IgG Glycan Hydrolysis Attenuates ANCA-Mediated Glomerulonephritis

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ABSTRACT

Anti-neutrophil cytoplasmic autoantibodies (ANCA) directed against myeloperoxidase (MPO) and proteinase 3 (Pr3) are considered pathogenic in ANCA-associated necrotizing and crescentic glomerulonephritis (NCGN) and vasculitis. Modulation of ANCA IgG glycosylation may potentially reduce its pathogenicity by abolishing Fc receptor–mediated activation of leukocytes and complement. Here, we investigated whether IgG hydrolysis by the bacterial enzyme endoglycosidase S (EndoS) attenuates ANCA-mediated NCGN. In vitro, treatment of ANCA IgG with EndoS significantly attenuated ANCA-mediated neutrophil activation without affecting antigen-binding capacity. In a mouse model of anti-MPO IgG/LPS-induced NCGN, we induced disease with either unmodified or EndoS-treated (deglycosylated) anti-MPO IgG. In separate experiments, we administered EndoS systemically after disease induction with unmodified anti-MPO IgG. Pretreatment of anti-MPO IgG with EndoS reduced hematuria, leukocyturia, and albuminuria and attenuated both neutrophil influx and formation of glomerular crescents. After inducing disease with unmodified anti-MPO IgG, systemic treatment with EndoS reduced albuminuria and glomerular crescent formation when initiated after 3 but not 24 hours. In conclusion, IgG glycan hydrolysis by EndoS attenuates ANCA-induced neutrophil activation in vitro and prevents induction of anti-MPO IgG/LPS-mediated NCGN in vivo. Systemic treatment with EndoS early after disease induction attenuates the development of disease. Thus, modulation of IgG glycosylation is a promising strategy to interfere with ANCA-mediated inflammatory processes.


Similar to autoantibodies in other autoimmune diseases, ANCA are considered to be pathogenic.4 The pathogenic effect of ANCA comprises activation of cytokine-primed neutrophils within the mi-
crovasculature through binding of ANCA IgG to surface-expressed antigens with their F(ab)\textsubscript{2} portions and ligating to constitutively expressed neutrophil Fcy receptors (FcyR), FcyRIIa, and FcyRIIb, with their Fc tail.\textsuperscript{5–7} The activated neutrophils release their granules, which contain tissue-degrading enzymes, and generate oxygen radicals, leading to bystander damage of endothelial cells. In addition, the alternative complement pathway is activated and the recruitment of neutrophils and monocytes is promoted, resulting in accelerated inflammation and eventually organ damage.\textsuperscript{4} The pathogenicity of ANCA is most convincingly proven by the finding that administration of murine anti-MPO antibodies to mice induces an acute glomerular inflammation that progresses to NCGN within days.\textsuperscript{8} Subsequent studies have shown that anti-MPO IgG–mediated NCGN in this model is severely aggravated upon co-administration of lipopolysaccharide (LPS), requires neutrophils as the main effector cells, and is complement and Fc receptor dependent.\textsuperscript{9–12}

To function properly, IgG molecules require glycosylation of a conserved asparagine residue (Asn297) in the CH\textsubscript{2} domains of both heavy chains of the Fc fragment. These Fc glycans are complex biantennary structures with a high degree of heterogeneity, depending on species, age, gender, and disease status.\textsuperscript{13} Modifications in the Fc glycans cause conformational changes of the IgG molecule, which affect the affinity of the Fc fragment for binding to FcyRs and complement factor C1q. Complete removal of the Fc glycans by glycoside hydrolases abolishes Fc-mediated effector functions, such as antibody-dependent cell-mediated cytotoxicity and complement-dependent cytotoxicity. IgG glycan hydrolysis is therefore a promising strategy for the treatment of (auto)antibody-mediated diseases.

Endoglycosidase S (EndoS), secreted by Streptococcus pyogenes, specifically hydrolyzes the conserved asparagine-linked glycans on the IgG heavy chains.\textsuperscript{14,15} EndoS hydrolyzes all subclasses of human IgG and almost completely abolishes IgG binding to FcyRs and IgG-dependent complement activation.\textsuperscript{16} In vivo, administration of EndoS to mice causes complete hydrolysis of circulating IgG within hours that lasts for several days.\textsuperscript{17} Importantly, in vivo EndoS administration does not affect glycosylated plasma proteins other than IgG and has no detectable adverse effects. Moreover, repeated EndoS injections induce only a minimal immune response against the enzyme that does not affect its activity.\textsuperscript{15} Interestingly, pretreatment of pathogenic autoantibodies with EndoS abrogates disease development in mouse models of arthritis and immune thrombocytopenic purpura.\textsuperscript{15,18} Systemic injection of EndoS also rescues mice from already established immune thrombocytopenic purpura and inhibits pathology in lupus-prone mice.\textsuperscript{15,17}

We hypothesized that Fc glycans of ANCA IgG are important for the development of ANCA-associated glomerulonephritis/vasculitis and that glycan hydrolysis by EndoS abrogates the pathogenic effects of ANCA. To test this hypothesis, we evaluated whether EndoS (pre)treatment diminishes glomerulonephritis development in experimental anti-MPO antibody/LPS-induced NCGN.

**RESULTS**

**EndoS-Mediated Glycan Hydrolysis Inhibits ANCA IgG–Induced Neutrophil Activation**

*EndoS Treatment Efficiently Hydrolyzes ANCA IgG Glycans* MPO- and Pr3-ANCA IgG isolated from ANCA-positive patients with active, biopsy-proven NCGN (Table 1) were treated with glutathione S-transferase (GST)-tagged EndoS or GST alone. Subsequent SDS-PAGE analysis showed that EndoS treatment reduced the molecular weight of the IgG heavy chain by approximately 3 kD (Figure 1), which corresponds to loss of the Fc glycans.\textsuperscript{15} Lens culinaris agglutinin lectin (LCA) blot analysis of the same samples showed that LCA could not bind to EndoS–treated ANCA IgG. It was previously shown by mass spectroscopy that the lack of LCA signal corresponds well with complete IgG glycan hydrolysis.\textsuperscript{19} For some patients, the LCA signal was very low (patient 8) or not detectable (patients 7 and 9) in untreated ANCA IgG. Nevertheless, the combined analysis of lectin blots and stained SDS-PAGE clearly demonstrates that EndoS completely deglycosylates the heavy chains of all patients’ ANCA IgG.

*EndoS Treatment of ANCA IgG Does Not Affect Antigen-Binding Capacity* To verify that EndoS treatment does not affect the antigen-binding capacity of ANCA IgG, we tested the samples in a

<table>
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<tr>
<th>Parameter</th>
<th>ANCA Specificity</th>
<th>ANCA Titer</th>
<th>Gender</th>
<th>Age (years)</th>
</tr>
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| **Patients**
| 1 | Anti-MPO | 160 | M | 66 |
| 2 | Anti-MPO | 320 | F | 23 |
| 3 | Anti-MPO | 80 | M | 43 |
| 4 | Anti-MPO | 320 | M | 56 |
| 5 | Anti-MPO | 80 | F | 73 |
| 6 | Anti-Pr3 | 160 | M | 66 |
| 7 | Anti-Pr3 | 320 | M | 66 |
| 8 | Anti-Pr3 | 80 | M | 34 |
| 9 | Anti-Pr3 | 40 | F | 28 |
| 10 | Anti-Pr3 | >640 | F | 73 |
| **Controls**
| 1 | Anti-GBM | Negative | F | 35 |
| 2 | Anti-GBM | Negative | M | 42 |
| 3 | None | Negative | F | 60 |
| 4 | None | Negative | M | 50 |
| 5 | None | Negative | M | 55 |

GBM, glomerular basement membrane.
direct anti-MPO or anti-Pr3 ELISA (Figure 2, C and F). Deglycosylated ANCA IgG showed similar dilution curves compared with unmodified ANCA IgG. Also, the perinuclear staining pattern of MPO-ANCA IgG and the cytoplasmic staining pattern of Pr3-ANCA IgG on ethanol-fixed neutrophils were not affected by EndoS treatment (Figure 2, A, B, D, and E). Together, these data indicate that glycan hydrolysis by EndoS does not affect the antigen-binding capacity of ANCA IgG.

**EndoS Treatment Inhibits ANCA IgG–Induced Neutrophil Respiratory Burst**

Next, we tested the ability of ANCA IgG samples to activate TNF-α–primed neutrophils, using the dihydrorhodamine (DHR) respiratory burst assay. Consistent with previous studies, oxidative burst was detected only when neutrophils were primed with TNF-α (data not shown). IgG from healthy control subjects and patients with anti–glomerular basement membrane disease did not activate TNF-α–primed neutrophils (data not shown). In contrast, all Pr3- and MPO-ANCA IgGs were able to activate TNF-α–primed neutrophils, although to a variable extent (range in change in mean fluorescence intensity was 57 to 1091 in one neutrophil donor). ANCA-mediated induction of the oxidative burst was Fc tail dependent because ANCA-derived F(ab’)2 fragments were not able to induce oxidative burst (Supplemental Figure 1, A and B). EndoS-mediated deglycosylation of MPO- and Pr3-ANCA IgG markedly attenuated ANCA-induced respiratory burst (Figure 3), demonstrating that IgG glycan hydrolysis by EndoS strongly attenuates the neutrophil-activating capacity of ANCA IgG.

**EndoS Treatment Inhibits ANCA IgG–Induced Neutrophil Degranulation**

In addition to respiratory burst induction, we investigated whether deglycosylation of IgG by EndoS attenuates ANCA IgG–induced neutrophil degranulation. Both MPO- and Pr3-ANCA IgG were able to induce release of lactoferrin and elastase (Figure 4). F(ab’)2 fragments from patient-derived ANCA IgG were not able to induce lactoferrin and elastase release from TNF-α–primed neutrophils (Supplemental Figure 1, C and D). Deglyco-

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**Figure 1.** EndoS-mediated hydrolysis of the glycan moiety of ANCA IgG. Patient-derived MPO- and Pr3-ANCA IgG are treated with GST-tagged EndoS or GST alone. In the top part (Stain), a Coomassie Brilliant Blue staining is shown of MPO-ANCA IgG (patients 1 through 5) and Pr3-ANCA IgG (patients 6 through 11) that were untreated (C), EndoS-treated (E), or GST control-treated (G). In the bottom part (LCA), an LCA blot is shown from the same ANCA IgGs as presented in the top part. The binding site for LCA is located in the IgG heavy-chain glycan.

**Figure 2.** EndoS-treated ANCA IgG retains antigen-binding capacity. (A, B, D, and E) Using indirect immunofluorescence on ethanol-fixed neutrophils, both deglycosylated (A) and unmodified (B) MPO-ANCA IgG show a perinuclear staining pattern, whereas deglycosylated (D) and unmodified (E) Pr3-ANCA IgG show a cytoplasmic staining pattern. Deglycosylated (C) and unmodified (F) MPO- and Pr3-ANCA IgG samples are serially diluted and tested in a direct ELISA for anti-MPO or anti-Pr3, demonstrating similar titration curves. (C and F) Representative examples of a MPO-ANCA IgG (C) and Pr3-ANCA IgG (F) are shown. Magnification, ×400.
sylation of MPO- and Pr3-ANCA IgGs significantly reduced lactoferrin and elastase release in two neutrophil donors (Figure 4). These results demonstrate that IgG glycan hydrolysis by EndoS attenuates ANCA IgG–induced neutrophil degranulation.

Deglycosylation of Anti-MPO IgG Markedly Diminishes Induction of Crescentic Glomerulonephritis in Mice

Next, we investigated whether deglycosylated anti-MPO IgG was able to induce NCGN in mice. EndoS treatment of mouse anti-MPO IgG efficiently hydrolyzed the heavy-chain glycans of the IgG, as was shown by a reduction in the molecular weight of the IgG heavy chain, and loss of LCA binding (Figure 5A). Mice that received deglycosylated anti-MPO IgG and LPS had similar levels of circulating anti-MPO antibodies compared with mice that received unmodified anti-MPO IgG and LPS after 1 and 7 days (Figure 5B). Mice that received unmodified anti-MPO IgG developed marked hematuria and leukocyturia that lasted from day 1 until being killed on day 7 (Figure 6, A and B). On day 1, hematuria in these mice reached the maximum value of 4+, whereas leukocyturia was highest on day 7. In contrast, mice that received deglycosylated anti-MPO IgG displayed less hematuria on day 1, whereas leukocyturia on day 7 was completely absent. Albuminuria was increased on day 1 and
with EndoS either early (3 hours) or late (24 hours) after disease induction. Consistent with previous studies, EndoS treatment 3 hours after disease induction resulted in an efficient deglycosylation of serum IgG 24 hours after NCGN induction, whereas on day 7, LCA-positive IgG had reappeared (Supplemental Figure 2). Systemic EndoS treatment did not affect anti-MPO antibody titers (Figure 9). Early or late EndoS treatment had no significant effect on hematuria and leukocyturia (Figure 10, A through D). EndoS treatment 3 hours after disease induction reduced albuminuria on days 1 and 7 compared with control mice (Figure 10E); however, EndoS treatment 24 hours after disease induction did not affect albuminuria (Figure 10F). Similarly, early EndoS treatment significantly reduced the percentage of glomerular crescents compared with control mice (Figure 11A, C, and D), whereas late EndoS treatment did not affect glomerular crescent formation (Figure 11B). Together, these results demonstrate that early (3 hours) but not late (24 hours) systemic EndoS administration rescues mice from disease progression.

**DISCUSSION**

In this study, we demonstrate that EndoS efficiently hydrolyzed the Fc glycans of patient ANCA IgG, thereby disrupting the neutrophil-activating capacity of ANCA in vitro. EndoS-mediated deglycosylation of mouse anti-MPO IgG almost completely prevented experimental anti-MPO IgG/LPS-mediated glomerulonephritis. Moreover, systemic EndoS administration 3 hours after disease induction rescued mice from disease progression, whereas disease development was not attenuated by EndoS treatment after 24 hours.

According to the widely accepted theory on the pathogenesis of ANCA-mediated vasculitis, ANCA-induced activation of primed neutrophils is crucial for disease development. Activation of neutrophils leads to oxygen radical production and the release of lytic granule constituents that are injurious to endothelial cells. ANCA-induced neutrophil activation is considered to involve simultaneous engagement of the F(ab’)_2 portion of ANCA with ANCA antigens and interaction of the Fc tail of ANCA with Fc receptors. Our in vitro observations revealed that ANCA-mediated neutrophil activation, in particular degranulation, was attenuated but not completely blocked after Fc glycan removal. This finding suggests that Fc receptor–independent pathways are involved in ANCA-induced neutrophil activation as well. Indeed, in some studies, ANCA F(ab’)_2 fragments could activate neutrophils how-

![Figure 5](image-url)
This phenomenon may also explain our findings that EndoS-mediated deglycosylation of patients’ ANCA IgG did not completely prevent neutrophil activation in vitro and that hematuria and crescent formation were not completely blocked after administration of deglycosylated anti-MPO IgG in vivo.

To establish further the beneficial effects of EndoS-mediated IgG deglycosylation, we used the mouse model of anti-MPO IgG/LPS-induced glomerulonephritis. In this model, co-administration of murine anti-MPO antibodies and LPS induces an acute glomerular inflammation that progresses to NCGN within days. One day after disease induction, signs of acute inflammation are present, such as hematuria and glomerular neutrophil influx, whereas leukocyturia, albuminuria, and glomerular crescents are important disease characteristics on day 7. Because the pathogenicity of anti-MPO antibodies in mice seems to be FcγR dependent,11,25 we hypothesized that EndoS-mediated deglycosylation of the anti-MPO IgG Fc tail could reduce the pathogenic effects of the antibodies. Indeed, we found that EndoS-mediated deglycosylation of anti-MPO IgG markedly diminished both early and late disease characteristics of this model.

After these promising findings with deglycosylated anti-MPO IgG, we investigated whether in vivo EndoS treatment was able to rescue mice from disease progression. Systemic injection of EndoS 3 hours after disease induction efficiently hydrolyzed circulating IgG and rescued mice from disease progression; however, systemic injection of EndoS 24 hours after disease induction did not significantly attenuate disease development. A possible explanation for this observation is that 24 hours after disease induction, mechanisms downstream of the antibody-mediated effects have already been initiated. Previous studies in this disease model demonstrated a crucial role for (alternative) complement pathway activation, most likely via C5a generation.12 C5a promotes recruitment of more neutrophils to the inflammatory site, leading to a vicious, self-enhancing process with production of proinflammatory cytokines and further activation of complement. When deglycosylated anti-MPO IgG was administered, the initial start of this process was blocked and glomerular neutrophil influx on day 1 was strongly diminished. Also, when EndoS was administered systemically 3 hours after disease induction, EndoS was able to interfere with this process. Apparently, after 24 hours, the process was activated to such an extent that EndoS was not able to rescue mice from disease progression.
already been formed. The hypothesis that complement activation is important after 24 hours is supported by the finding that complement inhibition in the same model via intervention with a C5-inhibiting mAb after 24 hours attenuates disease progression, illustrated by a decrease in urinary abnormalities and a strong reduction in glomerular crescent formation.26

A limitation of the mouse model of anti-MPO IgG/LPS-induced NCGN is that disease is induced by the passive, one-time transfer of anti-MPO IgG, which results in a rapid, monophasic renal disease. This is clearly different from ANCA-associated glomerulonephritis in humans, whereby ongoing disease activity results in the progressive accumulation of new lesions.27,28 This may be a particularly important limitation when evaluating the effect of EndoS treatment on established disease. It would therefore be interesting to evaluate how (pre)treatment with EndoS would affect the development of glomerulonephritis in the established rat model of anti-MPO NCGN. In this model, Wistar-Kyoto rats are immunized with human MPO, which leads to the generation of antibodies against human MPO that cross-react with rat MPO. In this chronic and progressive model, pauci-immune crescentic glomerulonephritis is observed after 8 weeks.29,30 Because it is not known yet how EndoS affects rat IgG, the feasibility of such experiments would need more investigation.

In patients with certain inflammatory diseases (e.g., rheumatoid arthritis, systemic lupus erythematosus, Crohn’s disease), the IgG glycan composition is different from that of healthy control subjects. Holland et al.31 showed that this also applies to patients with active ANCA-associated systemic vasculitis. ANCA IgG was shown to contain more agalactosylated IgG molecules (with glycans containing no galactose residues). The authors also showed that ANCA IgG from these patients were hypogalactosylated only in their Fc but not in their F(ab’)_2 part, indicating that there was no defect in the glycosylation or processing machinery.32 Also, in our patients’ ANCA IgG, we observed differences in glycosylation (Figure 1). For some patients, the LCA signal was very low (patient 8) or even absent (patients 7 and 9), indicating that these patients might have an altered glycosylation of their IgG. Another expla-
Mice were treated with EndoS or GST (as a control) after 24 hours. In both groups (EndoS [ ], GST control [ ]), anti-MPO titers decreased on day 7. Data are means ± SD of six mice. In both groups (EndoS [ ], GST control [ ]), anti-MPO titers decreased on day 7. Data are means ± SD of six mice.

Figure 9. EndoS treatment does not affect anti-MPO IgG titers. (A) After induction of NCGN by anti-MPO IgG and LPS, mice were systemically treated with EndoS or GST (as a control) after 3 hours. Mice of both groups (EndoS [ ] and GST control [ ]) had developed similar titers of anti-MPO IgG on day 1. In both groups (EndoS [ ] and GST control [ ]), anti-MPO titers decreased on day 7. Data are means ± SD of six mice. (B) After induction of NCGN by anti-MPO IgG and LPS, all mice had developed similar titers of circulating anti-MPO IgG after 24 hours. Mice of both groups (EndoS [ ] and GST control [ ]). Anti-MPO titers decreased on day 7. Data are mean ± SD of 12 (day 1) or six (day 7) mice.

CONCISE METHODS
Preparation of IgG Fractions from ANCA-Positive Patients and Control Subjects
Plasma samples, obtained from plasmapheresis material or freshly drawn blood, were collected from ANCA-positive patients with active, biopsy-proven NCGN (MPO positive n = 5; Pr3 positive n = 6, five of which are used in each assay). The patients received immunosuppressive treatment. Also, plasma samples from patients with anti–glomerular basement membrane disease and from healthy control subjects were collected (characteristics are shown in Table 1). Plasma samples were tested for the presence of anti-Pr3 or anti-MPO antibodies by capture ELISA. Plasma samples were tested for ANCA titers by indirect immunofluorescence assay on ethanol-fixed neutrophils. Plasma samples were tested for ANCA titers by indirect immunofluorescence assay on ethanol-fixed neutrophils. Until IgG isolation, the plasma samples were stored at ~20°C. Purified IgG fractions were prepared using a protein G column (Hi-Trap Protein G HP; GE Healthcare, Freiburg, Germany) according to the manufacturer’s instructions. Before use in the activation experiments, the IgG fractions were centrifuged for 15 minutes at 14,000 × g to remove aggregates.

EndoS Treatment of Patient ANCA IgG
Recombinant EndoS was produced in Escherichia coli and purified via a GST affinity tag as described previously. For all experiments, recombinant GST-tagged EndoS (GST-EndoS) or GST alone was used. For in vitro experiments, 100 μg of ANCA IgG was incubated with 1 μg of GST-EndoS in PBS at 37°C for 2 hours. The efficiency of GST-EndoS treatment was analyzed by SDS-PAGE and LCA blotting as described in Analysis of IgG Glycan Hydrolysis.

Analysis of IgG Glycan Hydrolysis
Two or 0.5 μg of purified IgG was separated on 10% SDS-PAGE and stained with Coomassie blue or electroblotted onto polyvinylidene difluoride membrane (Immobilon-P; Millipore, Bedford, MA), respectively. Glycosylated IgG was detected by using 1 μg/ml biotinylated LCA and 1 μg/ml of streptavidin-horseradish peroxidase (Vector Laboratories, Burlingame, CA) and SuperSignal West Pico peroxidase substrate (Pierce, Rockford, IL). Membranes were analyzed using a
Chemidoc XRS imaging system and Quantity One image analysis software (Bio-Rad, Hercules, CA).

**Indirect Immunofluorescence**

Freshly isolated healthy donor neutrophils were ethanol-fixed and sequentially incubated with ANCA IgG fractions (dilutions 1:20 to 1:640) and affinity-purified F(ab’)_2 rabbit anti-human IgG/FITC (diluted 1:400; Dako A/S, Copenhagen, Denmark) using a standard protocol.34,35

**Human Anti-MPO and Anti-Pr3 ELISA**

Untreated GST-EndoS- or GST control-treated ANCA IgG fractions were tested for the ability to bind to Pr3 or MPO by ELISA. Briefly, Nunc Maxisorp plates were coated with 135 μg/ml MPO or 10 μg/ml Pr3 (in the presence of 87 μg/ml PMSF) in 0.1 M carbonate buffer (pH 9.6) overnight at 4°C. After washing thoroughly with 25 mM Tris/HCl (pH 8.0) containing 0.15 M NaCl and 0.05% Tween-20 (washing buffer), the plates were incubated with 100 μl of IgG fractions (two-fold serial dilutions from 1:25 to 1:3200) in 50 mM Tris/HCl (pH 8.0) containing 0.25% Tween-20, 0.30 M NaCl, and 1% BSA (incubation buffer) for 2 hours at room temperature. After washing, bound antibody was detected by incubation with 167 ng/ml affinity-purified F(ab’)_2 goat anti-human IgG linked to alkaline phosphatase (American Qualex, San Clemente, CA), and p-nitrophenyl-phosphate disodium was used as a substrate. The OD at 405 nm was measured.

**Isolation of Neutrophils**

Neutrophils were isolated from heparinized venous blood of healthy control subjects by centrifugation on Lymphoprep (Axis-Shield, Oslo, Norway). Contaminating erythrocytes were lysed with ice-cold ammonium chloride buffer. Cells were washed with cold Hanks’ balanced salt solution (HBSS) without Ca²⁺/Mg²⁺ (HBSS/ supplementation) and resuspended in cold HBSS with Ca²⁺/Mg²⁺ (HBSS/; both from Invitrogen, Breda, Netherlands).

**Measurement of Respiratory Burst by Oxidation of DHR to Rhodamine**

The generation of reactive oxygen radicals by neutrophils was determined by measuring the oxidation of the nonfluorescent DHR to the green fluorescence rhodamine as described previously.37 Freshly isolated healthy donor neutrophils (final concentration 2.5 × 10⁶/ml) were gradually warmed to 37°C and incubated with 5 μg/ml cytochalasin B (Sigma-Aldrich, Zwijndrecht, Netherlands) for 5 minutes at 37°C to enhance oxygen radical production. Then cells were loaded with 0.05 mM DHR (D632; Molecular Probes, Eugene, OR) for 10 minutes at 37°C. Sodium azide (2 mM) was added to prevent intracellular breakdown of H₂O₂ by catalase. Then cells were primed with 2 ng/ml recombinant human TNF-α (rHuTNFα; Boeringher Ingelheim, Heidelberg, Germany) for 15 minutes at 37°C and finally incubated with IgG fractions (final concentration 200 μg/ml) as stimulus. After 1 hour at 37°C, samples were washed with ice-cold HBSS/ and resuspended in cold HBSS with Ca²⁺/Mg²⁺ (HBSS/; both from Invitrogen, Breda, Netherlands).

**Figure 10.** Early (3 hours) but not late (24 hours) EndoS treatment reduces albuminuria in anti-MPO IgG/LPS-induced NCGN. (A, C, and E) Early (3 hours) EndoS treatment. For hematuria (A) and leukocyturia (C), no differences are observed between GST-treated mice (□) and EndoS-treated mice (♦) after 1 and 7 days. (E) Albuminuria was significantly reduced in the EndoS-treated mice (♦) on days 1 and 7 compared with GST-treated mice (□). Bars represent means ± SD. *P < 0.05; **P < 0.01; ***P < 0.001. (B, D, and F) Late (24 hours) EndoS treatment. For hematuria (B) and leukocyturia (D), no differences are observed between GST-treated mice (□) and EndoS-treated mice (♦) after 7 days. Also for albuminuria (F), no differences are observed between GST-treated mice (□) and EndoS-treated mice (♦) after 7 days. Bars represent means ± SD. *P < 0.05; **P < 0.01; ***P < 0.001.
Degranulation Assays

Freshly isolated healthy donor neutrophils (final concentration 1 × 10⁶/ml) were gradually warmed to 37°C and treated with 5 µg/ml cytochalasin B (Sigma-Aldrich) for 5 minutes at 37°C. Then neutrophils were primed with 2 ng/ml rHuTNFα (Boeringher Ingelheim) for 15 minutes at 37°C. The primed neutrophils (0.2 × 10⁶ cells/well) were incubated in a 96-well round-bottom microtiter plate (Greiner) with the IgG fractions (final concentration 200 µg/ml) for 120 minutes at 37°C. At the end of this incubation period, cell-free supernatants were collected for the determination of lactoferrin and elastase.

The lactoferrin content of the supernatant was measured as described previously. 26 Briefly, Costar plates (Uden, Netherlands) were coated with an F(ab′)₂; rabbit anti-human lactoferrin polyclonal antibody (667 ng/ml; Jackson ImmunoResearch Laboratories, West Grove, PA) overnight at room temperature, then washed and incubated with serial (two-fold) dilutions of the samples, starting at a dilution of 1:25, for 1 hour at 37°C. After washing, a rabbit anti-human lactoferrin polyclonal antibody conjugated with horseradish peroxidase (400 ng/ml; Jackson) was incubated for 30 minutes at 37°C. Finally, TMB (3,3′,5,5′-tetramethylbenzidine; Sigma-Aldrich) substrate was incubated for 15 minutes. The color reaction was stopped with 2 N H₂SO₄. OD values were measured at 450 nm. The range of the lactoferrin standard (HK329; Hbt, Uden, Netherlands) was 0.16 to 100.00 ng/ml.

The elastase content of the supernatant was measured using a commercially available ELISA kit (HK319; Hbt), according to the manufacturer’s description. The range of the elastase standard was 0.39 to 25.00 ng/ml.

Mice

Mpo−/− mice were backcrossed to a C57BL/6 background seven times 89 and bred in-house. Female C57BL/6 wild-type mice were purchased from Harlan (Horst, Netherlands). All animal experiments were performed according to national guidelines and were approved by the local Animal Care and Experimentation Committee.

Production of Polyclonal Mouse Anti-MPO IgG

Murine MPO was purified from WEHI-3 cells and used for immunization of Mpo−/− mice as described previously. 9 Total IgG was isolated from pooled sera of immunized Mpo−/− mice, and the anti-MPO titer was analyzed by ELISA as reported previously. 9

Induction and Evaluation of Anti-MPO IgG-Induced NCGN

Wild-type C57BL/6 mice (8 to 10 weeks, body weight 16 to 18 g) received 1 mg of anti-MPO IgG intravenously, followed by an intraperitoneal injection of 150 EU/g LPS (Escherichia coli, serotype O26:B6; Sigma-Aldrich) 1 hour later. Mice were killed after 1 or 7 days, and kidneys were harvested, cut, and partly snap-frozen for immunohistochemistry and partly embedded in paraffin for histopathologic evaluation. Plasma and (18-hour) urine were collected at both time points. Plasma samples were tested for circulating MPO antibody titers by ELISA as described previously. 9 Urine samples were tested for hematuria (0 to 4+ score) and leukocyturia (0 to 3+ score) by Combur-Test strips (Roche Diagnostics BV, Almere, Netherlands) and albuminuria by ELISA (Bethyl Laboratories, Montgomery, TX). Periodic acid-Schiff staining was performed on paraffin sections, and the number of glomerular crescents was counted in 100 consecutive glomerular cross-sections in a blinded manner, as described previously. 26 Immunohistochemical staining for neutrophils was performed on acetone-fixed 3-µm cryosections using an anti-rabbit peroxidase-based Envision+ system (DakoCyto-

Figure 11. Early (3 hours) but not late (24 hours) EndoS treatment reduces glomerular crescent formation in anti-MPO IgG/LPS-induced NCGN. (A and B) Quantification of glomerular crescent formation in GST- and EndoS-treated mice expressed as the percentage of glomerular crescents. Early (3 hours; A) but not late (24 hours; B) EndoS treatment reduces the amount of glomerular crescents on day 7. Horizontal lines represent mean percentages in each group. ***P < 0.001. (C) Overview of renal cortical tissue 7 days after disease induction from a mouse that received early (3 hours) GST control treatment, representing the focal and segmental nature of the glomerulonephritis. Glomerular crescents are indicated by arrows. (D) Overview of renal cortical tissue 7 days after disease induction from a mouse that received early (3 hours) EndoS treatment; only a few glomerular crescents are seen. (C and D) Periodic acid-Schiff stain. Magnification, ×100 in C and D.
In Vivo EndoS Treatment

First, the effect of EndoS pretreatment of anti-MPO IgG was investigated. Mice were administered an intravenous injection of anti-MPO IgG that was preincubated (2 hours at 37°C) with GST-EndoS or GST (1 μg/100 μg IgG) to achieve deglycosylation. Mice were killed after 1 or 7 days (n = 6 per group) to evaluate disease development.

Then the effect of EndoS treatment at two different time points (3 hours [early] and 24 hours [late]) after disease induction was investigated. For this purpose, mice were administered an intravenous injection of 20 μg GST-EndoS or GST in 200 μl of PBS 3 or 24 hours after disease induction and killed on day 7 (n = 6 per group).

Statistical Analysis

Data are expressed as means ± SD and were analyzed using the unpaired two-tailed t test. All statistical analyses were performed using SPSS 16.0 (SPSS Inc., Chicago, IL) or GraphPad Prism 5.00 for Windows (Graphpad Software, San Diego, CA). P ≤ 0.05 was considered statistically significant.

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