Proteolysis Breaks Tolerance toward Intact \( \alpha \) 345(IV) Collagen, Eliciting Novel Anti–Glomerular Basement Membrane Autoantibodies Specific for \( \alpha \)345NC1 Hexamers

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*J Immunol* 2013; 190:1424-1432; Prepublished online 9 January 2013;
doi: 10.4049/jimmunol.1202204

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Proteolysis Breaks Tolerance toward Intact α345(IV) Collagen, Eliciting Novel Anti–Glomerular Basement Membrane Autoantibodies Specific for α345NC1 Hexamers

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Goodpasture disease is an autoimmune kidney disease mediated by autoantibodies against noncollagenous domain 1 (NC1) monomers of α345(IV) collagen that bind to the glomerular basement membrane (GBM), usually causing rapidly progressive glomerulonephritis (GN). We identified a novel type of human IgG4-restricted anti–GBM autoantibodies associated with mild nonprogressive GN, which specifically targeted α345NC1 hexamers but not α3NC1 monomers. The mechanisms eliciting these anti–GBM autoantibodies were investigated in mouse models recapitulating this phenotype. Wild-type and FcyRIIB−/− mice immunized with autologous murine GBM NC1 hexamers produced mouse IgG1-restricted autoantibodies specific for α345NC1 hexamers, which bound to the GBM in vivo but did not cause GN. In these mice, intact collagen IV from murine GBM was not immunogenic. However, in Cola3A+/+ Alport mice, both intact collagen IV and NC1 hexamers from murine GBM elicited IgG Abs specific for α345NC1 hexamers, which were not subclass restricted. As heterologous Ag in COLA4A3-humanized mice, murine GBM NC1 hexamers elicited mouse IgG1-restricted autoantibodies specific for α345NC1 hexamers, which bound to the GBM in vivo but did not cause GN. In these mice, intact collagen IV from murine GBM was not immunogenic. However, in Cola3A+/+ Alport mice, both intact collagen IV and NC1 hexamers from murine GBM elicited IgG Abs specific for α345NC1 hexamers, which were not subclass restricted. As heterologous Ag in COLA4A3-humanized mice, murine GBM NC1 hexamers elicited mouse IgG1, IgG2a, and IgG2b autoantibodies specific for α345NC1 hexamers and induced anti–GBM Ab GN. These findings indicate that tolerance toward autologous intact α345(IV) collagen is established in hosts expressing this Ag, even though autoreactive B cells specific for α345NC1 hexamers are not purged from their repertoire. Proteolysis selectively breaches this tolerance by generating autoimmunogenic α345NC1 hexamers. This provides a mechanism eliciting autoantibodies specific for α345NC1 hexamers, which are restricted to noninflammatory IgG subclasses and are nonnephritogenic. In Alport syndrome, lack of tolerance toward α345(IV) collagen promotes production of autoantibodies to α345NC1 hexamers, including proinflammatory IgG subclasses that mediate posttransplant anti–GBM nephritis. The Journal of Immunology, 2013, 190: 1424–1432.

G lomerulonephritis (GN), an important cause of kidney injury leading to end-stage renal disease, is often due to autoimmune responses against components of the glomerular filtration barrier. A prominent target is α345(IV) collagen, the major component of glomerular basement membranes (GBM). Formation of supramolecular collagen IV networks is driven by specific interactions among the noncollagenous domains, which direct the assembly of chains into trimeric molecules and then associate end-to-end forming noncollagenous domain 1 (NC1) hexamers. The NC1 domains of α345(IV) collagen are the targets of Abs associated with the most aggressive forms of rapidly progressive GN (1, 2). In anti–GBM Ab GN, including Goodpasture disease, IgG autoantibodies mainly target two conformational epitopes within α3NC1 monomers (3–6). The immunodominant α3NC1 autoepitopes abut the α4NC1 and α5NC1 subunits within native α345NC1 hexamers, which prevents the binding of autoantibodies until hexamers dissociate (7, 8). An alloimmune form of anti–GBM Ab GN, Alport posttransplant nephritis (APTN), occurs in some patients with Alport syndrome after a kidney transplant. These patients typically have mutations in COLA4A3, COLA4A4, or COLA4A5 genes that prevent the normal assembly of α345(IV) collagen. APTN is mediated by anti–GBM alloantibodies that bind to the kidney allograft, targeting α3NC1 and/or α5NC1 epitopes accessible within α345NC1 hexamers (6, 9–12).

In anti–GBM Ab GN, the hallmark findings on the renal biopsy are smooth linear GBM deposition of IgG (mostly IgG1 and IgG4) and often complement C3, with crescentic and necrotizing injury as a result of complement- and FcγR-mediated inflammation. Clinically, the disease usually manifests as rapidly progressive crescentic GN, sometimes accompanied by life-threatening pulmonary hemorrhage due to autoantibodies binding to alveolar basement membranes (13). Prompt diagnosis and treatment with immuno-
suppresses drugs and plasma exchange are critical because of the fulminating course of the disease, with mortality >50% in untreated patients (14). However, some patients exhibit only mild glomerular involvement with normal renal function despite biopsy-proven linear GBM IgG autoantibodies (15–18). The molecular basis of this atypical presentation is not fully understood.

Deciphering the mechanisms uncoupling the anti–GBM autoantibodies from rapidly progressive GN can provide insights into the pathogenesis of Ab-mediated disease and may identify new targets for therapeutic intervention. By characterizing serum autoantibodies from a patient with strong linear GBM deposition of IgG but atypically mild nephritis, we identified a novel type of IgG4 subclass–restricted anti–GBM autoantibodies that selectively targeted α345NC1 hexamers but did not bind to α3NC1 monomers, unlike typical Goodpasture autoantibodies. The mechanisms eliciting the novel anti–GBM autoantibodies were studied in mouse models recapitulating this phenotype. Our results showed that tolerance toward autologous intact α345(IV) collagen is established in hosts expressing this Ag, even though autoreactive B cells specific for α345NC1 hexamers are not purged from the repertoire. Proteolysis selectively breaches this tolerance by generating autoimmunogenic α345NC1 hexamers. This provides a mechanism eliciting autoantibodies specific for quaternary epitopes within α345NC1 hexamers, which are restricted to noninflammatory IgG subclasses and are nonnephritogenic.

Materials and Methods

Materials

Recombinant NC1 monomers of human collagen IV chains were expressed in HEK293 cells and purified as described; recombinant α345NC1 hexamers assembled in vitro from NC1 monomers were used in some experiments (19). NC1 hexamers of GBM collagen IV, comprising a mixture of α345NC1 and α12NC1 hexamers, were solubilized by collagenase digestion of human and mouse kidney cortex basement membranes and purified by an ion-exchange column and gel-filtration chromatography (20). The α12NC1 hexamers were separated from α345NC1 hexamers by affinity chromatography using immobilized α–NC1 mAbs (20).

Anti–GBM autoantibodies were analyzed in serum from a patient with mild nonprogressive GN, which tested negative for anti–GBM Abs using a commercial clinical immunoassay despite strong linear GBM staining for α2 in hosts expressing this Ag, even though autoreactive B cells specific for α345NC1 hexamers are not purged from the repertoire. Proteolysis selectively breaches this tolerance by generating autoimmunogenic α345NC1 hexamers. This provides a mechanism eliciting autoantibodies specific for quaternary epitopes within α345NC1 hexamers, which are restricted to noninflammatory IgG subclasses and are nonnephritogenic.

Immunoassays

ELISA was performed in MaxiSorp plastic plates coated overnight with NC1 monomers (100 ng/well) or NC1 hexamers (300 ng/well) and then blocked with 1% BSA. Sera were diluted 1:50 unless otherwise indicated. Secondary Abs were alkaline phosphatase–conjugated anti–human IgG (American Qualex, San Clemente, CA) and peroxidase-labeled mAbs HP6009, HP6014, HP6047 (Zymed, San Franscisco, CA), and HP6023 (SouthernBiotech, Birmingham, AL) specific for human IgG1, IgG2, IgG3, and IgG4 subclasses, respectively. Human IgG4 was also detected using biotin-labeled mAbs HP6025 (Sigma-Aldrich, St. Louis, MO). Subclass-specific secondary Abs were diluted to yield approximately equal absorbance binding when purified human myeloma IgG1–IgG4 (100 ng/well; Sigma-Aldrich) were used as standards. After addition of substrate for phosphatase (Sigma-Aldrich) or peroxidase (Bio-Rad, Hercules, CA), color development was measured with a SpectraMax Plus 384 ELISA plate reader ( Molecular Devices, Sunnyvale, CA).

Animal experiments

DBA/1 mice were purchased from The Jackson Laboratory. Fgr2b<−/− mice backcrossed onto the DBA/1 background for >12 generations (D1. Fgr2b<−/−) were obtained from Dr. Sandra Kleinau, B6.CdbA3<−/− mice (22) were obtained from Dr. Jeffrey Miner. CdbA3<−/− mice expressing a human COLA3/COLA4 YAC transgene (23) on a mixed genetic background were obtained from Dr. Laurence Heidt. The genotype of mutant mice was verified by PCR. Mice were housed in a specific pathogen-free facility with free access to food and water. All procedures were approved by the Institutional Animal Care and Use Committee and conducted in accordance with the Guidelines for Animal Care and Use Program of Vanderbilt University.

Mice of either sex, between 6 and 10 wk old, were used for experiments. For immunizations, intact collagen IV from mouse GBM (5 mg) or purified NC1 hexamers from mouse GBM (100 μg) in 50 μl PBS were emulsified in an equal volume of CFA (Sigma-Aldrich), then injected s.c. at two sites on the back. Mice were boosted 3 wk later with Ag in IFA (Sigma-Aldrich). In control mice, the Ag was replaced by PBS. Mice were regularly checked for signs of disease. Blood was collected every 2 wk from the saphenous vein. Spot urine was collected every 2 wk. Mice were killed at 10 wk postimmunization. Blood and kidneys were collected for further analyses.

Evaluation of kidney function and renal histopathology

Urinary albumin excretion was measured in spot urine samples by capture ELISA using a mouse albumin quantitation kit (Bethyl Laboratories, Montgomery, TX). Urine creatinine and blood urea nitrogen (BUN) were measured using Infinity creatinine and urea liquid stable reagents (Thermo Fisher Scientific, Middletown, VA) according to the manufacturer’s protocols. Albuminuria was expressed as the urinary albumin/creatinine ratio (ACR). Portions of mouse kidneys or lungs were fixed in 10% buffered formalin, dehydrated through graded ethanol, embedded in paraffin, and kidney sections (2 μm thick) were stained with periodic acid-Schiff. At least 50 glomeruli from each mouse were observed to assess lesions.

For transmission electron microscopy (EM), kidney cortex was fixed in 4% paraformaldehyde in 0.1 M cacodylate buffer (pH 7.4), postfixed in aqueous 1.25% osmium tetroxide, dehydrated through an ethanol series, embedded in plastic, sectioned with a diamond knife, and stained with 4% uranyl acetate and lead citrate.

Direct and indirect immunofluorescence

Portions of snap-frozen mouse kidneys or lung embedded in OCT cryosectioned (5 μm), fixed in acetone for 10 min at ~20°C, and blocked with 1% BSA. For direct immunofluorescence (IF), frozen sections were stained with FITC-conjugated goat anti–mouse IgG, IgG1, IgG2, IgG2b (BD Biosciences/Pharmingen, San Jose, CA) or FITC-conjugated goat anti–mouse C3c (Nordic Immunology, Tilburg, The Netherlands). Secondary Abs were Alexa Fluor 488–conjugated goat anti–rabbit and anti–rat IgG (Invitrogen, Carlsbad, CA) or FITC-goat anti–rat IgG (BD Biosciences/Pharmingen). Sections were mounted with anti-fade reagent (Invitrogen) and examined under a Nikon Eclipse E800 epifluorescence microscope. Photomicrographs were recorded with a charge-coupled device digital camera, using the same exposure settings for each primary Ab.

Analysis of circulating and kidney-bound mouse IgG Abs

Kidney-bound Abs were eluted from homogenized mouse kidney cortex with 0.1 M glycine (pH 2.8). Serum and kidney-eluted mouse IgG (mlgG) Abs were analyzed by ELISA, as described for human autoantibodies. Secondary Abs were alkaline phosphatase–conjugated goat anti–mouse IgG (Rockland Immunocochemicals, Gilbertsville, PA) and HRP-conjugated goat anti–mouse IgG1, IgG2a, IgG2a* (for C57Bl/6d mice), and IgG2b (Bethyl Laboratories). The dilutions of secondary Abs were chosen to yield comparable ELISA readings when standard amounts of murine IgG1, IgG2a, and IgG2b mAbs against mouse α345NC1 hexamers were used as positive controls in the same assay.

Statistical analyses

Data are shown as means ± SD. Statistical analyses were performed using GraphPad Prism version 5.01. The significance of differences among groups was evaluated by one-way ANOVA test, followed by post hoc tests for pairwise comparisons. A p value <0.05 was considered to be statistically significant.

Results

Novel anti–GBM autoantibodies associated with mild GN selectively target α345NC1 hexamers and are restricted to human IgG4 subclass

Serum from a patient with mild proteinuria, microscopic hematuria, and elevated serum creatinine but stable renal function, whose renal biopsy showed linear GBM deposition of IgG but no crescents, tested negative for anti–GBM autoantibodies using a commercial
clinical assay. Because serology implied the absence of IgG auto-
antibodies against α3NC1 monomers, a serum sample was analyzed
for the presence of autoantibodies to other collagen IV NC1 domains.
Indirect ELISA showed IgG autoantibodies selectively binding to
human α345NC1 hexamers, but not to α3NC1, α4NC1, or α5NC1
monomers (Fig. 1A); typical autoantibodies from Goodpasture sera
bound equally well to α3NC1 monomers and α345NC1 hexamers.
Specificity for α345NC1 hexamers was corroborated by inhibition
ELISA (Fig 1B). IgG binding to native NC1 hexamers from human
GBM was abolished by hexamer dissociation (Fig. 1C). Analysis of
subclasses of IgG binding to α345NC1 hexamers revealed that the
patient’s autoantibodies were restricted to human IgG4, consistent
with linear GBM staining for human IgG4 (hIgG4) in the renal bi-
opsy, whereas Goodpasture sera also contained IgG1 besides IgG4
autoantibodies (Fig. 1D). IgG4 autoantibodies binding to α345NC1
hexamers were not found in normal human sera (Fig. 1E). Overall,
these results identified a novel type of anti–GBM autoantibodies
binding to α345NC1 hexamers but not α3NC1 monomers, restricted
to hIgG4 subclass, and associated with mild GN.

We used mouse models to further investigate the mechanisms
eliciting the novel anti–GBM autoantibodies and breaking tolerance
toward α345(IV) collagen. Based on the specificity of autoanti-
body, we hypothesized that the α345NC1 hexamer is the culprit
autoantigen. In Col4a3−/− mice, which cannot assemble α345
(IV) collagen, immunization with native NC1 hexamers from normal
mouse GBM elicits IgG Abs selectively targeting α345NC1 hex-
amers, but not α3NC1 monomers (10). However, it is not known
whether mouse GBM NC1 hexamers can also induce IgG auto-
antibodies in mice with normal expression of α345(IV) collagen.
This was addressed next.

**Mice immunized with autologous GBM NC1 hexamers produce
mouse IgG1-restricted anti–GBM autoantibodies to α345NC1
hexamers, which do not cause GN**

We chose DBA/1 mice for experiments because this strain develops
severe Ab-mediated glomerular disease after immunization with
α3NC1 monomers (24–26). We also used congenic D1 Fcgr2b–/–
mice because the ablation of the inhibitory IgG Fc receptor FcγRIIB
heightens humoral autoimmunity and Ab-mediated inflammation
(27) and was required to induce GN in mice injected with human
anti–GBM allotriobodies (21). The findings were essentially identi-
cal in DBA/1 wild-type and Fcgr2b–/– mice. All mice immunized
with autologous mouse GBM NC1 hexamers had normal renal
function for 10 wk postimmunization, as judged from urinary
albumin excretion and blood urea nitrogen levels, similar to those in
control mice immunized with adjuvant alone (Fig. 2A). At 10 wk
after immunization, kidney histology was normal, indistinguishable
from that of control mice (Fig 2Bc–e). Direct IF staining revealed
linear GBM staining for mouse IgG (Fig 2Bd–f), but no glomerular
C3 deposition (Fig 2Bg–i). The GBM and podocytes appeared
normal by transmission EM, whereas electron-dense deposits were
completely absent (Fig. 2Bj, k). These findings contrast with those
in α3NC1-immunized DBA/1 mice, which develop massive albumi-
numuria, have granular deposits of IgG and C3 in the GBM, and
exhibit GBM thickening with subepithelial immune complexes and
podocyte foot process effacement by EM (24–26).

The specificity of circulating and kidney-bound mIgG auto-
antibodies in hexamer-immunized DBA/1 mice was analyzed by
ELISA. IgG from sera (Fig. 3A) and kidney eluates (Fig. 3B)
bound to mouse GBM NC1 hexamers; minimal binding to mouse
α12NC1 hexamers implied that the autoantibodies were specific
for α345NC1 hexamers. Unlike sera or eluates from α3NC1-
immunized mice, those from hexamer-immunized mice did not
bind to human α3NC1 monomers. As only human but not mouse
α3NC1 monomers were available, and for comparison with hu-
man anti–GBM autoantibodies, we further verified that mouse IgG
autoantibodies bind to native but not dissociated human GBM
hexamers and to α345NC1 hexamers assembled in vitro (Fig. 3C).

Analysis of IgG subclasses of autoantibodies binding to mouse
GBM NC1 hexamers from sera (Fig. 4A) and kidney eluates (Fig.
4B) revealed the presence of mIgG1 but not mIgG2a nor IgG2b
autoantibodies. In corroboration of the ELISA results, analysis of
IgG subclasses bound to GBM by direct IF staining showed linear
GBM deposition of mIgG1 but not mIgG2a and mIgG2b sub-
classes (Fig. 4C). In contrast, small amounts of mIgG2a and
mIgG2b accompany mIgG1 autoantibodies in the sera and kidneys
of α3NC1-immunized DBA/1 mice (24). Thus, autologous GBM
NC1 hexamers elicit autoantibodies restricted to mIgG1 subclass,
which bind to mouse GBM in vivo but do not cause GN. These
murine autoantibodies recapitulate the properties of human anti–
GBM autoantibodies (Fig. 1) with respect to specificity, subclass
restriction, and lack of nephritogenicity.

**Intact collagen IV from mouse GBM does not elicit anti–NC1
Abs in DBA/1 mice**

NC1 hexamers occur in tissues as parts of supramolecular collagen
IV networks. We next evaluated whether intact collagen IV from

![FIGURE 1](http://www.jimmunol.org/)

**FIGURE 1.** Atypical specificity and subclass restriction of anti–GBM autoantibodies from a patient with mild GN. (A) By indirect ELISA, IgG autoantibodies from patient serum (diluted 1:50) bind to human α345 NC1 hexamers, but not to α1NC1, α3NC1, α4NC1, or α5NC1 monomers (open bars). Typical Goodpasture (GP) autoantibodies (sera diluted 1:200) bind both α3NC1 monomers and α345NC1 hexamers (filled bars). (B) The binding of the patient’s IgG autoantibodies to immobilized α345NC1 hexamers was inhibited by soluble α345NC1 hexamers but not α1NC1 or α3NC1 monomers. (C) Binding of IgG from patient’s serum to native and dissociated NC1 hexamers from human GBM, compared with GP autoantibodies and APTN allotriobodies. (D) Indirect ELISA measuring the binding to immobilized α345NC1 hexamers of human IgG1, IgG2, IgG3, and IgG4 from patient’s serum (open bars) or typical GP sera (filled bars; n = 4). (E) IgG4 autoantibodies binding to immobilized human α345NC1 hexamers were found only in the patient’s serum (open symbol) but not in normal sera (filled symbols; n = 5). All sera were diluted 1:50.
mouse GBM also elicits autoimmune responses in DBA/1 and D1. Fcgr2b<sup>−/−</sup> mice. Renal function (urine ACR and BUN) remained normal for 10 wk after immunization (Fig. 5A). Kidney histology was normal (Fig. 5Ba). Direct IF staining (Fig. 5Bb–f) revealed weak-to-moderate staining for total mlgG and mlgG1 (but not mlgG2a and mlgG2b) in a mesangial pattern, potentially due to Abs against Ags other than α345(IV) collagen present in the mouse GBM preparation. Glomerular deposition of C3 was absent. Neither sera nor kidney eluates contained mlgG1, mlgG2a, or mlgG2b autoantibodies binding to mouse GBM NC1 hexamers (Fig. 5Ca–c). The presence of mlgG anti–GBT autoantibodies in kidney eluates was further evaluated by indirect IF staining. IgG staining the GBM of normal mouse kidneys was only present in kidney eluates from mice immunized with GBM NC1 hexamers (Fig. 5D). These results indicate that DBA/1 and D1. Fcgr2b<sup>−/−</sup> mice do not develop anti–GBT autoantibodies after immunization with intact collagen IV from mouse GBM.

**Intact collagen IV from mouse GBM elicits IgG Abs specific for α345NC1 hexamers in Col4a3<sup>−/−</sup> mice but not in wild-type mice**

To establish whether the low immunogenicity of intact collagen IV from mouse GBM is due to host (immune tolerance) or Ag properties (supramolecular assembly, insolubility), we compared immune responses to this Ag in wild-type C57BL/6 mice and congenic Col4a3<sup>−/−</sup> Alport mice. Circulating IgG Abs binding to mouse GBM NC1 hexamers were found in sera from Col4a3<sup>−/−</sup> mice but not wild-type mice immunized with intact mouse GBM collagen IV (Fig. 6A, left). In contrast, mouse GBM NC1 hexamers elicited comparable Ab responses in both Col4a3<sup>−/−</sup> and wild-type mice (Fig. 6A, right). Lack of IgG binding to mouse α1α2NC1 hexamers implies that anti–GBT Abs produced in Col4a3<sup>−/−</sup> mice were specific for α345NC1 hexamers. This specificity was confirmed by indirect IF staining of kidneys from Col4a3<sup>−/−</sup> mice expressing human COL4A3, showing IgG binding to the GBM (Fig. 6B). These results indicate that intact collagen IV from mouse GBM elicits Abs binding to the NC1 domains of α345(IV) collagen only in Col4a3<sup>−/−</sup> mice but not in wild-type mice, implying that immune tolerance to intact α345(IV) collagen is acquired.

Abs to α345NC1 hexamers produced in Col4a3<sup>−/−</sup> mice are not IgG subclass-restricted

If the subclass restriction of autoantibodies specific for α345NC1 hexamers is solely determined by intrinsic properties of the Ag (e.g., its quaternary structure), it should not be influenced by the
genetic background of the host. However, we found that Col4a3<sup>−/−</sup> mice immunized with intact collagen IV (Fig. 7A) or NC1 hexamers from normal mouse GBM (Fig. 7B) produced not only mouse IgG1 but also IgG2a and IgG2b Abs specific for α345NC1 hexamers. This result implies that the production of proinflammatory IgG2a/c and IgG2b Abs against α345NC1 hexamers can occur in Alport mice lacking α345(IV) collagen, but it is selectively suppressed in mice expressing α345(IV) collagen.

**Ab-mediated GN in COL4A3-humanized mice immunized with murine GBM NC1 hexamers is associated with murine anti-GBM autoantibodies of IgG2a/IgG2b subclasses**

The loss of mouse α3(IV) collagen in Col4a3<sup>−/−</sup> mice can be compensated by transgenic expression of human α3(IV) collagen in COL4A3-humanized mice (23). This allows assembly of hybrid human/murine α345(IV) collagen, which is deposited in the GBM but not tubular basement membranes of transgenic mice. In COL4A3-humanized mice, NC1 hexamers from normal mouse GBM (a heterologous Ag in this setting) induced autoantibodies specific for α345NC1 hexamers (Fig. 8A), comprising mouse IgG1, IgG2a, and IgG2b subclasses (Fig. 8B). COL4A3 humanized mice
immunized with mouse GBM NC1 hexamers developed significant albuminuria (Fig. 8C). Kidney histology showed crescentic GN and glomerular deposition of mouse IgG in a linear GBM pattern (Fig. 8D). This indicates that nephritogenic IgG subclasses of anti–GBM autoantibody can be induced by immunizing mice with heterologous NC1 hexamers.

**FIGURE 6.** Intact collagen IV from normal mouse GBM elicits anti–NC1 Abs in Col4a3−/− Alport mice but not in wild-type C57BL/6 mice. (A) Sera from Col4a3−/− (open bars), C57BL/6 (filled bars), and DBA/1 (gray bars) mice immunized with intact mouse collagen IV (left) or mouse NC1 hexamers (right) were analyzed by ELISA in plates coated with mouse α12NC1 and α345NC1 hexamers. Mouse sera were diluted 1:100. (B) Indirect IF staining of kidneys from COL4A3-humanized mice by sera from Col4a3−/− (a, b) mice and C57BL/6 wild-type mice (c, d) immunized with intact mouse GBM collagen IV (a, c) and mouse GBM NC1 hexamers (b, d). Original magnification ×200.

**FIGURE 7.** Abs to α345NC1 hexamers produced in Col4a3−/− mice are not IgG subclass restricted. (A and B) Analysis of mlgG1 (a), mlgG2a (b), and mlgG2b (c) autoantibodies binding to mouse GBM NC1 hexamers (300 ng/well) in sera from Col4a3−/− (open bars) and C57BL/6 (filled bars) mice immunized with intact collagen IV (A) or NC1 hexamers (B) from normal mouse GBM. Mouse sera were diluted 1:100 (open bars). The significance of differences between groups was assessed by t test. *p < 0.05, **p < 0.01, ***p < 0.001.

**Discussion**

Novel human anti–GBM autoantibodies target quaternary epitopes within α345NC1 hexamers

Anti–GBM Ab GN, including Goodpasture disease, is clinically important because of its fulminant course, causing significant morbidity and mortality if not promptly treated. However, some patients with anti–GBM Abs develop atypically mild GN. The molecular mechanisms underlying this unusual presentation are not known. In this study, we identified a novel type of anti–GBM autoantibodies associated with mild nonprogressive GN, which were restricted to human IgG4 subclass and selectively targeted α345NC1 hexamers but not α3NC1 monomers. These autoantibodies targeted epitopes dependent on the native quaternary structure of α345NC1 hexamers, as hexamer dissociation abolished binding. In contrast, reactivity with α3NC1 monomers is a hallmark feature of Goodpasture autoantibodies, which preferentially bind to α3NC1 subunits of dissociated α345NC1 hexamers (3). Thus, the novel autoantibodies more closely resemble anti–GBM autoantibodies from patients with APTN, which bind to α3NC1 or α5NC1 epitopes accessible in native α345NC1 hexamers (9, 10), and may also target quaternary α345NC1 epitopes (28).

Low nephritogenicity of human IgG4 anti–GBM autoantibodies

The novel anti–GBM autoantibodies to α345NC1 hexamers were restricted to IgG4 subclass, which best explains the atypically mild kidney disease. Human IgG4 are noninflammatory because they do not activate the classic complement pathway, do not bind IgG Fc receptors, and are functionally monovalent owing to an Fab arm exchange (29). Other patients with hlgG4-restricted anti–GBM autoantibodies have normal kidney function or mild GN (16, 18, 30). Sera from healthy people contain natural autoantibodies to α3NC1 monomers, restricted to noninflammatory IgG2 and IgG4 subclasses (31). In contrast, IgG1 autoantibodies are prevalent in patients with anti–GBM/Goodpasture disease, suggesting that proinflammatory IgG subclasses are required for development of severe GN (32–34).

*Autologous α345NC1 hexamers are autoimmunogenic and elicit mlgG1-restricted, nonnephritogenic anti–GBM autoantibodies in mice*

We found that mice immunized with autologous NC1 hexamers from mouse GBM developed autoantibodies specific for α345NC1 hexamers, restricted to mlgG1 subclass, which did not cause GN despite binding to the GBM in vivo. Mouse IgG1 is functionally equivalent to human IgG4 because it is associated with a Th2 type (or an IL-4–secreting T follicular helper type) response and does not activate the classic complement pathway. Because mlgG1 has comparatively high affinity for the inhibitory Fcγ receptor, and it only binds the activating IgG receptor FcγRIII but not FcγRIV, it is less inflammatory than mlgG2a or mlgG2b (35). However, mlgG1 autoantibodies can be pathogenic when inflammation is FcγRIII dependent but does not require FcγRIV. For instance, mlgG1 mAbs to collagen II induce arthritis in DBA/1 mice, which is abolished in FcγRII−/− mice and enhanced in FcγRII−/− mice (36). In our study, the binding of mlgG1 autoantibodies to the GBM was not sufficient to cause murine GN, as found in other models (21, 37, 38). Thus, we established a mouse model recapitulating the specificity and subclass restriction of the novel human anti–GBM autoantibodies and also the mild kidney phenotype.

A novel finding in our study was that FcγRII−/− mice were also resistant to GN induced by mlgG1-restricted anti–GBM autoantibodies. That ligation of FcγRIII by mlgG1 was insufficient to cause murine GN even when inhibitory signals from FcγRIIB

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were absent implies a critical role for FcγRIV. This is consistent with findings of another study, which showed that FcγRIII and FcγRIV are both required for induction of GN by mouse IgG2a or IgG2b Abs planted in the GBM (37). Besides activating Fcγ receptors, mlgG2a and mlgG2b may also promote the development of murine GN by activating complement (39). Although the ablation of FcγRIIB is critical for the development of murine GN after passive transfer of human anti–GBM Abs (21) or immunization with pepsin-solubilized collagen IV from bovine lens capsule (40), pathology in these models is likely mediated by mlgG2a and/or mlgG2b Abs. Indeed, production of mlgG2a and mlgG2b anti–GBM autoantibodies in COL4A3-humanized mice immunized with mouse GBM NC1 hexamers was associated with Ab-mediated GN. Overall, our results suggest that proinflammatory IgG subclasses of anti–GBM autoantibodies are necessary for the development of severe GN.

**Restriction of autoantibodies specific for α345NC1 hexamers to mouse IgG1 subclass**

The pattern of IgG subclasses of anti–α345NC1 autoantibodies (hlgG4 in the patient and mlgG1 in NC1 hexamer–immunized mice) implies that IL-4–secreting T cell help was involved. In renal disease, this has been attributed to an IL-4 Th2 type response (41), with class switching to IgG2a (in mice) being IFN-γ (Th1 or T follicular helper) mediated (42). Because restriction to mouse IgG1 subclass was observed when murine GBM NC1 hexamers were used as an autologous Ag (in wild-type mice) but not as a heterologous Ag (in Alport mice or COL4A3-humanized mice), the production of mlgG2a and mlgG2b autoantibodies against α345NC1 hexamers appears to be effectively suppressed in mice expressing α345(IV) collagen. This likely reflects the activity of a subset of regulatory T cells inhibiting Th1/IFN-γ–directed responses, although direct suppression of B cells is also possible (43).

**Tolerance toward intact autologous α345(IV) collagen is breached by proteolysis**

Because autoantibodies elicited by α345NC1 hexamers bind to the GBM, they must cross-react with epitopes with native collagen (IV) collagen, implying that intact collagen (IV) collagen is a fragment of rat collagen XVII, the major autoantigen for bullous pemphigus (46), as well as ectodomain shedding generating neoepitopes implicated in pemphigus foliaceus anddesmoglein-3 neoepitopes implicated in pemphigus vulgaris (47). Thus, proteolysis is emerging as a general mechanism breached immune tolerance, along with other types of post-translational modifications (48).

**Breach of tolerance to α345(IV) collagen by proteolysis is selective**

First, even though the immunogen used in our study comprised a mixture of α12NC1 and α345NC1 hexamers, mice did not

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**FIGURE 8.** GN in COL4A3-humanized mice immunized with mouse GBM NC1 hexamers is associated with the production anti–GBM IgG autoantibodies, which are not subclass restricted. (A) Specificity of serum mlgG Abs from COL4A3-humanized mice immunized with mouse GBM NC1 hexamers (open bars, n = 4) or CFA (filled bars, n = 3) was analyzed by ELISA in plates coated with recombinant α1NC1, α3NC1, α4NC1, or α5NC1 monomers (100 ng/well) and affinity-purified mouse (m) α12NC1 hexamers or total NC1 hexamers from mouse GBM (300 ng/well). Mouse sera were diluted 1:100. (B) Analysis of mlgG1 (a), mlgG2a (b), and mlgG2b (c) subclasses of autoantibodies binding to mouse GBM NC1 hexamers (300 ng/well) in sera from COL4A3-humanized mice immunized with mouse GBM hexamers (open bars). Sera from CFA-immunized were used as negative controls (filled bars). Mouse sera were diluted 1:100. The significance of differences between groups was assessed by t test. *p < 0.05, **p < 0.01, ***p < 0.001. (C) ACR (left) and BUN (right) were monitored as indicators of renal function in COL4A3-humanized mice immunized with mouse GBM NC1 hexamers (C) or CFA (Δ). Means and SD are shown (n = 4 mice). (D) Kidney histology in COL4A3-humanized mice immunized with CFA (a, c) or mouse GBM NC1 hexamers (b, d) was evaluated by light microscopy (H&E staining) (a, b) and glomerular deposition of mouse IgG (c, d) by direct IF staining. Original magnification ×400.
produce autoantibodies to α12NC1 hexamers. The robust tolerance toward self α2(IV) collagen may be due to its ubiquitous presence in all basement membranes, including in primary lymphoid organs where autoreactive lymphocytes are negatively selected. Second, the breach of tolerance was limited to a subset of autoepitopes dependent on the native quaternary structure of α345NC1 hexamers. Goodpasture-like autoantibodies binding to α3NC1 monomers were not produced by immunization with native GBM NC1 hexamers. Goodpasture autoantibodies are thought to be elicited by structural alterations or conformational changes of α345NC1 hexamers that unmask cryptic α3NC1 epitopes (6, 8, 21, 49). In mice, this can be induced by hexamer dissociation (10) or oxidation (50). Autologous rat GBM NC1 hexamers are autoimmunogenic in both LEW and WKY rats, but they induce anti-α3NC1 autoantibodies and GN only in WKY rats (51, 52), suggesting that the host genetic background also affects the production of nephritogenic anti-α3NC1 autoantibodies.

Implications for Alport posttransplant anti–GBM nephritis
Unlike wild-type mice, Col4a3−/− mice immunized with intact collagen IV from normal mouse GBM readily developed IgG Abs against α345NC1 hexamers, including proinflammatory IgG subclasses. Because production of class-switched IgG Abs requires help from cognate CD4+ T cells, tolerance toward α345(IV) collagen in Alport syndrome must be absent in both B cell and T cell compartments. Despite immunosuppression, Alport patients exposed to allogeneic α345(IV) collagen in the kidney allograft are prone to develop nephritogenic anti-GBM autoantibodies and posttransplant nephritis because they do not have tolerance toward α345(IV) collagen or mechanisms suppressing the production of proinflammatory IgG subclasses of anti-α345NC1 Abs.

Acknowledgments
We thank Selene Colon for technical support and Dr. Elena Tchekneva for access to the mouse urine collection station (53) described in the patent application available at http://www.freepatentsonline.com/20110239953.pdf.

Disclosures
The authors have no financial conflicts of interest.

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