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Glomerulopathy Induced by Immunization with a Peptide Derived from the Goodpasture Antigen α3IV-NC1

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Mouse experimental autoimmune glomerulonephritis, a model of human antiglomerular basement membrane disease, depends on both Ab and T cell responses to the Goodpasture Ag noncollagenous domain 1 of the α3-chain of type IV collagen (α3IV-NC1). The aim of our study was to further characterize the T cell–mediated immune response. Repeated immunization with mouse α3IV-NC1 caused fatal glomerulonephritis in DBA/1 mice. Although two immunizations were sufficient to generate high α3IV-NC1–specific IgG titers, Ab and complement deposition along the glomerular basement membranes, and a nephrotic syndrome, two additional immunizations were needed to induce a necrotizing/crescentic glomerulonephritis. Ten days after the first immunization, α3IV-NC1–specific CD4+ cells producing TNF-α, IFN-γ, or IL-17A were detected in the spleen. With the emergence of necrotizing/crescentic glomerulonephritis, ~0.15% of renal CD4+ cells were specific for α3IV-NC1. Using peptides spanning the whole α3IV-NC1 domain, three immunodominant T cell epitopes were identified. Immunization with these peptides did not lead to clinical signs of experimental autoimmune glomerulonephritis or necrotizing/crescentic glomerulonephritis. However, mice immunized with one of the peptides (STVKAGDLEKIISRC) developed circulating Abs against mouse α3IV-NC1 first detected at 8 wk, and 50% of the mice showed mild proteinuria at 18–24 wk due to membranous glomerulopathy. Taken together, our results suggest that autoreactive T cells are able to induce the formation of pathologic autoantibodies. The quality and quantity of α3IV-NC1–specific Ab and T cell responses are critical for the phenotype of the glomerulonephritis. The Journal of Immunology, 2015, 194: 3646–3655.

In humans, anti–glomerular basement membrane (GBM) glomerulonephritis (GN) is characterized by rapidly progressive renal failure due to crescentic GN and frequently lung hemorrhage (1, 2). Diagnosis is based on the detection of circulating autoantibodies and their deposition along the GBMs (3, 4). The major autoantigen is the noncollagenous domain 1 of the α3-chain of type IV collagen (α3IV-NC1), which is targeted by both Abs and T cells (5–7). The role of the T cells in the initiation and progression of crescentic GN is still incompletely understood (8, 9).

Anti–GBM GN has been studied in a number of animal models using immunizations with heterologous or homologous GBM preparations or α3IV-NC1 to breach tolerance against the autoantigen, often referred to as experimental autoimmune GN (EAG) (10–13). We have previously characterized the T cell response to heterologous human α3IV-NC1 in DBA/1 mice and found a biphasic disease course. Autoantibodies are deposited early along the GBM with a membranous phenotype whereas the necrotizing/crescentic GN develops rapidly during the late phase (14, 15). This is associated with the accumulation of Th1 and Th17 cells in the kidneys, suggesting that these cells are involved in GN initiation and/or progression.

The present study was designed to test homologous mouse (m) α3 IV-NC1 as the Ag, to define immunodominant T cell epitopes within m-α3IV-NC1, and to determine whether peptides restricted to these epitopes are sufficient to induce disease in the mice. We found that the number of immunizations had a major influence on the phenotype of the GN despite a similar evolution of the α3IV-NC1–specific Ab response. Whereas two immunizations were sufficient to induce a membranous glomerulopathy, the development of necrotizing/crescentic GN required additional immunizations. In m-α3IV-NC1, three T cell epitopes could be identified. Immunization with one of the epitopes generated a late Ab response to α3IV-NC1 resulting in membranous glomerulopathy. Our results suggest that primary T cell–mediated tissue damage participates in the pathogenesis of anti–GBM GN.

Materials and Methods
Cloning and expression of m-α3IV-NC1

mRNA was prepared from mouse renal cortex using TRIzol and reverse transcribed using oligo(dT)20 and SuperScript. m-α3IV-NC1 cDNA was amplified by PCR adding at the N terminus an Nhel restriction site, a thymidine, and a flag epitope, and at the C terminus an XhoI restriction site after the stop codon. Cloning and subcloning into the expression vector pCEP-Pu was performed as described (14). Plasmid DNA containing the correct sequence was transfected into 293EBNA cells, and
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with either PBS for 45 min and subsequently incubated with dilutions of sera from two patients with anti-GBM disease. It was not reactive to mAbs directed against the αIV- or α5IV-1 domain (Supplemental Fig. 1).

Animal experiments

All animal experiments were performed according to the German Animal Protection Law and were approved by the local Animal Care Committee. Male DBA/1 mice (Charles River Laboratories, Sulzbach, Germany) were housed under specific pathogen-free conditions.

To test the nephrotoxicity of immunodominant T cell epitopes, the peptides P05 (RHSQTAIPSCPEGT), P32 (MDMAPISGRALEPYI), and P71 (STVKAGDLEKIISRC) as well as a control peptide P15 (KRAHGQDLGTLGSCL; purity 95% by HPLC; JPT Peptide Technologies, Berlin, Germany) were dissolved in distilled water at a concentration of 5 mg/ml. Mice were immunized s.c. with a total of 50 mg peptide in 50 μl PBS emulsified in an equal volume of CFA. Subcutaneous booster immunizations were performed with the same dose emulsified in IPA on days 21, 35, and 49.

To test the nephrotoxicity of immunodominant T cell epitopes, sections were treated with 6 M urea in 0.1 M glycine (pH 2.2) for 10 min on ice (16). Sections were blocked with 5% normal horse serum in PBS for 45 min and subsequently incubated with dilutions of sera from EAG mice or with a goat anti-collagen IV Ab (SouthernBiotech; dilution 1:400) overnight. Binding of Abs was determined with Cy2-conjugated donkey anti-mouse IgG (H+L; Jackson Immunoresearch Laboratories, West Grove, PA) and Cy3-conjugated rabbit anti-goat Abs (Dako, dilution 1:100). Sections were counterstained with DNA dye DRAQ5 (Cell Signaling Technology) and Hoechst (14).

Isolation of spleen and renal cells, in vitro cytokine production, and FACS analysis

Mice were killed and organs were collected after perfusion with ice-cold PBS through the left ventricle. Cells from kidneys and spleens were isolated and cultured as previously described (15). Spleenocytes collected 10 d after immunization were cultured in vitro in the presence/absence of 50 μg/ml m-α3IV-NC1. Supernatants were collected at 40 h and tested for the secreted IFN-γ and IL-10 as described (14). A custom peptide set (Bio-chem, Germany) was used for mapping the T cell epitopes. The peptide set included 74 overlapping peptides spanning the whole m-α3IV-NC1 domain with a purity of ~80% as estimated by the manufacturer. Each peptide consisted of 15 aa with an overlap of 12 as peptide 1 (P01; ATGTRMRGHFLTTHS to peptide 74 (P74; EKISRCVQVCMKKHR). Peptide pellets were dissolved in distilled water with 10% acetic acid and 40% acetic anhydride with an estimated final concentration of 0.25 mM. Spleenocytes of m-α3IV-NC1–immunized mice collected at day 10 were stimulated in vitro with ~10 μg/ml peptide/well and supernatants were analyzed for secreted IFN-γ after 40 h of culture as described (14).

For detection of m-α3IV-NC1–specific T cells by FACS, 1 × 106 splenocytes or gradient-enriched kidney cells were incubated with 54 μg/ml m-α3IV-NC1 or with 10–3 M peptides for 8 h. To ensure Ag presentation, 1 × 106 CFSE-labeled spleenocytes of naive DBA/1 mice were added to cultures with kidney T cells. Brefeldin A (10 μg/ml) was added for the final 4 h. The polyclonal control stimulation was performed with PMA and ionomycin as described (15). Flow cytometry was performed as described excluding all CFSE+ cells (15). Prior to fixation and intracellular staining, cells were incubated with a fixable dead cell stain (Pacific Orange succinimidyl ester, Invitrogen Life Technologies, Darmstadt, Germany). Lymphocytes were identified according to their forward scatter/side scatter profile. Doublets and dead cells were excluded using the forward scatter area/forward scatter height profile and the Pacific Orange stain. CD4+ cells from immunized mice were identified as CD45+CD4+CFSE− cells. In these cells, activation in response to peptide, m-α3IV-NC1, or PMA and ionomycin was determined by the intracellular expression of CD40L/CD154 and of the cytokines TNF-α, IFN-γ, and IL-17A.

Results

EAG induced by m-α3IV-NC1

Groups of DBA/1 mice were immunized with m-α3IV-NC1 in CFA and boosted once at 3 wk (mNC1-2×) or three times (mNC1-4×) at 3, 5, and 7 wk. Six of six mice immunized twice and eight of nine mice immunized four times developed clinical signs of EAG between days 57 and 75 (Fig. 1A). Most of the mice presented with massive edema (mNC1-2×, four of six mice; mNC1-4×, six of nine mice). Proteinuria evaluated semiquantitatively at 4, 8, and 10 wk showed mild proteinuria at 8 wk and moderate to severe proteinuria at 10 wk (Fig. 1B, Table I). Mice with severe clinical symptoms were sacrificed and further assessed for kidney and lung pathology (mNC1-4×, median 81 d, range 72–84 d; mNC1-2×, median 75 d, range 64–84 d; p = 0.190). Kidney pathology showed a membranous glomerulopathy in all mice immunized with m-α3IV-NC1 with a superimposed necrotizing crescentic GN in the mNC1-4× group. Glomeruli had thickened peripheral capillaries, and protein deposits and spikes were visible at high magnification (Fig. 2A–E). By transmission electron microscopy, numerous subepithelial electron-dense deposits were seen (Fig. 2I–K). A striking difference in the number of glomerular crescents was apparent between the two groups (median mNC1-4× of 35% versus mNC1-2× of 3%, p = 0.009; Fig. 1D). In parallel to the crescents, an increasing amount of tubulointerstitial damage was noted (median mNC1-4× score of 2.04 versus mNC1-2× score of 1.25, p = 0.008; Fig. 1E). PBS-immunized control mice remained healthy throughout the experiment and no functional or histological abnormalities were found.
Repeat experiments performed with four immunizations showed comparable results (data not shown) and enabled us to look at proteinuria in more detail revealing a progressive increase in the albumin/creatinine ratio over 3 log steps beginning at 6 wk (Fig. 1C).

BUN was mildly elevated in EAG mice immunized four times (median mNC1-4 of 57.0 versus mNC1-2, 48.5 versus control, p = 0.012; mNC1-2 versus control, p = 0.093). BUN values correlated with the number of glomerular crescents and the tubulointerstitial damage score (Spearman correlation coefficient of 0.7 for both, p < 0.001, not shown). Pulmonary disease was not detected in any of the mice.

By immunohistochemistry, increased numbers of CD3+ lymphocytes and F4/80+ macrophages were found in the interstitium of immunized mice with significantly higher numbers in the mNC1-4 group (CD3+ cells, median mNC1-4 of 32.0 versus mNC1-2, 20.1 versus control, p = 0.018; F4/80+, median mNC1-4 of 16.0 versus mNC1-2 of 7.8 versus control 0.6 cells/high-power field, mNC1-4 versus mNC1-2, p = 0.049; Figs. 1G, 1H, 2F, 2G).

As described before, immunization with a3IV-NC1 caused only a marginal, nonsignificant increase in the number of glomerular CD3+ cells (Fig. 1I) (15). Interstitial CD3+ and F4/80+ cell counts correlated with the percentage glomerular crescents (r² = 0.537, p < 0.001; Figs. 1J, 2G).

Table I. Proteinuria in DBA/1 mice immunized with m-α3IV-NC1

<table>
<thead>
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<th>mNC1-2×</th>
<th>Control</th>
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<tbody>
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<td>4 wk</td>
<td>++ (n = 8; range, 0 to ++)</td>
<td>ND</td>
<td>0 (n = 6)</td>
</tr>
<tr>
<td>8 wk</td>
<td>++ (n = 4; range, + to ++)</td>
<td>++ (n = 4; range, + to ++)</td>
<td>0 (n = 4)</td>
</tr>
<tr>
<td>10 wk</td>
<td>+++ (n = 4; range, ++ to ++++)</td>
<td>+++ (n = 1)</td>
<td>ND</td>
</tr>
</tbody>
</table>

*Proteinuria was scored semiquantitatively on Coomassie-stained polyacrylamide gels scoring the albumin band as negative (0), little (+), moderate (++), or severe (+++).

Three of nine mice showed clinical signs of EAG at 10 wk. Urine could be assessed in four of the mice.

Four of five mice alive showed clinical signs of EAG at 10 wk. Urine could be preserved in only one of the mice.
correlated with the numbers of crescents and the tubulointerstitial damage score (Spearman correlation coefficient of 0.79 for all, \( p < 0.001 \); Fig. 1J, 1K and data not shown).

**Ab response to m-\( \alpha \)-3IV-NC1 and IgG deposition in the kidneys**

Immunized mice mounted a strong Ab response against \( \alpha \)-3IV-NC1 that in some mice was already detectable at day 10 (data not shown). At week 4, all mice had a high Ab titer, which remained high at 8 wk, and had diminished variably in the proteinuric mice at the time of sacrifice (Fig. 3A). There were no differences in the IgG titers between mice immunized four or two times at any time point. Testing for IgG isotypes at sacrifice revealed the presence of IgG1 and with lower titers of IgG2a and IgG2b (Fig. 3B). IgG1 levels were significantly lower in the mNC1-2\( \times \) group whereas there were no significant differences for IgG2a and IgG2b. IgG deposition was found in all m-\( \alpha \)-3IV-NC1–immunized mice with a granular pattern along the peripheral capillaries (Fig. 2H). A semiquantitative analysis revealed no difference in glomerular IgG deposition between mice immunized four or two times (Fig. 3C). Assessment of isotypes showed dominant deposition of IgG1 with

**FIGURE 2.** Histological findings in EAG mice at sacrifice. (A–E) High-power magnifications of GN induced by immunizations with m-\( \alpha \)-3IV-NC1. (A) Glomerular crescent and tubulointerstitial damage in mNC1-4\( \times \) mice. (B) Thickened GBM in mNC1-2\( \times \) and no tubulointerstitial damage. (C) Normal histology in PBS-immunized control mice. Irregularly thickened GBM, spike formation (D), and peripheral protein deposits (E; red color) in immunized mice. (F–H) Immunohistochemical staining of CD3\(^+\) (F: diaminobenzidine [DAB] brown color) and F4/80\(^+\) cells in the interstitium (G; 3-amino-9-ethylcarbazole, red color) and IgG deposition along the GBM in EAG mice (H; DAB, brown color). (I–K) Transmission electron microscopy shows subepithelial electron-dense deposits (arrows), formation of new basement membrane, and loss of podocyte foot processes in mNC1-4\( \times \) (l) and mNC1-2\( \times \) mice (J). A regular GBM structure was seen in the controls (K). (L–O) Deposition of IgG1 (L), IgG2a (M), IgG2b (N), and complement C3c (O) along the GBM in EAG mice (DAB, brown color; New Fuchsin, red color). Please note unspecific staining of erythrocytes in the IgG2a and IgG2b staining. (A–D) Periodic acid–Schiff stain; original magnification \( \times \)400 or \( \times \)630. (E) Trichrome stain; original magnification \( \times \)630. (F–H and L–O) Immunohistochemistry of paraffin sections; original magnification \( \times \)400 or \( \times \)630. (I–K) Transmission electron microscopy; original magnification \( \times \)3500 or \( \times \)1400).
much less IgG2a and IgG2b (Figs. 2L–N, 3C). By immunohistochemistry, both immunized groups showed comparable levels of complement C3c binding (Figs. 2O, 3D).

Diluted sera of both mNC1-4× and mNC1-2× mice did not bind well to mouse kidney cryosections under native conditions. Binding to the GBM was much enhanced after denaturing the sections with urea (Fig. 3E). However, we did not observe a difference in binding between sera from mNC1-4× and mNC1-2× mice.

Early and late T cell responses to m-α3IV-NC1

The Ag-specific cellular immune response was evaluated in splenocytes 10 d after immunization. In vitro stimulation with 50 μg/ml m-α3IV-NC1 resulted in the secretion of IFN-γ and IL-10 as shown by cytokine ELISA (Fig. 4A, 4B). FACS analysis revealed the presence of CD4+ T cells that upregulated CD154 (CD40L) and produced IFN-γ, TNF-α, or IL-17A in response to stimulation with m-α3IV-NC1 (Fig. 4C, 4D). Polyclonal PMA/ionomycin control stimulation revealed no difference between m-α3IV-NC1- and PBS-immunized mice (data not shown).

With established disease, very low frequencies of m-α3IV-NC1–specific T cells were detected in stimulated splenocytes (<0.02% of CD154+TNF-α+ cells, no CD154+IFN-γ+ or CD154+IL-17A+ cells; data not shown). In contrast, kidneys of EAG mice contained ~0.15% specifically activated T cells, with most of them producing TNF-α (Fig. 4E).

Mapping of T cell epitopes

To identify T cell epitopes within the m-α3IV-NC1 domain, splenocytes prepared 10 d after immunization with m-α3IV-NC1 were stimulated in vitro with a set of overlapping 15mer peptides spanning the whole m-α3IV-NC1 domain (Fig. 5). IFN-γ ELISA identified three clusters of peptides. From these clusters, the central peptides P05 (RHSQTTAIPSCPEGT), P32 (MDMAPISGRALEPYI), and P71 (STVKAGDLEKIISRC) caused relatively strong IFN-γ production in

FIGURE 3. m-α3IV-NC1–specific serum Abs and glomerular IgG and complement C3c deposition. (A and B) Indirect ELISA of m-α3IV-NC1–specific IgG in serum: total IgG over time (A) and isotypes IgG1, IgG2a, and IgG2b at sacrifice (500-fold serum dilution) (B). No significant differences were found between mNC1-4× and mNC1-2× in total m-α3IV-NC1–specific IgG. At sacrifice, IgG1 levels were significantly lower in the mNC1-2× group (mNC1-4× versus mNC1-2×, p = 0.036). (C and D) Semiquantitative scoring of glomerular IgG, IgG1, IgG2a, and IgG2b (C) as well as complement C3c (D) deposition revealed no significant differences between mNC1-4× and mNC1-2× mice. (E) Immune fluorescence staining of untreated (− urea) or urea treated (+ urea) kidney sections from naive mice with sera from immunized (mNC1-4×, mNC1-2× at sacrifice) and naive mice (control). Binding of Abs was detected with Cy2-conjugated donkey anti-mouse IgG Ab. (A–D) Each circle represents data of an individual mouse. The bars in (B)–(D) give the median. (E) Images show representative results; original magnification ×400.
four of five independent experiments. P32 showed the strongest induction of IFN-γ, followed by P71 and P05 (Table II).

Membranous glomerulopathy induced by P71 immunization

To test the nephritogenicity of the peptides, groups of six DBA/1 mice were immunized and boosted with the immunodominant peptides P05, P32, and P71 or a control peptide P15 (KRAHGQDLGTLGSCL). None of the mice developed clinical signs of EAG within the 6-mo observation period. However, three of six mice immunized with P71 developed mild proteinuria starting at week 18. No proteinuria was detected in the other groups of peptide-immunized mice (Table III).

Kidney histology revealed membranous glomerulopathy in the three proteinuric mice immunized with P71 (Fig. 6A, 6D). By immunohistochemistry, granular IgG deposition along the peripheral glomerular capillaries was seen, and electron microscopy confirmed the presence of multiple subepithelial electron-dense deposits (Fig. 6B, 6C, 6E, 6F). The remaining three mice immunized with P71 showed no pathology by light microscopy. However, immunohistochemistry revealed segmental peripheral IgG deposition in only some of the glomeruli (data not shown). The histological assessment of all other peptide-immunized groups showed no abnormalities (data not shown).

P71-immunized mice mount an Ab response against mα3IV-NC1

Serum samples obtained at weeks 2, 4, and 8 and at sacrifice were tested for peptide-specific and anti–mα3IV-NC1 Abs by indirect ELISA. No specific Abs were detected at 2 and 4 wk. At 8 wk and at sacrifice, all P71-immunized mice had Abs specific for mα3IV-NC1 with titers comparable to mα3IV-NC1–immunized mice in the three animals that developed proteinuria and membranous glomerulopathy. At the same time, Abs directed against the peptide had emerged in four of the six mice, but only two of these showed high titers. No peptide- or mα3IV-NC1–specific Abs could be detected in the other peptide-immunized groups (Fig. 7A and data not shown). The IgG isotype pattern was similar to the pattern in mα3IV-NC1–immunized mice. As with total IgG, the proteinuric P71 mice had higher titers than did the nonproteinuric mice (Fig. 7B). Ab binding to mα3IV-NC1 did not change after preincubation of the sera with excess P71 peptide. In contrast, binding to P71 was almost completely blocked (Fig. 7C).
lated in the kidney in the necrotizing/crescentic stage of disease. 3)

To adjust for interassay variations, IFN-γ concentrations were expressed as the percentage of the maximum concentration measured in an individual experiment.

Table II. IFN-γ production after in vitro stimulation of splenocytes with peptides P01–P74

<table>
<thead>
<tr>
<th>Peptide</th>
<th>No. Positive Experiments</th>
<th>Mean % of [IFN-γ]</th>
<th>Maximum</th>
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<tr>
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<td>3</td>
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<td>P73</td>
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Ag-specific T cells after peptide immunization

Splenocytes isolated 10 d after immunization with P71 were stimulated in vitro and analyzed for Ag-specific T cells by FACS. In immunized mice but not in control mice, a low frequency of CD4+ T cells that responded to P71 with upregulation of CD154 and the production of TNF-α, IFN-γ, and IL-17A could be identified in the spleen (Fig. 7D). A low frequency of P71-specific T cells was also detected in IFN-γ ELISPOT assays (data not shown). T cells responding to the whole m-α3IV-NC1 protein were below the detection level in P71-immunized mice (data not shown).

Discussion

The α3IV-NC1 domain of collagen type IV is the autoantigen responsible for anti–GBM GN in humans and has been used to induce EAG in susceptible mouse and rat strains. In the present study, homologous m-α3IV-NC1 was used to induce EAG in DBA/1 mice. Three important observations were made. 1) Immunization of DBA/1 mice with m-α3IV-NC1 caused fatal autoimmunity in the kidney. Depending on the number of immunizations, mice developed membranous glomerulopathy (two immunizations) or a focal necrotizing GN in mNC1-4 (16). Therefore, a solid Ab response appears to be sufficient to induce membranous glomerulopathy, which is mediated by immune complex deposition as well as in situ formation (17).

Progression of membranous glomerulopathy to necrotizing/crescentic GN in mNC1-4 mice may be due to an evolution of the Ab response with additional immunizations. This could result in the generation of Abs with higher affinity and avidity or a switch to “more inflammatory” IgG isotypes. Additionally, the Ab response could spread to other epitopes within the α3IV-NC1 domain or even other Ags within the GBM (18). Olaru et al. (19) recently demonstrated that in the DBA/1 EAG model, development of crescentic GN depends on the formation of IgG2a and IgG2b recognizing epitopes accessible only on α3IV-NC1 monomers but not on native α345-NC1 hexamers. Although we cannot exclude a “driver” role of both mechanisms in the progression from membranous glomerulopathy to necrotizing/crescentic GN, our data did not show major differences in the Ab responses between mNC1-2× and mNC1-4× with respect to IgG isotype profile, specificity, deposition, and complement binding. As shown for human Goodpasture autoantibodies, sera from both groups mostly targeted epitopes not accessible under native conditions (16).

Our results rather suggest that the transition to a necrotizing/crescentic GN in this EAG model may be T cell driven. Currently, we are not able to prove the functional relevance of α3IV-NC1–specific T cells in the kidneys during the late course of the disease. In a previous study using human α3IV-NC1, we have looked at the number of renal T cells after polyclonal stimulation and found increased numbers of IFN-γ-producing cells already early whereas IL-17A–producing cells were only seen in the late phase of the disease (15). The number of α3IV-NC1–specific cells in the kidneys is much lower than the number of T cells responding

Table III. Proteinuria in DBA/1 mice immunized with peptides P05, P32, P71, or P15 (control)

<table>
<thead>
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<th>P05</th>
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<th>P71</th>
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<td>8 wk</td>
<td>0 (n = 5)</td>
<td>0 (n = 4)</td>
<td>0 (n = 2)</td>
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<td>14 wk</td>
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<td>0 (n = 3)</td>
<td>0 (n = 2)</td>
</tr>
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<td>0 (n = 4)</td>
<td>0 (n = 2)</td>
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</tr>
<tr>
<td>24 wk</td>
<td>0 (n = 5)</td>
<td>0 (n = 3)</td>
<td>0 (n = 2)</td>
<td>++ (n = 6; range, 0 to ++++)</td>
</tr>
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Proteinuria was scored semiquantitatively on Coomassie-stained polyacrylamide gels scoring the albumin band as negative (0), little (+), moderate (++), or severe (+++).
Mice immunized with P71 mounted an Ab response against m-α3IV-NC1 first documented at 8 wk. Three of six mice analyzed developed proteinuria due to membranous glomerulopathy in the course. Interestingly, we did not observe an Ab response against P71 until the emergence of anti-α3IV-NC1 Abs. Blocking experiments also revealed recognition of P71-independent epitopes of α3IV-NC1 Abs. Short peptides are usually not immunogenic for B cells, unless they are coupled to a carrier. Detection of anti-P71 Abs together with anti-α3IV-NC1 Abs rather suggests that the Ab

with cytokine production after polyclonal stimulation. Even with established disease, it is barely above the detection limit. Although the renal CD3 cell count and its strong correlation with the percentage of glomerular crescents hint at a causal relationship, transfer experiments are necessary to prove this point. Indeed, T cell transfers in WKY rats or in HLA-DRB1*15:01 transgenic mice have also suggested a direct participation of T cells in the emergence of crescentic GN (20, 21). Our results also support studies in the EAG and the nephrotoxic nephritis model, which suggest a role of both Th1 and Th17 cells in the development of crescentic GN (15, 20–25).

With overlapping 15mer peptides, we could identify three T cell epitopes of m-α3IV-NC1 recognized in the context of the DBA/1 background. These epitopes differed from those recently described in a human HLA-DRB1*15:01–restricted transgenic mouse model of anti-GBM disease and from T cell epitopes identified in HLA-DR15+ patients with Goodpasture’s disease (7, 21). Interestingly, P05 includes the dominant epitope found in the WKY rat EAG model (26–28).

Nephritogenicity of the three T cell epitopes was tested by repeated peptide immunizations of DBA/1 mice. None of the peptides induced clinical signs of EAG and neither focal necrotizing nor crescentic GN was seen by histology. Our findings are in line with the results reported in the HLA-DRB1*15:01 transgenic mouse model, but the findings in mice contrast to the results obtained in WKY rats, where a single immunization with an immunodominant peptide is sufficient to induce crescentic GN (21, 26–28). Immune responses launch dose-dependently. Therefore, the amount of peptide used for the immunizations may have been a relevant factor. Compared to the immunizations with complete m-α3IV-NC1, we used a 50-fold peptide dose (∼1 nmol versus ∼50 nmol). It was comparable to the dose used in the transgenic mouse study, and related to the body weight it was higher than the ones used in the WKY rat studies (21, 26–28). It is interesting that the extreme susceptibility of WKY rats for EAG is dependent on non-MHC genes and in particular the Fcγr3 has been shown to be relevant in the context of crescentic GN (29, 30). In mouse EAG, Fcγr2b has a protective effect, as C57BL/6 mice deficient in Fcγr2b develop crescentic GN after immunization with α3IV-NC1 and peptide immunization in the HLA-DRB1*15:01 transgenic mice, which also carried the Fcγr2b knockout, resulted in focal segmental necrotizing GN (21, 31).

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response was secondary to damage inflicted by P71-specific T cells. α3IV-NC1 released from basement membranes could subsequently provoke autoantibodies against the protein, including anti-P71 Abs. In support of this hypothesis, we could detect P71-specific CD4+ T cells producing TNF-α, IFN-γ, or IL-17A in spleens of P71-immunized mice, although at relatively low frequencies. At this point, we failed to detect CD4+ T cells reacting against the complete α3IV-NC1 peptide, which was most likely due to technical limitation (data not shown). In contrast to peptides, protein requires uptake and processing by APCs, which restricts the sensitivity of the assay and prevents detection of very low T cell frequencies.

In our model, it is not clear where the primary tissue injury takes place. With the restricted tissue distribution of α3IV collagen, the kidneys or lungs are the most probable candidates (1). It is likely that this damage is temporary, as we did not see any kidney or lung pathology in our previous study prior to Ab deposition in the glomeruli (15). In the WKY model, the mechanism of α3IV-NC1–specific Ab generation has been studied in detail. Peptide immunization resulted in glomerular damage with the emergence of the specificities of the generated Abs have proven intra- and intermolecular epitope spreading in rat EAG model (18, 27).

Our results as well as T cell transfer assays in mouse and rat EAG suggest that T cells may be the principal mediators of crescent formation and subsequent/associated tubulointerstitial damage (15, 20, 21). However, we failed to detect crescentic GN in P71-immunized mice despite the induction of P71-specific CD4+ T cells. The most likely explanation could be the number of autoreactive T cells in the different experiments. In the transfer assays, 10^5 or 6 × 10^5 α3IV-NC1–specific T cells were given to recipient mice or rats, respectively. These numbers are probably several magnitudes above the numbers of P71-specific T cells induced by peptide immunization. In the same line, immunization with α3IV-NC1 most likely induces a stronger and broader T cell response than does P71 immunization. Particularly after several rounds of boost immunization, this T cell response could surpass a threshold level necessary for the induction of crescentic GN. Alternatively, the relatively low number of autoreactive T cells following P71 immunization may have still been sufficient to facilitate an Ab response against the GBM, which subsequently caused membranous glomerulopathy.

In conclusion, our results demonstrate that depending on the nature of the Ag used for immunization (peptide versus NC1 protein) and the schedule of immunization (one versus three booster immunizations) autoimmunity against α3IV-NC1 can result in membranous glomerulopathy or a transformation into a necrotizing/crescentic GN. Strong Ab responses are most likely a prerequisite for both outcomes, and the deposition of IgG along the GBM appears to be sufficient for the formation of membranous glomerulopathy. For T cells, our results suggest two functions: 1) T cells might facilitate or even cause the generation of pathogenic Abs, and 2) T cells appear to be responsible for the progression of disease to necrotizing/crescentic GN. Currently, the factors determining the outcome of renal disease are unclear. However, we would suggest that the number and probably the differentiation status of the α3IV-NC1–specific T cells might be decisive.

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Disclosures
The authors have no financial interests.

References


