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## Neuromyelitis optica: Aquaporin-4 based pathogenesis mechanisms and new therapies

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### Abstract

Neuromyelitis optica (NMO) is an autoimmune ‘aquaporinopathy’ of the central nervous system that causes inflammatory demyelinating lesions primarily in spinal cord and optic nerve, leading to paralysis and blindness. NMO lesions show loss of aquaporin-4 (AQP4), GFAP and myelin, infiltration of granulocytes and macrophages, and perivascular deposition of activated complement. Most patients with NMO are seropositive for immunoglobulin autoantibodies (AQP4-IgG) against AQP4, the principal water channel of astrocytes. There is strong evidence that AQP4-IgG is pathogenic in NMO, probably by a mechanism involving complement-dependent astrocyte cytotoxicity, causing leukocyte infiltration, cytokine release and blood-brain barrier disruption, which leads to oligodendrocyte death, myelin loss and neuron death. Here, we review the evidence for this and alternative proposed NMO pathogenesis mechanisms, such as AQP4-IgG-induced internalization of AQP4 and glutamate transporters, complement-independent cell-mediated cytotoxicity, and AQP4-IgG inhibition of AQP4 water transport function. Based on the initiating pathogenic role of AQP4-IgG binding to astrocyte AQP4 in NMO, selective blocker therapies are under development in which AQP4-targeted monoclonal antibodies or small molecules block binding of AQP4-IgG to astrocytes and consequent downstream pathology.

### Keywords

NMO; Devic’s disease; Aquaporin-4; Astrocyte; Complement; Autoimmunity

## 1. Introduction

The aquaporins (AQPs) are a family of related membrane water channels, most of which, including aquaporin-4 (AQP4), which is the focus of this review, are selective for water transport. Some AQPs, called aquaglyceroporins, also transport glycerol. The water-selective AQPs are involved in a wide range of biological functions including transepithelial fluid transport, cell migration and neuroexcitation (Verkman, 2011, 2012). The aquaglyceroporins are involved in cell proliferation, adipocyte metabolism and epidermal water retention (Hara-Chikuma and Verkman, 2008; Rojek et al., 2008). Loss-of-function mutations in human AQPs cause nephrogenic diabetes insipidus (AQP2) and congenital cataracts (AQP0). The AQPs are assembled in membranes as tetramers in which each ~30kDa monomer contains six transmembrane helical domains and two short helical segments surrounding cytoplasmic and extracellular vestibules connected by a narrow aqueous pore (Fujiyoshi et al., 2002; Walz et al., 2009). Water-selective AQPs are expressed

broadly in mammalian tissues involved in fluid transport, such as epithelial and endothelial cells in kidney, lung, eye and the gastrointestinal tract. The aquaglyceroporins are expressed in adipocytes, epidermal keratinocytes, and various cell types involved in tissue regeneration. Many tumors express AQPs as well.

The focus of this review is on cellular pathogenesis mechanisms of the AQP4-based neuroinflammatory disease, neuromyelitis optica (NMO). NMO was originally thought to be a variant of multiple sclerosis in that both diseases are associated with inflammatory, demyelinating lesions of the central nervous system (CNS). However, there are clear differences in the sites and pathology of lesions, and in their clinical course and response to therapies. NMO primarily affects optic nerve and spinal cord, causing blindness and paralysis, with relatively little brain pathology. The prevalence of NMO is ~2–4 per 100,000 individuals, and ~6–8 times more prevalent in women than in men (Wingerchuk et al., 2007). The incidence of NMO is greater in Asians and blacks than in Caucasians. The median age of onset is 39 years, and 80–90% of NMO patients have relapsing optic neuritis and myelitis rather than a monophasic course (Jarius and Wildemann, 2010). Current NMO therapies include immunosuppression, plasmapheresis and B-cell depletion. The clinical aspects of NMO are discussed in more detail in recent reviews (Fazio et al., 2011; Jarius and Wildemann, 2010; Wingerchuk et al., 2007).

A defining feature of NMO is the presence of serum autoantibodies against AQP4 (called AQP4-IgG or NMO-IgG), which is detected in 60–90% of NMO patients (Jarius and Wildemann, 2010; Lennon et al., 2005). AQP4-IgG seropositivity is highly specific for NMO. AQP4 is expressed on the plasma membrane of astrocytes throughout the CNS, as well as in skeletal muscle and in epithelial cells in kidney, stomach and exocrine glands (Frigeri et al., 1995a,b). Though AQP4-IgG was initially thought to be a serum marker of NMO, perhaps related to astrocyte damage, there is now strong evidence that AQP4-IgG is pathogenic in NMO. AQP4-IgG binding to AQP4 on astrocytes is thought to cause complement-dependent cytotoxicity, leading to leukocyte infiltration, cytokine release and blood-brain barrier breakdown. These initial events lead to oligodendrocyte death, myelin loss and neuron death, and consequent clinical neurological deficit.

Familial NMO occurs in approximately 3% of cases and seems to have a complex genetic basis (Matiello et al., 2010). Previous studies failed to establish significant correlation between polymorphisms in AQP4 sequence and NMO susceptibility (Crane et al., 2011b; Matiello et al., 2011). An increased risk of NMO has been associated with particular human leukocyte antigen alleles in specific populations (Wang et al., 2011; Zephir et al., 2009). It remains largely unknown why patients develop antibodies against AQP4. AQP4 autoimmunity frequently coexists with other autoimmune diseases, and it has been suggested in some NMO patients that AQP4 autoimmunity is initiated as an immune response to tumor antigens (Antoine et al., 2004; Ducray et al., 2007; Pittock and Lennon, 2008). In this review we evaluate potential pathogenic effects of AQP4-IgG binding to AQP4 and their relevance in NMO pathogenesis. How AQP4-IgG causes NMO pathology has important consequences for development of new therapies that target the underlying cause(s) of the disease.

## 2. AQP4 structure and function

AQP4 was originally cloned in 1994 by our lab based on its homology to known AQPs (Hasegawa et al., 1994). X-ray structure analysis showed that each AQP4 monomer consists of six helical, membrane-spanning domains and two short helical segments surrounding a narrow aqueous pore (Ho et al., 2009), similar to other AQPs. AQP4 monomers assemble in membranes as tetramers, which can further associate in the cell plasma membrane as large

aggregates called orthogonal arrays of particles (OAPs). OAPs are seen by freeze-fracture electron microscopy as regular square arrays of intramembrane particles (Landis and Reese, 1974; Rash et al., 1974; Wolburg et al., 2011). Our lab identified AQP4 as the major OAP protein from the appearance of OAPs in AQP4-transfected cells (Yang et al., 1996) and the absence of OAPs in AQP4 knockout mice (Verbavatz et al., 1997). AQP4 is present in two major isoforms: a long (M1) isoform with translation initiation at Met-1, and a short (M23) isoform with translation initiation at Met-23 (Fig. 1A) (Lu et al., 1996; Yang et al., 1995). Both isoforms are expressed in astrocytes, forming heterotetramers. OAP formation is stabilized by intermolecular N-terminus interactions involving residues just downstream of Met-23 (Crane and Verkman, 2009a). Our lab has applied biophysical methods, including quantum dot single particle tracking and super-resolution imaging, to study the structure, dynamics and regulation of AQP4 assembly in OAPs (Crane et al., 2008, 2009; Crane and Verkman, 2009a,b; Jin et al., 2011; Rossi et al., in press).

In the central nervous system, AQP4 is by far the most abundant water channel. AQP4 is concentrated in astrocyte membranes facing blood-brain and brain-CSF interfaces and in the basolateral membrane of ependymal cells (Nielsen et al., 1997; Rash et al., 1998). AQP4 is also expressed in astrocyte-like 'supportive cells' in sensory organs such as retinal Müller cells (Li et al., 2002). AQP4 facilitates movement of water between the blood and brain and the brain and CSF compartments. Knockout mice lacking AQP4 show reduced cytotoxic (cell swelling) brain edema following water intoxication or ischemic stroke (Manley et al., 2000), increased vasogenic (leaky vessel) brain edema in brain tumor (Papadopoulos et al., 2004) or abscess (Bloch et al., 2005), and increased ventricular enlargement in obstructive hydrocephalus (Bloch et al., 2006). Phenotype analysis of knockout mice has also implicated AQP4 involvement in neuroexcitation (Binder et al., 2006; Li et al., 2002; Li and Verkman, 2001; Lu et al., 2008), astrocyte migration (Auguste et al., 2007; Saadoun et al., 2005) and neuroinflammation (Li et al., 2011). Impaired AQP4 water permeability in astrocytes is likely responsible for each of these phenotypes.

### 3. Cellular consequences of AQP4-IgG binding to AQP4

When bound to their cellular target, antibodies can cause: (i) altered target function; (ii) target internalization, reducing cell surface expression; (iii) complement activation, causing cell death (complement-dependent cytotoxicity, CDC); and/or (iv) activation of effector cells, including natural-killer cells (NK-cells), causing cell death (antibody-dependent cellular cytotoxicity, ADCC). Available evidence implicates (iii) as the primary mechanism involved in NMO pathogenesis, though (iv) may contribute as well. Though conflicting data have been reported, mechanisms (i) and (ii) are probably not important in NMO pathogenesis.

#### 3.1. AQP4-IgG binds to extracellular epitopes on AQP4

AQP4-IgG is substantially larger in size than AQP4 (Fig. 1B). Ratio-imaging fluorescence microscopy measurements indicate that AQP4-IgG generally binds with higher affinity to M23-AQP4 than to M1-AQP4 (Fig. 1C), though some antibodies bind with similar affinity to both isoforms (Crane et al., 2011a; Nicchia et al., 2009). Because M23-AQP4 forms OAPs and M1-AQP4 does not, this suggests a preference for AQP4-IgG binding to OAPs. Studies with M23-AQP4 mutants that do not form OAPs confirmed preferential binding of AQP4-IgG to OAPs (Crane et al., 2011a). Measurements were done using NMO patient serum, as well as monoclonal recombinant AQP4-IgGs derived from clonally expanded plasma cells from cerebrospinal fluid of NMO patients (Bennett et al., 2009). The dissociation constant of the tightest binding monoclonal AQP4-IgG was 44 nM. Mutagenesis studies suggest that AQP4-IgGs bind to various three-dimensional epitopes on the extracellular surface of AQP4 (Pisani et al., 2011; Yu et al., 2011).

### 3.2. AQP4-IgG binding to AQP4 causes CDC and ADCC

It has been shown, using different cellular systems and cytotoxicity read-outs, that AQP4-IgG can cause CDC when complement is present, and ADCC when effector cells, such as NK-cells, are present (Hinson et al., 2007, 2009; Kalluri et al., 2010; Vincent et al., 2008). The ability of AQP4-bound AQP4-IgG to cause CDC and ADCC is not unexpected, as the IgG Fc region binds complement protein C1q and effector cell receptor FcR. AQP4-IgG in NMO is predominantly of the IgG1 subtype (Isobe et al., in press), which strongly activates complement and binds all classes of Fc receptors involved in ADCC (Capel et al., 1994). An interesting recent finding was that efficient CDC (but not ADCC) requires AQP4 assembly in OAPs (Phuan et al., 2012). Fig. 2A shows AQP4 immunofluorescence and immunoblot analysis of cells expressing M1-AQP4 or M23-AQP4. M1-AQP4, which does not form OAPs, shows a smooth pattern of cellular fluorescence and a single (tetramer) band by native gel electrophoresis; M23-AQP4, which forms OAPs, shows a punctate pattern of fluorescence and multiple higher-order bands. CDC, as measured by LDH release (Fig. 2B, left) and live/dead cell staining (Fig. 2B, right) assays, was substantial for M23-AQP4-expressing cells, but minimal or absent for M1-AQP4-expressing cells. Measurements of complement protein binding suggested a mechanism for the greatly enhanced CDC involving C1q binding to AQP4-IgG when clustered on AQP4 OAPs (Fig. 2C). C1q is a large, multivalent protein whose binding affinity to IgG is greatly enhanced with increased binding valency. OAP formation by AQP4 thus enhances CDC at two levels: AQP4-IgG binding affinity to AQP4, and C1q binding to clustered AQP4-IgG.

### 3.3. AQP4-IgG binding to AQP4 does not inhibit AQP4 water permeability

Because AQP4-IgG is large compared to AQP4, it is sterically not possible to accommodate more than one AQP4-IgG per AQP4 tetramer, which contains four monomers and thus four separate water pores. Significant inhibition of AQP4 water permeability by AQP4-IgG is thus theoretically unlikely. Further, despite considerable search, small-molecule AQP4 inhibitors have not been identified. Two studies of osmotic volume changes in cultured cells have concluded that AQP4-IgG does not inhibit AQP4 water permeability (Melamud et al., 2012; Nicchia et al., 2009). We also found no significant inhibition of AQP4 water permeability by high concentrations of serum from multiple NMO patients, as well as by monoclonal NMO antibodies (Rossi et al., 2012). For these studies AQP4 water permeability was measured by a stopped-flow light scattering method in plasma membrane vesicles from AQP4-expressing cells and in AQP4-reconstituted proteoliposomes, an approach that can detect differences in water permeability of less than 5% and is insensitive to effects of internalization, unstirred layers, and ion/solute transport. One conflicting study, however, reported inhibition of AQP4 water permeability by AQP4-IgG (Hinson et al., 2012) using a time-to-lysis assay of *Xenopus* oocytes, which is an inaccurate surrogate of osmotic water permeability. With the exception of the Hinson et al. study, the evidence supports the conclusion that AQP4-IgG does not inhibit AQP4 water permeability.

### 3.4. AQP4-IgG binding to AQP4 does not cause AQP4 internalization in vivo

An initial study demonstrated that AQP4-IgG addition to cells stably transfected with a GFP-AQP4 chimera caused rapid internalization and degradation of AQP4 (Hinson et al., 2007). Cellular internalization of AQP4 and AQP4-IgG, if it occurs in the CNS *in vivo*, would have important implications for NMO pathogenesis and therapy, as it would protect astrocytes from CDC and ADCC. Cellular internalization of AQP4 in response to AQP4-IgG has also been shown in cultures of rat and human primary astrocyte cultures (Hinson et al., 2008; Melamud et al., 2012; Vincent et al., 2008). Using a dark quencher to resolve extracellular vs. intracellular localization of a fluorescent AQP4-IgG, we found rapid and selective internalization of AQP4-IgG and AQP4 in transfected cell cultures, in agreement with prior findings; however, there was little or no internalization of AQP4-IgG or AQP4 in

primary cultures of mouse astrocytes (Ratelade et al., 2011a). *In vivo*, after injection of a fluorescent AQP4-IgG in mouse brain, AQP4-IgG was localized to perivascular astrocyte end-feet as long as 24 h after injection, indicating minimal or no AQP4-IgG internalization (Ratelade et al., 2011a). We conclude that AQP4 internalization, which occurs in some cell culture models, is probably not relevant to NMO disease *in vivo*.

## 4. Organ culture and rodent models of NMO

Cell culture studies show that AQP4-IgG can produce CDC when complement is present and ADCC when effector cells are present. An animal model of NMO should recapitulate the key pathological features of human NMO lesions, including loss of AQP4 and GFAP immunoreactivity, demyelination, perivascular deposition of activated complement, and inflammatory cell infiltration. The ideal model, which so far has not been developed despite considerable effort, should manifest an AQP4-IgG autoimmune response with spontaneous development of NMO pathology in spinal cord and optic nerve. Short of this goal, our lab has developed *ex vivo* and *in vivo* mouse models of NMO that have been useful in studying NMO pathogenesis and testing new therapies.

### 4.1. Spinal cord and optic nerve culture models

As an *ex vivo* model of NMO, 300  $\mu\text{m}$ -thick vibratome-cut transverse slices of mouse spinal cord were cultured on transwell porous supports (Fig. 3A) (Zhang et al., 2011). Spinal cord cellular structure, including astrocytes, microglia, neurons and myelin, were preserved in culture. After 7 days in culture, spinal cord slices were exposed to NMO inducers, such as AQP4-IgG and complement, for 2–3 days and analyzed by immunofluorescence. Slices exposed to AQP4-IgG and complement showed marked loss of GFAP, AQP4 and myelin (Fig. 3B), as well as deposition of activated complement and microglial cell activation. Lesions were not seen with AQP4-IgG or complement alone, or in spinal cord slices from AQP4 null mice exposed to AQP4-IgG and complement together. The slice culture model has been useful in examining the roles of specific cell types and soluble factors in NMO pathogenesis. For example, in slice cultures treated with submaximal AQP4-IgG and complement, lesion severity was increased with inclusion of neutrophils, eosinophils or macrophages, or the soluble factors  $\text{TNF}\alpha$ , IL-6, IL-1 $\beta$  or interferon- $\gamma$  (Zhang et al., 2011, in press). Interestingly, lesions without myelin loss were produced by exposure of spinal cord slides to AQP4-IgG and NK-cells in the absence of complement. Further studies using an *in vivo* mouse model described below implicated the involvement of neutrophils in early NMO lesions and provided evidence for the potential utility of the neutrophil elastase inhibitor Sivelestat for NMO therapy (Saadoun et al., 2012). The spinal cord slice model was also used to demonstrate efficacy of monoclonal antibody (Tradtrantip et al., 2012b) and small molecule (Tradtrantip et al., 2012a) blockers, as discussed further below. A similar *ex vivo* model of NMO optic neuritis was accomplished by optic nerve culture for 1 day (Fig. 3C), in which NMO lesions were produced by incubation of optic nerve cultures with AQP4-IgG and complement (Zhang et al., 2011).

### 4.2. Rodent models

Rodent models have provided important data supporting the conclusion that AQP4-IgG is pathogenic in NMO. Initial studies showed that peripheral administration of AQP4-IgG exacerbates CNS lesions in rats with pre-existing experimental autoimmune encephalomyelitis (Bennett et al., 2009; Bradl et al., 2009; Kinoshita et al., 2009) and in rats pre-treated with complete Freund's adjuvant (Kinoshita et al., 2010). In these models, AQP4-IgG caused perivascular astrocyte destruction with loss of AQP4 and GFAP, as well as perivascular deposition of IgG and activated complement. Pathology was not seen in rats administered IgG from non-NMO patients. However the background hyper-inflammatory

environment and presence of myelin protein-reactive T lymphocytes in these models preclude unambiguous interpretation of data in terms of a pathogenic role of AQP4-IgG in NMO.

Direct evidence of a pathogenic role of AQP4-IgG came from intracerebral injection of AQP4-IgG and human complement in mice, which produced the major features of human NMO lesions, including loss of AQP4 and GFAP (Fig. 4), inflammatory cell infiltration, loss of myelin, and perivascular deposition of activated complement (Saadoun et al., 2010). Loss of myelin was seen as early as 12 h after administration of AQP4-IgG and human complement. In these experiments human complement was used because of the relatively inefficient mouse complement system. The inflammatory cell infiltrate in this model includes primarily neutrophils at early time and macrophages at later times. Interestingly, NMO pathology, including demyelination, was reduced in neutropenic mice and increased in neutrophilic mice, providing evidence for involvement of neutrophils in NMO pathology, particularly early in lesion generation (Saadoun et al., 2012). NMO pathology in this mouse model was not seen in key controls, including injection of complement with IgG from non-NMO humans or with AQP4-IgG depleted NMO serum, injection of AQP4-IgG in the absence of complement or with complement together with complement inhibitor, or injection of AQP4-IgG and complement in AQP4-knockout mice (Fig. 4).

To date, there is no good animal model of NMO based on peripheral administration of AQP4-IgG. We found that intravenously administered AQP4-IgG accesses and binds rapidly to AQP4 in peripheral organs (kidney, stomach, trachea and skeletal muscle), but does not measurably penetrate the CNS except in circumventricular organs, such as the area postrema, which have a leaky blood-brain barrier (Ratelade et al., 2011b). Therefore reduced accessibility of AQP4-IgG to AQP4 cannot explain why AQP4-expressing peripheral organs are spared in NMO. Similarly, passive transfer of AQP4-IgG to naïve rats did not induce NMO pathology (Bradl et al., 2009), even in circumventricular organs or when AQP4-IgG was administered to young rats with a leaky blood-brain barrier. Perhaps inefficient activation of rodent complement in these models is responsible for the absence of pathology, or perhaps structural or functional differences between rodent and human astrocytes. Human astrocytes have a substantially greater volume than their rodent counterparts and a higher ratio of glia to neurons (Oberheim et al., 2006). The greater complexity of the astrocyte network in humans may translate to increased susceptibility to NMO pathology. Development of improved animal models of NMO is a high priority.

## 5. Evaluation of NMO pathogenesis mechanisms

As diagrammed in Fig. 5, it is thought that NMO pathogenesis involves binding of AQP4-IgG to AQP4 on astrocyte end-feet, which activates complement, leading to formation of membrane attack complex (MAC) and primary astrocyte injury. This initial event is followed by recruitment of inflammatory cells, first granulocytes (neutrophils and eosinophils) and later macrophages, which further disrupt the blood-brain barrier. Primary astrocyte injury and initiation of an inflammatory reaction (such as neutrophil degranulation) are thought to damage oligodendrocytes and neurons secondarily. Evidence for this chronology comes from observations in human lesions in which AQP4 and GFAP can be diminished without myelin loss (Misu et al., 2007), and from animal models showing myelin loss after initial astrocyte injury (Saadoun et al., 2010). Though CDC is probably the major initiating mechanism in NMO, other pathogenic mechanisms triggered by AQP4-IgG have been proposed as discussed in this section.

### 5.1. Role of ADCC

As mentioned above, AQP4-IgG together with NK-cells can cause death of AQP4-transfected cells and astrocyte cultures. Binding of NK-cells to the Fc region of AQP4-IgG leads to their degranulation and release of perforin and granzymes that cause astrocyte injury (Fig. 6A). We found that intracerebral injection of AQP4-IgG and NK-cells produces NMO-like lesions in mice with astrocyte injury, but without myelin loss (Ratelade et al., 2012). Minimal lesions were observed when AQP4-IgG was injected with NK-cells and an excess of a mutant AQP4-IgG lacking ADCC effector function, suggesting that ADCC is the pathogenic mechanism in this model. However, the relevance of ADCC to NMO pathogenesis is not clear. Though NK-cells are seen infrequently in human NMO lesions, their short life following activation precludes any conclusion about their involvement in NMO pathogenesis.

Several leukocyte types besides NK-cells express Fc receptors and can mediate ADCC, including macrophages, neutrophils and eosinophils, which are abundantly found in NMO lesions. We found in spinal cord slice cultures that macrophages and neutrophils exacerbate NMO lesions caused by submaximal AQP4-IgG and complement, which probably involves an ADCC mechanism (Zhang et al., 2011). Recent work supports an important pathogenic role of eosinophils in NMO, through complement-dependent and independent cell-mediated cytotoxicity mechanisms (Zhang et al., in press). Definitive quantification of involvement of ADCC in human NMO, which will be quite challenging, is of clinical importance in determining the need for NMO therapies targeting ADCC.

### 5.2. Role of glutamate excitotoxicity

Hinson et al. (2008) reported decreased glutamate uptake in astrocytes exposed to human NMO serum and internalization of the glutamate transporter EAAT2 together with AQP4 in transfected HEK293 cells following exposure to NMO serum. They postulated a direct interaction of EAAT2 with AQP4. In addition, NMO lesions in human spinal cord show loss of AQP4 and EAAT2 immunostaining. The authors proposed that NMO pathogenesis involves glutamate excitotoxicity by a mechanism involving AQP4-IgG-induced internalization of glutamate transporter EAAT2 on astrocytes and consequent impairment in glutamate uptake from the extracellular space following neuroexcitation, leading to oligodendrocyte damage and myelin loss (Fig. 6B). Supporting this hypothesis, Marignier et al. (2010) reported reduced oligodendrocyte loss in mixed primary glial cultures exposed to AQP4-IgG and a competitive NMDA receptor antagonist. Based on this mechanism, ceftriaxone, which upregulates EAAT2 (Rothstein et al., 2005), was proposed as a therapy for NMO.

In re-evaluation of the glutamate excitotoxicity mechanism, we found no significant internalization of glutamate transporter EAAT2 in astrocytes after exposure to high concentrations of AQP4-IgG, nor was glutamate uptake reduced (Ratelade et al., 2011a). The loss of EAAT2 immunoreactivity in NMO lesions reported by Hinson et al. (2008) is probably related to astrocyte injury and loss, as AQP4 and GFAP are lost as well. We conclude that glutamate excitotoxicity is not involved in NMO pathogenesis and thus EAAT2 upregulation by ceftriaxone would not have therapeutic utility in NMO. Mice deficient in EAAT2 show lethal spontaneous seizures and increased susceptibility to acute cortical injury due to elevated glutamate in brain (Tanaka et al., 1997). EAAT2 internalization, if it occurred, would be predicted to produce a seizure phenotype, which is not seen clinically in NMO.



### 5.3. Role of differential M1-AQP4 vs. M23-AQP4 internalization

Hinson et al. (2012) recently proposed a new mechanism of AQP4-IgG pathogenesis in which M1-AQP4 is rapidly internalized by astrocytes upon AQP4-IgG exposure, whereas M23-AQP4 resists internalization. They concluded that NMO pathogenesis involves AQP4-IgG-induced internalization of M1-AQP4, resulting in increased OAP size and enhanced CDC (Fig. 6C). If correct, their mechanism has important implications regarding the initiating events in NMO pathogenesis and new treatment strategies. However, differential internalization of M1-AQP4 and M23-AQP4 following AQP4-IgG exposure is quite unlikely *a priori*, as M1-AQP4 and M23-AQP4 form tight heterotetramers in cell plasma membranes (Crane et al., 2009; Neely et al., 1999). Differential internalization of M1-AQP4 and M23-AQP4 conflicts with an earlier report by Hinson et al. (2008) showing full internalization of AQP4 in astrocytes, and data from our laboratory showing rapid internalization of M1-AQP4 and M23-AQP4 in transfected cells (Ratelade et al., 2011a). Additional studies of internalization mechanisms in transfected cells show that M1-AQP4 is internalized slower than M23-AQP4 when expressed separately, but equally when coexpressed (Phuan et al., 2012; Rossi et al., 2012). These findings, though interesting, are probably not relevant to NMO pathogenesis because, as mentioned above, our prior study indicated minimal internalization of AQP4-IgG *in vivo* (Ratelade et al., 2011a). Hinson et al. (2012) also proposed that rapid internalization of AQP4-IgG bound to M1-AQP4 protected M1-AQP4 transfected cells from CDC. However, as discussed above, we found that the reduced CDC for M1-AQP4 is not due to preferential internalization of M1-AQP4 vs. M23-AQP4, but to assembly of M23-AQP4 in OAPs and efficient, multivalent binding of C1q to clustered AQP4-IgG (Phuan et al., 2012). Also, Hinson et al. (2012) reported that a consequence of preferential M1-AQP4 internalization was increased OAP size. By super-resolution imaging, we found that AQP4-IgG did not affect OAP size in astrocyte cultures (Rossi et al., in press), though clustering could be induced by non-selective toxic insults that may account for the results in Hinson et al. (2012). We conclude that preferential internalization of M1-AQP4 does not occur and is thus not involved in NMO pathogenesis.

### 5.4. Role of AQP4 water transport inhibition

Hinson et al. (2012) noted that intramyelinic edema can be seen near some brain lesions in NMO. They interpreted the edema in terms of reduced AQP4 water permeability by AQP4-IgG binding and/or AQP4 internalization, disrupting water homeostasis and leading to local edema (Fig. 6D). As discussed above, however, our lab and others do not find altered AQP4 water permeability or internalization in response to AQP4-IgG binding. Further, AQP4 knockout mice do not manifest baseline abnormalities in brain water content (Manley et al., 2000; Papadopoulos et al., 2004). The intramyelinic edema observed in some NMO lesions is likely a non-specific finding that is also seen in other CNS diseases, such as Alexander Disease, where AQP4-IgG is absent (Balbi et al., 2010).

## 6. NMO therapy based on blocking AQP4-IgG binding to AQP4

Fig. 7A summarizes the central features of NMO pathogenesis, showing AQP4-IgG generation and binding to AQP4 on astrocytes, which initiates CDC and ADCC, inflammation and demyelination. NMO therapies, as shown, can target various steps in the scheme. Currently used therapies include general immunosuppression (*e.g.* steroids, azathioprine), immunomodulation (*e.g.* B-cell depletion by rituximab) and plasmapheresis. An ongoing open-label clinical trial is in progress of a monoclonal antibody complement inhibitor (eculizumab). Theoretically, therapies directed at reducing cell surface AQP4 expression or OAP assembly may also be beneficial.

Because AQP4-IgG binding to AQP4 is likely the initiating event in NMO, we introduced a strategy to block, using monoclonal antibodies or small molecules, AQP4-IgG binding to AQP4. In the monoclonal antibody strategy, a non-pathogenic, high-affinity monoclonal antibody ('aquaporumab') was generated from a recombinant monoclonal NMO antibody that binds tightly to AQP4 by mutation of its Fc region to eliminate CDC and ADCC effector functions (Tradtrantip et al., 2012b) (Fig. 7B). Aquaporumab prevented binding of AQP4-IgG in NMO patient sera, as well as cytotoxicity in AQP4-expressing cell cultures (Fig. 7C), spinal cord slice cultures, and mice receiving intraparenchymal AQP4-IgG and complement *in vivo*. Because aquaporumab targeting of AQP4 is highly selective, minimal toxicity is anticipated. Aquaporumab therapy may be useful during acute disease exacerbation to stop disease progression when the blood-brain barrier at the lesion site is disrupted. In this context aquaporumab could prevent astrocyte killing by AQP4-IgG and complement, and allow migration of reactive astrocytes into the already formed lesion, with consequent glial scarring and prevention of further inflammation. Aquaporumab might also be used as a maintenance therapy to reduce the frequency and severity of exacerbations. In an alternative approach, high-throughput screening identified several small-molecule drugs and natural products, including the antiviral agent arbidol, that bind to AQP4 and sterically prevent AQP4-IgG binding (Tradtrantip et al., 2012a). Further pre-clinical evaluation and optimization of monoclonal antibody and small-molecule blockers is in progress.

## 7. Remaining questions and directions

Though substantial progress has been made recently in understanding the involvement of AQP4-IgG in NMO pathogenesis and the central role of CDC, major questions remain. It is not known why NMO lesions localize mainly in spinal cord and optic nerve rather than in brain, and why peripheral, AQP4-expressing organs are unaffected. It is not known how AQP4-IgG, which has been assumed to be produced peripherally, initially enters the CNS. The identification of AQP4-IgG-producing plasma cells in the cerebrospinal fluid raises the possibility of AQP4-IgG production in the CNS, perhaps as the initiating event in NMO lesion formation. The roles of specific inflammatory cell types and cytokines in NMO pathogenesis are largely unknown and have potentially important therapeutic implications. As the involvement of neutrophils in NMO has led to consideration of a neutrophil elastase inhibitor for NMO therapy, definition of the roles of eosinophils and macrophages may lead as well to new therapeutic strategies. New therapies are needed for NMO, as disease morbidity remains high with current therapies. Blocker therapy has considerable potential as it selectively targets the disease-initiating event without causing generalized immunosuppression. Perhaps therapies could be developed in which a patient's own pathogenic AQP4-IgG could be converted into therapeutic blocking antibodies, or in which tolerization to AQP4 prevents AQP4-IgG generation.

## Abbreviations

<b>ADCC</b>	antibody-dependent cellular cytotoxicity
<b>AQP</b>	aquaporin
<b>AQP4</b>	aquaporin-4
<b>AQP4-IgG</b>	anti-AQP4 immunoglobulin NMO autoantibody
<b>BN-PAGE</b>	blue-native polyacrylamide gel electrophoresis
<b>CDC</b>	complement-dependent cytotoxicity
<b>CNS</b>	central nervous system

<b>CSF</b>	cerebrospinal fluid
<b>EAAT2</b>	excitatory amino acid transporter 2
<b>GFAP</b>	glial fibrillary acidic protein
<b>GFP</b>	green fluorescent protein
<b>LDH</b>	lactate dehydrogenase
<b>MAC</b>	membrane attack complex
<b>MBP</b>	myelin basic protein
<b>NK-cell</b>	natural-killer cell
<b>NMDA</b>	N-methyl-D-aspartate
<b>NMO</b>	neuromyelitis optica
<b>OAP</b>	orthogonal arrays of particles
<b>TIRFM</b>	total internal reflection fluorescence microscopy

## References

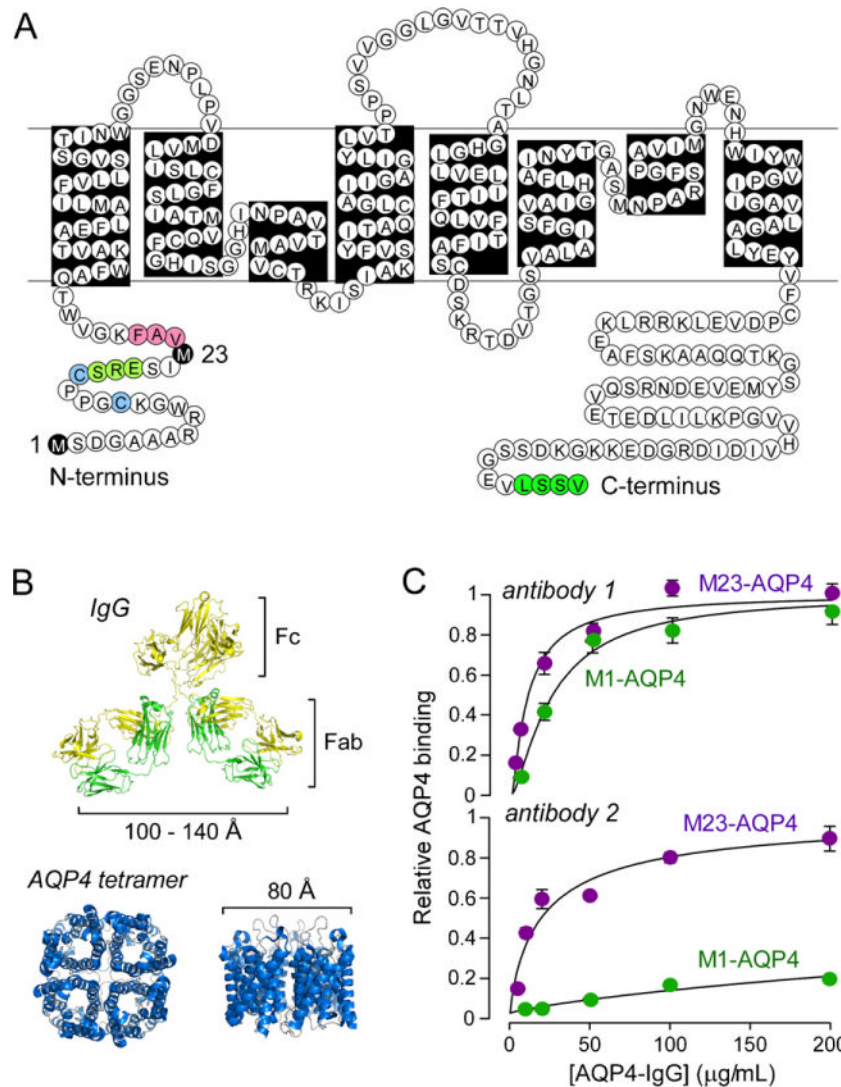
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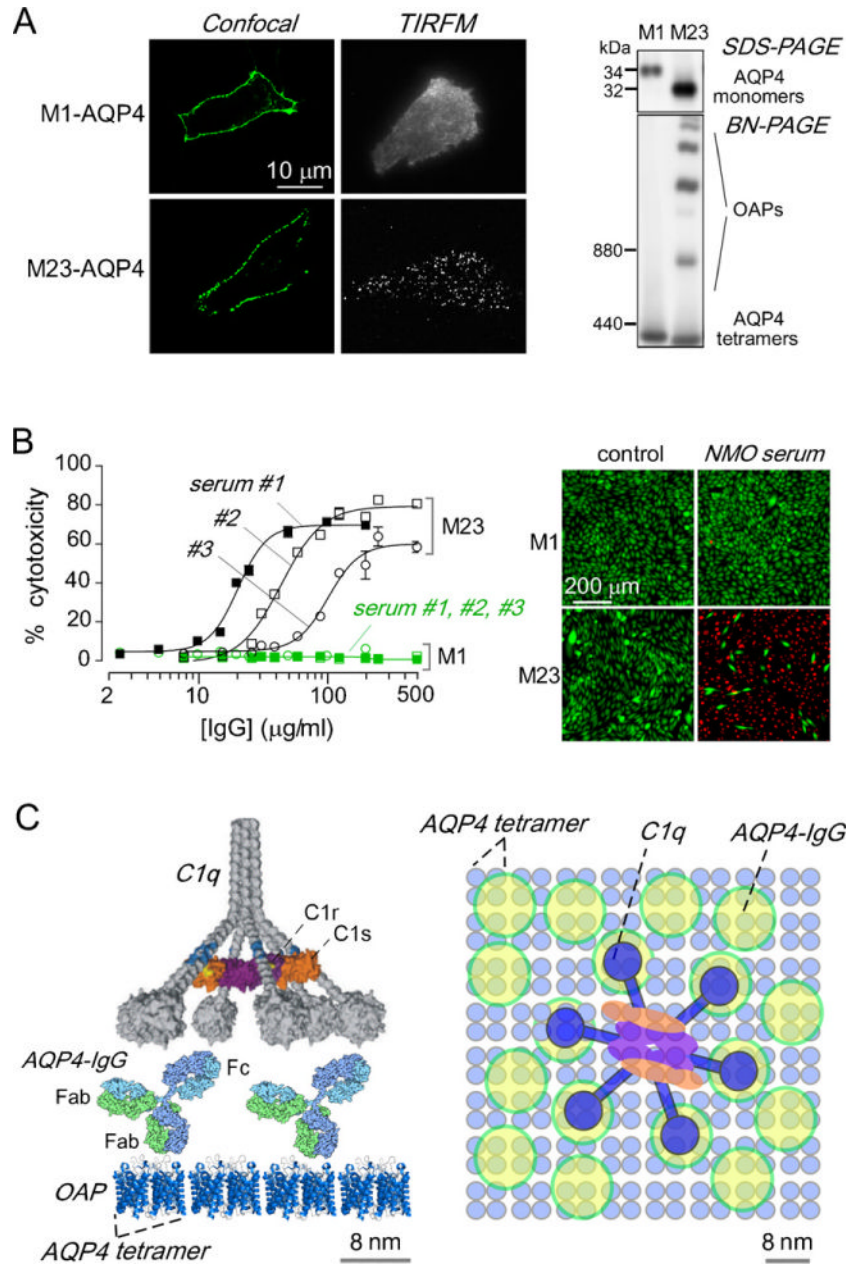
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**Fig. 1.** AQP4-IgG binding to its target, AQP4. (A) Amino acid sequence of human AQP4 showing Met-1 and Met-23 translation inhibition sites (black). Pink – residues involved in intermolecular N-terminus associations to form OAPs; light green – residues preventing OAP formation by M1-AQP4; blue – cysteine residues involved in palmitoylation-regulated OAP assembly; and dark green – C-terminus PDZ domain. (B) Crystal structure of human AQP4 (PDB, 3GD8) showing tetrameric association with central aqueous pore, along with structure of a generic IgG antibody shown on the same size scale. (C) Binding of two different monoclonal recombinant NMO autoantibodies to cells expressing M1-AQP4 or M23-AQP4.

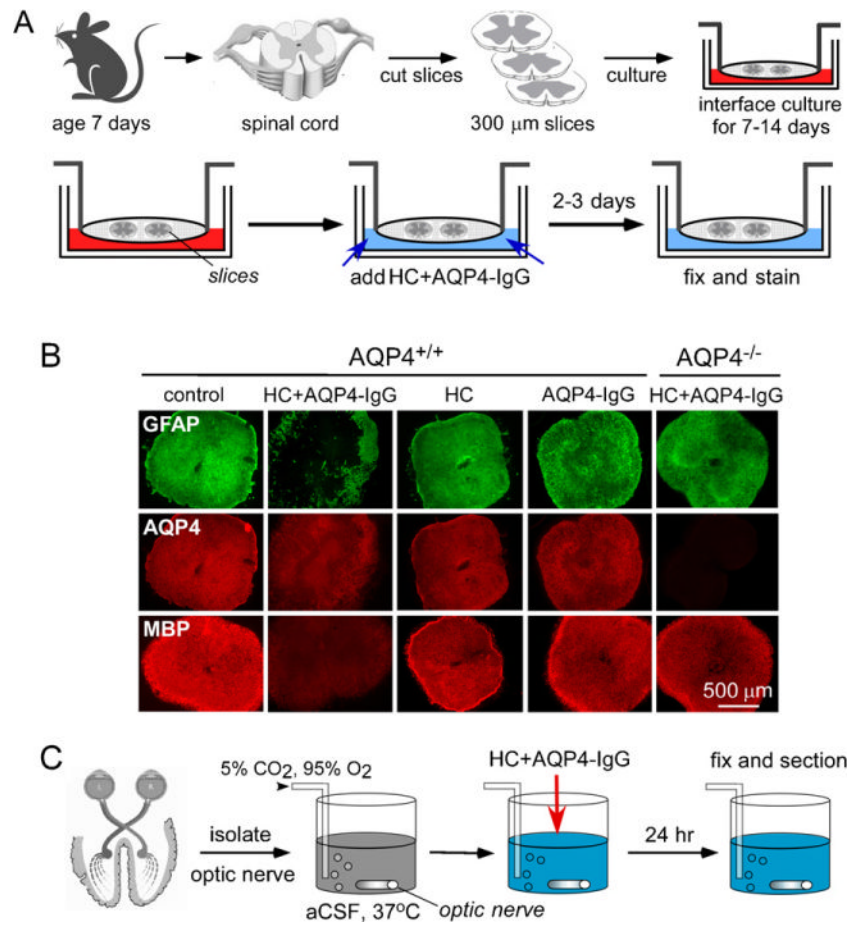
Parts of this figure were adapted from work originally published in the *Journal of Biological Chemistry* (Crane et al., 2011a) © the American Society for Biochemistry and Molecular Biology.





**Fig. 2.** Complement-dependent cytotoxicity requires AQP4 assembly in orthogonal arrays. (A) (left) Confocal and TIRFM of M1- and M23-AQP4 transfected CHO cells immunostained for AQP4 using a C-terminus anti-AQP4 antibody. (right) SDS-PAGE (top) and BN-PAGE (bottom) of cell homogenates. (B) Cells expressing M1-AQP4 are resistant to CDC caused by AQP4-IgG in human NMO sera. (left) Cytotoxicity plotted as a function of total IgG concentration (from NMO patient serum) in the presence of human complement. Cells were incubated for 1 h prior to LDH release assay. (right) Fluorescence micrographs showing live/dead (green/red) cell staining. (C) Multivalent binding of C1q to Fc regions of clustered AQP4-IgG bound to OAP-assembled AQP4. Side-view (left) and en-face view (right) shown.

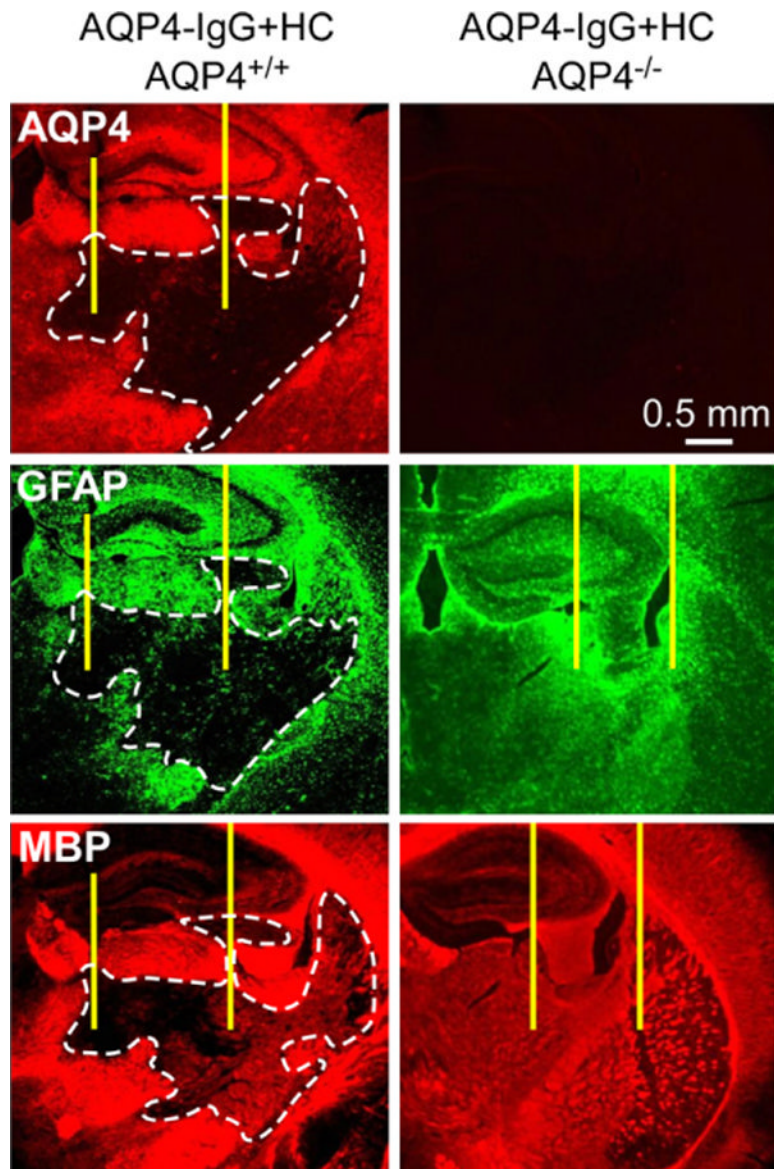
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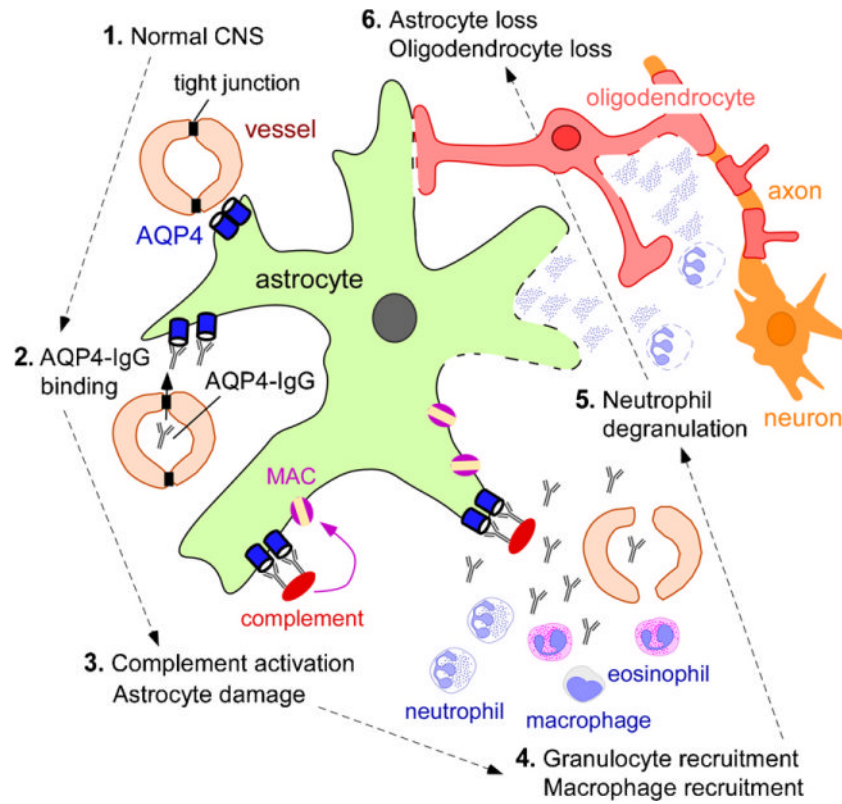
**Fig. 3. Ex vivo**

organ culture models of NMO. (A) Schematic showing spinal cord slices cultured on a semi-porous membrane at an air-medium interface. After 7 days in culture, spinal cord slices were incubated with human complement (HC) and/or AQP4-IgG for 2–3 days. (B) Immunofluorescence for GFAP (green), AQP4 (red) and myelin basic protein (MBP) (red) in wildtype (AQP4<sup>+/+</sup>) and AQP4 knockout (AQP4<sup>-/-</sup>) mice. ‘Control’ indicates no added AQP4-IgG or HC. (C) Schematic of optic nerve culture model showing 24 h incubation of freshly isolated optic nerves.

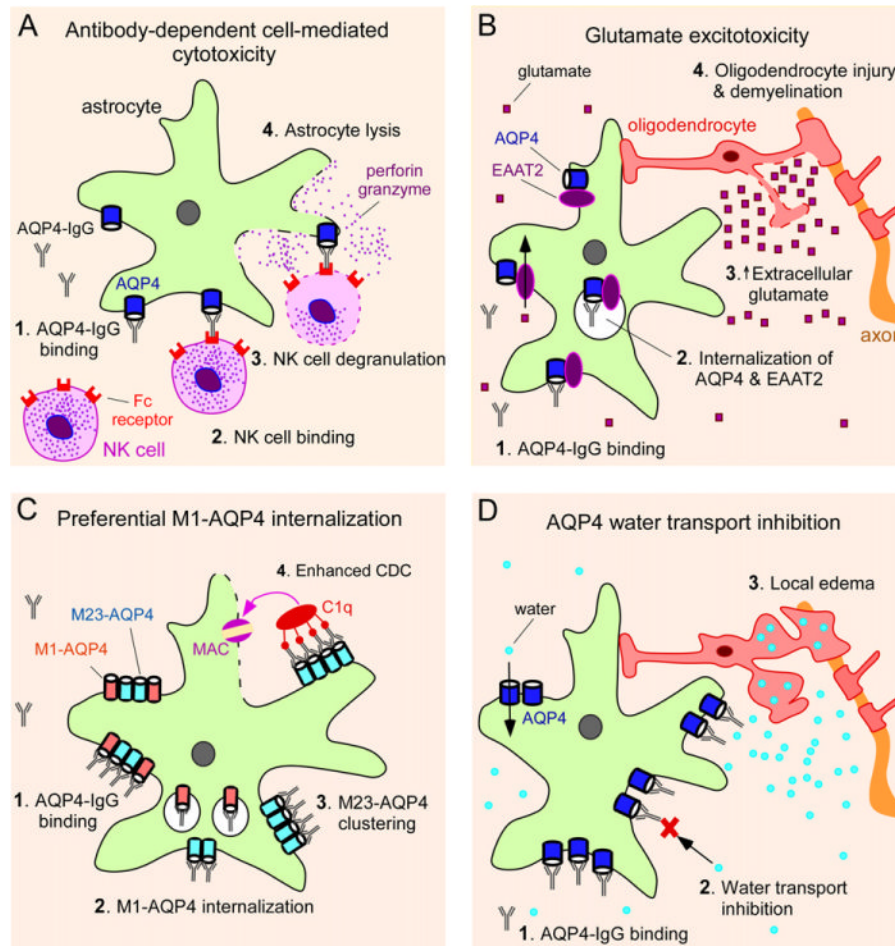
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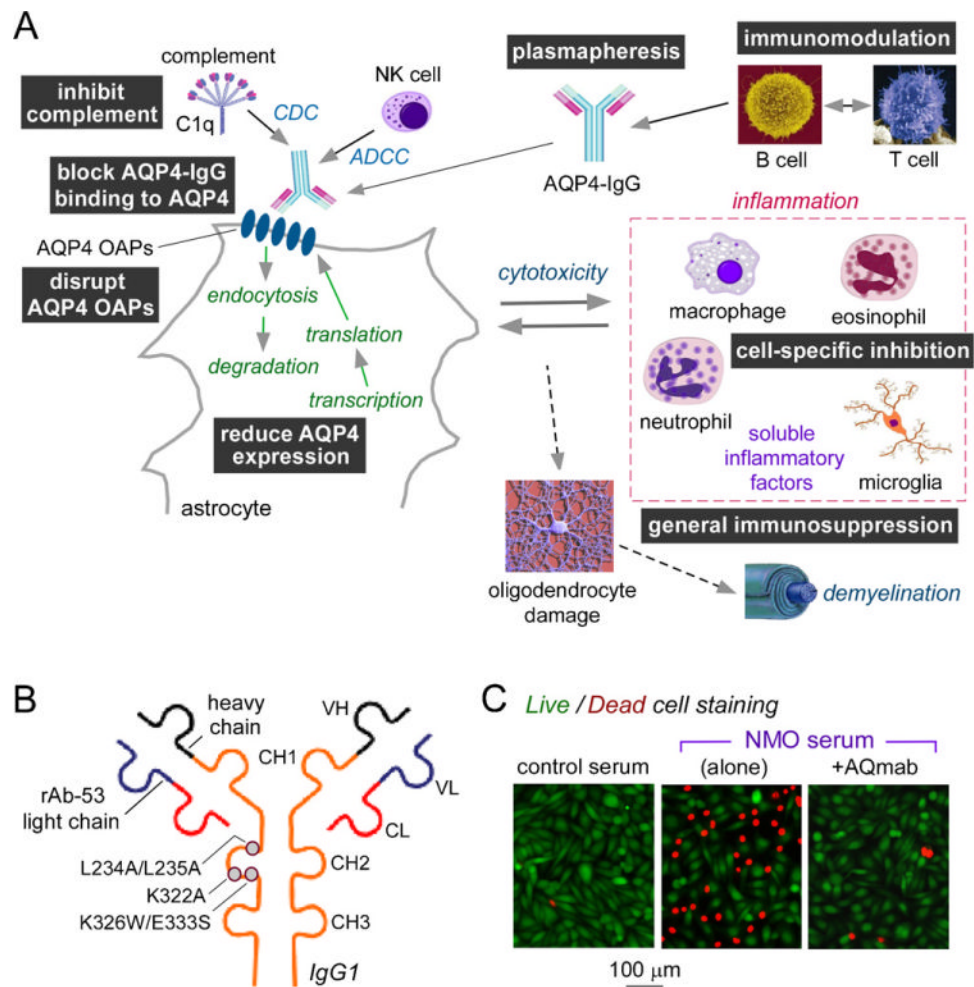
**Fig. 4.** Intracerebral injection of AQP4-IgG and human complement produces NMO lesions in mice. Wildtype (AQP4<sup>+/+</sup>) and AQP4 knockout (AQP4<sup>-/-</sup>) mice were killed 4 days after injection of AQP4-IgG and human complement (HC). Brain sections were immunostained for AQP4, GFAP and myelin basic protein (MBP). Yellow lines represent needle tracts. White dashed lines demarcate lesions.



**Fig. 5.** NMO pathogenesis mechanism. In the normal CNS, AQP4 is expressed at astrocyte end-feet facing the blood-brain barrier formed by endothelial cells connected by tight junctions (labeled '1'). In NMO, by an unknown mechanism, circulating AQP4-IgG crosses the blood-brain barrier and binds AQP4 on astrocytes (2). This leads to recruitment and activation of complement and deposition of the membrane attack complex (MAC), producing astrocyte damage (3). Complement activation and cytokine secretion by astrocytes recruit inflammatory cells (eosinophils, neutrophils and macrophages), which further disrupt the blood-brain barrier, allowing more entry of AQP4-IgG (4). Degranulating inflammatory cells (5) and astrocyte damage secondarily cause oligodendrocyte injury, myelin loss and axon damage (6).



**Fig. 6.** Alternative proposed NMO pathogenesis mechanisms based on AQP4-IgG binding to AQP4. (A) Antibody-dependent cell-mediated cytotoxicity. (B) Glutamate excitotoxicity. (C) Preferential M1-AQP4 internalization. (D) AQP4 water transport inhibition. See text for explanations.



**Fig. 7.** NMO therapy based on blocking of AQP4-IgG binding to AQP4 on astrocytes. (A) Overview of NMO pathogenesis showing AQP4-IgG production by lymphocytes, binding to AQP4 on astrocytes, CDC and (potentially) ADCC, and initiation of a cascade of inflammatory events. Therapeutic approaches shown in black boxes. (B) Schematic of AQP4-IgG antibody showing heavy (VH) and light (VL) chain variable regions, light chain constant region (CL), and heavy chain constant regions (CH1-CH3). Locations of amino acid mutations introduced in the CH2 domain of AQP4-IgG to reduce CDC (K322A), ADCC (K326W/E333S) or both (L234A/L235A). (C) Aquaporumab (AQmab) prevents CDC following exposure to AQP4-IgG and complement as shown by live/dead cell assay. Adapted with permission from Tradtrantip et al. (2012b).