (45). Because susceptibility to GP Ab-mediated nephritis is associated with GBM isoforms of α3(IV) collagen not stabilized by NC1 crosslinks, these isoforms are likely implicated in the pathogenesis of GP disease as targets for GP autoantibodies directed against cryptic epitopes. 

Since the α3NC1 autoepitopes targeted by GP autoantibodies are inaccessible in the mouse GBM, what are the targets of nephritogenic mIgG Abs in mouse models of anti-GBM glomerulonephritis? In mice immunized with α3NC1 from bovine GBM (46–48) or recombinant human α3NC1 (37, 49), which develop glomerulonephritis associated with serum anti-α3NC1 Abs and linear GBM deposition of mIgG, it is likely that some mIgG may be produced against non-cryptic mouse α3NC1 epitopes (Fig. 7F). Supporting this view, mIgG mAb 8D1, raised against human α3NC1 monomers, binds to native murine α3ε4ε5NC1 hexamers in vitro (23) and in vivo (35). More intriguing is the nature of autoantibodies mediating crescentic glomerulonephritis and hemorrhagic pneunonitis (GP syndrome) in FcγR2b−/− mice immunized with Cellmatrix-IV (36). Cellmatrix-IV consists of collagen IV fragments solubilized from bovine lens capsule basement membranes by limited pepsin digestion, a treatment that destroys the NC1 domains (50). Sera from mice immunized with Cellmatrix-IV contain mIgG Abs binding to pepsin-extracted murine collagen IV from Engelbreth–Holm–Swarm tumors, suggesting that their epitopes reside in the collagenous domain of α1ε2 (IV) collagen (Fig. 7G), a rare target of human anti-GBM autoantibodies (51). Mouse GBM Abs other than α3ε4ε5(IV) collagen are also targeted by anti-GBM Abs from sheep nephrotoxic sera (Fig. 7H). 

The new mouse model of glomerulonephritis described in this study is suitable for investigating the pathogenic role of allautoantbodies in APTN and for testing new experimental therapies, such as using decoy Ag to prevent the binding of pathogenic Abs to target tissues (25). For these purposes, the widely used murine model of nephrotoxic serum nephritis induced by heterologous anti-mouse GBM Abs—although useful for studies of the effector mechanisms of Ab-mediated glomerulonephritis—is inadequate, since anti-GBM Abs from nephrotoxic sera do not reproduce the restricted Ag and epitope specificity of IgG Abs mediating anti-GBM disease in patients. Whereas our model resembles murine nephrotoxic nephritis models with regard to the development of mIgG against the foreign globulin, which amplify glomerular injury in the autologous phase, much smaller amounts of GBM-specific patient Abs (0.1 mg of IgG per mouse) were sufficient to induce severe experimental nephritis in susceptible mice, thus mitigating against the limited availability of human anti-GBM Abs. Although the nephritogenicity of autoantibodies from GP patients cannot currently be evaluated by passive transfer into mice, this may become possible when mice unable to crosslink α3ε4ε5(IV) collagen are genetically engineered. Until then, primate models remain available for this purpose (20).

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References


Supplemental Figure 1: In vitro binding of human anti-GBM antibodies to kidneys from different mouse strains. Acetone-fixed cryostat sections of kidneys from C57BL/6j, 129/Sv, DBA/1, and SJL/J mice were stained with normal human IgG (a,f,k,p), APTN alloantibodies eluted from a kidney allograft (b,g,l,q), serum APTN alloantibodies (c,h,m,r), and with GP IgG autoantibodies on native conditions (d,i,n,s) and after acid urea treatment (e,j,o,t). Anti-human IgG conjugated with FITC was used as secondary antibody. Magnification 200X.
**Supplemental figure 2: In vivo and in vitro binding of anti-GBM antibodies to mouse lung basement membranes.**

A. Direct immunofluorescence staining showing hIgG deposition in the kidneys (a,c) but not the lungs (b,d) of wild type (a,b) and Fcgr2b-/-(c,d) mice, sacrificed at 8 weeks after the injection of APTN alloantibodies. B. Indirect immunofluorescence staining of lungs from the APTN-injected mice shows staining of alveolar basement membranes by a rat mAb to α5(IV) collagen (a,e) and by APTN alloantibodies (b,f). GP antibodies did not bind to alveolar basement membranes in native lung sections (c,g), but stained them after acid urea treatment (d,h). Magnification 200X.