Anti-Neutrophil Cytoplasmic Antibodies and Effector CD4+ Cells Play Nonredundant Roles in Anti-Myeloperoxidase Crescentic Glomerulonephritis


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

Most humans with microscopic polyarteritis and anti-myeloperoxidase (anti-MPO), anti-neutrophil cytoplastic antibodies (ANCA) develop “pauci-immune” crescentic glomerulonephritis. For dissection of the roles of ANCA and cell-mediated effectors in microscopic polyarteritis, experimental autoimmune anti-MPO glomerulonephritis was induced by immunizing C57BL/6 mice with human MPO. Autoimmunity to mouse MPO (ANCA and CD4+ cell reactivity) was induced. Challenge with anti-glomerular basement membrane globulin resulted in accumulation of neutrophils, CD4+ cells and macrophages, and significant numbers of crescentic glomeruli compared with similarly challenged control-immunized mice. MPO-deficient (Mpo−/−) mice immunized with MPO developed similar immune responses to MPO but failed to recruit effector cells to glomeruli or develop significant crescent formation, suggesting that MPO is acting as a planted glomerular autoantigen. Effector CD4+ cell depletion in this model attenuated crescentic glomerulonephritis and effector cell influx without altering ANCA titers. However, B cell–deficient mice, with no ANCA, still developed severe crescentic glomerulonephritis with accumulation of effector cells. Intravital microscopy studies demonstrated that passive transfer of sera from MPO-immunized Mpo−/− mice to LPS-primed mice rapidly induced glomerular neutrophil accumulation and release of MPO. These studies provide in vivo evidence in a relevant vascular bed for both humoral and cellular anti-MPO responses as key inducers of injury. ANCA induces glomerular neutrophil infiltration and MPO deposition. Subsequently, anti-MPO CD4+ cells recognize MPO as a planted glomerular antigen and act with macrophages to amplify severe glomerular injury.


Microscopic polyarteritis and Wegener granulomatosis are associated with circulating anti-neutrophil cytoplasmic antibodies (ANCA), usually directed against two proteins in neutrophil granules, myeloperoxidase (MPO) and proteinase-3 (1–4). Autoimmunity to MPO, detected by ANCA directed against MPO (MPO-ANCA), is the more common finding in patients with microscopic polyarteritis, who usually have renal involvement (5,6), usually in the form of “pauci-immune” necrotizing and crescentic glomerulonephritis (GN). Proof of the concept that immunity to MPO can induce crescentic GN was provided by Xiao et al. (7), who demonstrated that splenocyte transfer from MPO-immunized MPO-deficient (Mpo−/−) mice to Rag2−/− mice resulted in pauci-immune crescentic GN and circulating MPO-ANCA. These data implicate MPO-ANCA as a major determinant of disease pathology. Further studies demonstrated that sera from MPO-immunized Mpo−/− mice could induce crescent formation in an LPS- and neutrophil-dependent manner (8,9). In most autoantibody-mediated diseases, a key feature is the presence of the autoantibody at the sites of tissue injury. Paradoxically, ANCA-associated crescentic GN is uniquely characterized by an absence or minimal presence of antibodies in affected glomeruli, hence the historical description of the lesion as “pauci-immune” (10). However, both neutrophils and extracellular MPO are present in glomeruli (11). A further paradox is that the glomerular lesion is not “immune negative,” as almost all patients with active GN have glomerular CD4+ T cells and macrophages, cellular effectors of delayed-type hypersensitivity (DTH) (12–14). This finding suggests that cell-mediated immunity is a major effector pathway of injury. The hypothesis that links these disparate observations is that DTH effectors respond to MPO acting as a planted autoantigen in glomeruli.

A further hypothesis that draws together humoral and cellular effector arms of the autoimmunity to MPO present in these patients is that MPO-ANCA− and anti-MPO–specific CD4+ cells may work together in a unique way. A growing body of evidence implicates ANCA in neutrophil activation, favoring their vascular adherence and degranulation (11,15–20). The capacity for interactions between ANCA and neutrophils to induce injury has been widely explored and confirmed, particularly in vitro (15,21). One consequence of ANCA/neu-
trophil interactions is the deposition of degranulated MPO in small vessels such as glomeruli (11). Once released from intracellular neutrophil granules and deposited in small vessels, the autoantigen MPO can be processed by antigen-presenting cells and recognized in the target tissue by MPO-specific effector T cells. The induction of ANCA requires CD4^+ T cell help, and patients with MPO-ANCA have MPO-specific CD4^+ cell responses (22,23), although reactivity was measured not infrequently in control patients. Furthermore, vascular deposition of MPO by MPO-ANCA–activated neutrophils has the potential to induce an effector CD4^+ response. The full expression of MPO-ANCA–associated “pauci-immune” crescentic GN in humans therefore may be a three-step process involving (1) the loss of tolerance and acquisition of autoimmunity to MPO, (2) MPO-ANCA–induced glomerular localization and degranulation of neutrophils that release MPO and induce injury, and (3) CD4^+ effector responses to MPO inducing severe GN with crescent formation. These hypotheses are tested by these studies.

Materials and Methods

Mice

All mice were on a C57BL/6 background. Wild-type (WT) mice or mice with a targeted disruption of either MPO (Mpo^{-/-} mice; from Prof. A.J. Lucis, UCLA, Los Angeles, CA) (24) or μMT^{-/-} mice (25), lacking mature B cells (confirmed by flow cytometric analysis of B220^−/− mice, circulating B cells) (26), were immunized, and autoimmune anti-MPO responses were induced. Mice were bred at Monash University (Clayton, Victoria, Australia), and experiments approved by the Monash University, Monash Medical Centre Animal Ethics Committee.

Experimental Design

Experimental autoimmune anti-MPO crescentic GN was induced by sensitizing 8- to 10-wk-old male WT mice (n = 6, each group) with 10 μg of human MPO (hMPO; Sigma, St. Louis, MO) in complete Freund adjuvant (Sigma; 100 μl subcutaneously in each flank). Control mice (n = 6) were sensitized with BSA (Sigma) in complete Freund adjuvant. Ten days after immunization, mice were administered 32 mg of sheep anti-mouse glomerular basement membrane (GBM) globulin intravenously in two divided doses and killed humanely on days 4, 5, 7, and 10. Renal injury was assessed to determine the optimal time point at which injury was stimulated by sheep anti-mouse GBM globulin–induced release of neutrophil MPO was maximal and the autologous immune response to sheep globulin was minimal. Further experiments were performed on day 4. CD4^+ cells were depleted in C57BL/6 mice (n = 6) with autoimmune anti-MPO GN, by intravenous injection of 2 mg of rat anti-mouse CD4 mAb (GV1.5; ATCC, Manassas, VA) or nonimmune rat IgG, administered after MPO priming, 12 h before anti-GBM globulin. CD4^+ cell depletion was assessed by whole-blood flow cytometry and by splenic immunohistochemistry. As previously published (27), this protocol resulted in ≥90% CD4^+ cell depletion (day 4, anti-CD4 treated; CD4^+ cells 0.1 ± 0.03% of peripheral blood lymphocytes; rat IgG treated; 15.1 ± 0.2%). Significance of differences between groups was determined by t test, except for studies in intravital microscopy, in which ANOVA was used (GraphPad, San Diego, CA). Data were expressed as the mean ± SEM.

Assessment of Systemic Immune Responses to MPO

Native mouse MPO (mMPO) was purified from differentiated 32Dc13 cells as described previously (28). Mouse anti-hMPO antibody titers in sera were measured by ELISA using a modification of a previously described protocol (29) using 100 μl/well (1 μg/ml hMPO or 5 μg/ml mMPO). For indirect immunofluorescence and flow cytometry, neutrophils were harvested from mice 4 h after intraperitoneal injection of 0.5 ml of thioglycolate. For indirect immunofluorescence, neutrophils were coated onto slides and fixed. Mouse sera were diluted 1:10 in PBS and Fc block (1 μg/ml/10^6 cells in 100 μl; BD Biosciences) and incubated (4°C, 5 min). Sera were incubated on slides (30 min, room temperature), washed, then incubated with sheep anti-mouse Ig–FITC (Silenus, Hawthorn, Victoria, Australia; 1:100, 30 min, room temperature). For flow cytometric assessment of anti-MPO antibody reactivity to mouse neutrophils, a modification of methods used for human samples was used (30).

For assessment of dermal DTH to hMPO, mice with experimental autoimmune anti-MPO crescentic GN (or control-immunized mice) were challenged by intradermal injection of 2 μg of hMPO in 40 μl of PBS into the right plantar footpad, the same dose of the irrelevant antigen (BSA/ovalbumin [OVA]) that was injected into the contralateral footpad. Swelling was measured (24 h) using a micrometer (Mitutoyo, Kawasaki-shi, Japan); DTH was assessed as the difference in swelling between the two footpads and expressed in Δmm. IFN-γ production was determined by ELISA or enzyme-linked immunosorbent spot (ELISPOT; BD Biosciences). ELISPOT was performed according to the manufacturer’s instructions, using 4 × 10^5 splenocytes/well with 5 μg/ml heat-inactivated mMPO (16 h, 37°C), using anti-mouse IFN-γ (51–2525KC), biotinylated anti-mouse IFN-γ (51–1818KZ), avidin–horseradish peroxidase and 3-amino-9-ethylcarbazole. Spots were counted and assigned a value according to size: 1 = small, 2 = medium, 3 = large. Results are expressed as area of positive staining/10^6 CD4^+ cells (31). For ELISA, single-cell splenocyte suspensions (day 4) were prepared in DMEM/5% FCS (32); 4 × 10^5 splenocytes/ml in DMEM/10% FCS were incubated (72 h, 37°C) with sterile hMPO in PBS (5 μg/ml). IFN-γ in supernatants was measured by ELISA using a modification of a previously published technique (33,34), using GK1.5, FA11 (anti-mouse CD68, [35]), or R66–3CS (Gr-1; DNAx, Palo Alto, CA). A minimum of 20 glomeruli/animal were assessed, and results are expressed as cells per glomerular cross-section. For assessment of mouse antibody deposition in glomeruli, immunofluorescence was performed on 4-mm cryostat-cut tissue, using FITC-sheep anti-mouse Ig (1:200). Sections from two nonautoimmune forms of GN where mouse IgG is deposited in glomeruli (anti-horse spleen apoferritin and accelerated autologous anti-GBM GN) were positive controls.

Assessment of the Glomerular Microvasculature via Intravital Microscopy

The murine glomerular microvasculature was examined according to a previously published technique (36). Briefly, the left ureter was li-
gated under anesthesia to induce unilateral hydronephrosis. After allowing 12 wk for renal interstitial atrophy, the mouse was anesthetized (ketamine 150 mg/kg, xylazine 10 mg/kg), and the hydronephrotic kidney was exteriorized onto an optically clear viewing pedestal via a lateral incision. Urine was drained with a 30-G needle, and the kidney was extended over the optical window using silk sutures attached to its edges, kept warm and moist by superfusion of warmed bicarbonate-buffered saline (pH 7.4), and covered with a coverslip held in place with vacuum grease. Circulating leukocytes were visualized via intravenous injection of rhodamine 6G (50 μl, 0.05% in saline) and defined as adherent in glomerular capillaries when they remained stationary for >30 s. This approach has been used previously to assess glomerular leukocyte adhesion in rodent models (37,38). Mice were pretreated with LPS (1 μg, intraperitoneally), and 4 h later, the kidney was prepared for intravital microscopy. Three glomeruli were randomly selected and examined throughout the experimental protocol. After baseline recordings (time 0), 50 or 100 μl of pooled sera from Mpo−/− mice that had been immunized with mMPO or OVA was injected at 10 min and again at 25 min. Leukocyte adhesion was assessed before intravenous infusion of anti-MPO serum or anti-OVA serum (as a control) and then immediately after antiserum infusion and 15 and 30 min later.

Assessment of MPO Deposition and Co-Localization with Neutrophils in Glomeruli

Cryostat-cut, snap-frozen kidney tissue sections (6 μm) from contralateral kidneys of mice that underwent intravital microscopy were blocked (5% normal rat serum in 1% BSA/PBS), then incubated with rabbit anti-hMPO antibody (Labvision, Fremont, CA) and then sheep anti-rabbit IgG-FITC (Chemicon, Temecula, CA) at a final dilution of 1:50 (60 min, room temperature). Confocal images were collected using a Bio-Rad confocal inverted Nikon microscope with an air-cooled 25-mW argon/krypton laser (39).

Results

WT Mice Immunized with hMPO Develop Systemic Immunity to hMPO and Autoimmunity to mMPO

C57BL/6 WT mice immunized with hMPO developed high anti-hMPO antibody titers (Figure 1A) and dermal DTH responses to hMPO (Figure 1B), demonstrating the establishment of both B and T cell responsiveness to hMPO. Humoral responses to hMPO were absent in mice immunized with BSA as an irrelevant antigen. Compared with BSA-immunized results, dermal DTH to MPO was markedly increased in MPO-immunized mice, the minimal swelling in DTH responses in the control group reflecting the innate proinflammatory activity of mice that had been immunized with hMPO than from BSA-immunized mice (Figure 1C).

Mice immunized with hMPO made autoimmune responses to mMPO. Sera from hMPO-immunized mice stained neutrophils that expressed mMPO (Figure 2, A and B), demonstrating autoimmune responses to mouse neutrophils. Flow cytometric analysis of mouse sera from MPO- and control-immunized mice also demonstrated binding of immune sera to mouse neutrophils (Figure 2C). Sera from mice tested by ELISA against mMPO showed specific reactivity (Figure 2D). Therefore, using criteria similar to those used in the clinic to test humans for anti-MPO antibodies (30,40), mice developed MPO-ANCA. In addition, cell-mediated responses to mMPO were demonstrated by the presence of dermal DTH to mMPO and significant IFN-γ production (ELISPOT) by splenocytes from hMPO-immunized mice after stimulation with mMPO (Figure 2, E and F).

Experimental Autoimmune Anti-MPO Crescentic GN Depends on Murine MPO

To test the hypothesis that MPO deposition in glomeruli of MPO-immunized mice would result in crescentic glomerular injury, we immunized C57BL/6 WT mice with hMPO in FCA (day −10), then injected sheep anti-mouse GBM antibodies intravenously (on day 0). Injection of heterologous anti-GBM antibodies is a standard method of inducing glomerular neutrophil recruitment within hours (in these studies 6.2 ± 1.0 cells per glomerular cross-section; n = 5, 4 h) (41). Glomerular crescent formation was assessed on days 4, 5, 7, and 10 in separate groups of mice to determine the optimal time point for study at which crescentic injury could be attributed to the MPO immunization and not to any developing autologous response to sheep globulin present on the GBM. Crescent formation was significantly greater in MPO-immunized mice compared with

Figure 1. Immunization with myeloperoxidase (MPO) induces anti-human MPO B and T cell responses. (A) C57BL/6 wild-type (WT) mice sensitized with human MPO (hMPO) produced significant hMPO-specific circulating antibody titers (MPO-ANCA). Mice immunized with the control protein BSA did not develop MPO-ANCA. (B) Footpad swelling, dermal delayed-type hypersensitivity (DTH) to hMPO was significantly less than seen in MPO-immunized mice. (C) Cells from MPO-immunized mice produced significantly higher IFN-γ when stimulated with MPO than BSA-immunized mice. *P < 0.05 (t test).
BSA-immunized mice at all time points (Figure 3A). The optimal time point was day 4, when the generation of glomerular crescents in MPO-immunized mice was significant (17 ± 2% of glomeruli affected; Figure 3, B and D) but minimal in BSA-immunized mice (5 ± 1%; Figure 3, C and E). Immunofluorescence revealed minimal deposition of mouse IgG in glomeruli, confirming the lack of a significant autologous effector response to sheep globulin at day 4. Mpo−/− mice were studied to determine the dependence of this lesion on endogenous mMPO. WT mice with autoimmune anti-MPO crescentic GN developed significant accumulation of classical DTH effectors, CD4+ T cells, and macrophages in glomeruli (Figure 4, B and C), the site of neutrophil accumulation after anti-GBM challenge. Neutrophils also were present in glomeruli at day 4 (Figure 4D). The glomerular influx of effectors in these mice was significantly enhanced compared with BSA-immunized WT mice (all P < 0.05; Figure 4, dotted lines). Development of crescentic GN was dependent on mMPO, as crescent formation was all but absent in Mpo−/− mice (Figure 4A). Accumulation of CD4+ T cells, macrophages, and neutrophils in glomeruli from Mpo−/− mice was markedly reduced, demonstrating the requirement for glomerular mMPO deposition in disease development. However, a lack of mMPO expression did not impair systemic immune responses to immunization with hMPO, as Mpo−/− mice developed both anti-MPO antibody responses and dermal DTH responses to MPO, similar to WT mice (Figure 5).

**Effect of CD4+ Cells Play a Key Role in Experimental Autoimmune Anti-MPO Crescentic GN**

To determine the role of CD4+ cells in the effector phase of injury, mice were depleted of CD4+ cells 10 d after immunization with MPO but before triggering of disease with anti-GBM antibodies. Anti-CD4–treated mice developed similar MPO-ANCA titers to control rat IgG–treated mice, indicating that humoral anti-MPO immunity was not impaired (Figure 6A). In contrast, effector dermal DTH responses in these mice were impaired (Figure 6B). Despite circulating MPO-ANCA, glomerular crescent formation was attenuated in CD4-depleted mice compared with control-treated mice (Figure 7A). Accumulation of CD4+ cells and macrophages in glomeruli was reduced, but glomerular neutrophil numbers were similar (Figure 7, B through D). To test the requirement for circulating ANCA in this model, we studied B cell-deficient (and therefore antibody-
deficient) \(\mu MT^{-/-}\) mice. \(\mu MT^{-/-}\) mice failed to develop MPO-ANCA (Figure 8A), but dermal DTH responses were maintained at a similar level to WT mice, indicating that \(\mu MT^{-/-}\) mice develop similar anti-MPO CD4\(^+\) cell responses (Figure 8B). Glomerular crescent formation was not reduced in the absence of MPO-ANCA (Figure 9A). Similarly, the absence of MPO-ANCA did not affect the accumulation of DTH effectors in the glomeruli of \(\mu MT^{-/-}\) mice. These mice exhibited similar levels of accumulation of CD4\(^+\) cells, macrophages, and neutrophils in glomeruli compared with WT controls (Figure 9, B through D).

Mouse Anti-MPO Antibodies Trigger Neutrophil and MPO Accumulation in Glomeruli In Vivo

Having demonstrated the requirement for endogenous MPO in severe glomerular injury, it was hypothesized that under inflammatory conditions, MPO-ANCA would activate neutrophils, enabling their localization to susceptible vascular beds and planting MPO as an autoimmune target for effector CD4\(^+\)
cells. To test this hypothesis, we examined recruitment of neutrophils to glomeruli in vivo using intravital microscopy. WT mice that received injections of sera from MPO-immunized mice did not develop leukocyte accumulation in glomeruli (data not shown). WT mice were pretreated with LPS 4 h before, then injected with sera from Mpo−/− mice immunized with either MPO or OVA. LPS alone caused minimal accumulation of leukocytes in glomeruli (Figure 10A), but transfer of anti-MPO sera induced a rapid influx of leukocytes into glomeruli (Figure 10, B and C) that did not occur when anti-OVA control sera were administered. Anti-MPO antibody–induced leukocyte recruitment was dose dependent, with lower doses of sera inducing smaller increases in recruitment (Figure 10D). In cremasteric postcapillary venules, anti-MPO sera after LPS injection did not induce increases in leukocyte rolling or adhesion beyond control sera (data not shown).

MPO-ANCA–induced deposition of MPO in glomeruli was assessed using sections from the contralateral kidney at the end of experiments. Neutrophils (Gr-1+ cells) had accumulated, and MPO was deposited in glomeruli of mice that received injections of combined LPS and anti-MPO sera (Figure 11, A)

Figure 8. B cell–deficient mice lack humoral immune responses and do not develop circulating MPO-ANCA. (A) μMT−/− mice with autoimmune anti-MPO crescentic GN failed to produce MPO-ANCA compared with WT control mice. (B) The development of dermal DTH to MPO did not significantly differ from WT mice, demonstrating that T cell responses to hMPO remain intact in μMT−/− mice.

Figure 9. Despite the absence of MPO-ANCA, crescentic renal injury with the accumulation of intraglomerular leukocytes developed in μMT−/− mice with autoimmune MPO GN. (A) Glomerular crescent formation was significant in μMT−/− mice and increased compared with WT mice. There was no significant difference in the accumulation of DTH effectors, CD4+ T cells (B) and macrophages (C), or change in the accumulation of neutrophils (D) in glomeruli of μMT−/− mice. *P < 0.05 (t test).

Figure 10. Intravital microscopy demonstrates that after an inflammatory stimulus, MPO-ANCA induces leukocyte recruitment to glomeruli. (A) Four hours after injection of 1 μg of LPS intraperitoneally, kidneys of mice (rendered hydronephrotic 12 wk before) were exteriorized and subjected to intravital microscopy. Leukocyte adherence was minimal at baseline and remained low in mice that had received an injection only with LPS. Experiments were performed with two injections of sera (either 50 or 100 μl) from Mpo−/− mice immunized with either hMPO or ovalbumin (OVA) (timing of injections indicated by arrows). (B through D) Whereas anti-OVA sera had minimal effect, sera that contained MPO-ANCA induced significant, dose-dependent glomerular leukocyte adhesion. c/glom, cells that adhered in glomerular capillaries for >30 s of observation; *P < 0.05 (ANOVA).
and B). Some of the MPO co-localized to neutrophils, but MPO that was not associated with neutrophils also was present in glomeruli that existed as a planted autoantigen, indicating glomerular MPO deposition, presumably as a result of neutrophil degranulation in glomeruli (Figure 11C). MPO was not observed in cross-sections of glomeruli from mice given LPS alone or LPS and anti-OVA sera (data not shown).

Discussion

Autoimmunity to neutrophil cytoplasmic antigens is associated with small-vessel vasculitis. Our studies were designed to test the hypothesis that both anti-MPO humoral and cellular effector mechanisms contribute to injury. A technique of inducing murine anti-MPO autoimmunity with CD4$^+$ anti-MPO responses was defined. For testing the contribution of cellular effectors, glomerular MPO deposition, induced by an MPO-ANCA–independent mechanism (anti-GBM antibodies), precipitated “pauci-immune” crescentic GN that was mediated by CD4$^+$ cells independent of MPO-ANCA. Intravital microscopy studies confirmed that MPO-ANCA has the capacity to induce glomerular localization of neutrophils and MPO deposition in glomeruli in vivo. Collectively, the studies support a multistep induction of autoimmune anti-MPO crescentic GN, with key roles for both MPO-ANCA and CD4$^+$ anti-MPO effectors in the full expression of disease. They support a two-step mechanism: (1) Interactions between MPO-ANCA and neutrophils deposit the autoantigen (MPO) in glomeruli, and (2) recruitment of CD4$^+$ effectors directs a DTH-like lesion, resulting in crescentic GN.

Although humoral reactivity to MPO is observed in patients with microscopic polyangiitis and crescentic GN (6), a causal relationship between MPO-ANCA and crescentic GN until recently has been difficult to confirm (7). Because MPO is an enzyme in neutrophil granules, this disease is likely to be a systemic autoimmune process with glomeruli injured as passive bystanders. Neutrophils are present in glomeruli in autoimmune anti-MPO crescentic GN (11). Attention has focused on the hypothesis that ANCA induce injury by binding to neutrophils, leading to activation, glomerular localization, degranulation, and injury. It is likely that translocation of MPO to the neutrophil outer membrane to allow binding of MPO-ANCA is necessary. That microbial products have the capacity to induce neutrophil MPO translocation to the cell surface (8,15) may explain the link between sepsis and the onset/relapse of ANCA-associated vasculitis. ANCA activates neutrophils (15) in an Fc-dependent manner (16–18), altering their cytoskeleton and their deformability (19), enhancing their adhesion to the endothelium (20), and inducing endothelial injury (21).

We used renal intravital and confocal microscopy to observe directly the potential for passively administered serum from mMPO-immunized Mpo$^{-/-}$ mice to localize neutrophils and MPO to glomerular capillaries. Neutrophils rapidly accumulated in vivo after MPO-ANCA sera transfer. This effect required systemic LPS (likely because of the induction of MPO expression on neutrophil membranes) but was due to MPO-ANCA (neither control immune sera to mice given LPS nor injection of MPO-ANCA alone had significant effects). Recent
studies in mesenteric postcapillary venules showed that ANCA (induced in Wistar-Kyoto rats by hMPO immunization) augments neutrophil influx that is induced by topical application of CXCL1 (an IL-8 homologue) (42). The requirement for topical application of a chemokine to endothelia is inconsistent with a primary role for ANCA in the initiation of injury but does demonstrate the concept that MPO-ANCA can recruit leukocytes to an inflamed microvasculature. Our understanding of the mechanisms of leukocyte adhesion in glomerular capillaries, where, for example, neutrophil rolling does not occur (38), is limited compared with adhesion mechanisms in postcapillary venules. Our studies demonstrated that MPO-ANCA could localize neutrophils to glomeruli, key vascular targets.

Transfer of sera that contained MPO-ANCA not only induced neutrophil accumulation in glomeruli but also induced glomerular MPO deposition. Renal biopsies from patients with ANCA-associated disease have demonstrated extracellular MPO in affected kidneys (11). Other studies have demonstrated that under inflammatory conditions, neutrophils release MPO that binds to and is transcytosed across the endothelium (43,44). This exposure of nonsequestered target autoantigen should act as a local challenge for anti-MPO effectors (e.g., MPO-specific CD4+ cells) to induce glomerular injury in response to this planted autoantigen. Further experiments tested the potential contributions of humoral and cellular effector arms of the autoimmune anti-MPO response to crescentic injury in response to an MPO-ANCA–independent glomerular MPO challenge by comparing the effects of deletion/depletion of either MPO-ANCA or CD4+ cells. Determining the effector CD4+ cell contribution is complicated by observations that passive transfer of MPO-ANCA induces in vivo glomerular neutrophil influx. Therefore, depletion of MPO-ANCA potentially would prevent cellular effector responses by preventing deposition of the planted autoantigen (MPO), as well as attenuating any direct injurious effects of MPO-ANCA in glomeruli. Therefore, a surrogate trigger for inducing autoimmune effector responses in glomeruli was adopted, passive injection of anti-GBM antibodies, a recruitment stimulus for neutrophils (41).

C57BL/6 mice had both humoral and cellular responses to hMPO. Immunizing mice with hMPO induced autoactivity to mMPO with both MPO-ANCA− and anti-mMPO–specific CD4+ cells (strong dermal DTH and IFN-γ production to mMPO). Loss of tolerance to MPO that was induced by immunization with hMPO has been demonstrated in rats (42,45). Autoreactive CD4+ anti-MPO T cells in patients with humoral autoimmunity to MPO are expected, and human CD4+ reactivity to MPO has been demonstrated in patients with MPO-ANCA–associated crescentic GN (22,23). The demonstration of Th1 responses by CD4+ cells has not been described before, although this is a feature of human peripheral blood mononuclear cell responses in ANCA-associated vasculitis (46).

In the context of autoimmunity to mMPO, heterologous anti-GBM antibodies were used to induce glomerular neutrophil localization, degranulation, and endogenous MPO deposition. In nonimmune recipients, anti-GBM antibodies cause transient neutrophil-mediated proteinuria but relatively mild histologic injury without significant crescent formation or glomerular DTH effectors. After 7 to 10 d, autologous immunity to sheep globulin on the GBM results in substantial crescentic GN (47). Our experiments avoided this autologous phase by ending at day 4 (confirmed by the paucity of mouse antibody in glomeruli). At this time point, only MPO-sensitized mice developed substantial crescentic injury that was triggered by anti-GBM antibody–glomerular MPO challenge, in which a significant influx of CD4+ cells/macrophages occurred. BSA-immunized mice challenged with anti-GBM antibodies developed minimal crescentic GN and accumulation of DTH effectors in glomeruli, confirming that the extent of crescentic GN was not a consequence of anti-GBM antibody–induced GN and required previous sensitization to MPO. Although Mpo−/− mice developed systemic immunity to MPO, anti-GBM antibody challenge did not induce crescentic GN or T cell/macrophages in glomeruli, implicating MPO as a planted autoantigen that recruits DTH effectors and induces crescent formation.

The precise timing of the onset of autoimmune anti-MPO crescentic GN allowed for manipulations of the immune system that assessed the contribution of anti-MPO CD4+ effectors. CD4+ depletion immediately before the effector phase of injury resulted in reduced crescent formation and macrophage influx without affecting MPO-ANCA titers. The selective effects on DTH effectors suggest that effector CD4+ cells/macrophages (rather than MPO-ANCA) were responsible for crescentic GN. These data were supported by experiments in μMT−/− mice that developed T cell reactivity to MPO but not MPO-ANCA. When the requirement for MPO-ANCA to localize neutrophils (and MPO) to glomeruli is bypassed by using anti-GBM antibodies to plant MPO in glomeruli, these mice developed similar crescent formation (and T cell/macrophage influx) to WT mice. Therefore, the severity of this form of autoimmune anti-MPO crescentic GN is determined by glomerular DTH effectors (CD4+ cells and macrophages). The capacity of anti-GBM antibodies to trigger MPO deposition in glomeruli in this model is likely to have compensated for any potential loss of MPO deposition in μMT−/− mice as a result of the absence of MPO-ANCA.

Conclusion
These studies suggest that both MPO-ANCA− and MPO-specific effector T cells are important in “pauci-immune” crescentic GN, the former triggering disease by localizing MPO to the glomeruli and inducing a degree of neutrophil-mediated injury, and the latter enabling the full expression of the resulting crescentic lesion by the accumulation of effectors of DTH.

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