THERAPEUTIC CLEAVAGE OF ANTI-AQUAPORIN-4 AUTOANTIBODY IN NEUROMYELITIS OPTICA BY AN IgG-SELECTIVE PROTEINASE

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Running title: IdeS Proteinase Therapy for NMO

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Non-standard abbreviations: ADCC, antibody-dependent cell-mediated cytotoxicity; AQP4, aquaporin-4; CDC, complement-dependent cytotoxicity; IdeS, IgG-degrading enzyme of Streptococcus pyogenes; NMO, neuromyelitis optica; NMO-IgG, neuromyelitis optica immunoglobulin G; NMO-IgGIdeS, IdeS-treated NMO-IgG; NMO serumIdeS, IdeS-treated NMO serum
ABSTRACT

Neuromyelitis optica (NMO) is an inflammatory demyelinating disease of the central nervous system caused by binding of pathogenic IgG autoantibodies (NMO-IgG) to astrocyte water channel aquaporin-4 (AQP4). Astrocyte damage and downstream inflammation require NMO-IgG effector function to initiate complement-dependent cytotoxicity (CDC) and antibody-dependent cell-mediated cytotoxicity (ADCC). Here, we evaluated the potential therapeutic utility of the bacterial enzyme IdeS (IgG-degrading enzyme of Streptococcus pyogenes), which selectively cleaves IgG antibodies to yield Fc and F(ab’)2 fragments. In AQP4-expressing cell cultures, IdeS treatment of monoclonal NMO-IgGs and NMO patient sera abolished CDC and ADCC, even when IdeS was added after NMO-IgG was bound to AQP4. Binding of NMO-IgG to AQP4 was similar to that of the NMO-F(ab’)2 generated by IdeS cleavage. NMO-F(ab’)2 competitively displaced pathogenic NMO-IgG, preventing cytotoxicity, and the Fc fragments generated by IdeS cleavage reduced CDC and ADCC. IdeS efficiently cleaved NMO-IgG in mice in vivo, and greatly reduced NMO lesions in mice administered NMO-IgG and human complement. IgG-selective cleavage by IdeS thus neutralizes NMO-IgG pathogenicity, and yields therapeutic F(ab’)2 and Fc fragments. IdeS treatment, by therapeutic apheresis or direct administration, may be beneficial in NMO.
INTRODUCTION

Neuromyelitis optica (NMO) is an autoimmune disease affecting the central nervous system, with inflammatory demyelinating lesions in spinal cord and optic nerve, and, to a lesser extent, in brain (Wingerchuk et al., 2007; Jarius et al., 2008). A defining feature of NMO is the presence, in the majority of NMO patients, of serum immunoglobulin G (IgG) antibodies (NMO-IgG) directed against astrocyte water channel protein aquaporin-4 (AQP4) (Lennon et al., 2005; Jarius and Wildemann, 2010). AQP4 is concentrated in the foot-processes of astrocytes throughout the central nervous system, as well as in supportive cells in neurosensory organs such as Müller cells in retina, and in some peripheral organs including a subset of epithelial cells in kidney, stomach and the airways (Nielsen et al., 1997; Papadopoulos and Verkman, 2013). It is thought that NMO pathogenesis involves NMO-IgG binding to AQP4, which causes complement-dependent cytotoxicity (CDC) and antibody-dependent cell-mediated cytotoxicity (ADCC) (Lucchinetti et al., 2002; Kira, 2011; Papadopoulos and Verkman, 2012). The consequences of astrocyte damage include inflammation with cytokine release and infiltration of granulocytes and macrophages, disruption of the blood-brain barrier, and injury to oligodendrocytes and neurons. Current NMO therapies include immunosuppression, B cell-depletion with anti-CD20 monoclonal antibody, and plasma exchange (Collongues and de Seze, 2011; Cree, 2008; Sato et al., 2012; Kim et al., 2013). New therapeutics are needed with improved efficacy and reduced long-term side-effects.

NMO therapy targeting NMO-IgG binding to AQP4 – the presumed initiating event in NMO pathogenesis – is attractive because of its potential specificity. We previously reported an engineered monoclonal, anti-AQP4 antibody lacking CDC and ADCC effector functions (‘aquaporumab’, Tradtrantip et al., 2012b). Aquaporumab competes with pathogenic NMO-IgG for binding to AQP4, preventing downstream cytotoxicity and NMO pathology. As an alternative strategy, small-molecule
blocking compounds were identified that reduce NMO-IgG binding to AQP4 (Tradtrantip et al., 2012a). Recently, we reported that deglycosylation of patient NMO-IgG by the IgG-selective enzyme endoglycosidase S (EndoS) neutralized its pathogenicity without affecting its binding to AQP4 (Tradtrantip et al., 2013). EndoS is a 108 kdalton enzyme produced by *Streptococcus pyogenes* that digests asparagine-linked glycans on the heavy chain of all IgG subclasses (Collin and Olsen, 2001a).

Here, we investigated an alternative strategy to neutralize NMO-IgG pathogenicity using a proteinase from *Streptococcus pyogenes* that selectively cleaves IgG heavy chains in the hinge region to generate Fc and F(ab’)2 fragments (von Pawel-Rammingen et al., 2002; Vincents et al., 2004) (Fig. 1A). The enzyme, IdeS (IgG-degrading enzyme of *Streptococcus pyogenes*), also called Mac1, efficiently cleaves human IgGs of all subclasses without effect on other antibody classes or proteins. Compared to EndoS, IdeS: (i) has greater rate of IgG cleavage compared to the rate of EndoS deglycosylation; (ii) produces antibody fragments with zero residual effector function; and (iii) generates free Fc fragments that block Fc receptors on phagocytes. IdeS has shown efficacy in rodent models of experimental arthritis caused by anti-collagen antibodies (Nandakumar et al., 2007), idiopathic thrombocytopenic purpura caused by anti-platelet antibodies (Johansson et al., 2008), and glomerulonephritis caused by anti-glomerular basement membrane antibodies (Yang et al., 2010). IdeS represents a logical candidate to evaluate and potentially repurpose for NMO therapy, as another proteolytic enzyme from *S. pyogenes*, streptokinase, has been used for more than a decade as an effective, safe anticoagulant.
MATERIALS AND METHODS

Cell culture and antibodies

Chinese hamster ovary (CHO) cells stably expressing human M23-AQP4 (Phuan et al., 2012) were cultured at 37 °C in 5% CO2 / 95% air in F-12 Ham’s Nutrient mix medium supplemented with 10% fetal bovine serum, 200 µg/mL geneticin (selection marker), 100 units/mL penicillin and 100 µg/mL streptomycin. Recombinant monoclonal NMO antibodies rAb-53 and rAb-93 was generated from a clonally expanded plasma blast population from cerebrospinal fluid (CSF) of an NMO patient (Bennett et al., 2009; Crane et al., 2011). NMO serum was obtained from NMO-IgG seropositive individuals who met the revised diagnostic criteria for clinical disease (Wingerchuk et al., 2006). Non-NMO (seronegative) human serum was used as control. For some studies IgG was purified from NMO or control serum using a protein A resin (GenScript, Piscataway, NY) and concentrated using Amicon Ultra Centrifugal Filter Units (Millipore, Billerica, MA). Purified human IgM and IgA were purchased from Calbiochem (San Diego, CA), IgE from Abcam (Cambridge, MA), and IgG from Thermo Scientific Pierce (Rockford, IL).

IdeS treatment

IdeS (FabRICATOR®) and IdeS microspin column (FragIT™ Microspin) were purchased from Bulldog Bio Inc. (Rochester, NY). NMO-IgG or NMO serum (or control IgG/serum) was treated by incubation with IdeS (1-5 unit per 1 µg IgG) for up to 1 h at 37 °C; NMO serum was digested with IdeS using a microspin column containing IdeS covalently coupled to agarose beads. Treated antibody is referred to as NMO-IgG\textsuperscript{IdeS}. Treated NMO serum is referred to as NMO serum\textsuperscript{IdeS}. IdeS treatment was assessed by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) followed by staining with Coomassie Blue.
**NMO-IgG binding**

Cells were grown on glass coverslips for 24 h. After blocking with 1% BSA in PBS, cells were incubated with NMO-IgG or NMO serum (control or IdeS-treated) for 30 min at room temperature. Cells were washed with PBS and incubated with Cy3-conjugated AffiniPure goat anti-human IgG, F(ab’)2 fragment-specific, secondary antibody (1:200, Jackson ImmunoResearch, West Grove, PA). For AQP4 immunostaining cells were fixed in 4% paraformaldehyde (PFA) and permeabilized with 0.2% Triton-X. Rabbit anti-AQP4 antibody (1:200, Santa Cruz Biotech, Dallas, TX) was added followed by Alexa Fluor-488 goat anti-rabbit IgG secondary antibody (1:200, Invitrogen, Grand Island, NY) for quantitative ratio image analysis (Crane et al., 2011).

To test whether the F(ab’)2 fragments produced by IdeS cleavage compete with NMO-IgG for binding to AQP4, CHO-M23 cells were plated in black 96-well plates with clear plastic bottom (Corning-Costar) at a density of 25,000 cells per well for 24 h. After blocking with 1% BSA in PBS, cells were incubated with NMO-IgG and NMO-IgGIdeS or control-IgGIdeS for 30 min at room temperature. Cells were washed with PBS and incubated with HRP-conjugated goat anti-human IgG, Fc fragment-specific, secondary antibody (1:500, Invitrogen) for 30 min. After washing each well three times with PBS, 50 µl Amplex red substrate (100 µM, Sigma) and 2 mM H2O2 was added for measurement of HRP activity. Fluorescence was measured after 45 min (excitation 540 nm, emission 590 nm).

**CDC and ADCC assays**

For assay of CDC, cells were incubated for 60 min at 37 °C with NMO-IgG or NMO serum (control or IdeS-treated) with 2% human complement (Innovative Research, Novi, MI). In some experiments NMO-IgG was added 30 min before IdeS addition, followed 60 min later by complement. Cytotoxicity was measured by the Alamar Blue assay (Invitrogen). For assay of ADCC, NK-92 cells
expressing CD16 (Conkwest, San Diego, CA) were used as the effector cells. The AQP4-expressing CHO cells were incubated for 1 h at 37 °C with NMO-IgG and effector cells at an effector:target cell ratio of 4:1. To test the effect of Fc fragments generated by IdeS cleavage on CDC, AQP4-expressing CHO cells were incubated for 1 h human IgG Fc fragments (Calbiochem) together with NMO-IgG (3 μg/ml rAb-53) and 1% human complement. To test the effect on ADCC, human IgG Fc fragments (Calbiochem) were pre-incubated with NK cells for 30 min at 37 °C, then added together with NMO-IgG (3 μg/ml rAb-53) to AQP4-expressing CHO cells and incubated for 1 h.

**In vivo mouse models of NMO**

Adult wild type mice (30-35 g) were anesthetized with 2,2,2-tribromoethanol (125 mg/kg i.p.) and mounted in a stereotactic frame. Following a midline scalp incision, a burr hole of diameter 1 mm was made in the skull 2 mm to the right of bregma. A 30-gauge needle attached to 50-μL gas-tight glass syringe (Hamilton) was inserted 3-mm deep to infuse 0.6 μg NMO-IgG (control or IdeS-treated) and 3 μL of human complement in a total volume of 8 μL (at 2 μL/min) (Saadoun et al., 2010). In some experiments purified IgG from NMO serum (12 μg) was injected together with an excess of IdeS-treated IgG purified from NMO or control serum (48 μg) and 3 μL human complement in a total volume of 18 μL. In some experiments mice were injected with 0.6 μg NMO-IgG and 15 min later at the same site with 3 μL human complement with or without 16.75 U IdeS. After 3 days mice were anesthetized and perfused through the left cardiac ventricle with 5 mL PBS and then 20 mL of PBS containing 4% PFA. Brains were post-fixed for 2 hours in 4% PFA. Five μm-thick paraffin sections were immunostained at room temperature for 1 h with: rabbit anti-AQP4 (1:200, Santa Cruz Biotechnology, Santa Cruz, CA), mouse anti-GFAP (1:100, Millipore, Temecula, CA), and goat anti-myelin basic protein (MBP) (1:200, Santa Cruz Biotechnology) followed by the appropriate fluorescent secondary antibody (1:200, Invitrogen). Tissue sections were photographed using a Leica.
DM 4000 B fluorescence microscope at 25x magnification. AQP4, GFAP and MBP immunonegative areas were defined by hand and quantified using ImageJ. Data are presented as percentage of immunonegative area (normalized to total area of hemi-brain slice). Protocols were approved by the UCSF Committee on Animal Research.
RESULTS

*IdeS cleavage of NMO-IgG prevents CDC and ADCC*

Fig. 1A diagrams the binding of NMO-IgG to membrane-associated AQP4. IdeS cleavage occurs at the lower hinge / CH2 (heavy chain constant region 2) region of IgG-class antibody to produce an F(ab')2 fragment and two Fc fragments. SDS-PAGE with Coomassie blue staining shows loss of the antibody heavy chain and appearance of smaller fragments following IdeS cleavage of NMO-IgG (Fig. 1B). Fig. 1C verifies the IgG-selective action of IdeS. Purified human IgG, IgM, IgE and IgA were treated with a high concentration of IdeS (5 U IdeS/1 μg immunoglobulin). Whereas cleavage of IgG was essentially complete under these conditions, showing bands at the expected molecular sizes of heavy chain fragments (31 kDa) and light chains (25 kDa), no cleavage was seen for IgM, IgE and IgA.

Because IdeS separates F(ab')2 from Fc, it is anticipated that Fc-dependent effector functions of NMO-IgG should be abolished following IdeS cleavage. Fig. 2A shows loss of CDC, as measured by an Alamar Blue cytotoxicity assay, in AQP4-expressing cells incubated with control or IdeS-treated NMO-IgG, together with human complement. IdeS treatment prevented CDC caused by different monoclonal NMO-IgGs (left panel) and NMO patient sera (right panel). Fig. 2B shows the time- and IdeS concentration-dependence for reduction of CDC for two monoclonal NMO-IgGs. Fig. 2C shows that IdeS is effective when NMO-IgG is already bound to AQP4. AQP4-expressing cells were pre-incubated for 30 min with NMO-IgG, then IdeS was added, followed 30 min later by complement. IdeS treatment after NMO-IgG binding abolished CDC in concentration-dependent manner. Fig. 2D shows that IdeS cleavage abolished the ADCC effector function of NMO-IgG, as demonstrated in a cytotoxicity assay of AQP4-expressing cells incubated with NMO-IgG and human NK-cells.
IdeS-cleaved NMO-IgG binds to AQP4, competitively displacing pathogenic NMO-IgG

Binding of NMO-IgG to AQP4 was compared with that of the NMO-F(ab’)2 fragment generated by IdeS cleavage. Binding to AQP4-expressing cells was measured by a ratio imaging assay in which NMO-IgG was stained red (Cy3-conjugated F(ab’)2 fragment-specific anti-human secondary antibody) and AQP4 stained green (anti-C-terminus rabbit primary antibody, Alexa Fluor-488-anti-rabbit secondary antibody). Fluorescence micrographs show similar red fluorescence for control and IdeS-treated NMO-IgG, both for a recombinant NMO-IgG (Fig. 3A, left) and for NMO patient sera (Fig. 3B, left). Quantitative ratio image analysis showed little effect of IdeS cleavage on NMO-IgG binding (Fig. 3A and B, right).

The product NMO-F(ab’)2 fragments, which lack effector functions, compete with the original NMO-IgG for binding to AQP4. NMO-IgG binding was measured using a horseradish peroxidase-conjugated secondary antibody that recognizes the Fc fragment of the primary antibody (Fig. 4A). NMO-IgG binding was greatly reduced with increasing concentrations of IdeS-treated NMO-IgG (NMO-IgG<sub>IdeS</sub>), but not of IdeS-treated control antibody (control-IgG<sub>IdeS</sub>). Also, CDC was measured in AQP4-expressing cells treated with different monoclonal NMO-IgGs or NMO patient sera together with complement, and increasing concentrations of IdeS-treated NMO-IgG. Fig. 4B shows greatly reduced CDC with increasing concentrations of IdeS-treated NMO-IgG. IdeS cleavage thus converts pathogenic NMO-IgG into non-pathogenic, blocking NMO-F(ab’)2 fragments that interfere with binding of pathogenic NMO-IgG to AQP4 and downstream cytotoxicity.

Fc fragments released after IdeS cleavage reduce CDC and ADCC

To test whether the IgG Fc fragments generated by IdeS can protect against NMO-IgG-induced CDC, AQP4-expressing cells were incubated with NMO-IgG, human complement, and different concentrations of human IgG Fc fragments. CDC was greatly reduced with inclusion of IgG Fc
fragments (Fig. 4C). To test whether the IgG Fc fragments protect against NMO-IgG-induced ADCC, increasing concentrations of human IgG Fc fragments were added, together with NMO-IgG and human NK-cells, to AQP4-expressing cells. Figure 4D shows that IgG Fc fragments prevented NMO-IgG-induced ADCC. The reduced CDC and ADCC is probably related to Fc fragment binding to C1q and Fcγ receptors, respectively.

**IdeS treatment reduces NMO pathology in mice**

IdeS was also tested in a mouse model of NMO produced by intracerebral injection of NMO-IgG and human complement (Saadoun et al., 2010; 2012). In a first set of studies mice were injected with NMO-IgG (rAb-53), without or with IdeS pretreatment, together with complement. After 3 days there was marked loss of AQP4, GFAP and myelin around the injection site in mice administered untreated NMO-IgG (Fig. 5A, left), as found previously (Saadoun et al., 2010), with only small lesions in mice receiving IdeS-treated NMO-IgG. Higher magnification of the lesion in mice receiving untreated NMO-IgG shows well-demarcated areas of AQP4, GFAP and myelin loss in the ipsilateral hemisphere, with increased expression of GFAP and AQP4 in reactive astrocytes outside of the lesion (Fig. 5A, center). Loss of GFAP, AQP4 and myelin immunoreactivity was greatly reduced in the mice receiving IdeS-treated NMO-IgG (Fig. 5A, right).

In a second set of experiments mice were injected with untreated NMO-IgG (purified IgG from NMO patient serum) together with complement, without or with a 4-fold molar excess of IdeS-treated IgG from the same NMO patient. Fig. 5B (left) shows typical lesions in mice receiving untreated NMO-IgG and complement, with much reduced lesion size when excess IdeS-treated NMO-IgG was included. Areas of loss of immunoreactivity are summarized in Fig. 5B (right). IdeS-treated NMO antibody can thus compete with pathogenic NMO antibody in mouse brain *in vivo*. 

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In a third set of *in vivo* experiments, mice were administered NMO-IgG (rAb-53) followed 15 min later by IdeS and complement at the same site. Fig. 6A shows greatly reduced lesion size when IdeS was injected, with a summary of data in Fig. 6B. IdeS can thus cleave NMO-IgG already bound to astrocyte AQP4 in mouse brain *in vivo* at a sufficiently rapid rate to prevent the development of NMO lesions during exposure to complement.
DISCUSSION

Therapeutic cleavage of NMO-IgG by the IgG-selective proteinase IdeS adds to the list of new candidate therapies that target pathogenic NMO autoantibodies. In general, therapeutic approaches for autoimmune diseases caused by pathogenic autoantibodies include: (i) antibody removal by plasma exchange; (ii) prevention of autoantibody binding to its target; and (iii) antibody inactivation. Class ii therapeutics include aquaporumab blocking antibodies (Tradtrantip et al., 2012b) and small-molecule blockers (Tradtrantip et al., 2012a) that compete sterically with pathogenic NMO autoantibodies for binding to AQP4. The blocking strategy involves the engineering or identification of a non-pathogenic blocking molecule with sufficiently high AQP4 binding affinity and concentration at the target site to competitively displace polyclonal, pathogenic autoantibodies in NMO serum. The antibody inactivation strategy is conceptually simple and potentially more practical compared blocker strategies. The ideal antibody inactivation strategy would produce selective inactivation of NMO-IgG, though non-selective IgG inactivation has the potential benefit of inactivating alternative IgGs that might contribute to NMO pathology such as antibodies against myelin-oligodendocyte glycoprotein (Kitley et al., 2012). Because human NMO-IgG autoantibodies are of the IgG1 class, selective IgG1 inactivation would also be an attractive approach; however, the most selective available endoglycosidases and proteinases inactivate all IgG subclasses (Collin and Olsson, 2001a; von Pawel-Rammingen et al., 2002). We found here that IdeS neutralized NMO-IgG pathogenicity, abolishing CDC and ADCC effector functions, and yielding therapeutic F(ab’)2 and Fc fragments that blocked NMO-IgG binding to AQP4 and Fcγ receptors, respectively. IdeS efficiently cleaved both free and AQP4-bound NMO-IgG, without effect on AQP4 binding of the product NMO-F(ab’)2 fragment.

Data from animal models support the therapeutic utility of IdeS for autoimmune diseases caused by pathogenic autoantibodies. In mice, IdeS treatment has been shown to prevent collagen antibody-
induced arthritis (Nandakumar et al., 2007), anti-platelet IgG-induced thrombocytopenia (Johansson et al., 2008), and anti-glomerular basement membrane induced glomerulonephritis (Yang et al., 2010). The cleavage of IgG following intravenous administration of IdeS in mice and rabbits is rapid and efficient, and well-tolerated even at high IdeS concentration (Nandakumar et al., 2007; Johansson et al., 2008). As reported (von Pawel-Rammingen et al., 2002) and verified here, IdeS has high selectivity for IgG; other cysteine proteases such as Streptopain (SpeB) have broad proteolytic activity (Collin and Olssen, 2001b). Notwithstanding the successful use of IdeS when administered to animals, repeated intravenous administration of IdeS in humans would likely elicit an immune response. However, even though most humans who have been infected with S. pyogenes have antibodies against streptococcal proteins including IdeS (Akesson et al., 2004), these neutralizing antibodies do not interfere the IgG-cleaving action of IdeS (Johansson et al., 2008). An immune response against IdeS is unlikely to occur if patient serum is exposed to membrane-immobilized IdeS by extracorporeal circulation in therapeutic apheresis.

We found efficient cleavage of AQP4-bound NMO-IgG when administered by intracerebral injection. IdeS cleavage of NMO-IgG was sufficiently rapid to prevent NMO lesions in mouse brain after NMO-IgG was already bound to AQP4, in which IdeS and complement were coadministered 15 min after NMO-IgG. A similar experiment with EndoS (coadministration of EndoS + complement after NMO-IgG) was not successful (unpublished data), probably because of the lower enzymatic activity of EndoS compared to IdeS. The greater efficacy of IdeS compared to EndoS was also demonstrated in a mouse model of experimental glumerulonephritis (Yang et al., 2010).

In conclusion, IdeS cleavage represents a compelling therapeutic strategy for NMO autoantibody neutralization, as it efficiently and completely abolishes NMO-IgG effector functions, and produces therapeutic F(ab’)2 and Fc fragments. IdeS cleavage may be accomplished by therapeutic apheresis in
which patient blood is passed over surface-immobilized IdeS. Alternatively, notwithstanding potential concerns about immunogenicity, IdeS might be administered by intravenous injection, or by intrathecal or retro-orbital routes to target NMO lesions with minimal systemic exposure.
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AUTHORSHIP CONTRIBUTIONS.

Participated in research design: Tradtrantip, Asavapanumas, Verkman

Conducted experiments: Tradtrantip, Asavapanumas

Contributed new reagents or analytical tools: N/A

Performed data analysis: Tradtrantip, Asavapanumas

Wrote or contributed to the writing of the manuscript: Tradtrantip, Asavapanumas, Verkman
REFERENCES


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FIGURE LEGENDS

Figure 1. **IdeS cleavage of human NMO-IgG.**  A. Schematic showing F(ab’)2 and Fc fragments produced by IdeS cleavage of IgG heavy chains at lower hinge region.  HC, heavy chain; LC, light chain.  B. Coomassie blue SDS-PAGE of control and IdeS-treated NMO-IgG (rAb-53, 5 µg, 60 min incubation with 5 U IdeS at 37 °C).  C. Selective IgG cleavage by IdeS.  Coomassie blue SDS-PAGE of control and IdeS-treated antibodies (1 µg purified human antibodies incubated with 5 U IdeS for 30 min at 37 °C).

Figure 2. **IdeS treatment of NMO-IgG prevents CDC and ADCC.**  A. CDC in AQP4-expressing CHO cells incubated for 60 min with control and IdeS-treated monoclonal recombinant NMO-IgGs (rAb-53, rAb-93; each 0.2-20 µg/ml) (left) and NMO sera (5-200 µg/ml) (right), each together with 2% human complement.  Cytotoxicity quantified by Alamar Blue assay.  (inset) CDC for 3 different NMO sera (S.E., n=4).  B. Time course and concentration-dependence of IdeS action.  (left) CDC was measured as in A for NMO-IgG rAb-93 (1 and 3 µg/ml), which was incubated for 30 min with indicated concentrations of IdeS prior to addition to cells.  (right) CDC measurement in which NMO-IgG rAb-53 (1 and 3 µg/ml) was incubated with 1.68 U/ml IdeS for indicated times prior to addition to cells (S.E., n=4).  C. IdeS treatment of AQP4-bound NMO-IgG prevents CDC.  Cells were incubated with NMO-IgGs or NMO serum for 30 min, then treated with IdeS for 30 min, followed by 2% human complement for 1 hour (S.E., n=4).  D. ADCC in AQP4-expressing CHO cells incubated with 100,000 NK cells and 0.25-10 µg/ml untreated or IdeS-treated NMO-IgG (S.E., n=4).
Figure 3. **IdeS-treated NMO-IgG binds to AQP4.**  
**A.** Binding of recombinant NMO-IgG (rAb-93) to AQP4 in AQP4-expressing CHO cells. (left) Fluorescence micrographs showing F(ab')2 binding (red) to AQP4 (green). (right) Binding of NMO-IgG and NMO-IgG_{IdeS} showing red-to-green fluorescence ratio (R/G) as a function of NMO-IgG concentration (S.E., n=3).  
**B.** Binding of NMO patient sera to AQP4 in AQP4-expressing CHO cells. (left) Fluorescence micrographs showing F(ab')2 binding (red) to AQP4 (green). (right) R/G at IgG concentrations of 200 and 50 µg/ml for serum 1 and 2, respectively (S.E., n=3).

Figure 4. **IdeS-treated NMO-IgG competitively displaces pathogenic NMO-IgG, reducing cytotoxicity.**  
**A.** F(ab')2 fragments produced by IdeS cleavage of NMO-IgG competitively displaces NMO-IgG. Binding of NMO-F(ab')2 fragment on AQP4-expressing CHO cells incubated with NMO-IgG (1 µg/ml rAb-93) and NMO-IgG_{IdeS} or control-IgG_{IdeS}, followed by horseradish peroxidase (HRP)-conjugated anti-human IgG (Fc-specific) secondary antibody, as quantified by HRP activity assay.  
**B.** IdeS-treated NMO-IgG protects against CDC caused by (untreated) NMO-IgG. Cytotoxicity measured by Alamar Blue assay after 60 min incubation with NMO-IgG (2 µg/ml rAb-53; 1 µg/ml rAb-93) or NMO sera and 2% HC in AQP4-expressing cells, together with indicated concentrations of NMO-IgG_{IdeS} or NMO serum_{IdeS} (S.E., n=4).  
**C.** Fc fragments generated by IdeS cleavage reduce CDC. AQP4-expressing CHO cells were incubated for 60 min with NMO-IgG (3 µg/ml rAb-53) and 1% human complement with different concentration of human IgG Fc fragments.  
**D.** Fc fragments reduce ADCC. Human IgG Fc fragments were pre-incubated with NK cells for 30 min at 37 °C, then added together with NMO-IgG (3 µg/ml rAb-53) to AQP4-expressing CHO cells and incubated for 1 h (S.E., n=4).
Figure 5. **IdeS treatment of NMO-IgG prevents lesions in a mouse model of NMO.**  
A. (left) Brains of live mice were injected with 0.6 µg NMO-IgG or NMO-IgG<sup>Ides</sup> together with 3 µL human complement (HC). Representative GFAP, AQP4 and myelin (MBP) immunofluorescence at 3 days after injection. Yellow line represents needle tract. White line delimits the lesion with loss of AQP4, GFAP and myelin. (center) Higher magnification of brains injected with NMO-IgG and HC. White dashed line delimits the lesion (top). Contralateral hemispheres (non-injected) are shown (right). (right) Summary of lesion size from experiments as in A (S.E., 4 mice per group, **p < 0.01 by non-parametric Mann-Whitney test). 

B. Brains were injected with 12 µg of purified IgG from NMO serum and 48 µg of IdeS-treated IgG purified from the same NMO patient (NMO serum<sup>Ides</sup>) or a non-NMO control (control serum<sup>Ides</sup>), together with 3 µL HC. (left) Representative GFAP, AQP4 and MBP immunofluorescence at 3 days after injection. Yellow line shows the needle tract and white line delimits the lesion. (right) Summary lesion size (S.E., 4 mice per group, **p < 0.01).

Figure 6. **EndoS efficiently cleaves NMO-IgG in mice in vivo.** Injected IdeS reduces NMO lesions in mouse brain. Mice were injected with 0.6 µg NMO-IgG and 15 min later at the same site with 3 µL human complement (HC) without or with 16.75 U IdeS. 

A. Representative GFAP, AQP4 and myelin (MBP) immunofluorescence at 3 days after injection. Yellow line represents needle tract. White line delimits the lesion with loss of AQP4, GFAP and myelin. B. Summary of lesion size from experiments as in A (S.E., 4 mice per group, **p < 0.01 by non-parametric Mann-Whitney test).
Figure 1
Figure 2
Figure 3
Figure 4

A. Diagram of NMO-IgG binding assay with AQP4. Serum samples were incubated with NMO-IgG, followed by anti-Fc² Ab conjugated to HRP for detection.

B. Cytotoxicity assay with rAb-53 (2 µg/ml) and rAb-93 (1 µg/ml) against NMO-IgG in serum. Cytotoxicity was measured at different concentrations of NMO-IgG serum.

C. Diagram of C1q binding assay with AQP4. NK cells were incubated with AQP4 and NMO-IgG, followed by Fcγ receptor conjugated to HRP for detection.

D. Cytotoxicity assay with NK cells against AQP4. Excess Fc fragment was added to the assay to inhibit antibody binding.

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Figure 5
Figure 6

A

HC+NMO-IgG

AQP4

HC+NMO-IgG+IdeS

GFAP

MBP

1 mm

B

Loss of staining (% area)

30

20

10

0

20

10

0

AQP4

GFAP

MBP

IdeS - +

* **