Antineutrophil cytoplasmic autoantibodies (ANCAs) are identified in the circulation of approximately 80% of patients with pauci-immune necrotizing and crescentic glomerulonephritis and systemic small vessel vasculitis, such as microscopic polyangiitis and Wegener granulomatosis. The most common antigen target for ANCAs is myeloperoxidase (MPO), which is found in neutrophils and monocytes. We report definitive experimental animal evidence that ANCAs are pathogenic. MPO knockout (Mpo<sup>−/−</sup>) mice were immunized with mouse MPO. Splenocytes from these mice or from control mice were injected intravenously into recombinase-activating gene-2–deficient (Rag2<sup>−/−</sup>) mice, which lack functioning B lymphocytes and T lymphocytes. All mice that received splenocytes developed mild to moderate glomerular immune deposits, but only mice that received 1 × 10<sup>8</sup> or 5 × 10<sup>7</sup> anti-MPO splenocytes developed severe necrotizing and crescentic glomerulonephritis, granulomatous inflammation, and systemic necrotizing vasculitis, including necrotizing arteritis and hemorrhagic pulmonary capillaritis. To test the pathogenic potential of antibodies alone, purified anti-MPO IgG or control IgG was injected intravenously into Rag2<sup>−/−</sup> mice and wild-type mice. Mice that received anti-MPO IgG but not mice that received control IgG developed focal necrotizing and crescentic glomerulonephritis with a paucity of glomerular Ig deposition. Thus, anti-MPO IgG alone was able to cause pauci-immune glomerular necrosis and crescent formation in the absence of functional T or B lymphocytes in Rag2<sup>−/−</sup> mice and in the presence of an intact immune system […].
Antineutrophil cytoplasmic autoantibodies specific for myeloperoxidase cause glomerulonephritis and vasculitis in mice

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Antineutrophil cytoplasmic autoantibodies (ANCAs) are identified in the circulation of approximately 80% of patients with pauci-immune necrotizing and crescentic glomerulonephritis and systemic small vessel vasculitis, such as microscopic polyangiitis and Wegener granulomatosis. The most common antigen target for ANCAs is myeloperoxidase (MPO), which is found in neutrophils and monocytes. We report definitive experimental animal evidence that ANCAs are pathogenic. MPO knockout (Mpo−/−) mice were immunized with mouse MPO. Splenocytes from these mice or from control mice were injected intravenously into recombinase-activating gene-2–deficient (Rag2−/−) mice, which lack functioning B lymphocytes and T lymphocytes. All mice that received splenocytes developed mild to moderate glomerular immune deposits, but only mice that received 1 × 10^8 or 5 × 10^7 anti-MPO splenocytes developed severe necrotizing and crescentic glomerulonephritis, granulomatous inflammation, and systemic necrotizing vasculitis, including necrotizing arteritis and hemorrhagic pulmonary capillaritis. To test the pathogenic potential of antibodies alone, purified anti-MPO IgG or control IgG was injected intravenously into Rag2−/− mice and wild-type mice. Mice that received anti-MPO IgG but not mice that received control IgG developed focal necrotizing and crescentic glomerulonephritis with a paucity of glomerular Ig deposition. Thus, anti-MPO IgG alone was able to cause pauci-immune glomerular necrosis and crescent formation in the absence of functional T or B lymphocytes in Rag2−/− mice and in the presence of an intact immune system in wild-type C57BL/6J mice. This animal model offers strong support for a direct pathogenic role for ANCA IgG in human glomerulonephritis and vasculitis.


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Nonstandard abbreviations used: antineutrophil cytoplasmic autoantibody (ANCA); myeloperoxidase (MPO); proteinase 3 (PR3); recombinase-activating gene-2 (Rag2); wild-type (WT); immunofluorescence microscopy assay (IFA); blood urea nitrogen (BUN); hematoxylin and eosin (H&E); C57BL/6J (B6).
mune-competent mice. The resulting necrotizing and crescentic glomerulonephritis, pulmonary hemorrhagic capillaritis, and systemic necrotizing arteritis have remarkable pathologic similarity to human ANCA-associated glomerulonephritis and vasculitis.

Methods

Purification of mouse MPO. Mouse MPO was purified from WEHI-3 cells (a murine myeloid cell line purchased from American Type Culture Collection, Manassas, Virginia, USA) using a modification of the method of Hope et al. (8). Briefly, WEHI-3 cells were grown in McCoy5A medium with 10% FCS. Once the cells reached a density of 1.5 × 10^6 cells per milliliter, they were harvested by centrifugation and resuspended in buffer A (6.7 mM sodium phosphate, pH 6.0; 1 mM MgCl_2; 3 mM NaCl; 0.5 mM PMSF) at a ratio of 10 ml of buffer to 1 ml of cell pellet. The cells were lysed by Dounce homogenization on ice and then centrifuged at 20,000 g for 30 minutes. The pellets were resuspended in buffer A. Cetyltrimethylammonium bromide was added to a final concentration of 1%, and the mixture was stirred vigorously for 2 hours at 4°C. The insoluble material was removed by centrifugation at 20,000 g for 20 minutes at 4°C. The solubilized material was dialyzed against buffer B (100 mM sodium acetate, pH 6.3; 100 mM NaCl) for 5 hours at 4°C. CaCl_2, MgCl_2, and MnCl_2 were then added to a final concentration of 1 M each. The material was mixed end-over-end with 5 ml of concanavalin A-Sepharose (Amersham Pharmacia Biotech, Piscataway, New Jersey, USA) overnight at 4°C. The resin was poured into a Bio-Rad Econo-Column (Bio-Rad Laboratories, Hercules, California, USA) using a modification of the method of Hope et al. (8). Briefly, WEHI-3 cells were primed by intraperitoneal injection of 10 µg of purified MPO in incomplete Freund’s adjuvant on day 21 and day 36, and boosted by intravenous injection of 10 µg MPO without adjuvant 4 days before splenocytes were harvested. Development of antibodies was monitored by anti-MPO ELISA. Circulating anti-MPO was confirmed in selected animals by indirect immunofluorescence microscopy assay (IFA). For anti-MPO ELISA, microtiter plates were coated with 0.5 µg per well murine MPO, incubated with 100-fold dilutions of mouse sera, developed with alkaline phosphatase–conjugated goat antibodies specific for mouse IgG, and analyzed spectrophotometrically at OD 405 nm. Results were expressed as percentage of a positive control serum pool. For IFA, mouse neutrophils were harvested from the peritoneum of B6 or Mpo−/− mice 4 hours after intraperitoneal injection of 3% sterilized Protease peptone (Difco Laboratories, Detroit, Michigan, USA). Isolated cells were washed in PBS with 0.05 mM EDTA and adjusted to 1 × 10^6 cells per milliliter. More than 50% of isolated cells were polymorphonuclear neutrophils, and the remainder were mononuclear leukocytes. Cells were cytocentrifuged onto glass slides, air-dried, and fixed with "clean rooms" in autoclaved cages with microisolator tops. Mice lacking MPO (Mpo−/−) mice lack the ability to initiate V(D)J rearrangement and thus do not produce T or B lymphocytes with antigen receptors (10). Mpo−/− mice, 8–10 weeks old, were used for immunization and as donors of splenocytes and anti-MPO antibodies. Rag2−/− mice (10–12 weeks old), Mpo−/− mice (13 weeks old), and wild-type (WT) B6 mice (9–10 weeks old) were used as recipients for adoptive transfer experiments. Table 1 summarizes the characteristics of the experimental groups. The University of North Carolina Institutional Animal Care and Use Committee approved all animal experiments.

Table 1

Experimental animal groups

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<tr>
<th>Mouse strain</th>
<th>n</th>
<th>Male/Female</th>
<th>Received</th>
</tr>
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<tr>
<td>Rag2−/−</td>
<td>12</td>
<td>5/7</td>
<td>1 × 10^6 anti-MPO cells</td>
</tr>
<tr>
<td>Rag2−/−</td>
<td>4</td>
<td>2/2</td>
<td>5 × 10^6 anti-MPO cells</td>
</tr>
<tr>
<td>Rag2−/−</td>
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<td>2/2</td>
<td>1 × 10^6 anti-MPO cells</td>
</tr>
<tr>
<td>Rag2−/−</td>
<td>6</td>
<td>2/4</td>
<td>1 × 10^6 anti-BSA cells</td>
</tr>
<tr>
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<td>2/2</td>
<td>1 × 10^6 anti-BSA cells</td>
</tr>
<tr>
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<td>4/2</td>
<td>1 × 10^6 nonimmunized cells</td>
</tr>
<tr>
<td>Rag2−/−</td>
<td>3</td>
<td>2/1</td>
<td>5 × 10^6 nonimmunized cells</td>
</tr>
<tr>
<td>Mpo−/−</td>
<td>4</td>
<td>2/2</td>
<td>6.5 × 10^6 anti-MPO cells</td>
</tr>
<tr>
<td>Mpo−/−</td>
<td>5</td>
<td>0/5</td>
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</tr>
<tr>
<td>Mpo−/−</td>
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</tr>
<tr>
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<td>None</td>
</tr>
<tr>
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<td>0/6</td>
<td>50 µg/g anti-MPO IgG</td>
</tr>
<tr>
<td>WT B6</td>
<td>3</td>
<td>0/3</td>
<td>50 µg/g anti-BSA IgG</td>
</tr>
</tbody>
</table>
in PBS was injected via the tail vein at a concentration. Filled IgG was dialyzed against PBS. Sterile-filtered IgG using HiTrap protein G HP column affinity chro-
imunized with MPO or BSA by 50% ammonium sul-
unplacently from immunized and control
mice received anti-MPO antibodies. Nonimmunized
Mpo–/– mice that received 6.5 × 107 anti-MPO splenocytes
had mean anti-MPO ELISA titers of 12.1, 75.6, 90.6, and 98.9 at days 0, 3, 8, and 13, respectively. The presence of anti-MPO antibodies in Rag2–/– mice was
confirmed by indirect IFA using normal mouse neu-
trophils as positive substrate and Mpo–/– mouse neu-
trophils as negative control.

Development of necrotizing and crescentic glomerulonephritis,
granulomatous inflammation, and vasculitis after transfer of
anti-MPO splenocytes. Rag2–/– mice that received 1 × 108
or 5 × 107 anti-MPO splenocytes developed severe renal
failure that resulted in marked elevation of BUN and
serum creatinine (Figure 2). In contrast, Rag2–/– mice
that received 1 × 107 anti-MPO splenocytes, or any dose
of anti-BSA splenocytes or control splenocytes, devel-
orped minimal if any renal insufficiency. Urinalysis
revealed that all Rag2–/– mice that received splenocytes
developed urinary abnormalities (Figure 3). However,
Mpo–/– mice that received 6.5 × 107 anti-MPO splenocytes
developed no urine abnormalities (data not shown).

Gross examination revealed hemorrhagic dots on the
surface of the kidneys in mice that received 1 × 108 or

Results
Development of circulating MPO-ANCA after transfer of anti-
MPO splenocytes. Rag2–/– mice that received anti-MPO
splenocytes developed circulating anti-MPO (MPO-
ANCA) within 3 days (Figure 1). The titer continued to
rise until sacrifice at 13 days. There was a dose response
to anti-MPO splenocytes, with a dose of 1 × 108 spleno-
cytes producing a substantially lower induction of cir-
culating anti-MPO antibodies compared with a dose of
1 × 108 or 5 × 107 splenocytes. Mice that received
splenocytes from control mice that had been immu-
nized with anti-BSA or had not been immunized did not
develop anti-MPO antibodies. Nonimmunized
Mpo–/– mice that received 6.5 × 107 anti-MPO splenocytes
were used as negative controls.

Adoptive transfer of splenocytes and Ig. We isolated
splenocytes from immunized and control Mpo–/– mice
by disrupting the spleens into cold RPMI 1640 medi-
um and then washing twice with RPMI 1640. Red
blood cells were removed with lysis buffer (Sigma-
Aldrich, St. Louis, Missouri, USA) followed by washing
with RPMI 1640 and final suspension in sterile PBS.

Suspensions of 1 × 107, 5 × 107, or 1 × 108 splenocytes
in 500 μl PBS were administered via the tail vein to
Rag2–/– mice (n = 46) (Table 1). A group of Mpo–/– mice
(n = 4) received 6.5 × 107 anti-MPO splenocytes. No dif-
f erences were observed between males and females in
any of the experimental parameters measured.

γ-Globulins were isolated from serum of Mpo–/– mice
immunized with MPO or BSA by 50% ammonium sul-
fate precipitation. IgG was isolated from the γ-globulin
using HiTrap protein G HP column affinity chromato-
graphy (Amersham Pharmacia Biotech). The puri-

Laboratory and pathologic evaluation of disease induction.
Mice were placed in metabolic cages for 12 hours to
collect urine for analysis. Urine was tested by dipstick
test for hematuria, proteinuria, and pyuria (Roche Diag-
óstics Corp., Indianapolis, Indiana, USA). Serum cre-

anti-MPO splenocytes (closed diamonds), 5 × 107 anti-MPO
splenocytes (filled squares), 1 × 107 anti-MPO splenocytes
(filled triangles), 1 × 106 anti-BSA splenocytes (open triangles), or 1 × 106
splenocytes from nonimmunized mice (open circles). Mice that received
5 × 107 or 1 × 107 anti-BSA splenocytes had no values
above 15 (data not shown).

Figure 1
Anti-MPO antibody ELISA titers in Rag2–/– mice that received 1 × 108
anti-MPO splenocytes (closed diamonds), 5 × 107 anti-MPO
splenocytes (filled squares), 1 × 107 anti-MPO splenocytes
(filled triangles), 1 × 106 anti-BSA splenocytes (open triangles), or 1 × 106
splenocytes from nonimmunized mice (open circles). Mice that received
5 × 107 or 1 × 107 anti-BSA splenocytes had no values
above 15 (data not shown).
5 × 10⁷ anti-MPO splenocytes but not in mice that received other doses of splenocytes. All 16 Rag2⁻/⁻ mice that received 1 × 10⁸ or 5 × 10⁷ anti-MPO splenocytes developed severe necrotizing and crescentic glomerulonephritis (Figure 4; Figure 5, c and d). Crescents involved an average of 83.8% of glomeruli in mice that received 1 × 10⁸ anti-MPO splenocytes (range 35–99%) and 85.0% in mice that received 5 × 10⁷ anti-MPO splenocytes (range 48–99%) (Figure 4). None of the 30 Rag2⁻/⁻ mice that received anti-BSA or control splenocytes or 1 × 10⁷ anti-MPO splenocytes developed glomerular crescents (Figure 4). None of the 30 Rag2⁻/⁻ mice that received 1 × 10⁸ or 5 × 10⁷ anti-MPO splenocytes developed segmental or global glomerular necrosis, often in association with crescent formation. Necrosis involved an average of 63.3% of glomeruli in mice that received 1 × 10⁸ anti-MPO splenocytes (range 33–87%) and 82.5% in mice that received 5 × 10⁷ anti-MPO splenocytes (range 42–97%). Necrosis was rare in mice that received anti-BSA or control splenocytes or 1 × 10⁷ anti-MPO splenocytes (Figure 4).

Most mice that received 1 × 10⁸ or 5 × 10⁷ anti-MPO, anti-BSA, or normal control splenocytes developed mild to moderate granular glomerular localization of mouse Ig's (Figures 4 and 5b), C3, and MPO. In general, the relative intensity of staining was C3>IgG>IgM>IgA>MPO. The staining for MPO colocalized with the immune deposits and was accentuated at sites of necrosis. Glomerular staining was more intense in mice that received 1 × 10⁸ or 5 × 10⁷ splenocytes than in those that received 1 × 10⁷ splenocytes. Glomerular staining for MPO was no different in mice that received anti-MPO splenocytes compared with those that received anti-BSA or control splenocytes. Only mice that received 1 × 10⁸ or 5 × 10⁷ anti-MPO splenocytes developed marked focally variable glomerular staining for fibrin that corresponded to foci of glomerular necrosis and crescent formation (Figure 5e). No localization of Ig's or complement was identified in renal arteries.

Electron microscopy performed on kidney tissue from four mice that received 1 × 10⁸ or 1 × 10⁷ anti-MPO

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**Figure 2**
Mean BUN and serum creatinine in Rag2⁻/⁻ mice 13 days after they received 1 × 10⁸, 5 × 10⁷, or 1 × 10⁷ anti-MPO splenocytes, anti-BSA splenocytes, or nonimmunized control splenocytes. The normal mouse assay reference range was 18–29 mg/dl for BUN and 0.2–0.8 mg/dl for serum creatinine. Samples taken before injection of splenocytes were within the reference ranges (data not shown).

**Figure 3**
Urinalysis results in Rag2⁻/⁻ mice 13 days after they received 1 × 10⁸, 5 × 10⁷, or 1 × 10⁷ anti-MPO splenocytes, anti-BSA splenocytes, or nonimmunized control splenocytes. Samples taken before injection of splenocytes showed mean proteinuria 1.0+, hematuria 0.2+, and leukocyturia 0.0+ (data not shown).
splenocytes and two mice that received $1 \times 10^8$ anti-BSA splenocytes demonstrated mesangial immune complex–type electron-dense deposits in all mice and a few subendothelial deposits in one mouse in each group. Areas of segmental necrosis in the mice that received anti-MPO splenocytes had breaks in glomerular basement membranes, fibrin tactoids in capillary lumens and Bowman’s space, and cellular crescent formation.

Development of circulating MPO-ANCA after passive transfer of anti-MPO antibodies. A single intravenous dose of anti-MPO IgG resulted in an immediate high level of circulating anti-MPO antibodies that subsequently declined. The mean anti-MPO ELISA titer in Rag2−/− mice was 15.6 prior to injection of anti-MPO IgG, 136.4 one hour after injection, 102.8 after 3 days, and 76.8 after 6 days. Passive transfer of anti-BSA IgG resulted in no increase in anti-MPO reactivity in serum, with mean anti-MPO titers of 15.2, 15.5, 15.3, and 15.2 at 0 hours, 1 hour, 3 days, and 6 days, respectively. The mean anti-MPO titer in WT B6 mice that received anti-MPO IgG was 109.5 one hour after injection, 100.7 after 3 days, and 98.7 after 6 days. The mean anti-MPO titer in WT B6 mice that received anti-BSA IgG was 109.5 one hour after injection, 100.7 after 3 days, and 98.7 after 6 days. The presence of anti-MPO antibodies was confirmed by indirect immunofluorescence assay on normal mouse neutrophils.

Development of glomerulonephritis after passive transfer of anti-MPO antibodies. By day 3, mice that received anti-MPO IgG already had developed hematuria, proteinuria, and leukocyturia, which persisted until sacrifice at day 6 (Table 2). Mice that received anti-BSA IgG did not develop hematuria, leukocyturia, or proteinuria above background. Rag2−/− mice that received anti-

Figure 4
Pathologic findings in Rag2−/− mice 13 days after they received $1 \times 10^8$, $5 \times 10^7$, or $1 \times 10^7$ anti-MPO splenocytes, anti-BSA splenocytes, or non-immunized control splenocytes. The extent of glomerular crescent formation is expressed as the mean percent of glomeruli with crescents in each animal. The extent of glomerular necrosis is expressed as the mean percent of glomeruli with necrosis in each animal. The extent of glomerular endocapillary hypercellularity is expressed as the mean on a scale of 0 (none) to 4+ (severe). The extent of glomerular immunostaining for IgG is expressed as the mean on a scale of 0 (none) to 4+ (very intense). Normal Rag2−/− mice had no crescents, necrosis, endocapillary hypercellularity, or glomerular Ig staining (data not shown).

Figure 5
Glomerular lesions in Rag2−/− mice 13 days after they received splenocytes. (a) No light microscopic abnormality in a glomerulus from a mouse that received $1 \times 10^7$ anti-MPO splenocytes. (b) Moderate (2+) endocapillary hypercellularity in a mouse that received $1 \times 10^8$ anti-BSA splenocytes. (c) Segmental fibrinoid necrosis (arrow) in a mouse that received $1 \times 10^8$ anti-MPO splenocytes. (d) Cellular crescent (arrow) in a mouse that received $1 \times 10^8$ anti-MPO splenocytes. (e) Immunofluorescence staining for fibrin in a crescent in a mouse that received $1 \times 10^8$ anti-MPO splenocytes. (f) Predominantly mesangial moderate (2+) immunofluorescence staining for IgG in a mouse that received $1 \times 10^8$ anti-BSA splenocytes. Periodic acid Schiff stain for light microscopy is shown.
MPO IgG, which had the most severe renal lesions pathologically, were the only group to have slightly elevated BUN (Table 2). Serum creatinine did not differ between mice that received anti-MPO IgG (0.3 mg/dl) and those that received anti-BSA IgG (0.3 mg/dl).

All five Rag2–/– mice sacrificed 6 days after receiving anti-MPO IgG had focal necrotizing glomerulonephritis (mean 13.2% of glomeruli with necrosis) and crescents (mean 10.8% of glomeruli with crescents), whereas mice that received anti-BSA IgG had no histologic lesions (Table 2; Figure 7). Likewise, all six WT B6 mice sacrificed 6 days after receiving anti-MPO IgG had focal necrotizing glomerulonephritis (mean 4.7% of glomeruli with necrosis) and crescents (mean 3.3% of glomeruli with crescents), whereas WT B6 mice that received anti-BSA IgG had no histologic lesions (Table 2; Figure 8). Electron microscopy revealed no immune complex–type electron-dense deposits in the glomeruli of the five Rag2–/– mice that received anti-MPO IgG. Immunofluorescence microscopy demonstrated little or no glomerular staining for Ig’s, C3, or MPO in Rag2–/– and WT B6 mice that received anti-BSA IgG. There was trace mesangial staining for Ig’s that was no different between mice that received anti-MPO or anti-BSA IgG. Mice that received anti-BSA IgG lacked the focal segmental glomerular staining for IgG, C3, and MPO. Mice that received anti-MPO IgG had intense focal segmental glomerular staining for fibrin that corresponded to foci of segmental necrosis and crescent formation (Figures 7e and 8d). There was no staining for fibrin in glomeruli of mice that received anti-BSA IgG. The paucity of staining for Ig’s and complement in the glomeruli of mice with glomerulonephritis induced by anti-MPO IgG was identical to the pattern of staining seen with human ANCA-associated pauci-immune glomerulonephritis.

Focal pulmonary alveolar capillaritis was identified in two of the six WT B6 mice that received anti-MPO IgG (Figure 8f). Three of the WT B6 mice that received anti-MPO IgG had grossly discernible cutaneous lesions on the ears. Histologically, all three had focal ulceration and infarction. Necrotizing arteritis with fibrinoid necrosis and leukocytoclasia was identified in one specimen (Figure 8e). The vasculitic lesions caused by anti-MPO IgG in the WT B6 mice were histologically identical to alveolar capillaritis and necrotizing arteritis in humans with ANCA-associated vasculitis.

**Table 2**  
Renal abnormalities in Rag2–/– and WT B6 mice 6 days after they received anti-MPO or anti-BSA IgG

<table>
<thead>
<tr>
<th>Type of IgG and type of mouse</th>
<th>% Crescents (mean &amp; range)</th>
<th>% Necrosis (mean &amp; range)</th>
<th>BUN (mg/dl) (mean &amp; range)</th>
<th>Prot/Hem/Leu (0–4+)</th>
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</thead>
<tbody>
<tr>
<td>Anti-MPO IgG in Rag2–/– mice</td>
<td>10.8% (5–15)</td>
<td>13.2% (11–24)</td>
<td>47.4 (34–54)</td>
<td>2.0/2.7/1.8</td>
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<tr>
<td>Anti-BSA IgG in Rag2–/– mice</td>
<td>0%</td>
<td>0%</td>
<td>22.7 (21–25)</td>
<td>1.0/0.5/0.0</td>
</tr>
<tr>
<td>No IgG in Rag2–/– mice</td>
<td>0%</td>
<td>0%</td>
<td>21.4 (19–31)</td>
<td>1.0/0.0/0.0</td>
</tr>
<tr>
<td>Anti-MPO IgG in WT B6 mice</td>
<td>3.3% (2–6)</td>
<td>4.7% (3–7)</td>
<td>23.3 (21–27)</td>
<td>1.6/2.2/1.2</td>
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<tr>
<td>Anti-BSA IgG in WT B6 mice</td>
<td>0%</td>
<td>0%</td>
<td>25.7 (22–29)</td>
<td>1.0/0.0/0.0</td>
</tr>
</tbody>
</table>

Prot/Hem/Leu, proteinuria/hematuria/leukocyturia.
Rag2–/– mice that received 5 × 10^7 or 1 × 10^8 anti-MPO splenocytes and all five Rag2–/– mice and all six WT B6 mice that received 50 µg/g anti-MPO IgG developed glomerular crescents and necrosis. None of 17 Rag2–/– mice that received anti-BSA splenocytes or anti-BSA IgG developed glomerular crescents, nor did any of the mice that received only 1 × 10^7 anti-MPO splenocytes. None of three WT B6 mice that received anti-BSA IgG developed crescents.

All Rag2–/– mice that received splenocytes from immune-competent Mpo–/– mice developed low to moderate levels of glomerular immune complex localization. These immune deposits caused mild to moderate proliferative glomerulonephritis but, in the absence of anti-MPO, caused little or no renal insufficiency and no glomerular crescents or necrosis. The basis for the uniform production of glomerular immune deposits is not known but probably relates to the introduction of functioning lymphocytes into mice that previously had no functioning adaptive immune system. This low to moderate level of glomerular immune complex localization was the same in intensity and composition in mice irrespective of the transfer of anti-MPO, anti-BSA, or control splenocytes. The basis for this immune complex glomerulonephritis is unknown. Possibilities include reactions of the newly synthesized antibodies with circulating exogenous antigens, or production of autoantibodies possibly as a component of a graft-versus-host reaction. The latter is not likely, since all of the mice had the same B6 background and thus shared the same major histocompatibility antigens. Induction of an autoimmune response remains a possibility; however, preliminary cell extract immunoprecipitation analysis of serum from the mice that received splenocytes, performed by Hanno Richards at the University of Florida (Gainesville, Florida, USA), revealed no autoantibodies, including no antibodies against DNA, Sm, or ribonucleoproteins (data not shown).

Figure 7
Glomerular lesions in Rag2–/– mice 6 days after receiving anti-MPO IgG. (a) Glomerulus with no lesion. (b) Segmental fibrinoid necrosis (arrow). (c) Segmental fibrinoid necrosis with an adjacent small cellular crescent (arrow). (d) Large circumferential cellular crescent (between arrows) completely surrounding a glomerulus. (e) Immunofluorescence microscopy for fibrin showing prominent staining corresponding to segmental necrosis and crescent formation. (f) Immunofluorescence microscopy for IgG showing a paucity of segmental staining corresponding to an area of segmental necrosis. Masson trichrome staining for light microscopy is shown.

Figure 8
Vasculitic lesions in WT B6 mice 6 days after they received anti-MPO IgG. (a) Glomerulus with segmental fibrinoid necrosis (periodic acid Schiff stain). (b) Glomerulus with segmental fibrinoid necrosis and crescent formation (periodic acid Schiff stain). (c) Glomerulus with segmental fibrinoid necrosis and crescent formation (H&E stain). (d) Immunofluorescence microscopy for fibrin showing prominent staining corresponding to segmental necrosis and crescent formation. (e) Necrotizing arteritis with leukocytoclasis in the dermis of the ear (H&E stain). (f) Pulmonary alveolar capillaritis on the left and more normal lung on the right.
In striking contrast to Rag2−/− mice that received anti-BSA or normal control splenocytes, mice that received anti-MPO splenocytes developed very severe necrotizing and crescentic glomerulonephritis and small-vessel vasculitis. This disease induction was dose-dependent, since all mice that received 5 × 10⁷ or 1 × 10⁸ anti-MPO splenocytes developed severe crescentic glomerulonephritis but those that received 1 × 10⁷ developed no crescents at all. This dose effect is in accord with the serum titers of anti-MPO attained after the different doses of splenocytes, which were similarly high in mice that received 5 × 10⁷ or 1 × 10⁸ anti-MPO splenocytes and substantially lower in those that received 1 × 10⁷ anti-MPO splenocytes (Figure 1).

In addition to necrotizing and crescentic glomerulonephritis, mice that received anti-MPO splenocytes developed pulmonary hemorrhagic capillaritis and systemic necrotizing arteritis. The lesions were histologically identical to the pulmonary hemorrhagic capillaritis and systemic necrotizing arteritis that occurs in patients with ANCA-associated small-vessel vasculitis, such as microscopic polyangiitis and Wegener granulomatosis (1, 2). One mouse even had necrotizing granulomatous inflammation in a lymph node that resembled the granulomatous inflammation of Wegener granulomatosis. Only two or three levels of section of each organ specimen were evaluated for this study. Given the focal nature of the vasculitic lesions and granulomatous inflammation, many more lesions should be detected by evaluating additional tissue sections.

The transfer of splenocytes introduced both anti-MPO B lymphocytes and anti-MPO T lymphocytes into recipient mice. Thus, either MPO-specific T lymphocytes or antibodies produced by MPO-specific B lymphocytes could be mediating the glomerulonephritis and vasculitis in the mice that received splenocytes. To evaluate the pathogenicity of anti-MPO antibodies alone (i.e., MPO-ANCAs), purified anti-MPO IgG or anti-BSA IgG was injected into Rag2−/− mice and WT B6 mice. All five Rag2−/− mice and all six WT B6 mice that received anti-MPO IgG developed focal glomerular necrosis and crescent formation, whereas none of the six mice that received anti-BSA IgG did. This demonstrates that anti-MPO IgG causes glomerular necrosis and crescents in the absence of antigen-specific T and B lymphocytes in Rag2−/− mice and in the presence of a competent immune system in WT B6 mice. The glomerular lesions that were caused in mice by anti-MPO IgG were identical by light microscopy and immunofluorescence microscopy to the glomerular lesions of human ANCA-associated pauci-immune glomerulonephritis and small-vessel vasculitis (1, 2). Both mouse and human glomerular lesions have fibrinoid necrosis, crescent formation, and an absence or paucity of staining for Ig's by immunofluorescence microscopy. The WT B6 mice also developed vasculitic lesions identical to human ANCA-associated disease in other organs, including pulmonary alveolar capillaritis and cutaneous necrotizing arteritis.

The so-called pauci-immune characteristic of ANCA-associated glomerulonephritis is pathologically very distinct from the substantial vessel wall localization of Ig's in immune complex–mediated glomerulonephritis and glomerulonephritis induced by anti–glomerular basement membrane (anti-GBM) antibody. This suggests that the pathogenesis of pauci-immune ANCA-associated glomerulonephritis is distinct from that for immune complex glomerulonephritis or anti-GBM glomerulonephritis. This distinct mechanism appears to involve ANCA-induced activation of neutrophils and monocytes. ANCA IgG can activate neutrophils and monocytes in vitro (5, 6). For example, ANCA IgG stimulates cytokine-primed neutrophils to release injurious oxygen metabolites and proteinases (11–13). Activation of neutrophils by ANCA IgG also induces the release of numerous proinflammatory cytokines, such as IL-1, IL-8, and leukotrienes (14–16). Monocytes also have ANCA antigens, including MPO and PR3, and can be induced to undergo a respiratory burst and release of proteinases and cytokines after stimulation by ANCA IgG (17–19). Neutrophils that have been activated by ANCA IgG are capable of adhering to and killing endothelial cells in vitro (20, 21).

In vitro and in vivo experiments indicate that the activation of neutrophils and monocytes by ANCA and the induction of tissue injury by ANCA are facilitated by minor inflammatory stimuli that prime leukocytes to interact with ANCA (5–7). This priming causes increased expression of ANCA antigens, such as MPO, at the surface of neutrophils and monocytes where the antigens can interact with ANCA and cause leukocyte activation. The greater severity of the glomerulonephritis induced by anti-MPO splenocytes compared with that induced by anti-MPO IgG might be due to the synergistic presence of the glomerular immune complexes in the former acting as a priming factor for leukocytes. An alternative explanation is that anti-MPO T lymphocytes that are transferred into the recipients with the splenocytes might have an additive effect on the severity of the vascular inflammatory injury.

The in vitro data that document the ability of ANCA IgG to activate neutrophils and monocytes, combined with the current mouse model of ANCA-induced glomerulonephritis and vasculitis, strongly support a primary pathogenic role for ANCA in ANCA-associated glomerulonephritis and vasculitis. Using the Bradford Hill criteria for causation (22), the clinical and experimental evidence that is now available strongly indicates that ANCA's cause ANCA-associated glomerulonephritis and small-vessel vasculitis. The Bradford Hill criteria for concluding that an association is indicative of causation are: 1, strength; 2, specificity; 3, consistency; 4, temporality; 5, biological gradient; 6, experimental evidence; 7, coherence; and 8, analogy.

Criteria 1 and 2 are fulfilled by the very strong and specific clinical association between ANCA's and pauci-immune crescentic glomerulonephritis and small-vessel vasculitis (1, 2). Approximately 80–90% of active
untreated patients with pauci-immune necrotizing and crescentic glomerulonephritis and vasculitis have circulating MPO-ANCA or PR3-ANCA. Less than 10% of patients with other types of glomerulonephritis and vasculitis have ANCA, and less than 1% of the general population has ANCA.

Criterion 3 is fulfilled by the virtually complete consistency among research groups throughout the world in demonstrating the association between ANCs and pauci-immune glomerulonephritis and small-vessel vasculitis.

Criteria 4, 5, and 6 have been difficult to document clinically, but the experimental data in this article show that crescentic glomerulonephritis and small-vessel vasculitis develop within a few days after introduction of ANCA into experimental animals (temporality), and that the occurrence and magnitude of this effect is dose-dependent (gradient).

Criterion 7 is fulfilled by the coherence of the in vitro observations that ANCA IgG causes activation of neutrophils and monocytes with the in vivo pathologic observations that the acute phase of injury is characterized by necrotizing acute inflammation that is mediated by activated neutrophils and monocytes.

Criterion 8 is fulfilled by the analogy between MPO and PR3, which are the two major antigenic targets of ANCs. Although they are very different molecules with very different biological function, MPO and PR3 have the same locations in the cytoplasm of neutrophils and monocytes, are similarly displayed and released during varying phases of activation, and are both targeted by ANCs. These analogies between MPO and PR3 support the pathogenic potential not only of MPO-ANCA but also of PR3-ANCA.

Thus, based on the experimental model described here, we conclude that the association in patients between ANCs and pauci-immune glomerulonephritis and vasculitis is most likely due to causation. Therefore, we propose that the often-used terms “ANCA-associated glomerulonephritis” and “ANCA-associated vasculitis” should be changed to “ANCA glomerulonephritis” and “ANCA vasculitis.” The results of these studies suggest that therapeutic strategies that selectively eliminate or neutralize ANCs could be effective in treating ANCA glomerulonephritis and vasculitis.

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