Effector mechanisms of ANCA-associated glomerulonephritis
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Pathogenicity of monoclonal antibodies against myeloperoxidase in 129S6 mice

van der Veen BS, van Timmeren MM and Heeringa P
ABSTRACT

In patients with anti-neutrophil cytoplasmic autoantibody (ANCA)-associated vasculitis and in a mouse model of ANCA vasculitis, polyclonal antibodies against myeloperoxidase (MPO-ANCA) induce vasculitis and crescentic glomerulonephritis. It is unknown whether the pathogenic potential of MPO-ANCA IgG is dependent on a specific anti-MPO IgG subclass and/or a specific epitope. Previously, it was shown that systemic injection of monoclonal anti-MPO antibodies (anti-MPO moAbs) of different subclasses and epitope specificity did not induce glomerulonephritis in C57BL/6 mice, neither in the presence nor absence of bacterial lipopolysaccharide (LPS). Recently, it has been demonstrated that mice of the 129S6 background strain develop more severe glomerulonephritis upon injection of polyclonal anti-MPO antibodies when compared to C57BL/6 mice. Our objective was to examine whether monoclonal anti-MPO antibodies induce crescentic glomerulonephritis in 129S6 mice. 129S6 mice received either polyclonal anti-MPO IgG or a mix of three anti-MPO moAbs of different subclass and recognizing non-overlapping epitopes. In a subset of mice, the anti-MPO moAbs were followed by an injection of a moderate dose of LPS. We found induction of glomerulonephritis upon administration of polyclonal anti-MPO IgG in 129S6 mice, as evidenced by the occurrence of hematuria, albuminuria and glomerular crescent formation. Monoclonal anti-MPO moAbs induced glomerulonephritis in 129S6 mice as well, but only in combination with systemic LPS administration. Co-administration of isotype control moAbs and LPS did not induce glomerulonephritis. This preliminary study shows that monoclonal anti-MPO antibodies induce crescentic glomerulonephritis in 129S6 mice when co-administered with LPS. These results suggest that anti-MPO moAb-induced crescentic glomerulonephritis in 129S6 mice can potentially serve as a model for pathogenic analysis of specific ANCA IgG subclasses and antigen recognition patterns and for future therapeutic studies.
INTRODUCTION

Anti-neutrophil cytoplasmic autoantibody (ANCA)-associated small-vessel vasculitides are severe systemic autoimmune diseases with a high mortality that are frequently characterized by the development of glomerulonephritis. Patients with ANCA-associated vasculitis have circulating autoantibodies directed against neutrophilic enzymes, including myeloperoxidase (MPO). In C57BL/6 mice, polyclonal anti-MPO antibodies induce crescentic glomerulonephritis, demonstrating that anti-MPO antibodies are pathogenic. In this MPO-ANCA mouse model, co-administration of bacterial lipopolysaccharide (LPS) aggravates anti-MPO IgG-induced crescentic glomerulonephritis.

In both ANCA-associated vasculitis patients and in the mouse model of MPO-ANCA vasculitis, the anti-MPO antibody response involves all IgG subclasses, although some subclasses of patient ANCA IgG are more abundant (IgG1 and IgG4) or have more neutrophil-activating capacity in vitro (IgG3). It is however unclear whether the pathogenicity of ANCA IgG can be ascribed to a specific subclass and/or epitope. To investigate this, 11 monoclonal anti-MPO antibodies (anti-MPO moAbs) have been generated previously and tested for their pathogenicity in C57BL/6 mice. In these mice, the anti-MPO moAbs were not able to induce crescentic glomerulonephritis when administered alone or in several combinations, with or without LPS. However, some of the anti-MPO moAbs did have pathogenic potential, as demonstrated by their ability to aggravate mild glomerular injury induced by anti-glomerular basement membrane (GBM) antibody in C57BL/6 mice.

It has been suggested that genetic variation may, among other factors, determine the susceptibility for ANCA-associated glomerulonephritis. In mice, strain differences in susceptibility to immune-mediated glomerulonephritis have also been reported. Recent data suggest that mice of the 129S6 strain are more susceptible for development of crescentic glomerulonephritis induced by polyclonal anti-MPO antibodies than C57BL/6 mice.

In this study, we examined whether anti-MPO moAbs induce crescentic glomerulonephritis in 129S6 mice. To this end, we administered both polyclonal anti-MPO IgG and a combination of three anti-MPO moAbs, with or without LPS, to 129S6 mice and analyzed development of crescentic glomerulonephritis.

MATERIALS AND METHODS

Mice

Mpo−/− mice were backcrossed to a C57BL/6 background for seven times and bred in house. Female 129S6 mice were obtained from Taconic (Ejby, Denmark). All animal experiments were performed according to national guidelines and upon approval of the Institutional Animal Care and Use Committee.

Production of polyclonal anti-MPO antibodies

To produce polyclonal anti-MPO IgG, Mpo−/− mice were immunized with murine MPO that was purified from WEHI-3 cells, as described previously. Total IgG was isolated from pooled sera of
immunized $Mpo^{-/-}$ mice and the anti-MPO titer was checked by ELISA as reported previously.5

**Production of monoclonal anti-MPO antibodies (moAbs)**

The production and characterization of 11 anti-MPO moAbs has been described previously.8 In brief, $Mpo^{-/-}$ mice were immunized with mouse MPO. Spleens were harvested and a single-cell suspension was made. Cells were then fused with SP2/0 cells to generate anti-MPO IgG-producing hybridomas. Anti-MPO IgG-producing clones were selected by ELISA and subcloned 3-5 times to obtain monoclonal clones. Anti-MPO IgG was isolated from culture supernatants by protein G column affinity chromatography after concentration by ultracentrifugation. The isotype of each moAb was determined using a mouse isotyping test kit (Hbt, Uden, the Netherlands) and epitope specificity of the anti-MPO moAbs was determined by inhibition ELISA using biotinylated and non-biotinylated moAbs (Table 1).

**Administration of polyclonal and monoclonal anti-MPO antibodies to mice**

To induce glomerulonephritis, 129S6 mice (age 9-11 weeks; body weight 21.1 ± 1.5 g) received polyclonal anti-MPO IgG (1 mg; $n = 6$ mice) or an anti-MPO moAb mix consisting of three moAb clones (3F7 (IgG1), 8F11 (IgG2a) and 6D1 (IgG2b); 100 μg each; $n = 5$ mice) via an intravenous injection. In some mice, the anti-MPO moAb injection was followed by an intraperitoneal injection with 150 EU/g body weight of LPS ($Escherichia Coli$, serotype O26:B6; Sigma-Aldrich, St Louis, MO, USA) one hour later ($n = 9$ mice). As controls, additional groups of mice received an intraperitoneal injection with LPS alone (150 EU/g body weight; $n = 4$ mice) or an intravenous injection with an isotype control moAb mix consisting of three moAbs (MOPC-21 (IgG1), Cl.18 (IgG2a) and MPC-11 (IgG2b); 100 μg each; all from Bio X Cell, West Lebanon, NH, USA; $n = 4$ mice), followed by an intraperitoneal injection with LPS (150 EU/g body weight) one hour later.

**Laboratory and pathological evaluation of glomerulonephritis**

Urine was collected for 18 hours using metabolic cages at 1 and 7 day(s) after anti-MPO antibody administration and mice were sacrificed after 7 days. Hematuria (0-4+ score) was evaluated by Combur-Test® strips (Roche Diagnostics BV, Almere, the Netherlands) and albuminuria by ELISA (Bethyl Laboratories, Montgomery, TX, USA). Paraffin kidney sections (2 μm) were stained with hematoxylin and eosin (H&E) or periodic acid-schiff (PAS) stain. The number of glomerular crescents (≥2 cell layers in Bowman’s space) was determined by evaluating 100 consecutive glomerular cross sections in a blinded fashion.

**Statistical analysis**

Statistical significance was determined by 1-way ANOVA using GraphPad Prism 4.03 (Graphpad Software, San Diego, CA, USA).
RESULTS

We first determined the susceptibility of 129S6 mice for development of crescentic glomerulonephritis in response to polyclonal anti-MPO antibodies. Polyclonal anti-MPO IgG was isolated from MPO-immunized Mpo-/− mice and administered intravenously to 129S6 mice. As shown in Figure 1, administration of polyclonal anti-MPO IgG induced hematuria after 1 day and both hematuria and albuminuria after 7 days. In addition, pathological analysis of renal sections revealed that all mice had developed glomerular crescents (6.5 ± 2.3% of glomeruli, n = 6). These data demonstrate that 129S6 mice are susceptible for the development of crescentic glomerulonephritis in response to systemic administration of polyclonal anti-MPO antibodies.

Figure 1 Development of crescentic glomerulonephritis in 129S6 mice upon polyclonal anti-MPO IgG. 129S6 mice (n = 6) received 1 mg polyclonal anti-MPO IgG i.v. and were sacrificed after 7 days. Urine was analyzed for hematuria (A) and albuminuria (B) after 1 and 7 days. Bars represent mean ± sd. The horizontal line in B indicates baseline albumin levels (32.1 ± 10.4 μg/18h; n = 6). *** P < 0.001 compared to baseline and day 1 albumin levels. Kidneys were pathologically evaluated for presence of glomerular crescents upon polyclonal anti-MPO IgG administration (quantification: 6.5 ± 2.3% crescentic glomeruli). (C) A representative image of a kidney section is shown. Besides a glomerular crescent (white arrow), red blood cell and protein casts are present in some tubuli (black arrows). Original magnification, 200x. (D) shows a glomerular crescent at higher magnification (400x). (color image on page 186)
Next, we investigated whether monoclonal anti-MPO antibodies induce crescentic glomerulonephritis in 129S6 mice. Based on the previous characterization of 11 anti-MPO moAbs, we selected an IgG1, an IgG2a and an IgG2b moAb that do not cross-inhibit each other by ELISA, indicating that the moAbs recognize different epitopes (Table 1). As shown in Figure 2, administration of the mix of these three anti-MPO moAbs did not induce hematuria, albuminuria or glomerular crescent formation in 129S6 mice. As LPS is known to aggravate the development of glomerulonephritis induced by polyclonal anti-MPO antibodies in C57BL/6 mice, we examined whether anti-MPO moAbs induce glomerulonephritis in 129S6 mice in the presence of LPS. Co-administration of anti-MPO moAbs with LPS induced hematuria after 1 and 7 days and albuminuria after 7 days. In addition, all mice had developed glomerular crescents. To confirm that the observed disease characteristics were induced specifically by anti-MPO moAbs, we determined the effects of co-administration of isotype control moAbs and LPS and administration of LPS alone. None of the mice that received isotype control moAbs/LPS or LPS alone developed hematuria, albuminuria or glomerular crescents. These results demonstrate that anti-MPO moAbs induce crescentic glomerulonephritis in 129S6 mice when co-administered with LPS.

Table 1 Isotype specificity and cross-inhibition analysis of the 11 anti-MPO moAbs previously generated and characterized. The 3 anti-MPO moAbs highlighted in bold were used in this study. The table is adapted and modified from Huugen et al.8

<table>
<thead>
<tr>
<th>Clone</th>
<th>Isotype</th>
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<tbody>
<tr>
<td>8F4</td>
<td>IgG1</td>
<td>4H9</td>
</tr>
<tr>
<td>2D4</td>
<td>IgG1</td>
<td>8F4, 4H9, 6G4</td>
</tr>
<tr>
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<tr>
<td>6D1</td>
<td>IgG2b</td>
<td>1F1, 3B1, 1H4</td>
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Figure 2 Development of crescentic glomerulonephritis in 129S6 mice upon anti-MPO moAbs and LPS. 129S6 mice received a mix of three non-overlapping anti-MPO moAbs (IgG1, IgG2a and IgG2b; 100 μg each) or isotype control moAbs i.v., with or without i.p. co-administration of bacterial lipopolysaccharide (LPS; 150 EU/g). Mice were sacrificed after 7 days and urine was analyzed for hematuria on day 1 (A) and day 7 (B) and for albumin at both time points (C). Bars represent mean ± sd. The horizontal line in (C) indicates baseline albumin levels (66.3 ± 41.0 μg/18h; n = 25). *** P < 0.001 compared to baseline albumin levels and to the other groups on day 7. Kidneys were pathologically evaluated for glomerular crescent formation (D). Representative images of glomerular crescents (E and F) from mice that received anti-MPO moAbs and LPS are shown. (G) and (H) demonstrate no glomerular abnormalities upon administration of isotype moAbs and LPS. Original magnifications, 200x (E and G), 400x (F and H). (color image on page 187)
Chapter 5

DISCUSSION

This preliminary study shows that a selected combination of monoclonal anti-MPO antibodies induces crescentic glomerulonephritis in 129S6 mice when co-administered with LPS. The selected combination of anti-MPO moAbs was not able to induce glomerulonephritis when LPS was not administered. In contrast, polyclonal anti-MPO antibodies did induce glomerulonephritis in these mice without the necessity of LPS co-administration.

The concept of using combinations of moAbs against a single antigen for the development of models for autoantibody-driven diseases has been employed previously to create a mouse model for rheumatoid arthritis. In this collagen antibody-induced arthritis (CAIA) model, a cocktail consisting of 3-5 moAbs directed against collagen II induces arthritis in mice characterized by inflammatory injury to cartilage and the bone architecture. Although such a cocktail of anti-collagen II moAbs is pathogenic by itself, co-administration of LPS with the moAb cocktail is usually required to achieve severe persisting arthritis with rapid onset. LPS bypasses the required multiple epitope specificity, as it decreased the number of moAbs required for inducing arthritis from 4 to 2 clones. In addition, LPS reduced the threshold values of the arthritogenic dose of moAb (from 1 mg to 50 μg/clone per mouse). These results suggest that anti-MPO moAbs may induce glomerulonephritis in the absence of LPS when higher amounts of moAb are administered. Future experiments are necessary to optimize the dosing of anti-MPO moAbs to 129S6 mice in order to achieve a more robust model that reproducibly induces glomerulonephritis, either with or without LPS co-administration. For instance, additional moAbs recognizing more epitopes could be administered and/or higher doses of moAbs could be used. This optimized anti-MPO moAb-induced glomerulonephritis model can then potentially replace the current MPO-ANCA mouse model that is based on polyclonal anti-MPO IgG-induced glomerulonephritis in C57BL/6 mice. The use of moAbs would eliminate the need to raise polyclonal anti-MPO antibodies by immunization of Mpo-/- mice, which is a laborious procedure accompanied with apparent animal discomfort. Thus, optimization of glomerulonephritis induction by anti-MPO moAbs may provide a new, less laborious, model for MPO-ANCA glomerulonephritis that can be used for studying mechanisms of disease pathogenesis and experimental therapeutic interventions.

Patients with ANCA-associated vasculitis have circulating ANCA IgG molecules of all IgG subclasses (IgG1-IgG4) recognizing different epitopes. It is not known whether within this polyclonal batch of antibodies, the pathogenicity of ANCA IgG can be ascribed to a specific IgG subclass. Of the four subclasses, the IgG1 and IgG3 subclasses are generally considered the most pro-inflammatory due to their ability to fix and activate complement and to bind to protein antigens. In line with this, ANCA IgG molecules of the IgG1 and IgG3 subclass display a marked neutrophil-activating capacity in vitro. Moreover, clinical studies in patients with ANCA-associated vasculitis have suggested that the IgG3 subclass is associated with active disease and renal involvement. In mice, the most pro-inflammatory subclasses are IgG2a and IgG2b as compared to IgG1 and
IgG3 in humans.\(^2\) Interestingly, anti-MPO antibodies of the IgG2a and IgG2b subclass may also be most pathogenic, as IgG2a and IgG2b anti-MPO moAbs were found to aggravate anti-glomerular basement membrane (GBM) antibody-induced glomerulonephritis,\(^6\) whereas an IgG1 anti-MPO moAb did not (unpublished observations). More research is required to establish whether certain anti-MPO moAbs with specific subclasses are indeed more pathogenic than others. This can, for example, be studied by using IgG heavy chain switch variants. In addition, it is also unclear whether the pathogenicity of ANCA may be ascribed to one, or some, relapse- or disease-inducing epitopes. It has been shown that patient-derived MPO-ANCA and Pr3-ANCA recognize a restricted set of epitopes within the ANCA antigen (reviewed in reference 18), but to what extent antigen epitopes determine pathogenicity is unclear. The 129S6 model may provide a model to study the importance of specific IgG subclasses and epitope specificities for the pathogenicity of ANCA in more detail.

It has been suggested that genetic variation may underlie susceptibility to autoimmune diseases, including ANCA-associated vasculitides.\(^9\) Studies in animal models of MPO-ANCA glomerulonephritis have revealed differences in susceptibility for glomerulonephritis induced by anti-MPO antibodies between strains. In the case of rats, Wistar Kyoto (WKY) rats developed anti-MPO antibody-induced glomerulonephritis, whereas Wistar Furth, Lewis and Brown Norway rats did not.\(^2\)\(^3\) Also for mice, Xiao et al recently reported that 129S6 mice develop more severe glomerulonephritis in response to anti-MPO IgG (69% crescents, range 42-90%) as compared to C57BL/6 mice (8.56%, range 4-23%).\(^1\)\(^1\) A high susceptibility of the 129S6 mouse strain for glomerulonephritis development was also found in studies of anti-GBM antibody-induced glomerulonephritis, in which the 129S6 strain was compared with several other mouse strains, including C57BL/6.\(^1\)\(^0\), \(^2\)\(^4\) Unexpectedly, in our study, polyclonal anti-MPO IgG induced a substantial lower percentage of glomerular crescents in 129S6 mice (6.5% crescents, range 3-9%) as compared to 129S6 mice in the study by Xiao et al (69%, range 42-90%). As we did not include a direct comparison of C57BL/6 and 129S6 mice, we do not know whether 129S6 mice were relatively more susceptible to anti-MPO antibody-induced glomerulonephritis in our study. The difference in disease severity between our study and the study of Xiao et al may relate to a difference in pathogenicity of the anti-MPO IgG batch employed or in exposure of mice to pathogens, for instance due to animal housing conditions. More studies are required to determine whether 129S6 mice are indeed more susceptible to anti-MPO IgG-induced glomerulonephritis than C57BL/6.

In conclusion, this preliminary study shows that monoclonal anti-MPO antibodies induce crescentic glomerulonephritis in 129S6 mice when co-administered with LPS. When optimized, anti-MPO moAb-induced crescentic glomerulonephritis in 129S6 mice can potentially serve as a model for the analysis of the contribution of specific ANCA IgG subclasses and antigen recognition patterns to the pathogenesis of ANCA-mediated glomerulonephritis and vasculitis and for testing experimental therapies.
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