Murine Membranous Nephropathy: Immunization with α3(IV) Collagen Fragment Induces Subepithelial Immune Complexes and FcγR-Independent Nephrotic Syndrome

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Membrane nephropathy (MN) is a leading cause of nephrotic syndrome in adults and a significant cause of end-stage renal disease, yet current therapies are nonspecific, toxic, and often ineffective. The development of novel targeted therapies requires a detailed understanding of the pathogenic mechanisms, but progress is hampered by the lack of a robust mouse model of disease. We report that DBA/1 mice as well as congenic FcγRIIIa/−/− and FcγRIγ−/− mice immunized with a fragment of α3(IV) collagen developed massive albuminuria and nephrotic syndrome, because of subepithelial deposits of mouse IgG and C3 with corresponding basement membrane reaction and podocyte foot process effacement. The clinical presentation and histopathologic findings were characteristic of MN. Although immunized mice produced genuine anti-α3NC1 autoantibodies that bound to kidney and lung basement membranes, neither crescentic glomerulonephritis nor alveolitis ensued, likely because of the predominance of mouse IgG1 over IgG2a and IgG2b autoantibodies. The ablation of activating IgG Fc receptors did not ameliorate injury, implicating subepithelial deposition of immune complexes and consequent complement activation as a major effector pathway. We have thus established an active model of murine MN. This model, leveraged by the availability of genetically engineered mice and mouse-specific reagents, will be instrumental in studying the pathogenesis of MN and evaluating the efficacy of novel experimental therapies. The Journal of Immunology, 2012, 188: 3268–3277.

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Groups of four to six wild type, FcγRII+/− and FcγRI−/− DBA/1 mice, both males and females, between 6 and 10 wk old, were immunized s.c. at two sites on the back with αSNC1 (30 μg in 50 μl PBS) emulsified in equal volume of CFA (Sigma, St. Louis, MO), then boosted 3 wk later with the same amount of Ag in incomplete Freund’s adjuvant (Sigma). In control mice, the Ag was replaced with vehicle (PBS). Blood was collected every 2 wk from the saphenous vein. Spot urine was collected every 2 wk using a urine collection station (38). Mice were regularly checked for signs of disease and were euthanized if they developed edema, ascites, abnormality in body weight, high blood urea nitrogen (BUN) levels, or excessive loss of body weight (>10% in a week) or became lethargic. The remaining mice were killed at ~18 wk after immunization, except for several mice from each group sacrificed at 10 wk for comparison purposes. Blood, kidneys, and lungs were collected for further analysis. Three separate experiments were performed for each strain.

**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, Montgomery, TX). Urine creatinine and BUN were measured usingInfinity creatinine and urea liquid stable reagents (Thermo Fisher Scientific, Middletown, VA), according to the manufacturer’s protocols. Albuminuria was expressed as urinary albumin-to-creatinine ratio (ACR). The proteins in serum and urine samples were separated by SDS-PAGE electrophoresis under nonreducing conditions and stained with Coomasie Brilliant Blue. Total plasma cholesterol and triglycerides were measured with standard enzymatic assays at the Vanderbilt Mouse Metabolic Phenotyping Center. 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 (PAS) or Jones silver stain. Lung sections were stained with H&E. At least 50 glomeruli from each mouse were observed to assess lesions. Mesangial proliferation, global or segmental sclerosis, spike formation, necrosis, or crescents were assessed. For transmission EM, kidney cortex was fixed in 4% paraformaldehyde in 0.1 M cacodylate buffer (pH 7.4), postfixed in aqueous 1.25% osmium tetoxide, dehydrated through an ethanol series, embedded in plastic, sectioned with a diamond knife, and stained with 4% uranyl acetate and lead citrate. All assessments were made without knowledge of the experimental group.

**Direct and indirect IF**

Portions of snap-frozen mouse kidneys or lung embedded in OCT were cryosectioned (5 μm), fixed in acetone at −20°C, and blocked with 1% BSA. For direct IF, frozen sections were stained with FITC-conjugated goat anti-mouse IgG, IgG1, IgG2a, IgG2b (BD Bioscience Pharmingen, San Jose, CA), or FITC-conjugated goat anti-mouse C3c (Nordic Immunologicals, Tilburg, the Netherlands). For indirect IF staining of collagen IV chains, rat IgG mAbs were used as described (36). Staining with mAbs H11, H31, and RH34 was performed both with and without prior treatment of the sections with acid urea (6 M urea in 0.1 M glycine, pH 3.0). Rabbit anti-C5b9 (Abcam, Cambridge, MA) was used at 5 μg/ml. Secondary Abs were Alexa Fluor 488-conjugated goat anti-rabbit and anti-rat IgG (Invitrogen, Carlsbad, CA) or FITC-goat anti-rat IgG (BD Bioscience Pharmingen, San Jose, CA). Sections were mounted with anti-fade reagent (Invitrogen, Carlsbad, CA) 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 autoantibodies**

Kidney-bound Abs were eluted from homogenized mouse kidney cortex with 0.1 M glycine, pH 2.8. Serum and kidney-eluted mouse IgG (mIgG) Abs were analyzed by ELISA. Maxisorp plastic plates were coated overnight with rhαSNC1 (100 ng/well) or mouse GBM NC1 hexamers (300 ng/well) and blocked with 1% BSA. Mouse sera were diluted as indicated. Secondary Abs were alkaline phosphatase-conjugated goat anti-mouse IgG (Rockland Immunochemicals, Gilbertsville, PA) and HRP-conjugated goat anti-mouse IgG1, IgG2a, and IgG2b (Bethyl, Montgomery, TX). The dilutions of secondary Abs were chosen so that standard mAbs (TSF1 for mlgG1, TP90 for mlgG2a, TF86 for mlgG2b at 1 μg/ml each) yielded comparable ELISA readings when assayed for binding to total mouse IgG and mouse hexamers. Abs were considered positive if they yielded an OD reading >3 standard deviations above the mean background of appropriately treated culture plates. Plates were developed with 0.1 mg/ml p-nitrophenyl phosphate (Sigma, St. Louis, MO) or peroxidase (Biorad, Hercules, CA), color development was measured with a SpectraMax Plus 384 ELISA plate reader (Molecular Devices, Sunnyvale, CA).

**Materials and Methods**

**Materials**

The recombinant NC1 domain of human α3(IV) collagen (rhαSNC1) was expressed in HEK293 cells and purified as described (33). Mouse kidney (PeproTech, Rockville, MD) or rat lung (Rahman et al., 1999) cortex basement membranes (BMs), including the GBM, were digested with bacterial collagenase (Worthington, Lakewood, NJ). The solubilized NC1 hexamers were purified by passage through a DE-52 ion-exchange column and gel-filtration chromatography (34). The α3α4α5NC1 and α1α2α1NC1 hexamers from mouse GBM were affinity-purified using immobilized mAbs b14 (34) (NC1 domains from mouse GBM were separated by reverse-phase HPLC on a C18 column (Vydac 201TP C18 10μ) using a gradient of acetonitrile in 0.1% trifluoroacetic acid, as described previously (35). Rat IgG mAbs H11 (BD Bioscience Pharmingen, San Jose, CA), or FITC-conjugated goat anti-mouse C3 (Nordic Immunologicals, Tilburg, the Netherlands). For indirect IF staining of collagen IV chains, rat IgG mAbs were used as described (36). Staining with mAbs H11, H31, and RH34 was performed both with and without prior treatment of the sections with acid urea (6 M urea in 0.1 M glycine, pH 3.0). Rabbit anti-C5b9 (Abcam, Cambridge, MA) was used at 5 μg/ml. Secondary Abs were Alexa Fluor 488-conjugated goat anti-rabbit and anti-rat IgG (Invitrogen, Carlsbad, CA) or FITC-goat anti-rat IgG (BD Bioscience Pharmingen, San Jose, CA). Sections were mounted with anti-fade reagent (Invitrogen, Carlsbad, CA) 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.

**Animal experiments**

DBA/1 mice were purchased from The Jackson Laboratory. Breeding pairs of FcγRIII−/− and FcγRI−/− mice backcrossed onto the DBA/1 background for more than 12 generations were obtained from Dr. Sandra Kleinou. Col4a3−/− mice expressing a human COL4A3/COL4A4 YAC transgene (36) were obtained from Dr. Laurence Heidet. B6Col4a3−/− mouse (37) were provided by Dr. Jeffrey Miner (Washington University, St. Louis, MO). 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 Committee at the University of Pittsburgh in compliance with the U.S. Animal Welfare Act and guidelines or regulations of the United States Department of Agriculture, Federal Register, 59(9):17101, 1994.

**Results**

Polyclonal anti-NC1 sera were generated in rabbits immunized with the NC1 hexamer (34). The NC1 domain α3(IV) collagen, regardless of the expression of activating Fc receptors, developed massive albuminuria and nephrotic syndrome, associated with subepithelial IgG and C3 deposits by immunofluorescence (IF) and electron microscopy (EM) and characteristic GBM spike reaction without crescentic injury. We have thus established a novel active model of murine MN, which will be valuable for new studies of the disease mechanisms and preclinical studies of experimental therapies.

**Introduction**

Our goal was to determine the contribution of activating IgG Fc receptors to the pathogenesis of murine autoimmune GN induced by immunization with a well-defined GBM autoantigen. For this purpose, we chose to use DBA/1 mice because of their greater susceptibility to GN relative to C57BL/6 mice (30, 31) and the availability of congenic FcγRIII−/− and FcγRI−/− mice (32). Wild type, FcγRIII−/− and FcγRI−/− mice immunized with the NC1 domain of α3(IV) collagen, regardless of the expression of activating Fc receptors, developed massive albuminuria and nephrotic syndrome, associated with subepithelial IgG and C3 deposits by immunofluorescence (IF) and electron microscopy (EM) and characteristic GBM spike reaction without crescentic injury. We have thus established a novel active model of murine MN, which will be valuable for new studies of the disease mechanisms and preclinical studies of experimental therapies.
Results
Analyses of circulating anti-α3NC1 mIgG Abs

Circulating anti-α3(IV)NC1 mIgG Abs were detected in wild type, FcyRIII−/−, and FcRγ−/− DBA/1 mice immunized with rh-α3NC1 at ~2 wk after immunization, reached maximum titers at 6–8 wk, and declined (not shown). For all mIgG subclasses, serum levels of anti-α3NC1 Abs were essentially equal between wild type and FcγRIII−/− mice (Fig. 1A). Sera from FcγR−/− mice had significantly less mIgG1 and a trend toward lower mIgG2b, which is consistent with reduced Ab responses in FcγR−/− mice immunized with other Ags (29). In all groups of mice, serum anti-α3NC1 Abs of mIgG1 subclass had higher titers than mIgG2a or mIgG2b Abs (Fig. 1B), suggesting a predominantly Th2-polarized immune response.

Because mice were immunized with a heterologous Ag, we addressed whether the resulting mIgG Abs cross-reacted with endogenous autoantigen from mouse BMs. Sera from α3NC1-immunized but not control mice contained mIgG Abs binding to mouse GBM NC1 hexamers—a mixture of α1α2NC1 and α3α4α5NC1 hexamers in which the latter were the major target (Fig. 1C). Serum mIgG autoantibodies cross-reactive with mouse GBM NC1 hexamers were found in all groups of α3NC1-immunized mice, but their titers were lower in FcRγ−/− mice (Fig. 1D). By indirect immunofluorescence, mIgG from immunized mouse sera stained the GBM and tubular BMs in wild type mice and the GBM in COL4A3/A4-humanized mice, but did not bind to kidney BMs in Col4a3−/− mice (Fig. 1E). This pattern mirrors the expression of α3(IV) collagen in mouse kidney BMs (36). We thus established that mIgG autoantibodies can bind to α3α4α5(IV) collagen in mouse BMs under normal physiologic conditions.

Ablation of activating Fcy receptors does not ameliorate the development of heavy albuminuria and nephrotic syndrome

Urinary albumin excretion (Fig. 2A) and BUN levels (Fig. 2B) in experimental mice were monitored for up to 18 wk. A significant increase in albuminuria was first detected at 6 wk after immunization with α3NC1 (Fig. 2A). Between 6 and 10 wk post-immunization, ACR increased dramatically by several hundred-fold in all groups, rising slightly faster in FcγRIII−/− mice. In control mice immunized with adjuvant alone, ACR remained in the normal range during the experiment. After 10 wk, ACR reached a plateau at high values in the nephrotic range in all groups of mice. By this time, heavy nonspecific proteinuria and hypoalbuminemia developed in all α3NC1-immunized mice, but not in adjuvant-immunized control mice (Fig. 2C).

BUN levels, normal until ~10 wk after immunization, started rising progressively in some mice with heavy proteinuria (Fig. 2B), often in association with a sudden increase in body weight resulting from edema and ascites. At 10 wk, serum triglycerides (Fig. 2D) and total cholesterol (Fig. 2E) were also increased in α3NC1-immunized mice. Overall, these clinical findings are characteristic of nephrotic syndrome, and the affected mice were euthanized. Morbidity developed earlier in FcγRIII−/− mice, but the survival curves for wild type and FcγR−/− mice were not significantly different (Fig. 2F). Male mice suffered from these adverse consequences of prolonged heavy proteinuria earlier than females (Fig. 3). These results show that DBA/1 mice immunized with α3NC1 develop heavy albuminuria and eventually nephrotic syndrome by a mechanism that does not require expression of activating Fcγ receptors.
Kidney histopathology is typical of membranous nephropathy

PAS-stained kidney sections (Fig. 4Aa–h) from α3NC1-immunized mice showed GBM thickening, but minimal glomerular inflammation and few crescents (5 ± 3% in wild type mice and <2 ± 1% in FcγRIII−/− and FcRγ−/− mice; the difference was not significant by one-way ANOVA). Jones silver stain (Fig. 4Ai–l) showed prominent GBMs with capillary wall spikes (inset) characteristic of MN, with an underlying linear GBM deposits surrounded by the expanded matrix, and diffuse effacement of podocyte foot processes in α3NC1-immunized mice.

Glomerular deposition of mIgG and mC3 was analyzed by direct IF staining. In all groups of α3NC1-immunized mice, glomerular capillary loops showed intense staining (3+) for mIgG, in a granular pattern characteristic of MN, with an underlying linear GBM pattern typical for anti-GBM autoantibodies (Fig. 5b–d). Weak linear staining for mIgG was also observed in tubular BMs. C3 was deposited in a fine granular pattern along the glomerular capillary wall and focally in tubular BMs (Fig. 5f–h). Analysis of kidney-bound mIgG subclasses showed intense staining for mIgG1 (Fig. 5j–l) and weaker staining for mIgG2a (Fig. 5n–p) and mIgG2b (Fig. 5r–t).

Indirect IF staining revealed C5b-9 deposition in the capillary loops of α3NC1-immunized, but not CFA-immunized control mice (Fig. 6A). Urinary excretion of C5b-9 was evaluated by Western blot analysis. Rabbit Abs to mC5b-9 specifically stained an ∼250-kDa band (arrow) in urine samples collected at 8–10 wk (but not at 0–6 wk) from α3NC1-immunized mice, but not CFA-immunized control mice (Fig. 6B).

**Analyses of kidney-eluted mIgG autoantibodies**

We next characterized the specificity of mIgG Abs eluted from mouse kidneys. Compared with serum Abs, kidney-eluted Abs had greater reactivity toward total mouse GBM NC1 hexamers (Fig. 7A, right) and less toward rh-α3NC1 (Fig. 7A, left). The Abs in solution inhibited the binding of eluted Abs to rh-α3NC1 by 50–75% and to mouse GBM hexamers by ∼80%, confirming the cross-reactivity of kidney-bound mIgG autoantibodies. In contrast, mouse GBM hexamers in solution inhibited the binding of serum Abs to immobilized mouse GBM hexamers but not to rh-α3NC1, indicating that the bulk of circulating Abs specifically target rh-α3NC1. Using murine NC1 domains separated by reverse-phase HPLC, we further established that kidney-eluted mIgG Abs reacted exclusively with mouse α3NC1 (Fig. 7B), thus ruling out an epitope spreading to other NC1 domains. By indirect IF, kidney-eluted Abs bound in a linear pattern to kidney BM from wild type mice, but did not stain Col4a3−/− mouse kidneys (Fig. 7C). The latter suggests that intermolecular epitope spreading to normal podocyte Ags, other than α3(IV) collagen, is unlikely. However, we cannot rule out the production of autoantibodies to podocyte Ags induced or upregulated during disease, or to neo-epitopes generated by oxidative modification of podocyte or GBM proteins. By ELISA, kidney-eluted IgG autoantibodies binding to mouse GBM NC1 hexamers were predominantly mIgG1 (Fig. 7D), as found for serum Abs (Fig. 1) and consistent with the analysis of kidney-bound mIgG subclasses by direct IF staining (Fig. 5). Overall, these results show that kidney-bound mIgG autoantibodies are a subset of serum Abs that specifically target α3NC1 epitopes shared by the human and mouse Ags, and which are accessible in native NC1 hexamers of α3a4α5(IV) collagen.

**Expression of collagen IV chains and glomerular deposition of extrinsic Ag**

Increased production of collagen IV or other BM components by injured podocytes alters the GBM composition in experimental MN (39, 40). We analyzed the expression of collagen IV chains by indirect IF. In α3NC1-immunized mice, the GBM was stained more intensely by mAbs to α3, α4, and α5(IV) collagen chains, whereas the staining of tubular BMs was largely unchanged compared with control mice (Fig. 8d–f, j–l, p–r, v–x). Glomerular expression of α1(IV) collagen, mostly in a mesangial pattern, was not altered (Fig. 8h, n, t). These results reveal that α3-immunized mice have increased production or reduced turnover, or both, of α3α4α5(IV) collagen in the GBM, whereas α1α2(IV) collagen does not appear to contribute to GBM expansion in this model.

Unexpectedly, we found that mAb H31 specifically stained the GBM (but not tubular BMs) in native kidney sections from α3NC1-immunized mice (Fig. 8i, u). As the staining pattern in kidneys from control mice shows, mAb H31 targets an α3NC1 epitope that is normally cryptic in native NC1 hexamers in tissues (Fig. 8c, n), but is unmasked by acid-urea treatment (Fig. 8d, p). This finding implies that endogenous α3(IV) collagen in the GBM is altered during the disease or that rh-α3NC1 monomers deposit in the glomeruli—a possibility addressed next.

Indirect immunofluorescence staining using mAb RH34, which specifically binds to human but not mouse α3NC1 (36), showed granular deposition of rh-α3NC1 in the glomerular capillary loops, but no staining of tubular BMs (Fig. 9b–d). Staining with
GP autoantibodies, which do not bind to \( \alpha3(IV) \) collagen in native mouse BMs, produced the same results (Fig. 9f–h). Overall, the colocalization of rh-\( \alpha3NC1 \) with IgG and C3 in the capillary loops suggests that the extrinsic Ag used for immunization may participate in the formation of subepithelial IC deposits.

**Absence of lung inflammation despite mIgG binding to alveolar BMs**

Because \( \alpha3(IV) \) collagen is a normal component of alveolar BMs and is targeted by autoantibodies mediating pulmonary hemorrhage in GP patients, we assessed the lung phenotype in experimental mice. In \( \alpha3NC1 \)-immunized and control mice, lungs appeared grossly normal without overt hemorrhage. By light microscopy, H&E-stained lung sections showed normal alveoli with no inflammation (Fig. 10d, g, j). Direct immunofluorescence staining showed strong linear mIgG deposition along the alveolar BMs in \( \alpha3NC1 \)-immunized mice, but not in control mice (Fig. 10e, h, k). Negative staining by mAb RH34 indicated the absence of rh-\( \alpha3NC1 \) in the lungs (Fig. 10f, i, l). These results show that mIgG autoantibodies bound to alveolar BMs in vivo, but did not induce inflammation.

**Discussion**

Membranous nephropathy is a common cause of nephrotic syndrome in adults. Up to 40% of patients develop end-stage renal disease (41). Current treatments are nonspecific, toxic, and often ineffective (42, 43). Development of novel targeted therapies requires a thorough understanding of the pathogenic mechanisms. Rat Heymann nephritis has emerged as the best model for studying the disease mechanisms, because it faithfully recapitulates human MN (44). However, further progress is hampered by the lack of a mouse model that can take full advantage of the availability of genetically engineered strains and mouse-specific reagents.

In this study, we describe an active mouse model of membranous nephropathy induced by immunization with a fragment of \( \alpha3(IV) \) collagen, a normal GBM component, and target of GP autoantibodies. Wild type, FcyRIII\(^{-/-} \), and FcR\( \gamma \)-DBA/1 mice immunized with rh-\( \alpha3NC1 \) developed massive albuminuria followed by hypoalbuminemia, hyperlipidemia, and often edema—typical features of nephrotic syndrome. Evaluation of kidney histopathology revealed GBM thickening with subepithelial spikes, granular deposits of mIgG and mC3 along the glomerular capillary
and FcR
immunization with rh-
mIgG and mC3 and electron-dense subepithelial deposits at 11 wk
postimmunization. A higher proportion of crescents (∼23 versus
∼20% in our model) may be the result of a more aggressive im-
munization protocol (mice were immunized in the footpad and
subcutaneously three times). Whether the kidney phenotype in mouse
strains studied by Hopfer et al. (30) recapitulates human MN
immunized mice with rh-α3NC1, thickened GBM, and podocyte foot
process effacement in α3NC1-immunized wild type (a) and
FcγRIII−/− (b) and FcRγ−/− (c) mice. Original magnification ×2850.

FIGURE 4. GBM thickening with the formation of spikes, and sub-
epithelial electron-dense deposits are characteristic of membranous ne-
phropathy. (A) Kidney sections from adjuvant-immunized control mice
and wild type. FcγRIII−/− and FcRγ−/− mice immunized with rh-α3NC1,
stained with PAS (a–h) or Jones silver (i–l) show GBM thickening but
little glomerular inflammations or crescents, with occasional proteinaceous
casts and mild interstitial fibrosis and inflammation. Jones silver stain
shows GBM spikes (inset, arrows). Original magnification ×200 (a–d),
×400 (e–h), ×630 (i–l). (B) Transmission EM shows subepithelial elec-
tron-dense deposits (red arrowheads), thickened GBM, and podocyte foot
process effacement in α3NC1-immunized wild type (a), FcγRIII−/− (b)
and FcRγ−/− (c) mice. Original magnification ×2850.

loops by IF, with subepithelial electron-dense immune complexes
deposits, and diffuse effacement of podocyte foot processes by
EM. These clinical and histopathologic findings recapitulate the
hallmark features of membranous nephropathy.

The susceptibility of mouse strains to experimental GN induced
by immunization with rh-α3NC1 has been compared by Hopfer
et al. (30). C57BL/6 develop milder glomerular injury than do
DBA/1 mice, whereas AKR and NOD mice are relatively resis-
tant, despite mlgG deposition in the kidneys. Consistent with our
findings, DBA/1 mice exhibit linear-granular GBM staining for
mIgG and mC3 and electron-dense subepithelial deposits at 11 wk
postimmunization. A higher proportion of crescents (∼23 versus
∼5% in our model) may be the result of a more aggressive im-
munization protocol (mice were immunized in the footpad and
boosted three times). Whether the kidney phenotype in mouse strains
studied by Hopfer et al. (30) recapitulates human MN
immunized mice with rh-α3NC1, thickened GBM, and podocyte foot
process effacement in α3NC1-immunized wild type (a) and
FcγRIII−/− (b) and FcRγ−/− (c) mice. Original magnification ×2850.

Properties of anti-α3NC1 mlgG Abs: effect on the renal
phenotype

Because IgG autoantibodies are central mediators of both anti-
GBM/GP disease and MN, we have characterized in detail the
properties of circulating and tissue-bound mlgG Abs elicited by
immunization with rh-α3NC1. We showed that bona fide mlgG
anti-α3NC1 autoantibodies are produced, which cross-react with
native mouse GBM NC1 hexamers in vitro and bind to mouse
GBM and alveolar BM in vivo. Hence, these mlgG autoantibod-
ies must bind to α3NC1 epitopes accessible in the murine
α3α4ε5NC1 hexamers, similar to mlgG Abs produced in Co-
H4a3γ−/− mice immunized with rh-α3NC1 and to human IgG

FIGURE 5. Granular deposition of mlgG and mC3 in the glomerular
capillary loops. Direct IF microscopy shows kidneys from adjuvant-im-
munized control mice (a, e, i, m, q) and wild type (b, f, j, n, r). FcγRIII−/−
(c, g, k, o, s) and FcRγ−/− (d, h, l, p, t) mice euthanized at 10 wk after
immunization with rh-α3NC1. Sections were stained with fluorophore-
conjugated Abs to mlgG (a–d), mC3c (e–h), mlgG1 (i–l), mlgG2a (m–p),
mlgG2b (q–t). Original magnification ×400. The granular staining pattern
is apparent at high magnification shown in the inset (d, h). Indirect im-
munofluorescence staining of kidney sections from the CFA control mice
with mlgG1 mAb TF51 (i, inset), mlgG2a mAb TF90 (m, inset) and
mlgG2b mAb TF86 (q, inset), all specific for mouse α3α4ε5α5 NC1
domains, provided a reference for assessing the intensity of staining for
each mlgG subclass deposited in the kidneys.

FIGURE 6. Kidney deposition and urinary excretion of mC5b-9. (A)
Indirect IF staining shows granular GBM deposition of mC5b-9 in α3NC1-
immunized wild type (a) and FcγRIII−/− (b) mice, but not in adjuvant-
immunized mice (c). Staining was negative when the primary Ab was
omitted (d). Original magnification ×400. (B) Western blot analysis of
mouse urine samples (5 μl/lane) separated by SDS-PAGE under nonre-
ducing conditions using Abs to mC5b-9 showed specific staining of an
∼250-kDa band (arrow) in urine collected at 8 wk (f), but not at 0, 2, 4, or
6 wk after immunization with α3NC1 (b–e, respectively), nor in urine
collected at 8 wk after immunization with CFA (a). Urine from a 7-mo-old
B6.Co4a3γ−/− mouse with heavy albuminuria (ACR = 103) was used as an
additional control. The band at ∼150 kDa was also stained when the pri-
mary Ab was omitted, suggesting it is due to secondary Ab cross-reacting
with excreted mlgG in mouse urine.
alloantibodies mediating posttransplant anti-GBM nephritis in autosomal recessive Alport syndrome (16). In contrast, GP autoantibodies cross-react only with isolated mouse α3NC1 monomers or dimers, but do not bind to native mouse α3α4α5NC1 hexamers, which are extensively cross-linked and thus protected against autoantibody-induced dissociation (45–47). Although we cannot rule out that mice also produced GP-like autoantibodies, such autoantibodies would not bind to mouse tissues in vivo. Production of mlgG autoantibodies that bind in vivo to murine BMs containing α3α4α5(IV) collagen indicates that immune self-tolerance toward endogenous α3(IV) collagen is breached in mice immunized with α3NC1 monomers.

Although anti-α3NC1 mlgG autoantibodies bound to GBM and alveolar BMs, they did not mediate crescentic glomerulonephritis or alveolitis, suggesting a limited ability to elicit inflammation. The simplest explanation is the prevalence of mlgG1 over mlgG2a and mlgG2b among anti-α3NC1 autoantibodies. Only mlgG2a and mlgG2b but not mlgG1 mediate neutrophil influx and glomerular injury in a mouse model of acute neutrophil-mediated glomerular inflammation (25). In particular, anti-α3NC1 mAbs of the mlgG1 subclass that bind to mouse GBM in vivo are not nephritogenic (48). The lattice structure of collagen IV limits the maximum amount and density of autoantibodies that can bind to the NC1 domains. Therefore, competition by mlgG1 could reduce the density of GBM-bound IgG2a and IgG2b autoantibodies be-

FIGURE 7. Specificity of kidney-bound mlgG autoantibodies. (A) ELISA shows the binding of serum (open bars) and kidney-eluted (closed bars) mlgG Abs to immobilized rh-α3NC1 (hAg, left) and total NC1 hexamers from mouse GBM (mAg, right), and the inhibition of this binding by hAg (5 μg/ml) or mAg (50 μg/ml) in solution. (B) NC1 domains from mouse GBM were separated by reverse-phase HPLC on a C18 column (top), and the composition of the major peaks (a–d) was analyzed by Western blotting with chain-specific mAbs (bottom). Kidney-eluted mlgG autoantibodies reacted only with the fraction containing murine α3NC1. (C) Indirect IF staining shows the binding of mlgG eluted from the kidneys of α3NC1-immunized mice (a, b) or control mice (c, d) to kidney cryostat sections from wild type mice (a, c) and Col4a3−/− Alport mice (b, d). (D) ELISA analysis of subclasses of mlgG eluted from kidneys at ~10 wk after immunization with rh-α3NC1 (diagonal lines) or CFA (solid bars) showed the prevalence of mlgG1 autoantibodies binding to immobilized mouse GBM NC1 hexamers. Anti-NC1 mAbs TF51, TF90, TF86 (1 μg/ml each) were used as standards for mlgG1, mlgG2a, and mlgG2b, respectively (open bars). A similar distribution of mlgG subclasses was found for kidney-eluted autoantibodies binding to rh-α3NC1 (not shown). D, dimers; M, monomers.

FIGURE 8. Increased collagen IV deposition in the GBM. Kidneys from wild type (a–l) and FcRγ−/− (m–x) mice at 10 wk after immunization with adjuvant (a–f, m–r) or rh-α3NC1 (g–l, s–x) were analyzed by indirect IF microscopy. Acetone-fixed cryostat sections were stained with mAbs H11 specific for α1NC1 (a, g, m, s), H31 specific for α3NC1 (c, i, n, u), RH42 specific for α4NC1 (e, k, q, w), and b14 specific for α5NC1 (f, l, r, x). Some sections were treated with acid urea (AU) before staining with mAbs H11 (b, h, n, t) and H31 (d, j, p, v). Original magnification ×400.

FIGURE 9. Deposition of rh-α3NC1 in the glomerular capillary loops. Indirect IF staining shows the binding of mAb RH34 (a–d) and affinity-purified human GP autoantibodies (e–h) to kidney sections from adjuvant-immunized control mice (a, e) and from wild type mice (b, f). FcγRIII−/− mice (c, g), and FcγRI−/− mice (d, h) at 10 wk after immunization with rh-α3NC1. A detailed view (insets) illustrates the granular staining pattern. Control sections stained with secondary Ab but omitting the primary Ab were negative (not shown). Binding of mAb RH34 was slightly reduced but not abolished by acid urea treatment (not shown). Original magnification ×400.
Comparison with other models of experimental MN

Our mouse model recapitulates hallmark features of human MN as well as rat Heymann nephritis, the “gold standard” for experimental MN. Compared with the widely used passive model, active Heymann nephritis more closely resembles human disease because it involves the host’s own immune system. Its major disadvantages are longer duration, much greater variability, and animal discomfort because of footpad immunizations (67). By comparison, in our active mouse model, nephropathy is induced more rapidly and reliably. In addition, unlike rats, mouse models can take advantage of existing transgenic mice and mouse-specific reagents.

The development of a robust murine model of MN has been a work in progress for decades (67). Early models had various...
limitations, such as minimal or transient proteinuria or complement limitations, such as minimal or transient proteinuria or complement limitations, such as minimal or transient proteinuria or complement limitations, such as minimal or transient proteinuria or complement

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Disclosures

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References


