IL-23, not IL-12, Directs Autoimmunity to the Goodpasture Antigen


Centre for Inflammatory Diseases, Monash University Department of Medicine, Monash Medical Centre, Clayton, Victoria, Australia

ABSTRACT

The autoantigen in Goodpasture disease is the noncollagenous domain of \( \alpha 3(IV) \) type IV collagen \( \alpha 3(IV)NC1 \). We previously demonstrated that IL-12p40\(-/-\) mice are protected from experimental autoimmune anti–glomerular basement membrane (anti-GBM) glomerulonephritis, seemingly defining a role for IL-12 in this disease; however, the recent identification of IL-23, a heterodimer composed of IL-12p40 and IL-23p19 subunits, raises the possibility that IL-23, rather than IL-12, may modulate this disease instead. We immunized wild-type, IL-12p35\(-/-\) (IL-12 deficient, IL-23 intact), IL-12p40\(-/-\) (deficient in both IL-12 and IL-23), and IL-23p19\(-/-\) (IL-12 intact, IL-23 deficient) mice with recombinant mouse \( \alpha 3(IV)NC1 \). Wild-type mice developed autoreactivity to \( \alpha 3(IV)NC1 \): Humoral responses, cellular responses, renal histologic abnormalities, leukocyte accumulation, autoantibody deposition, and IL-17A mRNA expression (a cytokine produced by the IL-23–maintained Th17 subset). IL-23 but not IL-12 was detected in the immune system. Regardless of the presence of IL-12, mice deficient in IL-23 were protected, but mice with IL-23 were not. Both IL-23–deficient strains exhibited lower autoantibody titers, reduced cellular reactivity, diminished cytokine production (IFN-\( \gamma \) [Th1], IL-17A [Th17], TNF, and monocyte chemoattractant protein 1), and less renal disease and glomerular IgG deposition. The deficient responses in the absence of IL-23 were not due to increased regulatory T cells; IL-12p40\(-/-\) and IL-23p19\(-/-\) mice did not show increased proportions of CD4\(^+\)CD25\(^+\)FoxP3\(^+\) cells or IL-10 levels early in the immune response. In conclusion, autoreactivity to the Goodpasture antigen is directed primarily by IL-23, absence of which results in hyporeactivity including but extending beyond a deficient Th17 response.


Some forms of glomerulonephritis are caused by immune responses against autoantigens that are dependent on a CD4\(^+\) autoimmune response. Arguably the best characterized antigen involved in glomerulonephritis is the Goodpasture antigen, the noncollagenous domain of the \( \alpha 3 \) chain of type IV collagen \( \alpha 3(IV)NC1 \). Loss of tolerance in humans to \( \alpha 3(IV)NC1 \) results in anti–glomerular basement membrane (anti-GBM) glomerulonephritis. Substantial evidence exists for the involvement of both cellular and humoral effectors directed against \( \alpha 3(IV)NC1 \). The detection of anti-GBM antibodies is required for diagnosis. Antibodies binding to the GBM can activate complement and recruit macrophages and neutrophils. Passive transfer of anti-GBM antibodies can induce disease in monkeys, rats, and mice. Patients with anti-GBM also exhibit the effectors of delayed-type hypersensitivity (DTH), infiltrating CD4\(^+\) cells and macrophages in glomeruli together with promi

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Correspondence: Dr. Richard Kitching, Centre for Inflammatory Diseases, Monash University Department of Medicine, Monash Medical Centre, 246 Clayton Road, Clayton, VIC 3168, Australia. Phone: 61-3-9994-5550, Fax: 61-3-9994-6495; E-mail: richard.kitching@med.monash.edu.au

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nant fibrin deposition. Effector T cells can induce injury in experimental autoimmune anti-GBM glomerulonephritis. CD4+ T helper (Th) cells tend to be polarized into subsets characterized by the cytokines that they produce. IFN-γ is the signature cytokine of Th1 cells, IL-4 is the signature cytokine of Th2 cells, and IL-17A is the signature cytokine of Th17 cells. Th cells help determine patterns of the effector immune responses, patterns that aid in host defense but also play a key role in inflammatory tissue damage. Th1 responses classically result in CD4+ T cell/macrophage mediated cellular responses and drive IgG subclass switching toward complement-fixing and macrophage-recruiting subclasses. Th2 responses promote IgE-mediated humoral responses. A more recently described subset, Th17, acts against extracellular pathogens, and there is increasing evidence that it drives organ-specific autoimmunity.

A number of factors underpin the differentiation and maintenance of Th cell subsets, but the cytokine milieu during differentiation and effector CD4+ cell expansion is particularly important. IL-12, a heterodimer composed of IL-12p40 and IL-12p35 subunits, is the key cytokine in Th1 differentiation. Studies using neutralizing antibodies and IL-12p40−/− mice, including studies in experimental glomerulonephritis, seemed to define an important role for IL-12 in both autoimmune and nonautoimmune glomerulonephritis; however, the subsequent discovery of IL-23, a newer IL-12 family member that is also a heterodimer (composed of IL-12p40 and IL-23p19 subunits), prompted a reevaluation of studies that used IL-12p40−/− mice and anti–IL-12p40 antibodies. Other lines of evidence have defined IL-23 as important for the maintenance of the Th17 subset and implicated Th17 in the pathogenesis of organ-specific autoimmune disease.

We previously showed that in experimental autoimmune anti-GBM glomerulonephritis, IL-12p40−/− mice are protected from disease but IFN-γ−/− mice develop worse injury. With the knowledge of the structure of IL-12 and IL-23, together with the recent delineation of the Th17 subset, we sought to define definitively the individual and collective roles of the IL-12 family members IL-12 and IL-23 in the genesis, maintenance, and pattern of autoimmune responses to the Goodpasture antigen. C57BL/6 mice were immunized with recombinant mouse (rm) α3(IV)NC1. Our hypothesis was that IL-23 would drive pathogenetic Th17 responses and mice that were deficient in IL-23, by virtue of lacking either IL-23p19 or IL-12p40, would develop a selective defect in Th17 responses and less glomerulonephritis.

RESULTS

Cellular Immune Responses to α3(IV)NC1 Are IL-23 Dependent

Cellular immune responses were measured 19 d after immunizing mice with rmα3(IV)NC1 in Freund’s complete adjuvant (FCA) early in the autoimmune response to α3(IV)NC1 and at 7 mo after first immunization, when autoactivity is well established and histologic evidence of renal disease is present. Nineteen days after immunization, responses were reduced in the absence of endogenous IL-23 (in either IL-12p40−/− mice or IL-23p19−/− mice). The total number of cells recovered from the same five draining inguinal lymph nodes was decreased in IL-12p40−/− and IL-23p19−/− mice but not in mice deficient only for IL-12 (IL-12p35−/−; wild-type [WT] 5.8 ± 0.4 × 105 cells, IL-12p40−/− 3.1 ± 0.2, IL-12p35−/− 6.0 ± 0.6, IL-23p19−/− 3.4 ± 0.4; P < 0.01 versus WT). Proportions of CD4+ cells were not different (proportion of CD4+ cells within mononuclear cell population, WT 22.3 ± 1.5%, IL-12p40−/− 21.9 ± 0.7, IL-12p35−/− 22.6 ± 2.2, and IL-23p19−/− 21.4 ± 0.4). The proportions of CD4+ cells proliferating (by bromodeoxyuridine [BrdU] incorporation) or expressing the early activation marker CD69 were reduced in IL-12p40−/− and IL-23p19−/− mice compared with WT mice (Figure 1, A and B). This reduction in proliferation was also significant when compared with IL-12p35−/− mice. DTH responses to subcutaneous α3(IV)NC1 injection were reduced in IL-12p40−/− and IL-23p19−/− mice at 19 d compared with either WT or IL-12p35−/− mice, and reductions were sustained when immune responses were more established (Figure 1C); therefore, cellular and cell-mediated responses to the Goodpasture antigen are impaired in the absence of both IL-12 and IL-23 or in the absence of IL-23 alone but not in the absence of IL-12 alone.

Substantial Amounts of IL-23 but not IL-12 Are Produced by Immune Cells in Response to α3(IV)NC1

To determine whether IL-12 and/or IL-23 is secreted during the immune response, IL-12 and IL-23 were measured in supernatants from antigen-stimulated splenocytes from individual mice. IL-23 was easily detected in WT and IL-12p35−/− mice (Figure 1D), both early and during the established immune response; however IL-12 was not detected in any mouse (detection limit of ELISA 31 pg/ml) at either time point. As expected, IL-23 was not detected in IL-23p19−/− mice; produced by immune cells in response to α3(IV)NC1.

Th Cell Subset Cytokines and Proinflammatory Mediators Were Decreased in the Absence of IL-23

The Th1, Th17, and Th2 signature cytokines IFN-γ, IL-17A, and IL-4, respectively (Figure 2, A through C), were measured in antigen-stimulated splenocyte supernatants. IFN-γ production, as expected, was reduced in IL-12p35−/− mice, given IL-12’s role in Th1 responses; however, the reduction in IFN-γ was more substantial in IL-12p40−/− mice (which share the inability to produce heterodimeric IL-12p70 with IL-12p35−/−) and in IL-23p19−/− mice (deficient only in IL-23, not IL-12). These reductions persisted at a later time point. Both strains of mice deficient in IL-23 produced less IL-17A throughout disease when compared with WT mice, whereas...
Endogenous IL-23 Stimulates Humoral Responses and Anti-α3(IV)NC1 Autoantibody Production

Humoral responses to the Goodpasture antigen were determined by measuring CD19⁺ B cell proliferation and activation and titers of α3(IV)NC1-specific IgG, IgG1, IgG2b, and IgG3. Proportions of B cells (draining lymph nodes) in IL-12p40−/− and IL-23p19−/− mice at day 19 were similar (proportion of CD19⁺ cells within mononuclear cell population, WT 39.8 ± 1.1%, IL-12p40−/− 37.9 ± 0.9%, IL-12p35−/− 38.2 ± 0.6%, and IL-23p19−/− 41.2 ± 1.5%). In vivo CD19⁺ cell proliferation and activation (CD69 expression) after α3(IV)NC1 immunization were reduced in IL-12p40−/− and IL-23p19−/− mice when compared with WT and IL-12p35−/− mice (Figure 3, A and B). Furthermore, α3(IV)NC1-specific IgG titers were reduced in both IL-12p40−/− and IL-23p19−/− mice when compared with WT and IL-12p35−/− mice, but titers in IL-12p35−/− mice were similar to those in WT mice (Figure 3C). The hyporeactive humoral response in IL-12p40−/− and IL-23p19−/− mice persisted at 7 mo (Figure 3, D and E).

IL-12p35−/− mice displayed similar levels of IL-17A secretion (Figure 2B). All four groups of mice produced similar amounts of IL-4 early in the autoimmune response, but IL-4 production was reduced in IL-12p40−/− and IL-23p19−/− mice at the later time point, consistent with the development of a nonselective deficit in T cell cytokine production (Figure 2C). The proinflammatory/Th17 cytokine TNF and the chemokine monocyte chemoattractant protein 1 were also reduced in both IL-12p40−/− and IL-23p19−/− mice but not in IL-12p35−/− mice when compared with WT mice (Figure 2D), measured early in the immune response.
Histologic Renal Disease Is Attenuated in the Absence of IL-12p40 or IL-23p19

WT mice developed renal disease characterized by relatively mild glomerular and interstitial abnormalities (Figure 4, A and E) and linear Ig staining (GBM and tubular basement membrane; Figure 4, I and M). Quantifying these abnormalities in WT mice at 7 mo showed a mean of approximately 30% abnormal glomeruli (Figure 5A) together with modest interstitial infiltrates (Figure 5B), glomerular leukocytes (Table 1), and interstitial leukocyte infiltration (Table 2). Illustrative glomerular abnormalities in WT and IL-12p35−/− mice are shown in Supplemental Figure 8. The absence of endogenous IL-12 alone (in IL-12p35−/− mice) did not lessen disease (Figure 4, C, G, K and O). Glomerular findings, including histology (Figures 4C and 5A) and Ig (Figures 4K and 5C) were unchanged, although there were reduced numbers of CD8+ cells in glomeruli (Table 1); however, mice deficient in IL-23 (either both IL-12 and IL-23: IL-12p40−/− mice or IL-23p19−/− mice) were significantly protected compared with WT and IL-12p35−/− mice (Figure 4, B, E, F, H, J, L, N, and P). There were fewer abnormal glomeruli; less glomerular Ig; and fewer glomerular CD4+ cells, CD8+ cells, and macrophages. Neutrophil numbers were not significantly reduced. A similar protective pattern was evident in the interstitium of IL-12p40−/− or IL-23p19−/− mice except that an apparent reduction in interstitial infiltrate of IL-23p19−/− mice did not reach significance compared with WT mice (but was significant when compared with IL-12p35−/− mice; P < 0.001; Figure 5B). Intrarenal IL-17A mRNA expression was detectable in all eight WT mice, all six IL-12p35−/− mice, but only two of six IL-12p40−/− mice and one of five IL-23p19−/− mice (Figure 5D). IL-12p40−/− and IL-23p19−/− mice expressed significantly less IL-17A mRNA than IL-12p35−/− mice, but the apparent reduction compared with WT mice did not reach statistical significance. Mice did not develop abnormal proteinuria or elevated serum creatinine levels (data not shown).

Hyporeactivity to a3(IV)NC1 in the Absence of IL-23 Is not Due to Enhanced T Regulatory Cells

Th17 cell responses have been reciprocally linked to regulatory T cells (Tregs). To determine whether a3(IV)NC1 hyporeactivity observed in the absence of IL-23 was due to the upregulation of Tregs, we assessed the proportion of CD4+CD25+FoxP3+ cells in the draining lymph nodes 19 d after immunization. The proportion of CD4+CD25+FoxP3+ was not increased in IL-12p40−/− or IL-23p19−/− mice (Figure 6A), suggesting that the hyporeactivity observed in these mice was not due to increased Tregs. IL-12p40 and IL-10 may play reciprocal roles in the induction of immune responses (and IL-10 is a soluble product of some Tregs), but, as with most other cytokines, IL-10 levels were reduced in IL-12p40−/− and IL-23p19−/− mice (Figure 6B). We measured production of key cytokines [day 19, a3(IV)NC1-stimulated splenocytes] responsible for Th17 cells and Treg induction (TGF-β alone promotes Tregs, whereas IL-6 and TGF-β act

Antigen-specific IgG and IgG1, IgG2b, and IgG3 titers were significantly reduced in IL-12p40−/− and IL-23p19−/− mice compared with both WT and IL-12p35−/− mice, providing further evidence for nonselective effects of IL-12p40 or IL-23p19 deficiency on autoreactivity to a3(IV)NC1. Later in the immune response, IL-12p35−/− mice demonstrated a modest reduction in a3(IV)NC1-specific IgG and IgG2b but similar levels of IgG1 and IgG3 compared with WT mice.

Figure 3. Humoral responses and anti-a3(IV)NC1 autoantibody production. (A and B) a3(IV)NC1-specific CD19+ proliferation (A) and activation (B) were decreased in IL-12p40−/− and in IL-23p19−/− mice compared with WT or IL-12p35−/− mice. A and B show both representative FACS plots from single animals and a graph that summarizes data from all animals. Numbers within the FACS plot quadrants represent percentage of all cells analyzed. Numbers in top right quadrants represent the percentage of CD19+ cells that were either BrdU+ or CD69+. (C) Early a3(IV)NC1-specific IgG autoantibody titers were reduced in IL-12p40−/− and in IL-23p19−/− mice when compared with WT or IL-12p35−/− mice (sera dilution 1:2000). (D and E) The reduction in antibody titers persisted during established disease (D) and included a3(IV)NC1-specific IgG1 (1:10,000), IgG2b (1:10,000), and IgG3 (1:250) titers (E). (D) IgG and IgG2b titers were modestly reduced in IL-12p35−/− mice during established disease. *P < 0.05, **P < 0.01, ***P < 0.001 versus WT. P values for IL-12p40−/− and IL-23p19−/− mice are compared with WT and IL-12p35−/− mice.
together in Th17 differentiation.\textsuperscript{20} WT mice and IL-12p35\textsuperscript{−/−} mice made both TGF-β and IL-6, in similar proportions. Whereas both TGF-β and IL-6 production was reduced in IL-12p40\textsuperscript{−/−} and IL-23p19\textsuperscript{−/−} mice (in IL-12p40\textsuperscript{−/−} mice TGF-β was undetectable) (Figure 6, C and D), there were no changes that would favor the induction of Treg cells. In addition, the expression of FoxP3 mRNA in the renal draining lymph node 19 d after immunization was unchanged between the groups (Figure 6E).

**Alterations in Immune Responses to α3(IV)NC1 Reflect Antigen-Specific Differences**

Total Ig titers were similar between groups (Supplemental Figure 7A). Using the same immunization protocol as the 19-d α3(IV)NC1 studies but using ovalbumin as a model foreign antigen, immune responses (CD4\textsuperscript{+}CD69\textsuperscript{+} cells, dermal DTH responses, and ovalbumin-specific IgG titers) were reduced in the absence of IL-12, IL-23, or both IL-12 and IL-23 (Supplemental Figure 7, B through D). The reduced immune response to ovalbumin in the absence of IL-12p35, not seen in α3(IV)NC1 studies, demonstrates that results in α3(IV)NC1 are due to reactivity to α3(IV)NC1. To ensure that autoreactivity was to the Goodpasture antigen and not to a possible impurity in the insect cell-derived rmα3(IV)NC1 immunogen, serum IgG responses in rmα3(IV)NC1-immunized mice were measured against an alternatively prepared rhα3(IV)NC1 (a gift from Prof. Billy Hudson; Vanderbilt University, Nashville, TN). Reactivity to this rhα3(IV)NC1, prepared with a FLAG tag in HEK 293 cells, were similar to results when rmα3(IV)NC1 was used (Figure 3C and Supplemental Figure 7E).

**DISCUSSION**

In these studies, we aimed to determine the role of IL-12 and IL-23 (IL-12 family cytokines with a common IL-12p40 chain) in autoimmune responses against a well-defined renal autoantigen, α3(IV)NC1. The different roles for IL-12 and IL-23 in
The intensity of glomerular Ig staining was reduced in IL-12p40-deficient mice compared with WT or IL-12p35–/– mice. IL-23p19−/− mice had moderately reduced interstitial infiltrate (P = 0.05) compared with WT mice, which was significantly reduced compared with IL-12p35–/– mice (P < 0.001). (C) The intensity of glomerular Ig staining was reduced in IL-12p40−/− and IL-23p19−/− mice compared with WT or IL-12p35–/– mice. (D) Intrarenal IL-17A mRNA was reduced in IL-12p40−/− and IL-23p19−/− mice compared with IL-12p35–/– mice. **P < 0.01, ***P < 0.001 versus WT; †P < 0.01 versus IL-12p35–/–. Unless otherwise stated, P values for IL-12 p40−/− and IL-23p19−/− mice are true when compared with both WT and IL-12p35–/– mice.

Figure 5. Renal disease in mice with autoimmune anti-GBM glomerulonephritis. (A) Glomerular abnormalities were reduced in IL-12p40−/− and IL-23p19−/− mice compared with WT or IL-12p35–/– mice. (B) Interstitial infiltrate was reduced in IL-12p40−/− mice when compared with WT and IL-12p35–/– (P < 0.001) mice and increased in IL-12p35–/– mice compared with WT mice. IL-23p19−/− mice had moderately reduced interstitial infiltrate (P = 0.05) compared with WT mice, which was significantly reduced compared with IL-12p35–/– mice (P < 0.001). (C) The intensity of glomerular Ig staining was reduced in IL-12p40−/− and IL-23p19−/− mice compared with WT or IL-12p35–/– mice. (D) Intrarenal IL-17A mRNA was reduced in IL-12p40−/− and IL-23p19−/− mice compared with IL-12p35–/– mice. **P < 0.01, ***P < 0.001 versus WT; †P < 0.01 versus IL-12p35–/–. Unless otherwise stated, P values for IL-12 p40−/− and IL-23p19−/− mice are true when compared with both WT and IL-12p35–/– mice.

The induction/maintenance of Th1 and Th17 responses,21 together with data from other forms of experimental autoimmune disease,22–24 led us to hypothesize that IL-23 plays a significant role and selectively induces Th17 responses. These studies show that autoreactivity to α3(IV)NC1 is driven by IL-23, because (1) IL-23 but not IL-12 was detectable in the immune system in the course of the autoimmune response; (2) mice deficient in any of the subunits of IL-23, IL-23p19, or IL-12p40 (but not mice deficient in IL-12p35) developed substantially less autoreactivity to α3(IV)NC1; and (3) this reduced reactivity translated into less histopathology (although renal disease is relatively mild in WT mice in this model, in contrast to typical Goodpasture disease in humans). Although our data show that IL-23 does drive autoimmunity to α3(IV)NC1, we found a nonselective and substantial deficit in almost all immune response parameters measured. We had hypothesized that T cell proliferation would be diminished and that IL-17A production would be decreased in the absence of IL-12p40 or IL-23p19. In the absence of IL-23 (or IL-12 and IL-23) but not IL-12, our studies showed reduced IL-17A production systemically and reduced IL-17A mRNA expression in the kidney, demonstrating an impaired Th17 response; however, there was more widespread hyporeactivity to α3(IV)NC1 in the absence of IL-23, including decreased B cell activation and proliferation, diminished autoantibody titers, less cytokine production, and fewer regulatory cells. This translated into diminished renal disease, autoantibody deposition, and leukocyte infiltration (excluding neutrophils).

The cellular autoimmune response against α3(IV)NC1, including T cell proliferation and activation; production of Th1, Th2, and Th17 cytokines; and DTH to α3(IV)NC1, was impaired in the absence of both IL-12 and IL-23 and of IL-23 alone (IL-4 production was similar early but impaired later). In the absence of IL-12 alone, IFN-γ was decreased, but other responses remained intact, demonstrating that IL-23 and not IL-12 is a key to α3(IV)NC1-specific cellular responses. The absence of IL-23 resulted in diminished B cell activation and anti-α3(IV)NC1 autoantibody production. Whereas humoral responses to T cell–dependent foreign antigens are impaired in IL-23p19−/− mice,25 little is known about IL-23 in autoantibody production. This study demonstrates that IL-23 promotes autoantibody production. All antigen-specific IgG subclass titers were reduced, most pronounced in the IgG2b and IgG3, subclasses that (especially IgG3), when compared with IgG1, have a greater capacity to recruit macrophages at sites of injury. In the absence of IL-12 alone, total IgG responses were modestly reduced in the established immune response.

CD4+CD25+FoxP3+ Tregs are important in maintaining peripheral tolerance in autoimmune disease.26,27 There is evidence for their involvement in human Goodpasture disease.28 T cells from patients with Goodpasture disease produce IFN-γ28,29 and produce IL-10 in remission.29 When CD25+ cells are depleted ex vivo, IFN-γ production increases. These data suggest a role for T cell reactivity in disease and a role for Tregs and IL-10 in remission. In human anti-GBM disease, little is known as to whether IL-23 plays any role, although, if it is valid to extrapolate studies in other human autoimmune diseases to anti-GBM disease, then it may be important.30–33 The generalized hyporeactivity to α3(IV)NC1 in IL-12p40− and IL-23p19−/−-deficient mice, together with studies linking the development of Th17 responses reciprocally with impaired Treg responses,34 led us to examine Tregs. We found no

Table 1. Leukocytes in glomeruli of mice with autoimmune anti-GBM glomerulonephritis

<table>
<thead>
<tr>
<th>Parameter</th>
<th>CD4+ T Cells</th>
<th>CD8+ T Cells</th>
<th>Macrophages</th>
<th>Neutrophils</th>
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<td>C57 WT</td>
<td>2.40 ± 0.02</td>
<td>1.74 ± 0.21</td>
<td>2.81 ± 0.39</td>
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<td>IL-12p40−/−</td>
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<tr>
<td>IL-12p35−/−</td>
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<td>1.12 ± 0.19</td>
<td>3.10 ± 0.51</td>
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<tr>
<td>IL-12p19−/−</td>
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<td>0.82 ± 0.14b</td>
<td>0.84 ± 0.09b</td>
<td>0.86 ± 0.05</td>
</tr>
</tbody>
</table>

*Data are expressed as number of cells per 10 glomeruli (minimum of 50 glomeruli per mouse), compared with WT mice.

bP < 0.001.

cP < 0.01.
IL-12p19 in IL-12p40 through D) The Treg-associated cytokine IL-10 was not increased in the context of autoreactivity to α3(IV)NC1, IL-23, apart from maintaining the Th17 cell subset, has a more general effect on immune responses.

In murine autoimmune anti-GBM glomerulonephritis, variable degrees of severity of renal disease have been reported using different immunization protocols, different immunogens, and different mouse strains. Murine autoimmune anti-GBM to date has usually been induced by a heterologous form of α3(IV)NC1, for example, bovine α3/α5 dimers or recombinant human α3(IV)NC1. In these models, autoimmunity to mouse α3(IV)NC1 is induced initially by responses against a xenogeneic autoantigen with a very similar protein structure. In these studies, mice developed only a relatively mild form of renal disease but did develop easily measurable autoimmunity induced by affinity-purified rmα3(IV)NC1 and directed against mouse α3(IV)NC1. The mechanisms by which modulation of the autoreactive process limits the severity of end-organ disease in the mouse remain to be determined. It is not known whether exactly the same results would be obtained using IL-23p19−/− deficient mouse strains with different MHC genes.

In most parameters, IL-12p35−/− were similar to WT mice, although stimulated splenocytes made less IFN-γ, and later in the immune response, there was a modest reduction in serum anti-α3(IV)NC1 titers and fewer glomerular and interstitial CD8+ T cells. We found only modest reductions in IFN-γ in IL-12p35−/− mice. Although IL-12 has been considered critical for IFN-γ production, not all studies have shown marked impairment of IFN-γ production in IL-12p35−/− mice. Conclusions from these studies that IL-12 is not relevant to autoimmunity to α3(IV)NC1 should be tempered by the recent report of IL-12p35 as one chain of the newly described cytokine IL-35, thought to be important in regulatory cell function. Therefore, IL-12p35−/− mice are likely to be deficient in both IL-12 and IL-35. These data plus our previous published data in a similar model using IFN-γ−/− mice that developed worse disease17 (less in IL-12p40−/− mice) confirm that there is no significant role for IFN-γ in experimental autoimmune glomerulonephritis. This is in contrast to glomerulonephritis induced by an immune response against a planted foreign antigen, where IFN-γ is important, as are Th1 responses, because mice lacking T-bet (the Th1-defining transcription factor) are protected.

In summary, the IL-12 family member IL-23, an IL-12p40
and IL-23p19 heterodimer, plays an important role in α3(IV)NC1 autoreactivity that goes beyond a selective Th17 deficiency; however, the IL-12 heterodimer (IL-12p40 and IL-12p35 subunits) that defines Th1 responses is of limited importance. The results of these studies suggest that IL-12p40 might be a therapeutic target worth pursuing in the treatment of several forms of glomerulonephritis. IL-12p40 seems to hold a central position as both a component of IL-12, important in Th1 responses that are important in some forms of glomerulonephritis, and part of IL-23, a key cytokine in the induction and maintenance of autoimmune responses.

**CONCISE METHODS**

**Experimental Design**

Autoimmunity to mouse α3(IV)NC1 was studied in C57BL/6 mice (Monash University Animal Services, Clayton, Victoria, Australia), IL-12p40−/− mice, IL-12p35−/− mice, backcrossed 10 generations with C57BL/6 mice (Jackson Laboratories, Bar Harbor, ME; bred at Monash University), and IL-23p19−/− mice backcrossed using speed congenics and are >95% C57BL/6 (generated at Genentech by Drs. de Sauvage and Ghilardi; bred at Monash University). Autoimmune responses in 8-wk-old mice were induced by subcutaneous immunization at day 19 to study the immune responses in the early phase (n = 5 per group) or at day 14, day 28, and at 14 d before killing at 7 mo (WT, n = 8; IL-12p40−/− and IL-12p35−/−, n = 6; IL-23p19−/−, n = 5) to study immune responses and renal injury during the established phase of disease. For studying immune responses to ovalbumin, mice (WT, IL-12p40−/−, IL-12p35−/−, and IL-23p19−/−, n = 4 each group) were immunized and boosted with 25 µg of ovalbumin in FCA (Sigma-Aldrich, St. Louis, MO) using an identical protocol to that of disease. For measurement of cytokine production, splenocytes (4 × 106 cells/ml per well) were cultured in RPMI/10% FCS with α3(IV)NC1 (10 µg/ml; 48 h at 37°C). IFN-γ and IL-4 in splenocyte supernatants were measured by ELISA, as described previously. IL-17A, TGF-β1, IL-12, and IL-23 were measured by ELISA: IL-17A and TGF-β1 (R&D Systems, Minneapolis, MN) and IL-12 and IL-23 (eBioscience) per the manufacturers’ protocols. IL-6, IL-10, monocyte chemotactic protein 1, IFN-γ, TNF, and IL-12p70 were also measured by flow cytometry using a cytometric bead array mouse inflammation kit (BD Biosciences), as per the manufacturer’s protocol.

**Circulating Antibody Titers**

Circulating serum α3(IV)NC1-specific IgG titers were assessed by ELISA using hors eradish peroxidase–conjugated sheep anti-mouse IgG (1:2000; Amersham Biosciences, Rydalmere, Australia) and goat anti-mouse IgG1, IgG2b, and IgG3 antibodies (1:2000; Southern Bio-technology Assoc., Birmingham, AL). Total Ig titers were measured as described previously.

**Assessment of Renal Disease and Leukocyte Accumulation**

Glomerular abnormalities and renal tubulointerstitial injury were assessed on periodic acid-Schiff–stained, Bouin’s-fixed, 3-µm-thick, snap-frozen tissue sections using coded slides. The proportion of glomeruli affected was determined by examination of a minimum of 50 glomeruli per mouse for abnormalities according to a previously published method. Abnormalities included segmental proliferation, capillary wall thickening, glomerular hypercellularity, periglomerular cell infiltrates, and occasional crescent formation. Tubulointerstitial infiltrates were assessed on a minimum of 15 medium-power fields. A score of 1 was given when a peripheral, interstitial, or peritubular infiltrate was observed. Renal histology was compared with age- and gender-matched nonimmunized mice. Linear Ig staining of the GBM was assessed on 6-µm-thick, snap-frozen tissue sections using FITC–sheep anti-mouse Ig (1:100; Silenus, Hawthorn, Victoria, Australia) antibodies. A minimum of 40 glomeruli were scored 0 to 3+ according to intensity. CD4+ T cells, CD8+ T cells, macrophages, and neutrophils were demonstrated by immunoperoxidase staining of 6-µm-thick, snap-frozen tissue sections using coded slides.

The expression of CD4+, CD25+, and FoxP3+ . Antibodies (from BD Biosciences, North Ryde, Australia, unless otherwise stated) used were allophycocyanin-Cy7–conjugated anti-CD4, phycoerythrin (PE)-conjugated anti-CD4, FITC-conjugated anti-CD4, PE-conjugated anti-CD19, Allophycocyanin-conjugated anti-CD19, FITC-conjugated anti-Brdu with DNase, PE-conjugated anti-CD69, anti–CD25–FITC, and anti-mouse FoxP3 (eBioscience, San Diego, CA). Propidium iodide–positive cells were excluded from analyses. Flow cytometric analyses were performed on a BD FACScan to flow cytometer (BD Biosciences).
thick, periodate lysine paraformaldehyde-fixed, frozen kidney sections. The primary mAbs used were GK1.5 for CD4⁺ T cells (American Type Culture Collection, Manassas, VA), 53-6.7 for CD8⁺ T cells (American Type Culture Collection), FA/11 for macrophages (anti-mouse CD68; from Dr. Gordon L. Koch, MRC Laboratory of Molecular Biology, Cambridge, England), and RB6-8C5 for neutrophils (anti-Gr-1; DNAX Research Institute, Palo Alto, CA). A minimum of 50 consecutive glomeruli were viewed, and results were expressed as percent of 10 glomerular cross-sections. Proteinuria and serum creatinine levels were measured as described previously.38

Reverse Transcriptase–PCR for IL-17A and FoxP3 mRNA Expression
RNA extracted from kidney or renal nodes was reverse-transcribed to produce cDNA as described previously.46 Analysis of renal IL-17A expression was conducted using TaqMan gene expression assays mM00439618_m1 (IL-17A) and Hs99999901_s1 (ribosomal 18S; Applied Biosystems, Foster City, CA). Reactions were performed using the TaqMan universal PCR master mix (Applied Biosystems). Analysis of FoxP3 expression in the renal node was done using gene-specific primers for FoxP3 (forward primer cgccaatccctctggactct; reverse primer ttggctggcctagggttg) and 18S (forward primer gtaacccgttgaac- ccctcc; reverse primer gcctcaactatactacactaagc) using Power SYBR Green PCR master mix (Applied Biosystems), using a Rotor Gene RG-3000 (Corbett Research, Mortlake, New South Wales, Australia). IL-17A and FoxP3 expression was normalized with the reference gene 18S and expressed relative to the WT group as a fold change.

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DISCLOSURES
None.

REFERENCES
22. Cua DJ, Sherlock J, Chen Y, Murphy CA, Joyce B, Seymour B, Lucian

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