The HLA-DRB1*15:01–Restricted Goodpasture’s T Cell Epitope Induces GN


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ABSTRACT

Human anti-glomerular basement membrane (GBM) disease strongly associates with HLA-DRB1*15:01. The target autoantigen in this disease is the noncollagenous domain of the α3 chain of type IV collagen, α3(IV)NC1, but critical early T cell epitopes presented by this human MHC class II molecule are unknown. Here, by immunizing HLA-DRB1*15:01 transgenic mice with whole recombinant α3(IV)NC1 and with overlapping α3(IV)NC1 peptides, we defined a HLA-DRB1*15:01–restricted α3(IV)NC1 T cell epitope (α3136–146) with four critical residues. This peptide was not immunogenic in HLA-DRB1*01:01 transgenic or C57BL/6 mice. The T cell epitope is naturally processed from α3(IV)NC1. CD4+ T cell clones, generated from HLA-DRB1*15:01 transgenic mice and specific for α3136–146, transferred disease into naive HLA-DRB1*15:01 transgenic mice, evidenced by the development of necrotizing crescentic GN, albuminuria, renal impairment, and accumulation of CD4+ T cells and macrophages in glomeruli. Because Fcγ receptors are implicated in disease susceptibility, we crossed HLA transgenic mice onto an FcγRIIb-deficient background. Immunization with either α3136–146 or α3(IV)NC1 induced GN in HLA-DRB1*15:01 transgenic FcγRIIb-deficient mice, but HLA-DRB1*01:01 transgenic FcγRIIb-deficient mice were unaffected. Taken together, these results demonstrate that the HLA-DRB1*15:01–restricted T cell epitope α3136–146 can induce T cell responses and injury in anti-GBM GN.

may even be protective (e.g., HLA-DRB1*01:01 [odds ratio, 0.6; 95% confidence interval, 0.3–1.0]). The strong HLA-DRB1*15:01 association and the accuracy of diagnostic criteria allow meaningful studies on how the MHC II molecule confers risk in mechanistic terms.

Key human B cell epitopes in anti-GBM disease have been defined. Autoantibodies bind to the conformational α(3)(IV)NC1 epitope “E_A” α3127–141 and “E_B” α3127–141 (all amino acid numbering, including other cited epitopes, follows Netzer et al.). Studies examining T cell epitopes in human anti-GBM disease are less common, although an important study showed reactivity to two peptides, α3128–89 and α3129–148 (close to the E_a autoantibody epitope), in all six patients studied. However, in the Wistar Kyoto (WKY) rat, T cell reactivity occurs in several different areas. T cell–mediated disease can be induced by α314–26 and α311–2515.17 and there is evidence of epitope spreading to involve B cell epitopes.16,18

Our hypothesis was based on studies in humans with anti-GBM GN showing strong MHC II association and suggesting key epitopes. We hypothesized that either the regions in or around α3128–89 or α3129–148 (or both) would be immunogenic in the context of HLA-DRB1*15:01 but not other MHC II molecules, and could be defined as nephritogenic CD4+ T cell epitopes. Therefore, HLA-DRB1*15:01 would permit binding of one or both of these peptides at an appropriate avidity, allowing naïve autoreactive CD4+ cells to escape negative selection in the thymus, thus explaining why people who express HLA-DRB1*15:01 are more susceptible to anti-GBM GN. We studied mice that were entirely deficient in mouse MHC II,19,20 but that coexpressed the essentially nonpolymorphic HLA-DRA1*01:01, with either HLA-DRB1*15:01 or DRB1*01:01. HLA transgenic mice, especially those lacking all murine MHC II elements, have been powerful tools in studying the pathogenesis of autoimmune disease,20,21 because their CD4+ T cell repertoire is shaped by the presence of human (but not mouse) MHC II molecules. We found that in anti-GBM GN, α3136–146 is an immunodominant and nephritogenic CD4+ T epitope in DRB1*15:01 Tg mice, but not in DRB1*01:01 Tg mice or C57BL/6 mice. Importantly, CD4+ responses to this epitope induce severe anti-GBM GN.

RESULTS

Mice with a Human HLA II Susceptibility Allele Exhibit T Cell Responses to α3(IV)NC1 133–152

To determine immunogenic regions within the α3(IV)NC1 molecule, we immunized mouse MHC II–deficient mice transgenic for the Goodpasture disease susceptibility gene HLA-DRB1*15:01 or the protective HLA-DR1*01:01. Three pools of murine α3(IV)NC1 20-mers were used (each peptide overlapping by 12 aa; Supplemental Table 1), with each pool spanning one-third of the α3(IV)NC1 molecule. After 10 days, draining lymph node cells were re-stimulated with individual peptides within the pool and recall responses measured by [3H]-thymidine proliferation assays and IFN-γ and IL-17A enzyme-linked immunosorbent spots (ELISPOTs). Several regions within α3(IV)NC1 were immunogenic in both strains, but the striking difference was the strong reactivity to α3129–148 and α3137–156 in DRB1*15:01 Tg mice, but not in DRB1*01:01 Tg mice (Figure 1A). No spontaneous reactivity to any peptide could be detected in unimmunized DRB1*15:01 Tg or DRB1*01:01 Tg mice (proliferation assay, IFN-γ and IL-17A ELISPOTs; Supplemental Figure 1). We immunized mice with whole recombinant murine α3(IV)NC122 and re-stimulated cells with each peptide individually. We found a similar, but more restricted pattern of responses, with cells from DRB1*15:01 Tg mice, but not from DRB1*01:01 Tg mice responding when stimulated with α3129–148 and α3137–156 (Figure 1B). Therefore, we immunized groups of mice with either α3129–148 or α3137–156 alone. Although there was no reactivity to these peptides in DR1*01:01 Tg mice, in DRB1*15:01 Tg mice both α3129–148 and α3137–156 induced similar responses to each other, and to recombinant murine α3(IV)NC1 (rma3(IV)NC1) (Figure 2A). The murine and human α3(IV)NC1 sequences are very similar, with only three differences at α3135, α3136, and α3138 (murine numbering). We immunized DRB1*15:01 Tg mice with either murine α3133–152 or the equivalent sequence in humans (hα3132–151). Responses to each could be induced by the other species’ peptide and human α3132–151–immunized mice responded to rma3(IV)NC1 (Figure 2B).

We then defined reactivity in DRB1*15:01 Tg mice compared with C57BL/6 mice (murine 1-Aβ). After peptide pool immunization, several peptides were immunogenic, similar to the comparison between DRB1*15:01 Tg and DRB1*01:01 Tg mice (Figure 3A). Although responses were seen in DRB1*15:01 Tg mice after re-stimulation with α3129–148 or α3137–156, there were, as with 0101 Tg mice, no responses in C57BL/6 mice. After α3129–148 or α3137–156 immunization, DRB1*15:01 Tg mice, but not C57BL/6 mice, responded to either peptide or to rma3(IV)NC1 (Figure 3B). Because α3129–148 and α3137–156 both induce responses in DRB1*15:01 Tg mice, we then used a 20-mer derived from the common 12 aa of both peptides with 4 aa at each end (α3133–152).

The DRB1*15:01 Restricted T Cell Epitope Is Immunogenic after Whole Antigen Immunizations

To define anti-α3(IV)NC1 responses using whole proteins, we used chimeric human α3/α1(IV)NC1 domains, with the conformational α3(IV)NC1 autoantibody epitopes E_A or E_B substituted into the α1(IV)NC1 domain, which is not a common or primary autoimmune target in humans.11 DRB1*15:01 Tg mice were immunized with α1(IV)NC1, α3(IV)NC1, or the E_A chimera (containing hα3127–141) or the E_B chimera (containing hα3127–141 overlapping the restricted T cell epitope), and lymphocytes were then re-stimulated with either murine α39–29 or α3129–148. Mice immunized with α1(IV)NC1 or the E_A chimera responded to neither peptide (Figure 4A), whereas mice
immunized with the E\textsubscript{B} chimera or full-length \textalpha3(IV)NC1 responded to \textalpha3\textsubscript{129–148} but not \textalpha3\textsubscript{9–28}. Further groups of mice were immunized with \textalpha3(IV)NC1, the E\textsubscript{A} chimera, or the E\textsubscript{B} chimera and re-stimulated with chimeric and whole proteins. Mice immunized with \textalpha3(IV)NC1 responded to \textalpha3(IV)NC1 and to the E\textsubscript{B} chimera, but not to the E\textsubscript{A} chimera (Figure 4B). Immunization with the E\textsubscript{A} chimera did not induce recall responses, whereas E\textsubscript{B} chimera immunization induced recall responses.

Figure 1. Distribution of the \textalpha3(IV)NC1 T cell epitopes in HLA-DRB1*15:01 and HLA-DRB1*01:01 transgenic mice. Mice deficient in murine MHC II but possessing human DRB1*15:01 (1501 Tg) or DRB1*01:01 (0101 Tg) after immunization with either (A) peptide pools (with each pool encompassing a third of \textalpha3(IV)NC1, dotted lines) or (B) whole \textalpha3(IV)NC1. Reactive peptides are defined by re-stimulation of draining lymph node cells by ex vivo proliferation, IFN-\gamma ELISPOT, and IL-17A assays. Each dot represents the response from a mouse (n=5 per group). Numbers on the x axis represent the \textalpha3(IV)NC1 N-terminal number of a 20-mer. Several areas of reactivity are present, but one area of strong reactivity, \textalpha3\textsubscript{129–148} and \textalpha3\textsubscript{137–156} (corresponding to one of two epitopes found in humans with anti-GBM disease), is present only in 1501 Tg mice.
both to itself and to full-length human α3(IV)NC1. By 10 days, serum anti-human α3(IV)NC1 and anti-murine α3(IV)NC1 autoantibodies were detectable in mice immunized with either human α3(IV)NC1 or the EB chimera (Figure 4C).

The Core Immunogenic HLA-DRB1*15:01 Epitope Is α3136–146, with Four Critical Residues
We identified the core immunogenic region within α3133–152 by immunizing DRB1*15:01 Tg mice with α3133–152, and then stimulating draining lymph node cells at 10 days with shortened 16-mers. The 4 aa N-terminal end of α3133–152 (α3133–136) was required but the C-terminal 4 aa (α3149–152) was redundant (Figure 5A). Immunization with the 16-mer α3133–148 and re-stimulation with sequential 12-mers overlapping by 11 aa defined the nonredundant sequence DWVSLWKGSF (α3136–146) (Figure 5B). The critical residues within α3136–146 were delineated by immunizing DRB1*15:01 Tg mice with DWVSLWKGSF and re-stimulating lymphocytes with individual alanine substituted 11-mers, with each peptide having one residue substituted for alanine in sequential order. Valine138, tryptophan141, glycine143, and phenylalanine144 were most critical for responses (Figure 5C).

α3136–146–Specific CD4+ Cell Clones Induce Anti-GBM GN in DRB1*15:01 Tg Mice
To determine whether α3136–146–specific T cells mediate GN in the context of HLA-DRB1*15:01, α3136–146–specific IFN-γ–secreting CD4+ T cell clones were generated from DRB1*15:01 Tg mice and expanded in vitro. We transferred α3136–146–specific T cells into naïve DRB1*15:01 Tg mice, with 0.5 μg/g of LPS. Control mice received LPS and 107 CD4+ cells derived from a DRB1*15:01 Tg mouse immunized with Freund’s complete adjuvant alone. By 14 days, α3136–146–specific clone recipients had developed albuminuria (baseline, normal...
mice $0.01 \pm 0.00$, day 14 control cell recipients $0.01 \pm 0.00$, day 14
$\alpha_{3136-146}$-specific clone recipients $0.57 \pm 0.08 \text{ mg albumin/}\mu\text{mol creatinine; } P<0.001$, continuing until the end of experiments (35 days; Figure 6A). Analyses at 35 days showed GN only in the recipients of $\alpha_{3136-146}$-specific clones, with renal impairment (Figure 6B), necrotizing and crescentic GN (Figure 6, C–G), and features of delayed type hypersensitivity (DTH), including glomerular fibrin deposition (Figure 6, H–J) with CD4+ cells and macrophages in glomeruli (Figure 6, K–P). Glomerular neutrophil recruitment was not a feature (control cell recipients $0.01 \pm 0.0$, $\alpha_{3136-146}$-specific clone recipients $0.1 \pm 0.0 \text{ cells/glomerular cross-section}$). Glomerular pathology was prominent in recipients of $\alpha_{3136-146}$-specific clones, but tubulointerstitial infiltrates and disease were relatively modest.

$\alpha_{3133-152}$-Immunized HLA-DRB1*15:01 Tg Mice Develop Nephritogenic Autoimmunity

DRB1*15:01 Tg mice were immunized with $\alpha_{3161-180}$ (a control peptide, not shown to induce responses), $\alpha_{39-28}$ (which overlaps with the E3 region), or $\alpha_{3133-152}$. After 42 days, mice immunized with either control peptide or $\alpha_{39-28}$ did not develop lymphocyte proliferative responses to the immunogen (Figure 7A), and they did not develop albuminuria (Figure 7B). However, mice immunized with $\alpha_{3133-152}$ developed both reactivity to $\alpha_{3133-152}$ and significant albuminuria, but not renal impairment (Figure 7C). Glomerular changes were mild in all groups of mice with occasional proliferation and accumulation of periodic acid–Schiff (PAS)-positive material (Figure 7, D–F).

**Anti-GBM GN Is Induced by $\alpha_{3136-146}$ in DRB1*15:01, but not DRB1*01:01 Transgenic Mice**

Although mice do not easily develop severe autoimmune anti-GBM GN, the WKY rat is susceptible. This susceptibility is not MHC mediated, but several genetic elements have been identified, including FcγR abnormalities.23,24 We introduced an additional susceptibility element to MHCII−/− DRB1*15:01 Tg and DRB1*01:01 Tg mice by intercrossing them with mice that lack the inhibitory FcγR, FcγRIIb. After immunization of both strains with $\alpha_{3136-146}$, mice expressing DRB1*15:01 (mouse MHC−/−.DRB1*15:01 Tg, FcγRIIb−/− mice) developed measurable T cell autoimmunity to $\alpha_{3136-146}$ and to rma3(IV)NC1 at 10 days (Figure 8), but mice expressing DRB1*01:01 (mouse MHC−/−.DRB1*01:01 Tg, FcγRIIb−/− mice) did not. After repeated immunization, significant anti-GBM GN including albuminuria and renal impairment, with focal glomerular necrosis, crescent formation, fibrin deposition, and glomerular CD4+ cell and macrophage accumulation had developed in DRB1*15:01-expressing mice at 42 days, but glomeruli in immunized DRB1*01:01-expressing mice were
In DRB1*15:01-expressing mice, there were serum anti-a3(IV)NC1 autoantibodies and IgG deposition in glomeruli (Figure 10) that included both linear and granular components, as recently described. Although DRB1*01:01-expressing mice exhibited comparable serum anti-a3(IV)NC1 autoantibody titers to DRB1*15:01-expressing mice, IgG was not detected in glomeruli of a3(136–146)-immunized DRB1*01:01-expressing mice. Furthermore, mice expressing DRB1*15:01 that were immunized with full-length rmo3(IV)NC1 developed GN, but DRB1*01:01-expressing mice did not (Supplemental Figure 2).

**DISCUSSION**

HLA-DRB1*15:01 is a key susceptibility element in anti-GBM disease. By using mice expressing this risk allele or the protective HLA-DRB1*01:01 allele (but no mouse MHC II), we have defined an immunodominant CD4+ T cell epitope in α3(IV)NC1 (α3(136–146)) that also triggers autoantibody production. Importantly, α3(136–146)-specific CD4+ cells induce GN in naïve DRB1*15:01 mice and when additional FcyR-related susceptibility elements are introduced, active autoimmunity and disease is markedly enhanced.

Our initial comparative studies in DRB1*15:01 Tg, DRB1*01:01 Tg, and C57BL/6 mice showed several α3(IV)NC1 peptide sequences corresponding to reactivity in previous rat studies, and in some humans. However, only HLA-DRB1*01:01 Tg mice responded to one region spanning α3(129–156) (later shortened to α3(136–146)). Immunization with α3(IV)NC1 induced a similar, but somewhat more restricted, pattern of responses. The nonresponsiveness of HLA-DRB1*01:01 Tg mice was not a quirk of the DR1 allele, because C57BL/6 mice (I-Ab) were also nonresponsive to this peptide. Because this region overlaps a B cell epitope (Eβ), we used human α3/α1 chimeric domains. Findings were consistent with a dominant initial role for α3(136–146) because HLA-DRB1*01:01 Tg mice immunized with the Eβ chimera, but not the Eβ chimera, developed T and B cell responses similar to mice immunized with α3(IV)NC1. There is evidence for destructive processing within the α3(136–146) epitope, because the leucine140 to tryptophan141 (murine numbering) bond is cleaved by cathepsin D. However, that HLA-DRB1*01:01 Tg mice seem to present peptides derived from the Eβ chimera implies that destructive processing may not be a dominant tolerogenic mechanism in DRB1*15:01-positive individuals. It is unclear whether antigen-presenting cells have similar levels of processing enzymes and their inhibitors as other tissues, although inflammatory stimuli, such as LPS used in our clone transfer studies, can influence the net activity of these systems. The role of destructive processing in tolerance to α3(IV)NC1 requires further study.

We defined the 11 aa peptide α3(136–146) as the core immunogenic region, and identified the amino acids critical for immunoreactivity, with findings similar to a previous study.
The absence of $\alpha_{3,136}$ reduced ex vivo recall responses in the core region mapping experiments. However, $\alpha_{3,137-156}$ induced strong recall responses after immunization with either $\alpha_{3,137-156}$ or $\alpha_{3,129-148}$ suggesting that additional flanking residues on the carboxy-terminal end of $\alpha_{3,137-156}$ help to stabilize the peptide-DRB1*15:01 complex. Whereas HLA-DRB1*15:01 Tg mice express human MHC II, they express murine $\alpha_{3,IV}$NC1. However, the murine and human $\alpha_{3,133-152}$ sequences are well conserved, with only two differences between the cross-reactive human and murine $\alpha_{3,136-146}$ sequences. The murine aspartic acid at 136 is a glycine (not a critical amino acid) and valine at 138 is an isoleucine, a similar branched chain aa. The core sequence being $\alpha_{3,136-146}$ helps explain why responses occurred when mice were immunized with the E8 chimeric domain. Although the critical Goodpasture’s T cell epitope is $\alpha_{3,136-146}$, the E8 chimeric domain includes only the human $\alpha_{3,127-141}$ sequence (homologous to murine $\alpha_{3,128-142}$), with the $\alpha 1$ sequence comprising the remaining 4 aa. In contrast, in these C-terminal aa, the $\alpha 1$ and $\alpha 3$ sequences are similar ($\alpha 1$: GYSF, $\alpha 3$: GFSF), with the single difference being a tyrosine for phenylalanine, amino acids that possess very similar properties.

The $\alpha_{3,IV}$NC1 protein is present within the thymus, so one mechanism by which HLA-DRB1*0101 mice do not respond to $\alpha_{3,136-146}$ may be thymic deletion of CD4+ cells with high affinity to DR1-$\alpha_{3,136-146}$. However, peripheral tolerance is also important in Goodpasture’s disease, regulatory cells could also be relevant. Studies using a B cell transgenic mouse specific for a mouse $\alpha_{3,IV}$NC1 B cell epitope suggest that bone marrow expression of $\alpha_{3,IV}$NC1 (or a related protein) is relevant to B cell tolerance. The critical $\alpha_{3,136-146}$ epitope identified in our studies was not identified as an important T cell epitope in WKY rats. This may be due to CD4+ T cell thymic selection being guided by the binding of $\alpha 3$-derived peptides to the human anti-GBM disease susceptibility gene HLA-DRB1*15:01, not the MHC II RT.1Bl expressed by the WKY rat. The likely consequence of this is that because they were derived in the context of different MHC II molecules, the CD4+ T cell repertoires in the two rodents are different.

Whereas anti-GBM antibodies, especially when eluted from kidneys, are pathogenic in this autoimmune disease, cellular immunity also plays a role. We generated $\alpha_{3,136-146}$-specific CD4+ Th1 clones from HLA-DRB1*15:01 Tg mice and showed that transfer of clones to naïve HLA-DRB1*15:01 Tg mice induced necrotizing crescentic GN. Whereas T cell lines from an $\alpha_{3,IV}$NC1 immunized WKY rat have transferred disease in a previous study, these studies use a clone generated in mice containing the most relevant MHC II gene HLA-DRB1*15:01, not the MHC II RT.1Bl expressed by the WKY rat. The likely consequence of this is that because they were derived in the context of different MHC II molecules, the CD4+ T cell repertoires in the two rodents are different.

Whereas $\alpha_{3,IV}$NC1 is relevant to B cell tolerance, $\alpha_{3,IV}$NC1 B cell epitope identity as a mouse $\alpha_{3,IV}$NC1 B cell epitope suggests that bone marrow expression of $\alpha_{3,IV}$NC1 (or a related protein) is relevant to B cell tolerance. The critical $\alpha_{3,136-146}$ epitope identified in our studies was not identified as an important T cell epitope in WKY rats. This may be due to CD4+ T cell thymic selection being guided by the binding of $\alpha 3$-derived peptides to the human anti-GBM disease susceptibility gene HLA-DRB1*15:01, not the MHC II RT.1Bl expressed by the WKY rat. The likely consequence of this is that because they were derived in the context of different MHC II molecules, the CD4+ T cell repertoires in the two rodents are different.

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Our studies focused on defining the relevance of the \(\alpha_{3136-146}\) epitope detected only in HLA-DRB1*15:01 transgenic mice (the human susceptibility MHC II). However, there are other epitopes, including \(\alpha_{397-124}\) and \(\alpha_{3193-212}\) (found after immunization with peptide pools or whole \(\alpha_{3(IV)NC1}\)) and \(\alpha_{31-28}\) and \(\alpha_{365-92}\) (reactive after peptide pool but not \(\alpha_{3(IV)NC1}\) immunization), that could be nephritogenic. With the exception of \(\alpha_{39-28}\), tested due to its proximity to the EA region, which did not induce autoimmunity in HLA-DRB1*15:01 Tg mice, testing other possible epitopes in further studies could define their pathogenicity in the context of HLA-DRB1*15:01. Supplemental Table 2 contains a comparison of our studies with those of Cairns et al.\(^{12}\) in six humans with anti-GBM disease. Importantly, although there were notable similarities (and some differences), human peptides within the \(\alpha_{3129-156}\) region examined in our studies overlapped with the core epitope, bound with high affinity to DR15, and in the case of the human \(\alpha_{3(IV)NC1}\) IPPCPHHGWISLWKGFSFIMF sequence contained the entire core sequence and stimulated response \(\text{ex vivo}\) in all six patients tested. The identification of a key epitope paves the way for studies that examine the nature of the intra- and intermolecular epitope spreading seen in this disease.\(^{2,16,40,41}\)

Although autoimmune anti-GBM GN has been modeled in a number of animal species,\(^{5,6,42,43}\) the most commonly used model injects human \(\alpha_{3(IV)NC1}\) or rat \(\alpha_{3(IV)NC1}\) peptides into WKY rats\(^{7,15,16}\) that carry genetic susceptibilities rendering them vulnerable to various aspects of experimental GN.\(^{23,24}\) Although mice develop autoimmunity to \(\alpha_{3(IV)NC1}\) and may develop disease after approximately 10 weeks,\(^{9,39,44}\) severe disease in mice has been more difficult to establish. Immunization of HLA-DRB1*15:01 Tg mice with \(\alpha_{3136-146}\) broke tolerance to \(\alpha_{3(IV)NC1}\) and induced mild albuminuria, but \(\alpha_{39-28}\) immunization did not. Although HLA-DRB1*15:01 is an important susceptibility factor in Goodpasture’s disease, this allele is common in human populations, so other factors must also be involved in autoimmunity to \(\alpha_{3(IV)NC1}\). Because autoimmune diseases involve failures of multiple checkpoints,\(^{45}\) we bred another susceptibility element, deficiency of an inhibitory Fc\(\gamma\)R, Fc\(\gamma\)RIIb, into DRB1*15:01 Tg and DRB1*01:01 Tg mice. We chose Fc\(\gamma\)Rs because alterations in this system are associated with glomerular disease in rodents and humans.\(^{23,25,46,47}\) This additional susceptibility

glomeruli in \(\alpha_{3136-146}\)-specific clone show areas of segmental necrosis (C and F) and approximately 30% of glomeruli are affected by crescent formation (D and G), but recipients of naive CD4\(^+\) cells have normal histology (E). (H–J) Glomerular fibrin deposition is present only in mice that received the \(\alpha_{3136-146}\)-specific CD4\(^+\) clone. Within glomeruli, CD4\(^+\) cells (K–M) and macrophages (N–P) are present only in recipients of the \(\alpha_{3136-146}\)-specific CD4\(^+\) clone. Cells/gcs represents cells per glomerular cross-section, dotted lines represent mean values in normal mice (\(n=3\)), and arrows represent positively stained cells (black reaction product). *\(P<0.05\); ***\(P<0.001\) versus control cell recipients.

Figure 6. Transfer of CD4\(^+\) cells specific for \(\alpha_{3136-146}\) induces crescentic GN in HLA-DRB1*15:01 Tg mice. Naive DRB1*15:01 Tg mice are injected with 0.5 \(\mu\)g/g of LPS and either with 10\(^7\) CD4\(^+\) cells of a clone specific for \(\alpha_{3136-146}\) or nonspecific CD4\(^+\) cells derived from adjuvant immunized mice. Additional exogenous antigen or peptide is not administered. After 14 days, mice receiving the \(\alpha_{3136-146}\)-specific clone develop functional renal injury, manifested by pathologic albuminuria (A) and a raised serum BUN (B) (dotted lines represent values in normal mice). Mice receiving naive cells are not affected. The majority of glomeruli in \(\alpha_{3136-146}\)-specific clone show areas of segmental necrosis (C and F) and approximately 30% of glomeruli are affected by crescent formation (D and G), but recipients of naive CD4\(^+\) cells have normal histology (E). (H–J) Glomerular fibrin deposition is present only in mice that received the \(\alpha_{3136-146}\)-specific CD4\(^+\) clone. Within glomeruli, CD4\(^+\) cells (K–M) and macrophages (N–P) are present only in recipients of the \(\alpha_{3136-146}\)-specific CD4\(^+\) clone. Cells/gcs represents cells per glomerular cross-section, dotted lines represent mean values in normal mice (\(n=3\)), and arrows represent positively stained cells (black reaction product). *\(P<0.05\); ***\(P<0.001\) versus control cell recipients.
enhanced injury in HLA-DRB1*15:01–expressing mice after α3(IV)NC1 immunization, without promoting initial T cell autoimmune responses or injury in HLA-DRB1*01:01 Tg mice. By 42 days after α3,33-152 immunization, FcγRIIb-deficient HLA-DRB1*01:01 Tg mice did develop serum anti-α3(IV)NC1 antibodies (suggesting also some T cell autoreactivity), but these were not deposited in the kidney. Possible explanations for this observation include that the autoantibodies from 0101 Tg mice may have lower affinity, or different, non-pathogenic epitopes, or that the lack of cell-mediated injury in 0101 Tg mice means that native α3(IV)NC1 is not conformationally accessible to the anti-α3(IV)NC1 autoantibodies and binding does not occur. Previous studies have suggested that both are possible: certainly autoantibodies binding to the GBM eluted from kidneys are much more potent that those found in the serum,24 suggesting the presence of some anti-GBM antibodies that are nonpathogenic (or only marginally injurious). In addition, several reports have suggested that in vivo, injury to the GBM (by chemical [experimentally by reactive oxygen species]46 or immunologic [autoreactive T cells]26 means) may alter the structure of type IV collagen hexamers, exposing B cell α3(IV)NC1 epitopes, thus allowing and enhancing antibody binding.

Figure 7. Immunization of HLA-DRB1*15:01 Tg mice with α3,33–152 induces autoreactivity to α3(IV)NC1 but only mild disease. Groups of DRB1*15:01 Tg mice (n=4 per group) are immunized with one of three 20-mers: an irrelevant peptide from within α3(IV)NC1 (α3,33–152), a peptide from within the Hudson Eα epitope α3,28–28, or the α3,33–152 sequence. After 42 days, mice immunized with α3,33–152, but not the two other peptides, develop T cell reactivity to α3(IV)NC1 (A). All mice immunized with α3,33–152 develop albuminuria (B), indicating some renal injury, but mice immunized with the control peptide or α3,28–28 are unaffected. However, α3,33–152 does not induce renal impairment (C). There is no histologic evidence of glomerular disease in control peptide and α3,28–28 immunized mice (D and E), and alterations in glomerular histology induced by α3,33–152 are modest, with no areas of segmental necrosis or crescent formation (F). *P<0.05; ***P<0.001.

These studies used HLA humanized mice to identify a key pathogenic epitope in Goodpasture’s disease. This epitope is important in the initial autoreactivity to α3(IV)NC1 as well as GN. Our studies provide the basis for a mechanistic understanding of how the strongest genetic risk factor in anti-GBM disease confers risk, based on new humanized models of cell-mediated autoimmunity anti-GBM GN. These studies assist in moving toward more specific therapies by developing relevant preclinical models.

CONCISE METHODS

α3(IV)NC1 Peptides and Proteins

The murine α3(IV)NC1 peptide library used to determine immunoreactivity was synthesized as a PepSet (Mimotopes, Clayton, Australia). There were 28 peptides synthesized according to the published sequence.49 Each peptide was 20 aa long and overlapped by 12 aa (Supplemental Table 1). Control peptide, OVA323–339, was used in some experiments. Individual peptide immunization and re-stimulation assays were performed with 11–20 aa >90% pure peptides by HPLC (Mimotopes or AusPep, West Melbourne, Australia), confirmed for some peptides by further HPLC analysis at Monash University. Recombinant murine α3(IV)NC1 was generated using a baculovirus system as previously described.22 Recombinant FLAG-tagged human α1(IV)NC1, α3(IV)NC1, α2(IV)NC1, and α3/α1(IV)NC1 chimeric proteins were expressed in HEK 293 cells as previously described.11

Mice

Mouse MHC class II−/−, HLA-DRB1*15:01 Tg mice, and mouse MHC class II−/−, HLA-DRB1*01:01 Tg mice were generated by intercrossing mouse MHC class II−/−, HLA DRB1*15:01 Tg mice50 with mouse MHC class II−/−, HLA DRB1*01:01 Tg mice.51 The expression of mouse MHC class II was screened using Alexa Fluor 488
Figure 9. The T cell Goodpasture epitope induces anti–GBM GN in mice with human HLA-DRB1*15:01. DRB1*15:01 Tg or DRB1*01:01 Tg mice deficient in FcγRIIb (1501 Tg FcγRIIb−/− and 0101 Tg FcγRIIb−/−, respectively) are immunized (n=8 per group) with α3383-146. After 42 days, 1501 Tg FcγRIIb−/− mice immunized with α3383-146 develop pathologic albuminuria (A) and a raised BUN (B), but α3383-146 immunized 0101 Tg FcγRIIb−/− mice, or nonimmunized mice (n=8 per group) of either strain, have normal urinary albumin and normal renal function. Similarly, only α3383-146 immunized 1501 Tg FcγRIIb−/− mice show glomeruli with areas of segmental necrosis (C), crescent formation (D), or glomerular fibrin deposition (E). (F–I) Representative PAS-stained glomeruli (N) and macrophages (O) are present only in α3383-146 immunized 1501 Tg FcγRIIb−/− mice. Results are representative of two independent experiments. ***P<0.001.
5\times10^5 \text{cells/well}, [\text{H}]\text{-thymidine}, (0.5 \mu\text{Ci/well}, \text{PerkinElmer, Glen Waverley, Australia}), was added to culture for the last 16 hours and results expressed as a stimulation index (SI). To control for the potential immunogenicity of the FLAG component within the recombinant proteins in re-stimulation proliferation assays, mice were re-stimulated with α2(IV)/NC1 with and without the FLAG component (re-stimulation with media alone or α2(IV)/NC1 without FLAG both showed an SI of 1).

**T Cell Clone Transfer Model**

α3_{36–146}-specific CD4+ clones were generated by immunizing DRB1*15:01 Tg mice with 10 μg of α3_{36–146} subcutaneously in Freund’s complete adjuvant. Draining LN cells were harvested after 10 days, and then cultured at a concentration of 3\times10^6 cells/well in 6-well plates with 10 μg/ml of α3_{36–146} in supplemented RPMI media. Well isolated colonies of proliferating cells were identified by light microscopy and by repeated micromanipulation. Single cells were transferred into individual wells in a 96-well plate with 10^6 spleenocytes/well from naïve DRB1*15:01 Tg mice. Splenocytes were CD4⁺ depleted (by mouse CD4 [L3T4] MACS Microbeads with LD columns; Miltenyi Biotec, North Ryde, Australia) and mitomycin C (Kyowa Hakko Kogyo, Tokyo, Japan) treated (5\times10^6 cells/ml with 50 μg/ml mitomycin C in PBS for 30 minutes at 37°C). Cells were cultured in supplemented RPMI media with 50 U/ml recombinant murine IL-2 (Jomar Bioscience, Kensington, Australia) and 50 μg/ml α3_{36–146} changed weekly, and cell viability assessed at 4 weeks by \[^{[3]}\text{H}\]-thymidine proliferation assay. Viable clones were expanded in 24-well plates with weekly media changes, fortnightly division and addition of CD4⁺ depleted, mitomycin C-treated splenocytes. The T cell clone used for experiments was selected based on consistent IFN-γ secretion. As control, nonspecific CD4⁺ T cells from a mouse immunized with Freund’s complete adjuvant were selected and expanded in vitro by the same method. The TCR Vβ expression was screened by flow cytometry using the Mouse Vβ TCR Screening Panel (BD Biosciences). The TCR Vα expression was screened by flow cytometry using anti-Vα2, 3.2 and 8 specific antibodies (Biolegend).

In a pilot experiment (n=4 per group), recipient DRB1*15:01 Tg mice received 10^7 clones intravenously, 100 μg of α3_{36–146} subcutaneously in Freund’s complete adjuvant, and 0.5 μg/g of LPS (Sigma) intraperitoneally, and renal disease was assessed 2 weeks later. Recipient DRB1*15:01 Tg mice that received α3_{36–146}-specific clones developed GN as well as dermal DTH, measured by the change in footpad thickness 24 hours after injecting 10 μg of rmα3(IV)NC1 in the footpad. In the second experiment (Figure 6), recipient DRB1*15:01 Tg mice received 10^7 clones intravenously and 0.5 μg/g of LPS on day 0 and were killed 5 weeks later.

**Murine Model of Induced Autoimmune Anti-GBM GN**

Mice were immunized with 100 μg of peptide or rmα3(IV)NC1 subcutaneously on days 0, 7, and 14; first in Freund’s complete, and then Freund’s incomplete adjuvant. Mice were killed on day 42.

**Assessment of Renal Injury and Immune Cell Infiltration**

Glomerular necrosis and crescent formation were assessed on 3 μm thick, PAS-stained, formalin fixed, periodate-lysine-paraformaldehyde-fixed, frozen kidney sections. The primary mAbs used were GK1.5 (anti-murine CD4; American Type Culture Collection, Manassas, Virginia), FA/11 (macrophages, anti-murine CD68; from Dr. Gordon Montgomery, TX) and expressed as milligrams per micromoles of creatinine. BUN was measured using standard laboratory methods. CD4⁺ T cells, macrophages, and neutrophils were detected by immunoperoxidase staining of 6 μm thick, periodate lysine paraformaldehyde-fixed, frozen kidney sections. The primary mAbs used were GK1.5 (anti-murine CD4; American Type Culture Collection, Manassas, Virginia), FA/11 (macrophages, anti-murine CD68; from Dr. Gordon
Detection of Anti-α3(IV)NC1–Specific IgG

ELISA plates were coated with 5 μg/ml of recombinant human or murine α3(IV)NC1, and then blocked with 2% casein in PBS. Mouse serum diluted 1:200 was added in duplicate and incubated for 1 hour followed by HRP conjugated sheep anti-murine IgG antibody. Images were captured using a Nikon C1 confocal laser (488 nm) attached to a Nikon Ti-E inverted microscope.

Statistical Analyses

Where there were ≥3 groups, one-way ANOVA followed by Tukey’s post test was used to access differences. Where there were only two groups, a t test was used. Means and SEM are shown.

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DISCLOSURES

None.

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