Crescentic Glomerulonephritis

Renal Injury, but Attenuates T Cell Immunity Inducing Crescentic Glomerulonephritis

Dragana Odobasic, A. Richard Kitching, Timothy J. Semple, and Stephen R. Holdsworth

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

Myeloperoxidase (MPO) is an enzyme that is found in neutrophils and monocytes/macrophages. Intracellularly, it plays a major role in microbial killing, but extracellularly, it may cause host tissue damage. The role of endogenous MPO was studied during neutrophil-mediated (heterologous) and T helper 1 (Th1)/macrophage-mediated (autologous) phases of crescentic glomerulonephritis. Glomerulonephritis was induced in C57BL/6 wild-type (WT) and MPO-deficient (MPO−/−) mice by intravenous injection of sheep anti-mouse glomerular basement membrane globulin. MPO activity was increased in kidneys of WT mice during both the heterologous and autologous phases of glomerulonephritis. During the heterologous phase of glomerulonephritis, proteinuria was decreased, whereas glomerular neutrophil accumulation and P-selectin expression were enhanced in MPO−/− mice. In the autologous, crescentic phase of glomerulonephritis, MPO−/− mice had increased accumulation of CD4+ cells and macrophages in glomeruli compared with WT mice. However, no difference in renal injury (crescent formation, proteinuria, and serum creatinine levels) was observed. Neutrophils and macrophages from MPO−/− mice exhibited reduced production of reactive oxygen species. Assessment of systemic immunity to sheep globulin showed that MPO−/− mice had increased splenic CD4+ cell proliferation, cytokine production, and dermal delayed-type hypersensitivity, as well as enhanced levels of circulating IgG, IgG1, and IgG3. MPO−/− mice also had an augmented Th1:Th2 ratio compared with WT mice (IFN-γ/IL-4 and IgG3/IgG1 ratios). These results suggest that endogenous MPO locally contributes to glomerular damage during neutrophil-mediated glomerulonephritis, whereas it attenuates initiation of the adaptive immune response inducing crescentic, autologous-phase glomerulonephritis by suppressing T cell proliferation, cytokine production, and Th1:Th2 ratio.

MPO protein and/or its products (e.g., HOCl-modified proteins) are present in renal disease, including pyelonephritis, membranoproliferative GN, and membranous GN (17,18), implicating MPO in the pathogenesis of these conditions. Infusion of MPO into the renal artery of experimental animals has demonstrated a pathogenic role of exogenous MPO locally in glomerular dysfunction (19,20). In addition, systemic autoimmunity to MPO is associated with pauci-immune crescentic GN in which the presence of circulating autoantibodies to MPO (anti-neutrophil cytoplasmic antibodies [ANCA]), which are likely to have a pathogenic role (21), strongly correlates with disease (22). However, in vivo evidence demonstrating a functional role of endogenous MPO in glomerular inflammation (not associated with autoimmunity to MPO) has not been provided. This study examined the role of endogenous MPO during the heterologous (neutrophil-mediated) and autologous (Th1/macrophage-mediated) phases of crescentic anti-GBM GN using MPO−/− mice.

Materials and Methods

Experimental Design
Eight- to 10-wk-old male mice were used for experiments. C57BL/6 wild-type (WT) mice were obtained from Monash University Animal Services (Melbourne, Australia). MPO−/− mice on C57BL/6 background (23) were provided by Prof. A.J. Lusis (UCLA, Los Angeles, CA) and were bred at Monash Medical Centre (Melbourne, Australia). Studies adhered to the National Health and Medical Research Council of Australia guidelines for animal experimentation. GN was induced in studies as described by Hillegass (24). Kidneys were fixed in Bouin’s fixative, 3-μm-thick paraffin sections. Glomeruli were considered to be crescentic when two or more cell layers were observed in Bowman’s space. At least 50 glomeruli per mouse were counted to determine the percentage of crescent formation. Proteinuria (mg/24 h) was measured by a modified Bradford method (26) on urine that was collected during the first 24 h after anti-GBM Ig injection (heterologous phase) or during the final 24 h of experiments (autologous phase). Serum creatinine concentrations (μM) were measured by an enzymatic creatininase assay.

MPO Activity in Mouse Kidney and Spleen

MPO activity in whole mouse kidneys was assessed using an adaptation of the protocol described by Hillegass et al. (24). Kidneys were removed from normal WT mice without GN (n = 6) and WT mice 2 h (n = 8) or 21 d (n = 9) after anti-GBM Ig administration to assess renal MPO activity during the heterologous or autologous phase of GN, respectively. They were frozen in liquid nitrogen and stored at −70°C. After thawing, kidneys were weighed and homogenized on ice in 50 mM potassium phosphate buffer (pH 6.0; 1:40 wt/vol). Homogenates were centrifuged (20,000 × g, 15 min, 4°C), and lysates were sonicated and incubated twice (30 min, 4°C) and then centrifuged (20,000 × g, 15 min, 4°C). Supernatant was collected and assayed for MPO activity using an adaptation of the protocol described by Bradley et al. (25). Kidney extract (200 μl) was added to 2 ml of 50 mM potassium phosphate buffer (pH 6.0) that contained 0.167 mg/ml α-dianisidine (Sigma) and 0.0005% H2O2 (Sigma), and the absorbance at 460 nm (A460) was measured. One unit of MPO activity was defined as a change in A460 of 1.0 after 2 min, and results are expressed as mU of MPO activity per mg of kidney (mU/mg). Kidneys from MPO−/− mice with heterologous- or autologous-phase GN were used to detect background peroxidase activity.

For measurement of MPO activity in the spleen, spleens were removed from normal WT mice without GN (n = 6) and WT mice 21 d after anti-GBM Ig administration (n = 9) and frozen in liquid nitrogen. After thawing, they were weighed and homogenized in potassium phosphate buffer (pH 6.0) that contained 1% cetyltrimethylbromide (Sigma; 1:50 wt/vol). Extracts were then sonicated, incubated (30 min, 4°C), centrifuged (20,000 × g, 15 min, 4°C), and assayed for MPO activity as already described. Results are expressed as mU of MPO activity per mg of spleen tissue (mU/mg). Spleens from MPO−/− mice that were administered an injection of anti-GBM Ig were used to detect background peroxidase activity.

Renal Injury, Glomerular Leukocytes, and Mediators of Renal Inflammation

Crescent formation was assessed on periodic acid-Schiff–stained, Bouin’s-fixed, 3-μm-thick paraffin sections. Glomeruli were considered to be crescentic when two or more cell layers were observed in Bowman’s space. At least 50 glomeruli per mouse were counted to determine the percentage of crescent formation. Proteinuria (mg/24 h) was measured by a modified Bradford method (26) on urine that was collected during the first 24 h after anti-GBM Ig injection (heterologous phase) or during the final 24 h of experiments (autologous phase). Serum creatinine concentrations (μM) were measured by an enzymatic creatininase assay.

Macrophages, CD4+ cells and neutrophils were demonstrated by immunoperoxidase staining of periodeate lysine paraformaldehyde-fixed frozen 6-μm-thick kidney sections, as described previously (3), using FA/11 (anti-CD68 [27]) for macrophages, GK1.5 (anti-CD4; American Type Culture Collection, Manassas, VA) for CD4+ T cells, and RB6-8C5 (anti-Gr-1; DNAX, Palo Alto, CA) for neutrophils. For assessment of neutrophil accumulation during the heterologous phase of GN, kidneys from WT (n = 8) and MPO−/− (n = 7) mice were collected 2 h after anti-GBM Ig injection. A minimum of 20 glomeruli per mouse were assessed, and results are expressed as cells per glomerular cross-section (c/gcs).

P-selectin and intercellular adhesion molecule-1 (ICAM-1) were identified in 6-μm-thick frozen kidney sections. For P-selectin, kidney sections were blocked with the avidin-biotin blocking system (DakoCytomation, Glostrup, Denmark) followed by 10% normal swine serum (5% BSA/PBS) and incubated with rabbit anti-human P-selectin mAb, which cross-reacts with mouse P-selectin (28) (provided by Prof. M.C. Berndt, Monash University) followed by biotin-conjugated swine anti-rabbit Ig (DakoCytomation; 1:100 in 10% normal mouse/sheep serum) then streptavidin-FITC (DakoCytomation; 1:50). ICAM-1 was identified as described previously (29). Intraglomerular P-selectin and ICAM-1 expression was scored from 0 to 3: 0, background staining; 1, low positive staining; 2, moderate positive staining; and 3, strong positive staining. A minimum of 20 glomeruli per mouse were assessed to determine the average score per gcs.

For assessment of intrarenal expression of IFN-γ and TNF mRNA, whole-kidney RNA was extracted from randomly selected WT (n = 4) and MPO−/− mice (n = 4) with autologous-phase GN, and expression of IFN-γ and TNF mRNA was measured by real-time PCR, as described previously (30,31). Results are expressed as arbitrary units relative to β-actin. Urinary concentrations (μM) of nitrite (reflecting nitric oxide production) were determined by Griess assay, as described previously (32).

Intracellular Production of Reactive Oxygen Species by Thiglycollate-Elicited Peritoneal Neutrophils and Macrophages from WT and MPO−/− Mice

Cells were harvested from the peritoneum of WT (n = 4) and MPO−/− mice (n = 4) 4 h after intraperitoneal injection of 0.5 ml of }
thioglycolate. Cells (5 × 10^5) were incubated (37°C, 10 min) with 0, 0.2, or 100 ng/ml phorbol myristate-13-acetate (PMA; Sigma). Dihydrorhodamine123 (DHR123; Invitrogen Australia Pty Ltd.; Victoria, Australia) was then added (200 ng/ml, 37°C, 10 min). The conversion of nonfluorescent DHR123 to fluorescence rhodamine123 via interaction with reactive oxygen species (ROS) that were produced within cells was analyzed by flow cytometry, gating for neutrophils or macrophages on forward- and side-scatter characteristics. Cells were separately labeled with allophycocyanin-conjugated anti-CD45 (Pharmaningen, San Diego, CA), PE-conjugated anti-Gr-1 (Pharmaningen), and Alexa 488–conjugated anti–Mac-1 mAb (M1–70; American Type Culture Collection) and analyzed by flow cytometry. CD45^-/Gr-1^-/Mac-1^- cells were considered to be neutrophils (≥98% from scatter prediction), whereas CD45^-/Gr-1^-/Mac-1^+ cells were considered to be macrophages (≥80% from scatter prediction). Results are expressed as percentage of cells that are rhodamine123 positive.

**Splenocyte Cytokine Production and Dermal DTH**

Spleens were removed from mice, and single-cell suspensions were obtained. Splenocytes (4 × 10^6 cells/ml) were cultured in DMEM (10% FCS) with 10 ng/ml sheep globulin for 72 h. Concentrations of IFN-γ and IL-4 in supernatants were measured by ELISA, as described previously (34), using FITC-conjugated anti-mouse CD4 (Roche Diagnostics, Penzberg, Germany), as described previously (35), and PE-conjugated anti-CD46 mAb (Pharmaningen). Results are expressed as percentage of CD4 cells that are CD4^-/-/Gr-1^-/-/Mac-1^- mice that were immunized with mMPO served as a positive control. Sera from normal, nonimmunized WT mice provided baseline Ab levels for all ELISA. All results are expressed as the mean OD_{450} ± SEM.

**Results**

**Renal MPO Activity Is Increased during the Heterologous and Autologous Phases of GN**

MPO activity was increased in kidneys of WT mice during the heterologous phase of GN, 2 h after anti-GBM Ig administration, compared with normal WT mice without GN (Figure 1). Similarly, renal MPO activity was increased in WT mice during the autologous phase of GN, 21 d after anti-GBM Ig injection, compared with normal WT mice without GN (Figure 1). No MPO activity was detected in kidneys of MPO^-/- mice that received anti-GBM Ig.

**MPO Deficiency Reduces Renal Injury during the Heterologous Phase of GN**

WT mice that received anti-GBM Ig developed marked proteinuria (13.7 ± 0.7 mg/24 h) during the heterologous phase of GN (normal WT mice without GN 1.6 ± 0.2 mg/24 h). MPO^-/- mice had significantly reduced proteinuria compared with WT mice during the heterologous phase of the disease (Figure 2A). Neutrophils accumulated in glomeruli of WT mice 2 h after anti-GBM Ig injection (2.8 ± 0.4 c/gcs; normal WT mice without GN 0.08 ± 0.01 c/gcs). In this heterologous phase, glomerular neutrophil accumulation was increased in MPO^-/- compared with WT mice (Figure 2, B through D).

**Effect of MPO Deficiency on Glomerular Expression of Adhesion Molecules during Heterologous-Phase GN**

ICAM-1 was expressed constitutively at low levels in interstitial and glomerular areas in kidneys of normal WT mice.

**Circulating Ab Levels**

Circulating titers of mouse anti-sheep globulin IgG (1:100 to 1:6400 dilutions) and IgG subclasses, IgG1 (1:100 dilution), and IgG3 (1:50 dilution), were assessed by ELISA on serum that was collected at the end of experiments, as described previously (30). Circulating titers of mouse anti-mouse (m)MPO IgG (1:100 to 1:6400 dilutions) were assessed by ELISA on serum that was collected at the end of experiments, as described previously (36) using native mMPO purified from differentiated 32Dc3 cells (37). Pooled serum from MPO^-/- mice that were immunized with mMPO served as a positive control. Sera from normal, nonimmunized WT mice provided baseline Ab levels for all ELISA. All results are expressed as the mean OD_{450} ± SEM.
without GN and was upregulated in glomeruli of WT mice with heterologous-phase GN 2 h after anti-GBM Ig injection. Glomerular expression of ICAM-1 was not different between WT and MPO−/− mice with heterologous-phase GN (Figure 3A). P-selectin was not detected in kidneys of normal WT mice without GN but was expressed in glomeruli of WT mice during the heterologous phase of disease 2 h after anti-GBM Ig administration. MPO−/− mice had significantly increased intraglomerular expression of P-selectin compared with WT mice with GN (Figure 3, B through D).


effect of MPO Deficiency on Renal Injury, Glomerular Leukocyte Accumulation, and Inflammatory Mediators of Injury during Autologous-Phase GN

WT mice developed functional renal injury during the autologous phase of GN as demonstrated by abnormal proteinuria (5.9 ± 0.4 mg/24 h; normal WT mice without GN 1.6 ± 0.2 mg/24 h) and serum creatinine levels (25.6 ± 2.2 μM; normal WT mice without GN 13.9 ± 0.8 μM). During the autologous phase of GN, proteinuria (Figure 4A) and serum creatinine levels (Figure 4B) were not significantly different between WT and MPO−/− mice. Histologically, WT mice developed crescentic GN (normal WT mice without GN 0% crescents), which was not affected by MPO deficiency (Figures 4C and 5).

During the autologous phase of GN, both CD4+ T cells (Figure 6A) and macrophages (Figure 6B) accumulated in glomeruli of WT mice. Glomerular accumulation of CD4+ cells (Figures 6A and 7, A and B) and macrophages (Figures 6B and 7, C through F) was enhanced in MPO−/− compared with WT mice with GN. Although in this autologous phase of GN neutrophils accumulated in glomeruli, numbers were similar between WT and MPO−/− mice (2.4 ± 0.1 versus 2.5 ± 0.1 c/gcs).

Mediators of renal inflammation were assessed in autologous-phase injury. Compared with WT mice with GN, MPO−/− mice had increased urinary concentrations of nitrite (reflecting nitric oxide production; Figure 8A), as well as a trend toward augmented intrarenal expression of IFN-γ (Figure 8B) and TNF mRNA (Figure 8C). However, immunostaining demonstrated that WT and MPO−/− mice with autologous-phase GN had similar intraglomerular expression of adhesion molecules, P-selectin (1.5 ± 0.1 versus 1.5 ± 0.1 average score/gcs; normal WT mice without GN 0.0 average score/gcs), and ICAM-1 (2.0 ± 0.2 versus 2.1 ± 0.1 average score per gcs; normal WT mice without GN 0.5 ± 0.1 average score per gcs).

**Figure 2.** Proteinuria and glomerular neutrophil accumulation in WT and MPO−/− mice during the heterologous phase of GN. (A) MPO−/− mice developed less proteinuria compared with WT mice with heterologous-phase GN. (B) Glomerular neutrophil accumulation was enhanced in MPO−/− compared with WT mice. (C and D) Representative photomicrographs of glomerular neutrophil accumulation in WT (C) and MPO−/− mice (D) with heterologous-phase GN are shown (neutrophils were demonstrated by immunoperoxidase staining [hematoxylin counterstain]; brown reaction product represents Gr-1+ cells). Dotted lines (in A and B) represent mean values from normal WT mice without GN. c/gcs, cells per glomerular cross-section. *P < 0.05; **P < 0.0001. Magnification, ×400.

**Figure 3.** Intraglomerular expression of intercellular adhesion molecule-1 (ICAM-1) and P-selectin in WT and MPO−/− mice during the heterologous phase of GN. ICAM-1 and P-selectin were detected by immunofluorescence and scored semiquantitatively from 0 (background staining) to 3 (strong staining). Results are expressed as the average score per glomerular cross-section ± SEM. (A) Glomerular expression of ICAM-1 was similar in WT and MPO−/− mice with GN. The dotted line represents mean glomerular expression of ICAM-1 in normal WT mice without GN. (B) P-selectin was upregulated in glomeruli of MPO−/− compared with WT mice with GN (and was absent from kidneys of normal WT mice without GN). (C and D) Representative photomicrographs of glomerular P-selectin expression in WT (C) and MPO−/− mice (D) with heterologous-phase GN are shown. *P < 0.05. Magnification, ×400.
Peritoneal Thioglycollate-Elicited Neutrophils and Macrophages from MPO⁻/⁻ Mice Have Reduced ROS Production

For determination of the capability of phagocytes from MPO⁻/⁻ mice to produce ROS, leukocytes were isolated from the peritoneum of MPO⁻/⁻ mice 4 h after intraperitoneal injection of thioglycollate. The proportions of neutrophils (Figure 9A) and macrophages (Figure 9B) that were capable of producing ROS in response to PMA were significantly and markedly lower in MPO⁻/⁻ compared with WT mice, implicating MPO in the generation of damaging ROS in renal disease.

MPO Activity in the Spleen during the Development of Systemic Nephritogenic Immunity

For determination of whether MPO has the potential to influence the generation of adaptive immune responses, MPO activity was assessed in a secondary lymphoid organ, the spleen. MPO activity was present both in spleens from normal WT mice without GN (84.9 ± 6.9 mU/mg) and in spleens that were isolated from WT mice 21 d after anti-GBM Ig administration (77.5 ± 3.4 mU/mg). Low background peroxidase activity was detected in spleens that were isolated from MPO⁻/⁻ mice that were administered an injection of anti-GBM Ig (5.1 ± 1.5 mU/mg).

Systemic Immune Responses to Sheep Globulin Are Enhanced in MPO⁻/⁻ Mice

The production of IFN-γ (Figure 10A) and IL-4 (Figure 10B) by sheep globulin–stimulated splenocytes was enhanced in MPO⁻/⁻ compared with WT mice. The effect on IFN-γ production was greater than on IL-4, resulting in an increased Th1:Th2 cytokine (IFN-γ:IL-4) ratio in MPO⁻/⁻ compared with WT mice (Figure 10C). Similarly, proliferation of splenic CD4⁺ T cells was increased in MPO⁻/⁻ mice that were administered an injection of anti-GBM Ig (5.1 ± 1.5 mU/mg).

Circulating titers of anti-sheep globulin IgG (Figure 12A), as the peritoneum of MPO⁻/⁻ mice 4 h after intraperitoneal injection of thioglycollate. The proportions of neutrophils (Figure 9A) and macrophages (Figure 9B) that were capable of producing ROS in response to PMA were significantly and markedly lower in MPO⁻/⁻ compared with WT mice, implicating MPO in the generation of damaging ROS in renal disease.

MPO Activity in the Spleen during the Development of Systemic Nephritogenic Immunity

For determination of whether MPO has the potential to influence the generation of adaptive immune responses, MPO activity was assessed in a secondary lymphoid organ, the spleen. MPO activity was present both in spleens from normal WT mice without GN (84.9 ± 6.9 mU/mg) and in spleens that were isolated from WT mice 21 d after anti-GBM Ig administration (77.5 ± 3.4 mU/mg). Low background peroxidase activity was detected in spleens that were isolated from MPO⁻/⁻ mice that were administered an injection of anti-GBM Ig (5.1 ± 1.5 mU/mg).

Systemic Immune Responses to Sheep Globulin Are Enhanced in MPO⁻/⁻ Mice

The production of IFN-γ (Figure 10A) and IL-4 (Figure 10B) by sheep globulin–stimulated splenocytes was enhanced in MPO⁻/⁻ compared with WT mice. The effect on IFN-γ production was greater than on IL-4, resulting in an increased Th1:Th2 cytokine (IFN-γ:IL-4) ratio in MPO⁻/⁻ compared with WT mice (Figure 10C). Similarly, proliferation of splenic CD4⁺ T cells was increased in MPO⁻/⁻ mice that were administered an injection of anti-GBM Ig (5.1 ± 1.5 mU/mg).

Circulating titers of anti-sheep globulin IgG (Figure 12A), as
well as IgG subclasses, IgG1 (Figure 12B) and IgG3 (Figure 12C), were increased in MPO−/− mice compared with WT mice. MPO deficiency resulted in an enhanced IgG3:IgG1 (Th1:Th2) ratio (Figure 12D). However, splenic B cell proliferation (7.3 ± 0.8 versus 7.5 ± 0.6% BrdU+ CD19 cells) and apoptosis (6.0 ± 0.1 versus 5.7 ± 0.5% Annexin V–positive CD19 cells) were similar between WT and MPO−/− mice.

WT and MPO−/− Mice with Sheep Globulin–Induced Anti-GBM GN Do Not Develop Autoimmunity to Mouse MPO

For assessment of whether WT and MPO−/− mice that were immunized with sheep anti-GBM Ig and developing crescentic GN developed autoimmunity to mMPO, serum mouse anti-mMPO IgG levels were measured. Figure 13 demonstrates that neither WT nor MPO−/− mice that were immunized with sheep Ig developed circulating ANCA-like anti-mMPO antibodies.

Discussion

Neutrophils and monocytes/macrophages contain MPO, an enzyme that, when released extracellularly, can cause host tissue damage via formation of toxic reactive intermediates, such as HOCl (7). MPO is implicated in the pathogenesis of kidney disease because both MPO itself and its products have been demonstrated in diseased renal tissue (17,18). Functional studies that have examined the role of endogenous MPO in renal inflammation are limited. This study investigated the role of endogenous MPO during heterologous and autologous phases of experimental crescentic anti-GBM GN using MPO−/− mice. Renal injury during the heterologous phase of GN is mediated by neutrophils (1). Their infiltration is initiated by binding of the foreign anti-GBM Ig to the GBM and peaks 2 h after disease induction (2). During the heterologous phase of the disease, WT mice developed proteinuria, associated with glomerular neutrophil accumulation and increased MPO activity in the kidney. Proteinuria was decreased in MPO−/− mice, suggesting that MPO that is present in the kidney locally contributes to renal injury during neutrophil-mediated GN. Proof of concept that exogenous MPO can induce glomerular injury in association with H2O2 and chloride came from studies that infused these compounds into the renal artery of rats (19,20). Subsequent experiments showed activation of the MPO/H2O2/halide system in experimental immune-complex GN (38). This study demonstrated a functional pathogenic role of endogenous MPO in kidney damage during experimental neutrophil-mediated GN.
MPO is involved in the generation of a variety of injurious reactive intermediates, including HOCl, which has been shown to induce glomerular damage in vitro (39), and ROS, such as the hydroxyl radical and singlet oxygen (7). Previous studies showed that neutrophils contribute to glomerular injury by ROS production (40). Neutrophils that were isolated from the peritoneum of MPO knockout mice had markedly decreased generation of ROS in response to PMA compared with WT mice, suggesting that reduced ROS levels may contribute to the attenuated renal injury in MPO knockout mice during the heterologous phase of GN. Because humans have higher levels of MPO in neutrophils than rodents (10), neutrophil-derived MPO may contribute more to neutrophil-mediated renal disease in humans than in mice.

Glomerular neutrophil accumulation was assessed in WT and MPO knockout mice with heterologous phase GN to determine whether reduced injury in MPO knockout mice correlated with decreased neutrophil infiltration in the kidney. Neutrophil accumulation in glomeruli of MPO knockout mice was significantly increased compared with WT mice. This is not likely to be due to enhanced numbers of circulating neutrophils in MPO knockout mice because these mice have similar numbers of neutrophils in the blood compared with WT mice (23). Because exogenous MPO can cause endothelial cell lysis (20), a potential mechanism by which MPO might limit neutrophil accumulation in glomeruli is by affecting endothelial cell function and consequent adhesion molecule expression. Neutrophil influx in glomeruli depends on P-selectin and ICAM-1 (1). One of the mechanisms by which endogenous MPO may enhance glomerular neutrophil accumulation is by upregulating P-selectin in glomeruli, because the expression of P-selectin but not ICAM-1 was increased in glomeruli of MPO knockout mice. Taken together, these results demonstrate multiple roles of endogenous MPO during the heterologous phase of anti-GBM GN, involving neutrophil recruitment, adhesion molecule expression, and generation of injurious ROS.

The autologous phase of anti-GBM GN is mediated by a DTH-like effector response in which infiltrating T cells and macrophages induce renal injury (3–5). MPO knockout mice had increased numbers of CD4+ T cells and macrophages in glomeruli compared with WT mice with GN, correlating with increased urinary nitric oxide levels and a trend toward augmented renal mRNA expression of proinflammatory cytokines.
IFN-γ and TNF. However, no difference in injury (crescent formation, proteinuria, and serum creatinine levels) was observed between WT and MPO−/− mice with autologous-phase GN. MPO activity was enhanced in kidneys of WT mice with autologous injury compared with normal WT mice without GN and was absent in kidneys of MPO−/− mice. It is likely that MPO contributes to kidney damage locally during the autologous phase of GN. In addition, macrophages from MPO−/− mice are not fully activated because they produced less ROS in response to PMA. Therefore, increased glomerular accumulation of injurious effector leukocytes may be counterbalanced by the lack of MPO and injurious ROS in kidneys of MPO−/− mice, accounting for the similar degree of injury that is observed in WT and MPO−/− mice with autologous-phase GN.

It is possible that these assessments are complicated by WT mice developing injurious autoimmune responses to mMPO, which would not be expected to occur in MPO−/− mice (because of genetic deletion of the potential endogenous autoantigen). Some patients with anti-GBM GN develop circulating autoantibodies (ANCA) against MPO (41), and several murine models of MPO-ANCA–associated crescentic GN have been recently described (21,36,42,43). However, WT (and MPO−/−) mice with sheep globulin–induced anti-GBM GN did not develop (auto)immunity to mMPO as neither group of animals developed circulating autoantibodies to mMPO.

Systemic adaptive immune responses were assessed to examine potential mechanisms by which MPO might increase CD4+ cell accumulation in glomeruli. MPO activity was detected in the spleen 21 d after anti-GBM Ig injection, suggesting
that MPO has the potential to play a role in the induction of adaptive immunity. Compared with WT mice, MPO−/− mice exhibited increased proliferation of splenic CD4+ T cells with no effect on cell surface activation markers or apoptosis. One of the mechanisms by which endogenous MPO inhibits T cell accumulation in glomeruli is by suppressing T cell proliferation during the induction of adaptive nephritogenic immune responses in secondary lymphoid organs.

The development of crescentic anti-GBM GN is augmented by Th1 cytokines such as IFN-γ and inhibited by Th2 cytokines including IL-4 (44,45). Splenocyte production of both IFN-γ and IL-4 was enhanced in MPO−/− mice, suggesting that MPO downregulates Th1 and Th2 cytokine production. However, MPO−/− had an increased IFN-γ:IL-4 ratio compared with WT mice, indicating that MPO has a greater effect on Th1 than Th2 responses. Attenuated Th1:Th2 ratio is another mechanism by which endogenous MPO plays a protective role during the induction of adaptive nephritogenic immunity, inducing crescentic GN. Consistent with enhanced T cell proliferation and Th1:Th2 ratio, MPO−/− mice had augmented dermal DTH, providing further in vivo evidence that MPO downregulates antigen-specific T cell immunity.

Humoral responses to sheep globulin were also enhanced in the absence of MPO. Consistent with the cytokine data, the IgG3:IgG1 (Th1:Th2) ratio was enhanced in MPO−/− mice. Enhanced humoral immunity in MPO−/− mice is most likely due to the augmented T cell responses (proliferation, cytokine production) because no effect on B cell proliferation or apoptosis was observed. Although autologous Ab is not required for severe crescentic injury in this model (6), this study demonstrates that endogenous MPO plays an important in vivo role in downregulating antigen-specific humoral immune responses, thereby providing an additional link between innate and adaptive immunity.

Although some in vitro evidence has suggested that toxic products of the MPO system can have stimulatory effects on T cells (46), this study demonstrates that MPO downregulates adaptive T cell responses in vivo, which is supported by previous reports. Lymphocytes that are isolated from lymph nodes of MPO−/− mice with experimental autoimmune encephalomyelitis have increased proliferative capacity, leading to exacerbation of disease (15). Similarly, enhanced lung inflammation in MPO−/− recipients after allogeneic bone marrow transplantation is associated with increased numbers of inflammatory cells in the bronchoalveolar fluid (16). Downregulation of nephritogenic T cell responses by MPO could be due to effects on antigen-presenting cells and/or T cells themselves. In vitro studies have shown that taurine chloramine, a long-lived MPO-derived oxidant (47), can directly inhibit the function of both antigen-presenting cells and T cells (48,49).

**Conclusion**

This study demonstrates that endogenous MPO plays differential roles during the heterologous (neutrophil-mediated) and autologous (Th1/macrophage-mediated) phases of anti-GBM GN. Enzymatically active MPO is enhanced in the kidney during immune injury, where its local actions contribute to disease in passively induced heterologous injury. During the active, autologous, crescentic phase of anti-GBM GN, endogenous MPO, while still acting locally in the kidney, paradoxically attenuates systemic nephritogenic immunity (T cell proliferation, cytokine production, and Th1:Th2 ratio) and limits accumulation of T cells and macrophages in glomeruli.

**Acknowledgments**

This work was supported by grants from the National Health and Medical Research Council of Australia.

We thank Prof. A.J. Lusis (UCLA, Los Angeles, CA) for providing MPO−/− mice; Leon Moussa for performing Griess assay; Kim O’Sullivan, Alice Wright, and Lynelle Jones for technical assistance; James Ngui and Paul Hutchinson for assistance with flow cytometry; Prof. M.C. Berndt (Monash University, Clayton, Victoria, Australia) for anti-P-selectin mAb; and DNAX (Palo Alto, CA) for anti-Gr-1 mAb.

**Disclosures**

None.

**References**


4. Huang XR, Tipping PG, Shuo L, Holdsworth SR: Th1 responsiveness to nephritogenic antigens determines suscep-
36. Ruth A, Kitching A, Kwan R, Odobasic D, Ooi J, Timo-


