Anti-myeloperoxidase nephritogenic autoimmunity induces severe glomerulonephritis. To assess the therapeutic potential of monoclonal antibodies targeting T helper (Th) subset differentiation determining cytokines, we studied a murine model of anti-myeloperoxidase glomerulonephritis. The temporal participation of T helper subsets was determined by quantitating gene expression of CD4<sup>+</sup> T-cells isolated from nephritic kidneys and cytokine production by lymphocytes from nodes draining myeloperoxidase immunization sites. Th17 cytokines (IL-17A and IL-6) rose rapidly but declined as autoimmunity matured when Th1 cytokines (IL-12 and TNF) predominated. Therefore, T helper subset participation in anti-myeloperoxidase autoimmunity is biphasic, with Th17 early and Th1 late. To confirm the functional relevance of this biphasic pattern, we compared systemic anti-myeloperoxidase autoimmunity in wild type, Th17 deficient and Th1 deficient mice. Early, Th1 deficient mice developed similar autoimmunity and glomerulonephritis to wild type mice. However, Th17 deficient mice had significantly reduced anti-myeloperoxidase autoimmunity. In late autoimmunity, Th1 deficient mice developed reduced autoimmunity and were protected from anti-myeloperoxidase glomerulonephritis. The therapeutic potential of these findings were demonstrated by neutralizing monoclonal antibodies. Targeting IL-23p19 attenuated early Th17 dominated anti-myeloperoxidase autoimmunity and glomerulonephritis but not late phase disease. Targeting IL-12p35 attenuated late phase Th1 dominated anti-myeloperoxidase autoimmunity and glomerulonephritis but not early autoimmunity or glomerulonephritis. Targeting both T helper subsets with an anti-IL-12p40 monoclonal antibody was effective during both early and late phases of anti-myeloperoxidase glomerulonephritis. Thus, definition of dominant T helper differentiating subsets in anti-myeloperoxidase glomerulonephritis by renal CD4<sup>+</sup> T-cell cytokine gene expression allows effective proper phase monoclonal antibody treatment of anti-myeloperoxidase glomerulonephritis.


KEYWORDS: ANCA; autoimmune disease; biologicals; chronic kidney disease; focal segmental glomerulonephritis; glomerulonephritis; myeloperoxidase

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Translational Statement

Autoimmune anti-myeloperoxidase (MPO) glomerulonephritis is a major cause of chronic kidney disease, and current therapies are nonspecific and highly toxic. Renal injury in this disease is initiated by autoimmunity to MPO and mediated by cytokines. This study explored the phenotypic and temporal patterns of signature T helper cell 17 (Th17) and T helper cell 1 (Th1) cytokines in draining lymph nodes of MPO immunization sites, the pattern of cellular effectors, and the gene profile of CD4<sup>+</sup> T cells isolated from nephritic kidneys. It demonstrated that the development of anti-MPO autoimmunity is biphasic, with Th17 early and Th1 late. Administration of anti-cytokine biologicals targeting key Th17 and Th1 differentiating cytokines—interleukin-23 p19 subunit and interleukin-12 p35 subunit, respectively—confirms that therapeutic efficacy is dependent on identifying the dominant Th cell—differentiating pathways driving anti-MPO autoimmunity.

Antineutrophil cytoplasmic antibody (ANCA)–associated vasculitis (AAV) is the major cause of rapidly progressive crescentic glomerulonephritis (GN). Current treatments are nonspecific with poor safety profiles (despite treatment, after 5 years 30% of patients are dead or
on dialysis). In many autoimmune diseases, there is increasing successful adoption of biological strategies that target injurious immune pathways, as biologicals offer more specificity and lower toxicity than does conventional immunosuppression. However, the application of biologicals to AAV has been slow and limited. Currently, there is insufficient knowledge of the immunopathogenesis of myeloperoxidase (MPO)–AAV to confidently plan a clinical cytokine intervention trial. The primary autoantigen-driving nephritogenic autoimmunity is MPO and that proteinase 3 has been well defined. Experimental MPO-AAV models have confirmed a high level of homology between nephritogenic immunodominant MPO T-cell peptides and human MPO residues recognized by patients with active disease. Much of our understanding of the pathogenic role of both cellular and humoral autoimmunity in MPO-AAV comes from animal studies.

As CD4+ T cells play a central role in the development of both humoral and cellular autoimmunity, defining critical cytokine targets in the development and maintenance of anti-MPO autoimmunity is essential for the application of novel biological therapies that target cytokines. A critical step in the development of autoimmunity is proliferation and differentiation of antigen-specific CD4+ T cells into specific Th helper (Th) cell subsets, which differ in their effector phenotype and function. CD4+ Th cell differentiation and maturation is directed by a limited number of lineage-specific cytokines, which make Th cell–lineage-specific cytokines attractive therapeutic targets. Immunoneutralization of individual cytokines has shown their nonredundant importance in Th subset differentiation and maintenance. In humans, the limited information as to which Th subsets participate in the generation and maintenance of MPO autoimmunity in MPO-AAV suggests the participation of T helper cell 1 (Th1) and/or T helper cell 17 (Th17). Several articles have reported Th1 and Th17 signature cytokines in peripheral blood mononuclear cells from patients with AAV. Analysis of biopsies of patients with MPO-AAV have found interleukin-17A (IL-17A)—producing cells. The presence of renal interferon-γ (IFN-γ)–producing cells has not yet been assessed, although MPO-stimulated peripheral blood mononuclear cells from patients with AAV have been reported to produce IFN-γ. Moreover, monocytes/macrophages (the major Th1 effector leukocytes) are the dominant leukocytes in glomeruli and interstitium of patients with severe MPO-AAV. These data suggest that the key Th subsets in human MPO-AAV are Th1 and/or Th17. The major cytokines that direct and maintain Th17 and Th1 responses are IL-23 and IL-12, with the effector cytokines IFN-γ and IL-17A considered the signature cytokines for Th1 and Th17, respectively.

We have developed a murine model of MPO-AAV in which induced nephritogenic anti-MPO autoimmunity results in the same pattern of GN (pauci-immune focal segmental necrotizing GN) that occurs in humans. The nephritogenic-dominant peptide in this model is MPO peptide 409–428 (MPO409–428). This bears close homology with the dominant MPO peptide recognized by patients with active disease. Moreover, in mice with established nephritogenic anti-MPO autoimmunity, treatment with nasal insufflation of MPO409–428 induces tolerance. The strategy used in this study was first to define the timing and relative participation of Th17 and Th1 subsets in directing nephritogenic MPO autoimmunity and its induced GN. We then compared the development of anti-MPO autoimmunity and GN in wild-type (WT) mice, Th17-deficient (interleukin-23 p19 subunit [IL-23p19]−/−) mice, or Th1-deficient (interleukin-12 p35 subunit [IL-12p35]−/−) mice. We then used monoclonal antibodies (mAbs) to demonstrate the therapeutic potential of targeting the confirmed Th cell–lineage-specific cytokines, IL-23 and/or IL-12.

**RESULTS**

**Determination of Th17 and Th1 dominance by cytokine profiling**

The temporal patterns of anti-MPO Th17 and Th1 subset dominance during the development of induced autoimmunity was determined by assessing signature Th effector cytokines in draining lymph node (LN) cells from MPO immunization sites, stimulated ex vivo by MPO. This was done at 2 time points: early (day 20) and when autoimmunity was established (day 32). Early in the development of anti-MPO autoimmunity, Th17 cytokines—IL-17A and IL-6—were dominant but production of these cytokines declined as the anti-MPO autoimmune response matured. Conversely, the concentrations of Th1 effector cytokines—IFN-γ and tumor necrosis factor (TNF)—increased to be the dominant effector response as the autoimmune response matured (Figure 1a–d).

GN can be induced in mice with established anti-MPO autoimmunity by planting MPO in the kidney using a subnephritic dose of anti–glomerular basement membrane (GBM) globulin, resulting in an initial wave of glomerular neutrophil influx within the first 2 to 4 hours and the subsequent deposition of the target antigen MPO. GN was triggered at days 18 and 28 in the development of anti-MPO autoimmunity during early and established time points, respectively. As autoimmunity became established, the intensity of glomerular damage increased with increased frequency of segmental necrosis, glomerular leukocyte infiltration, and increasing albuminuria. No difference in blood urea nitrogen was observed between groups (Figure 1e and h). In early anti-MPO autoimmunity, neutrophils, the major Th17 effector leukocytes, were the dominant glomerular leukocytes, whereas in established anti-MPO autoimmunity, macrophages (Th1 effectors) and T cells dominated (Figure 1i). These changes in macrophage and neutrophil influx support early Th17 and late Th1 effector cell dominance. Intrarenal CD4+ T-cell mRNA analyses also showed a biphasic Th subset response, with expression of Th17–specific genes (Cxcl6, Il17a, and Tgfb1) being greater than that of Th1–specific genes (Stat4, Cxcr3, and Ifng) at an early time point (day 20), while Th1 genes became increasingly expressed as the autoimmune response matured (Figure 1j).
These data suggest that the development of nephritogenic anti-MPO autoimmunity is biphasic, with an initial Th17 dominance followed by Th1 dominance as the autoimmune response matures.

The nature of extended kidney injury was observed by triggering GN during the Th17-dominant anti-MPO autoimmunity phase (day 16) and assessing GN on day 32 using a non-nephritogenic mouse anti-mouse GBM globulin.
At day 32, compared to MPO-immunized mice receiving OVA-8D1, planting MPO 409-428 in glomeruli using MPO-8D1 enhanced glomerular injury with increased proportions of infiltrating glomerular cell–mediated effector cells (macrophages and CD4+ T cells) and enhanced anti-MPO–specific IFN-γ responses. These experiments demonstrate that kidney injury at day 32 during established anti-MPO autoimmunity and GN is Th1 predominant (Supplementary Figure S1).

Figure 2 | Development of early (day 20) anti-myeloperoxidase (MPO) autoimmunity in wild-type (WT), interleukin-23 p19 subunit deficient (IL-23p19−/−), and interleukin-12 p35 subunit deficient (IL-12p35−/−) mice. Compared with WT mice (n = 7), IL-23p19−/− mice (n = 9) were protected from the development of a systemic anti-MPO autoimmune response with reduced MPO–specific delayed-type hypersensitivity (DTH) footpad swelling (a) and reduced MPO-reactive interferon-γ (IFN-γ)–producing (b) and interleukin-17A (IL-17A)–producing (c) draining lymph node cells. No difference in serum MPO–antineutrophil cytoplasmic antibody (ANCA) IgG production was observed between groups (d). IL-12p35−/− mice (n = 7) developed similar anti-MPO autoimmunity (e–h) and glomerular damage (i–k) to WT mice (n = 6). In established anti-MPO autoimmunity (day 32), IL-12p35−/− mice had reduced DTH (i) and MPO–specific IFN-γ production by draining lymph node cells (j). The production of IL-17A was reduced in both IL-23p19−/− (n = 8) and IL-12p35−/− (n = 7) mice compared with WT mice (n = 6) (k). No difference in MPO-ANCA IgG production was observed between groups (l). Error bars represent mean ± SEM, with statistical analysis performed using the unpaired t test (a–h) and 1-way analysis of variance (WT vs. IL-12p35−/− vs. IL-23p19−/− mice) (i–l).

*P < 0.05, **P < 0.01, ***P < 0.001. OD450nm, optical density 450nm.

(clone 8D1)6,19,20 conjugated to MPO409-428 (MPO-8D1). At day 32, compared to MPO-immunized mice receiving OVA-8D1, planting MPO409-428 in glomeruli using MPO-8D1 enhanced glomerular injury with increased proportions of infiltrating glomerular cell–mediated effector cells.
Biphasic evolution of Th17 and Th1 subsets in anti-MPO autoimmunity: studies using IL-23p19−/− and IL-12p35−/− mice

When anti-MPO GN was triggered at the early time point, compared with WT mice, IL-23p19−/− mice developed less MPO-specific delayed-type hypersensitivity (DTH) and a lower frequency of MPO-responsive IFN-γ and IL-17A-producing lymphocytes from draining LNs (Figure 2a–c). Serum MPO-ANCA IgG production was similar in both groups (Figure 2d). Conversely, IL-12p35−/− mice developed anti-MPO autoimmune responses consistently similar to that found in WT mice (Figure 2e–h). These observations of developing anti-MPO autoimmunity in mice deficient in Th17 or Th1 subset strongly suggest that Th17 cells dominate during early developing anti-MPO autoimmunity.

Established anti-MPO autoimmunity was assessed on day 32 in WT, IL-23p19−/−, and IL-12p35−/− mice. Compared with WT mice, IL-12p35−/− mice exhibited significant reductions in all the Th1 components of anti-MPO autoimmunity (DTH and IFN-γ) and IL-17A responses from MPO

Figure 3 | Glomerulonephritis induced during early (day 20) and established (day 32) anti-myeloperoxidase (MPO) autoimmunity in interleukin-12 p35 subunit deficient (IL-12p35−/−) and wild-type (WT) mice. At day 20, no difference in glomerular injury between WT (n = 6) and IL-12p35−/− (n = 8) mice was observed 4 days after triggering glomerulonephritis (a–c). At day 32, IL-12p35−/− mice (n = 7) had glomerulonephritis with decreased CD4+ and neutrophil influx (d) and reduced frequency of segmental necrosis (e) and albuminuria (f) compared with WT mice (n = 6). Error bars represent mean ± SEM, with statistical analysis performed using the unpaired t test. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. c/gcs, cells per glomerular cross section; Mφ, macrophage; PMN, neutrophil.
challenged draining LN cells. However, IL-23p19−/− mice had intact Th1 anti-MPO autoimmunity. Both IL-12p35−/− and IL-23p19−/− mice produced significantly reduced IL-17A and developed similar humoral autoimmunity to that found in WT mice (Figure 2i–l). These observations strongly suggest that the dominant Th subset in established anti-MPO autoimmunity is Th1.

Functional role of Th1 in anti-MPO GN: studies using IL-12p35−/− mice

The development of GN induced during early developing anti-MPO autoimmunity in IL-12p35−/− mice was not reduced, consistent with the dominant role of Th17 autoimmunity at an early time point (Figure 3a–c). Whereas in the phase of established anti-MPO autoimmunity, GN was significantly reduced as observed by reduced glomerular leukocyte recruitment, attenuated frequency of glomerular segmental necrosis, and reduced albuminuria (Figure 3d–f). Thus, the study of anti-MPO GN in mice with genetic deficiency in Th1 capacity confirmed that the development of established Th1 anti-MPO autoimmunity and GN are dependent on the nonredundant role of IL-12.

The use of IL-23p19−/− mice for the assessment of the development of GN was confounded by our observation that IL-23p19−/− mice have significantly reduced capacity to recruit neutrophils to inflamed glomeruli (Supplementary Figure S2A). In the absence of glomerular neutrophil deposition of MPO, GN does not occur in this model.21 IL-23p19−/− mice with both early and established anti-MPO autoimmunity did not develop GN (Supplementary Figure S2C–H). To assess the capacity of Th17 autoimmunity to induce GN, we used mAb neutralization of IL-23p19.

Neutralizing IL-23p19 and IL-12p35 during early phase anti-MPO GN

The capacity of mAbs specific for IL-23p19 and IL-12p35 to attenuate early anti-MPO autoimmunity and its associated GN was assessed in vivo. Unlike IL-12p19−/− mice, anti–IL-23p19 mAb did not induce impairment of neutrophil recruitment induced by either anti-GBM globulin (Supplementary Figure S2B) or early, Th17-dominant, induced anti-MPO autoimmunity, allowing assessment of the effect of neutralization of IL-23 not only on anti-MPO autoimmunity but also on GN.

Anti–IL-23p19 and anti–IL-12p35 mAb were administered to WT mice, with early developing anti-MPO autoimmunity 1 day before GN being triggered and the severity of GN and intensity of systemic anti-MPO autoimmunity assessed on day 20. Neutralization of IL-23 significantly reduced systemic IL-17A, IFN-γ, and DTH. No difference was observed in serum MPO-ANCA IgG production between groups. The diminution of the adaptive anti-MPO Th17 immune response was associated with attenuation of kidney injury compared to untreated WT mice with GN (Figure 4a–g). On the contrary, although therapeutic administration of anti–IL-12p35 mAb reduced systemic IFN-γ production by draining LN cells, treatment did not reduce either IL-17A responses or DTH responses. Surprisingly, anti–IL-12p35 mAb treatment significantly reduced serum MPO-ANCA IgG. However, no attenuation of GN was observed (Figure 4h–n). These results are consistent with Th17 dominance at this time point and demonstrate the therapeutic benefit of neutralization of IL-23 at this time point. The inability of anti–IL-12p35 mAb therapy to attenuate GN is consistent with Th17 dominance.

Neutralizing IL-23p19 and IL-12p35 during established anti-MPO autoimmunity

Anti–IL-12p35 mAb significantly reduced DTH and humoral anti-MPO autoimmunity. Its use also reduced glomerular leukocyte infiltration, glomerular segmental necrosis, and albuminuria (Figure 5a–e). Given that IL-23p19−/− mice with established anti-MPO autoimmunity were protected from the development of GN at day 32 (because of their inability to recruit glomerular neutrophils), we used anti–IL-23p19 mAb (which does not affect glomerular neutrophil localization) to confirm that at day 32, Th17 responses are not able to induce GN. Inhibition of anti-MPO Th17 responses using anti–IL-23p19 mAb during mature autoimmunity did not alter systemic anti-MPO autoimmunity nor attenuate renal damage (Figure 5g–k). The lack of the effect of anti–IL-23p19 mAb on GN in this context confirms that mature anti-MPO autoimmune responses are mediated by Th1 cells and that only blocking Th1 responses using anti–IL-12p35 mAb is beneficial at this late time point.

Neutralizing both Th17 and Th1 anti-MPO autoimmunity attenuates both early and established anti-MPO GN

Anti–interleukin-12 p40 subunit (IL-12p40) mAb binds the common p40 chain of both IL-12 and IL-23, thus limiting differentiation and interrupting the maintenance of both Th1 and Th17 subsets, potentially attenuating GN at both early and late time points.

In early phase anti-MPO autoimmunity, anti–IL-12p40 mAb was administered 1 day before inducing GN. Compared to untreated MPO-immunized mice, anti–IL-12p40 mAb attenuated GN with reduced albuminuria, glomerular leukocyte accumulation, and segmental necrosis. No reduction in MPO-specific DTH or IFN-γ production was observed between groups, but fewer IL-17A–producing cells were observed. Anti–IL-12p40 mAb did not reduce MPO-ANCA IgG titers (Figure 6a–g). These observations are consistent with Th17-dominant anti-MPO autoimmunity early and confirm that anti–IL-12p40 mAb attenuates both Th17 CD4+ cellular anti-MPO autoimmunity and effector responses inducing GN.

Neutralizing IL-12 1 day before triggering GN during established anti-MPO autoimmunity significantly reduced glomerular effector leukocyte accumulation and segmental necrosis, although albuminuria was not affected (Figure 6h–j). Treatment diminished MPO-specific DTH and was associated with reduced IFN-γ–producing draining LN cells. No
Monoclonal antibody therapy during early developing anti-MPO GN, day 20

Figure 4 | Wild-type (WT) mice with early (day 20) or established (day 32) anti-myeloperoxidase (MPO) autoimmunity were treated with anti–interleukin-23 p19 subunit (IL-23p19) monoclonal antibody (mAb) or anti–interleukin-12 p35 subunit (IL-12p35) mAb 1 day before triggering glomerulonephritis (GN) and assessed 5 days later. Administration of anti–IL-23p19 mAb (n = 10) on day 15 significantly decreased MPO-specific interleukin-17A (IL-17A)–producing (a) and interferon-γ (IFN-γ)–producing (b) draining lymph node cells and delayed-type hypersensitivity (DTH) responses (c) compared with IgG2b-treated controls (Ctrls; n = 10), while no difference in serum MPO–antineutrophil cytoplasmic antibody (ANCA) IgG production was observed between groups (d). Albuminuria was significantly attenuated (e) as was glomerular leukocyte influx (f) and glomerular segmental necrosis (g) (with representative photomicrographs of glomerular segmental necrosis on periodic acid–Schiff [PAS]–stained kidney sections, original magnification ×400; bar = 20 μm). Neutralization of IL-12 (n = 6) with anti–IL-12p35 mAb reduced IFN-γ production from lymph nodes (h) and did not reduce the amount of IL-17A (i) or anti-MPO–specific DTH footpad swelling (j) compared to rat IgG Ctrl (n = 5). However, serum MPO-ANCA IgG production was significantly reduced compared to Ctrl (k). Comparable functional renal injury (albuminuria) (l) and structural glomerular damage (glomerular leukocyte influx and segmental necrosis) (m,n) was observed between groups. PAS-stained micrographs were taken at original magnification ×400. Bar = 20 μm. Error bars represent mean ± SEM, with statistical analysis performed using the unpaired t test. *P < 0.05, ***P < 0.001. c/gcs, cells per glomerular cross section; OD450nm, optical density 450nm. To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.
Monoclonal antibody therapy during established anti-MPO GN, day 32

a) DTH (Δmm) for Anti-IL-12/35 mAb

b) MPO-ANCA IgG (OD$_{	ext{450nm}}$) for Ctrl and anti-p35

c) Glomerular leukocytes (c/gps) for CD4⁺, Mφ, PMN

Ctrl and anti-p35

f) DTH (Δmm) for Anti-IL-23p19 mAb

g) MPO-ANCA IgG (OD$_{	ext{450nm}}$) for Ctrl, anti-p19

h) Glomerular leukocytes for T cells, Mφ, PMN

i) Ctrl and anti-p19

j) Glomerular segmental necrosis (%) for Ctrl and anti-p19

Albuminuria (µg/24 h) for Ctrl and anti-p19
difference in the numbers of IL-17A–producing cells or serum MPO-ANCA IgG was observed between groups (Figure 6k–n). These observations are consistent with dominance of Th1 in established anti-MPO autoimmunity. Collectively, these studies show that therapeutic administration of anti–IL-12p40 mAb during either early developing or established anti-MPO autoimmunity is effective.

**DISCUSSION**

Biological interventions to neutralize key cytokine-directed pathways in autoimmune AAV are potentially more selective and safer than conventional immunosuppressive drugs. There has been little progress in developing these biological treatments in AAV. Little is known about the cytokine-directed pathways involved in initiating and maintaining nephritogenic anti-MPO autoimmunity. Development of autoimmune responses involves antigen-specific CD4+ T-cell proliferation and differentiation into specific cytokine-directed pathways that produce different effector Th subsets. Most established autoimmune responses have a limited number of dominant Th subsets induced by a limited number of nonredundant “signature” effector cytokines. Defining the dominant Th subset in AAV would reveal the key cytokine(s) driving the autoimmune response and thus suggest potential therapeutic targets. In human MPO-AAV, the dominant Th subset is unclear, but the available data suggest the involvement of both Th17 and Th1 cells. In the present study, we used a murine model of induced anti-MPO autoimmunity that shares many similar immunological pathophysiological features with human disease.

In C57BL/6 mice with induced anti-MPO autoimmunity, we studied the developmental evolution of anti-MPO autoimmunity early (day 20 after MPO immunization) and when this autoimmunity was established (day 32). Th subsets were identified by the cytokine profile of induced MPO-specific CD4+ T cells, the pattern of cellular effectors, and the gene profile of CD4+ T cells isolated from nephritic kidneys. These observational studies revealed that (i) Th17 cytokines and gene signatures are prominent early and then decline, (ii) Th1 cytokines are dominant as maturity is established, (iii) patterns of glomerular effectors show initial neutrophil prominence early but DTH predominance later, (iv) GN becomes progressively more severe as Th1 becomes dominant, and (v) renal CD4+ T-cell gene profiling suggests Th17 dominance early and then Th1 dominance as MPO autoimmunity matures.

This biphasic participation of Th17 and Th1 reflects the importance of neutrophils and Th17 as early responders. Th1 development mediates “delayed”-type hypersensitivity, which becomes dominant if foreign or autoantigen persists. This pattern of biphasic participation also occurs in another form of crescentic GN—anti-GBM GN—with initial Th17 dominance followed by Th1 dominance.24,25

To define the relative temporal and functional importance of Th17 and Th1 subsets in MPO-AAV, we used IL-23p19−/− and IL-12p35−/− mice, which are incapable of directing the differentiation and proliferation of injurious Th17 and Th1 responses, respectively. Triggering GN in IL-23p19−/− and IL-12p35−/− mice with early and established anti-MPO–induced autoimmunity could potentially demonstrate the relative functional role of these cytokines in GN. This was feasible in IL-12p35−/− mice. However, although IL-23p19−/− mice have normal capacity to develop anti-MPO–induced autoimmunity, they lack the capacity to recruit neutrophils to glomeruli to trigger GN. They are therefore not useful to assess the development of GN in mice with either early or established anti-MPO autoimmunity. During early developing anti-MPO autoimmunity, IL-23p19−/− mice had significant reduction in Th17 anti-MPO autoimmunity. However, during established disease, IL-12p35−/− mice were protected from Th1 anti-MPO autoimmunity. These observations are consistent with Th17 subset dominance in the early development of anti-MPO autoimmunity and Th1 subset dominance in established autoimmunity. This suggests the nonredundant roles of IL-23 and IL-12 in directing Th17 and Th1 anti-MPO autoimmunity, respectively. This also suggests that these Th subset differentiation-inducing cytokines would play key roles in developing and directing GN.

The translational focus of this research was to define the dominant Th subsets, to determine their differentiation-defining cytokine(s), and then to assess the therapeutic effect of neutralizing mAbs targeting these cytokines in both early and established anti-MPO autoimmunity and GN. We used validated neutralizing mAbs targeting IL-23p19 and IL-12p35 as potential therapeutics in mice with early developing and established anti-MPO autoimmunity to treat GN. GN induced in mice with early developing anti-MPO autoimmunity treated with anti–IL-23p19 mAb showed significant reduction in anti-MPO autoimmunity (without the confounding effect on glomerular neutrophil recruitment seen in IL-23p19−/− mice) and were protected from GN. However, at this time point, anti–IL-12p35 mAb treatment was ineffective.

**Figure 5** | Established anti-myeloperoxidase (MPO) autoimmunity and glomerulonephritis (GN) was significantly reduced by neutralizing the T helper cell 1 (Th1) subset using anti–interleukin-12 p35 subunit (IL-12p35) monoclonal antibody (mAb). MPO-specific delayed-type hypersensitivity (DTH) swelling was significantly reduced (n = 7) compared with control-treated mice (Controls; n = 7) (a). Anti–IL-12p35 mAb significantly reduced serum MPO–antineutrophil cytoplasmic antibody (ANCA) IgG (b), glomerular accumulation of all effector leukocytes (CD4+ T cells, macrophages, and neutrophils) (c), glomerular segmental necrosis (d), and albuminuria (e) compared to Controls. The administration of anti–IL-12p35 mAb (n = 7) during established anti-MPO autoimmunity to neutralize the Th17 subset did not reduce systemic anti-MPO autoimmunity (f,g) or renal injury (h–j), and all measures were similar to Controls (n = 7). Periodic acid–Schiff-stained micrographs were taken at original magnification ×400. Bar = 20 μm. Error bars represent mean ± SEM, with statistical analysis performed using the unpaired t test. *P < 0.05, **P < 0.01, c/gcs, cells per glomerular cross section; OD400nm optical density 450nm. To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.
Figure 6 | Therapeutic administration of anti-interleukin-12 p40 subunit (IL-12p40) monoclonal antibody (mAb) to concomitantly neutralize T helper cell 17/T helper cell 1 subsets during early and established anti-myeloperoxidase (MPO) glomerulonephritis (GN). During early anti-MPO GN, the administration of anti-IL-12p40 mAb 1 day before inducing GN (n = 6) significantly reduced renal injury: albuminuria (a), glomerular leukocyte accumulation (b), and glomerular segmental necrosis, with representative photomicrographs of periodic acid–Schiff (PAS)–stained glomeruli (c) compared with control-treated mice (Ctrls; n = 7). Anti-IL-12p40 mAb treatment had no effect on MPO–specific delayed-type hypersensitivity (DTH) responses and IFNγ responses (d,e), while IL-17A production was significantly reduced (f) and serum MPO-antineutrophil cytoplasmic antibody (ANCA) IgG (g) was significantly elevated compared to control-treated mice. (Continued)
in Th17 anti-MPO autoimmunity (IL-17 and DTH) and did not reduce GN. These observations were consistent with Th17 dominance of early nephrogenic cell-mediated autoimmunity and the nonredundant role of IL-23 in GN at this time point. These observations confirm the importance of IL-23 in the early Th17-dominant phase of anti-MPO GN and the lack of benefit of IL-12p35 mAb treatment.

Triggering GN in mice with established anti-MPO autoimmunity treated with anti–IL-23p19 mAb did not significantly reduce Th1 autoimmunity or GN. However, at this time point, anti–IL-12p35 mAb treatment significantly reduced cellular and humoral anti-MPO autoimmunity and GN. These observations are consistent with established anti-MPO autoimmunity being Th1 dominant and IL-12 playing a powerful nonredundant role in the development of GN. Anti–IL-12p35 mAb immune neutralization is a potential biological therapeutic for established disease.

Interestingly, we found that only anti–IL-12p35 mAb treatment limits MPO-ANCA production whereas anti–IL-23p19 and anti–IL-12p40 mAb therapy did not alter MPO-ANCA production. In the model studied, ANCA production does not reach titers that can induce significant renal injury. This is because a major loss of humoral tolerance does not occur unless MPO\(^{-/-}\) are used where deletion of MPO-reactive lymphocytes does not occur.\(^{26}\) Consequently, the reduction in ANCA production induced by anti–IL-12p35 mAb treatment is not associated with the reduction in glomerular injury. However, ANCA is key inducer and perpetuator of injury in human disease and the additional benefit of anti–IL-12p35 mAb capacity to attenuate both cell-mediated and humoral anti-MPO autoimmunity makes anti–IL-12p35 mAb an attractive potential therapeutic for this disease.

Collectively, these studies demonstrate that the development of anti-MPO autoimmune response over time is biphasic. Th17-dominant responses occur early, which results in a neutrophil-rich glomerular lesion, and using anti–IL-23p19 mAb attenuates renal injury. However, maturing anti-MPO autoimmunity is Th1 driven, resulting in a macrophage-rich glomerular lesion, and only anti–IL-12p35 mAb is beneficial at this later time point. The current studies emphasize the importance of defining the dominant effector pathways in selecting a biological therapeutic strategy. The successful interventions with appropriate anti-cytokine mAbs also show that greater selectivity of immune targeting can be achieved if key pathways can be identified. Relevant to human disease, the current studies show that profiling the dominant Th subset can be achieved using data derived from peripheral blood mononuclear cells or renal biopsies at presentation. Current available pathological data from semiquantitation of effector cell profiles of renal biopsies with prominent inflammatory macrophages and fibrin, characteristic of DTH effector responses, strongly suggest Th1 dominance in human disease. The effectiveness of anti–IL-12p35 mAb to attenuate both T-cell and humoral autoimmunity suggests that this biological may be effective in human disease. Given the relatively long period from symptom onset to diagnosis in most people with AAV, it would be likely that Th17 responses are waning by the time patients present. However, these observations require much more data on the natural history of human anti-MPO autoimmunity before these hypotheses are confirmed.

**METHODS**

**Mice**

C57BL/6 (WT), IL-12p35\(^{-/-}\) (The Jackson Laboratory, Bar Harbor, ME), and IL-23p19\(^{-/-}\) (provided by N. Ghiardi and F. De Sauvage, San Francisco, CA\(^{17}\)) mice were used in this study. Mice were bred and housed under specific pathogen-free conditions at Monash Medical Centre Animal Facilities, Monash University, Clayton, Victoria, Australia. All knockout mice were on a C57BL/6 background. The study was approved by Monash University Animal Ethics Committee.

**Experimental design**

**Early developing experimental anti-MPO GN, day 20.** Mice were immunized s.c. with 20 \(\mu\)g of recombinant murine MPO\(^{27}\) in Freund’s complete adjuvant (Sigma-Aldrich, Louis, MO) and boosted (day 7) s.c. with 10 \(\mu\)g of MPO in Freund’s incomplete adjuvant. GN was triggered by i.v. injection of 3 mg of sheep anti-GBM globulin on day 16. Anti-MPO GN was assessed on day 20.

**Late, established experimental anti-MPO GN, day 32.** GN was triggered (day 28) in mice with induced anti-MPO autoimmunity (similar to day 20 protocol; MPO immunization, days 0 and 7), and experiments ended on day 32, a time point at which we showed and have previously demonstrated\(^{28}\) that serum MPO-ANCA IgG production plateaued (day 20, optical density [OD] 450nm [OD\(_{450nm}\)] = 1.13 \(\pm\) 0.14 vs. day 32, OD\(_{450nm}\) = 1.16 \(\pm\) 0.11; \(n = 7\) per group; \(P = 0.89\)).

**In vivo cytokine blockade.** Anti-cytokine mAbs were administered 1 day before triggering GN; for early anti-MPO GN, day 15; and for established anti-MPO GN, day 27. Antibodies used were mouse anti-mouse IL-23p19 mAb (100 \(\mu\)g per mouse; clone MMp19B2 [Biolegend, San Diego, CA]); controls received mouse IgG2b (Bio X Cell, West Lebanon, NH). Rat anti-mouse IL-12p35 mAb (250 \(\mu\)g per mouse; clone C18.2 [Thermo Fisher Scientific, Victoria, Australia]) and rat anti-mouse IL-12p40 mAb (1 mg per mouse; clone C17.8 [Bio X Cell]) were used; controls received rat IgG.

*Figure 6* | (Continued) Anti–IL-12p40 mAb administered during established anti-MPO GN (\(n = 9\)) did not reduce albuminuria (h) but significantly decreased the numbers of accumulating glomerular CD4\(^+\) T cells and macrophages (i) and attenuated glomerular segmental necrosis (j) compared toCtrls (\(n = 9\)). Anti–IL-12p40 mAb administration attenuated systemic anti-MPO autoimmunity as measured by dermal DTH swelling (k) and interferon-\(\gamma\) (IFN-\(\gamma\)) production of MPO-stimulated draining lymph node cells (l). No difference in interleukin-17A (IL-17A) (m) production and serum MPO-ANCA IgG (n) was observed between anti–IL-12p40 mAb–treated mice and Ctrls. PAS-stained micrographs were taken at original magnification \(\times 400\). Bar = 20 \(\mu\)m. Error bars represent mean \(\pm\) SEM, with statistical analysis performed using the unpaired \(t\) test.

\(*P < 0.05, **P < 0.01, \text{gels, cells per glomerular cross section; Mø, macrophage; OD}_{450nm}, \text{optical density 450nm; PMN, neutrophil. To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.} \)
Glomerular neutrophil recruitment in response to anti-GBM globulin. Mice were injected with 3 mg of sheep anti-mouse GBM immunoglobulin and killed 2 hours later.

Experimental anti-MPO GN model with GN triggered using MPO409-428-conjugated non-nephritogenic mouse anti-mouse GBM antibody. Mice were immunized with MPO as described above (days 0 and 7). GN was triggered by i.v. injection of 150 μg of MPO409-428-conjugated mouse anti-mouse GBM IgG1 (clone 8D1) on days 16 and 23. To conjugate MPO409-428 or OVA peptide 323-339 (OVA323-339) to 8D1, 5 mg/ml of 8D1 (grown in-house) was reacted with 0.1 mg/ml of N-succinimidyl-6-maleimidocaproate (Sigma-Aldrich) for 2 hours. MPO409-428 or OVA323-339 (10 mg/ml; Mimotopes, Victoria, Australia) was combined with 8D1 at 10-fold molar excess for 3 hours. The reaction was stopped by adding 2 mM cysteine. Unconjugated peptide was removed by dialysis in phosphate-buffered saline. Anti-MPO GN was assessed on day 32.

Renal CD4+ T-cell isolation

Kidney tissues were homogenized and digested in 4 mg/ml of collagenase D (Roche Diagnostics, Indianapolis, IN) and 100 μg/ml of DNase I in Hanks balanced salt solution without Ca2+ and Mg2+ (Sigma-Aldrich) for 30 minutes at 37 °C on a rotator. Cells were erythrocyte lysed and the CD45+ leukocyte population isolated using mouse CD45 microbeads (Miltenyi Biotec, San Diego, CA). CD4+ cells were stained using anti-CD4+ (clone; GK1.5, fluorochrome; phycoerythrin [PE]) and sorted for CD4+ T cells on the BD Influx 1 Cell Sorter (BD Biosciences, Victoria, Australia). RNA from CD4+ T cells was extracted using the RNeasy Micro Kit (Qiagen, Hilden, Germany) and reverse transcribed using the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific). Real-time polymerase chain reaction was performed at the Monash Health Translational Precinct Medical Genomics Facility (Clayton, Victoria, Australia). Briefly, preamplification was performed to increase the number of copies of each gene to detectable levels, as detailed in Gene Expression Preamplification with Fluidigm Preamp Master Mix and TaqMan Assays Quick Reference PN 100-5876B1 (Thermo Fisher Scientific). A no-template control was included, and all samples were preamplified for 14 cycles. Genes for analysis were chosen from a TaqMan assay library of mouse genes that were classified as either Th1 or Th17 specific. Assays and samples were combined in a 48.48 Dynamic Array IFC according to Fluidigm 48.48 Real-Time PCR Workflow Quick Reference PN 6800088 (Fluidigm, San Francisco, CA). The reaction was run according to the GE 48×48 Standard v2 Biomark Protocol (Fluidigm). Analysis of quantitative polymerase chain reaction (qPCR) data was performed using the Fluidigm Real-Time PCR analysis software (V4.1.1, Fluidigm). The delta-delta Ct method was used to approximate the expression levels of each mRNA expressed as a fold increase from baseline (measured from OVA-immunized controls) and normalized to expression of the ribosomal subunit mRNA 18S.

Assessment of renal disease and glomerular immune cell infiltration

Urine was collected by housing mice in individual metabolic cages over the final 24 hours of the experiment. Albuminuria was assessed by enzyme-linked immunosorbent assay (Bethyl Laboratories, Montgomery, TX) and expressed in micrograms per 24 hours. Urine creatinine and serum blood urea nitrogen were measured using standard methods at the Monash Medical Centre biochemistry laboratory. Histological assessment of renal injury was performed on 3-μm-thick, formalin-fixed, paraffin-embedded, and periodic acid–Schiff-stained kidney sections. A minimum of 30 glomeruli per mouse were examined, and the results are expressed as the proportion of each glomerulus affected by segmental necrosis per glomerular cross section. Glomerular CD4+ T cells, macrophages, and neutrophils were assessed using an immunoperoxidase-staining technique on 6-μm-thick, periodate-lysine-paraformaldehyde (PLP)–fixed kidney sections. Primary antibodies were as follows: GK1.5 for CD4+ T cells (American Type Culture Collection, Manassas, VA), FA/11 for macrophages (anti-mouse CD68 from Dr. Gordon L. Koch, Cambridge, UK), and RB6-8C5 for neutrophils (anti-Gr-1, DNAX, Palo Alto, CA). A minimum of 30 glomeruli were assessed, and the results are expressed as cells per glomerular cross section.

Systemic autoimmune responses to MPO

An enzyme-linked immunosorbent assay was used to detect serum anti-MPO IgG, 1 μg/ml of MPO, and hors eradish peroxidase–conjugated sheep anti-mouse IgG (GE Healthcare,Amersham,United Kingdom). To assess MPO-specific dermal DTH, mice were challenged by intradermal injection of 10 μg/30 μl of MPO in saline in the right footpad (contralateral footpad received saline). DTH was quantified 24 hours later by measuring the difference between footpad thickness (∆mm) using a micrometer. MPO-specific cell proliferation was performed by culturing draining LN cells at 5 × 10^5 cells/200 μl, restimulated with 10 μg/ml of MPO, and incubated for 72 hours. During the last 16 hours of culture, 0.5 μCi of [3H]thymidine (PerkinElmer, Waltham, MA) was added. [3H]Thymidine incorporation was measured as previously described. IFN-γ and IL-17A production was assessed by the ELISPOT assay (BD Biosciences, Mouse IFN-γ and IL-17A ELISPOT Kit), with draining LN cells seeded at 5 × 10^5 cells/200 μl restimulated with 10 μg/ml of MPO for 18 hours. IFN-γ– and IL-17A–producing cells were enumerated with an automated ELISPOT reader system. Cytokine production was detected in the cultured supernatants of draining LNs by using a cytometric bead array kit (mouse Th1/Th2/Th17; BD Biosciences).

Statistics

Data were analyzed with GraphPad Prism version 6 (GraphPad Software Inc.,San Diego,CA). The results are expressed as mean ± SEM. An unpaired t test was used when comparing 2 groups and 1-way analysis of variance for comparing between >2 groups.

DISCLOSURE

All the authors declared no competing interests.

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AUTHOR CONTRIBUTIONS

PYG and SRH designed the study. PYG, AC, JDO, JD, KN, KMO, and VO carried out the experiments. PYG, JDO, AC, ARK, and SRH analyzed the data. PYG made the figures. PYG, SRH, and ARK drafted and revised the article. All authors approved the final version of the manuscript.

SUPPLEMENTARY MATERIAL

Figure S1. Glomerulonephritis (GN) triggered during an anti-myeloperoxidase (MPO) T helper cell 17 (Th17)–dominant response (day 16) and GN evaluated on day 32 results in a T helper cell 1 (Th1)–predominant glomerular lesion. GN was triggered in mice with anti-MPO autoimmunity using MPO-8D1 (controls received OVA-8D1).
Depositing MPOO9-428 in glomeruli using MPO-8D1 (n = 7) induced significantly increased percentage of glomerular segmental necrosis and glomerular leukocyte accumulation (CD4+ T cells, macrophages, and neutrophils) compared to mice receiving OVA-8D1 (n = 6) (A–D). No difference in albuminuria or anti-MPO–specific DTH swelling was observed between groups (E,F). Compared to OVA-8D1–administered mice, MPO-8D1 resulted in significantly increased anti-MPO Th1 autoimmunity (interferon-γ [IFN-γ] ELISPOT) and no difference in anti-MPO Th17 autoimmunity (interleukin-17A [IL-17A] ELISPOT) or MPO-specific DTH footpad swelling (F-H). Error bars represent mean ± SEM, with statistical analysis performed using the unpaired t test. *P < 0.05, **P < 0.01, c/gcs, cells per glomerular cross section.

**Figure S2.** Inadequate glomerular neutrophil recruitment in IL-23p19−/− confounds anti-myeloperoxidase (MPO)–induced glomerular injury. Two hours after the administration of anti–glomerular basement membrane (GBM) globulin, IL-23p19−/− mice recruit significantly less numbers of neutrophils to glomeruli compared with wild-type (WT) mice (n = 7 per group). Photomicrographs were taken at original magnification ×400. Bar = 20 μm (A). Neutralization of IL-23 (n = 4) did not affect glomerular neutrophil recruitment in response to anti-GBM globulin compared to that found in untreated WT mice (n = 5) (B). During both early and established anti-MPO autoimmunity, all markers of IL-23p19−/− have significantly reduced glomerulonephritis (GN) compared to that found in WT mice (C–H). Error bars represent mean ± SEM, with statistical analysis performed using the unpaired t test and 1-way analysis of variance (WT vs IL-12p35−/− vs IL-23p19−/− mice). *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. c/gcs, cells per glomerular cross section; Mφ, macrophage; PMN, neutrophil. Supplementary material is linked to the online version of the paper at www.kidney-international.org.

**REFERENCES**