

## Original Article

# Elevation of AQP4 and selective cytokines in experimental autoimmune encephalitis mice provides some potential biomarkers in optic neuritis and demyelinating diseases

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**Abstract:** Idiopathic optic neuritis (ION) is an inflammation of the optic nerve that may result in a complete or partial loss of vision. ION is usually due to the immune attack of the myelin sheath covering the optic nerve. ION acts frequently as the first symptoms of multiple sclerosis (MS) and neuromyelitis optica (NMO), or other inflammatory demyelinating disorders. The pathogenic progression of ION remains unclear. Experimental autoimmune encephalitis (EAE) is a commonly used model of idiopathic inflammatory demyelinating disorders (IIDDs); the optic nerve is affected in EAE as well. The specific mediators of demyelination in optic neuritis are unknown. Recent studies have indicated that T-cell activation in peripheral blood is associated with optic neuritis pathogenesis. The object of the present study was to determine whether certain cytokines (IL-6, IL-17A, and IL-23) and AQP4 contribute to the demyelinating process using EAE model. We have found that IL-6R, AQP4 and IL-23R are significantly increased in mRNA and protein levels in optic nerves in EAE mice compared to control mice; serum AQP4, IL-6, IL-17A, IL-23 are increased whereas transforming growth factor beta (TGF- $\beta$ ) is decreased in EAE mice. These results suggest that AQP4 and selective cytokines in serum are associated with ION pathogenesis in the animal model, and these results shine light for future clinical diagnosis as potential biomarkers in ION patients.

**Keywords:** AQP4, cytokines, experimental autoimmune encephalitis, idiopathic optic neuritis

## Introduction

Idiopathic inflammatory demyelination disease (IIDD) includes a broad spectrum of central nervous system disorders, including optic neuritis and multiple sclerosis (MS).

Optic neuritis is an acute inflammatory disease of the optic nerves caused by demyelination [1]. Optic neuritis is called idiopathic optic neuritis (ION) when there are no other systemic diseases. ION is frequently the early symptom of MS, causing neurological dysfunction [2]. The other major type of optic neuritis is neuromyelitis optica (NMO), which is distinct in prognosis and clinical features from ION [3]. NMO is a heterogeneous inflammatory disorder characterized by optic neuritis and longitutive extensive transverse myelitis (LETM) [4, 5]. Experimental auto-

immune encephalomyelitis (EAE) is a widely used animal model of MS, NMO or other IIDDs exhibiting many neurological dysfunctions because of demyelination. These dysfunctions include optic neuritis, which can be induced by immunization with myelin antigens [5]. Inflammatory demyelination and axonal injury in the optic nerve are commonly seen in MS patients and are present in EAE affected mice. Optic nerve inflammation and neuronal loss, including retinal ganglion cell (RGC) apoptosis in eyes with optic neuritis, were reported in human MS patients [2, 6]. These similar observations occur in EAE mice. The underlying mechanisms are unclear and the current medications have limited effects on MS patient long-term disability [5, 7], thus the underlying new agents and targets that occur during optic nerve inflammatory phase will be beneficial in

**Table 1.** Antibody information

AQP4	Abcam	Ab46182
IL-6R	Santa Cruz	Sc-374259
IL-23R	Abcam	Ab22059
GAPDH	CST	5174

medications. EAE is accompanied by infiltration of reactive T cells into the central nervous system. Similar symptoms make the correct diagnosis of NMO, MS and ION difficult in the early phase [8]. Misdiagnosis will result in different immune regulation therapy, and different therapies would have different effects on ION and NMO. Thus correct diagnosis is critically essential.

Cytokine-associated pathogenic mechanisms have been extensively studied in optic neuritis. The involvement of T helper (Th)-type immunities have been implicated in autoimmune encephalitis and NMO. Th17 cells are proinflammatory effector T cells that can produce high amount of interleukin 17 (IL-17) and IL-23 and other cytokines [5]. TGF- $\beta$ , another key cytokine involved in T cell differentiation and it is associated with MS and NMO [9, 10]. IL-6 level is elevated in serum and cerebrospinal fluid (CSF) in NMO patients. In NMO, the astrocytic water channel aquaporin-4 (AQP4) specific T cells exhibit Th17 polarization, and monocytes produces more IL-6, a Th17-polarizing cytokine [11, 12]. AQP4 antibody has been a most reliable marker to assist NMO diagnosis [13]. In other words, AQP4 antibody could be used to distinguish NMO from MS, AQP4 participates in optic neuritis pathogenesis and causes cytotoxicity and immune cell infiltration [4, 12, 14]. Among several tests, IL-6 appears to be the major cytokine associated with AQP4 autoimmunity. However, the mechanism underlying Th17 cells and AQP4 antibody generation is unclear. The immunological factors in humoral immunity underlying optic neuritis have not been elucidated.

Th17, IL-6, and IL-17 have been reported in autoimmune central nervous system (CNS) disorders, specifically the association between IL-17/Th17 has been well documented [15, 16], and IL-17 plays an essential role in the development of EAE in the mouse model [17]. Thus, we hypothesized that changes in the T cells subtypes and cytokines may contribute to the

pathophysiology of optic neuritis. In this study, we used EAE mice as a model of optic neuritis to examine the pathological changes of these cytokines. We also examined AQP4 levels to determine whether it is involved in the pathological changes in EAE mice. Together, we hope to provide one immunologic mechanism during demyelinating processes that affect the optic nerve, to understand the progression of these demyelinating diseases including optic neuritis.

## Materials and methods

### *Animal model of EAE*

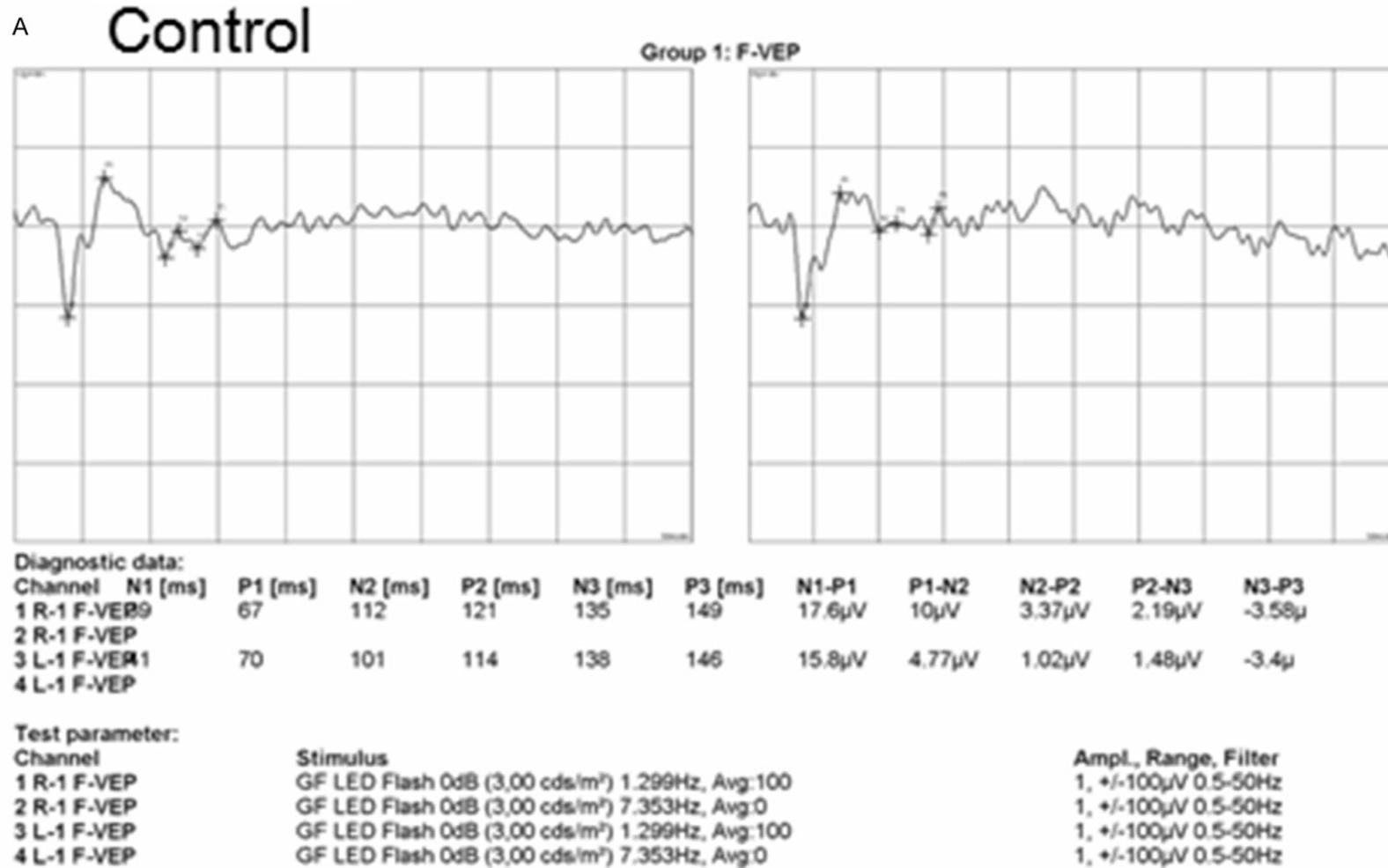
The mouse EAE model was induced as previously described (Stromnes and Goverman 2006). Total 20 female C57BL/6 mice were injected with an emulsion of MOG35-55 peptide, complete Freund's adjuvant (CFA) and Mycobacterium tuberculosis H37Rsubcutaneously, as the EAE group. PBS was injected into another 10 female mice as control group. Disease score was monitored daily after the immunization as follows: 0, no sign of disease; 1, loss of tone in the tail; 2, abnormal walking, partial hind limb paralysis; 3, unilateral hind limb paralysis; 4, bilateral hind limb paralysis with unilateral or bilateral front limb paralysis; 5, moribund or death [18]. Relapse was defined as the recurrence after the first onset, and the clinical scores of the recurrence were higher than previous time, or the same scores lasted more than 3 days. Mice weight was monitored from the immunization day. Injection and scoring were performed double-blinded. Electrophysiological measure (VEP) and histology were performed to evaluate the establishment of EAE model.

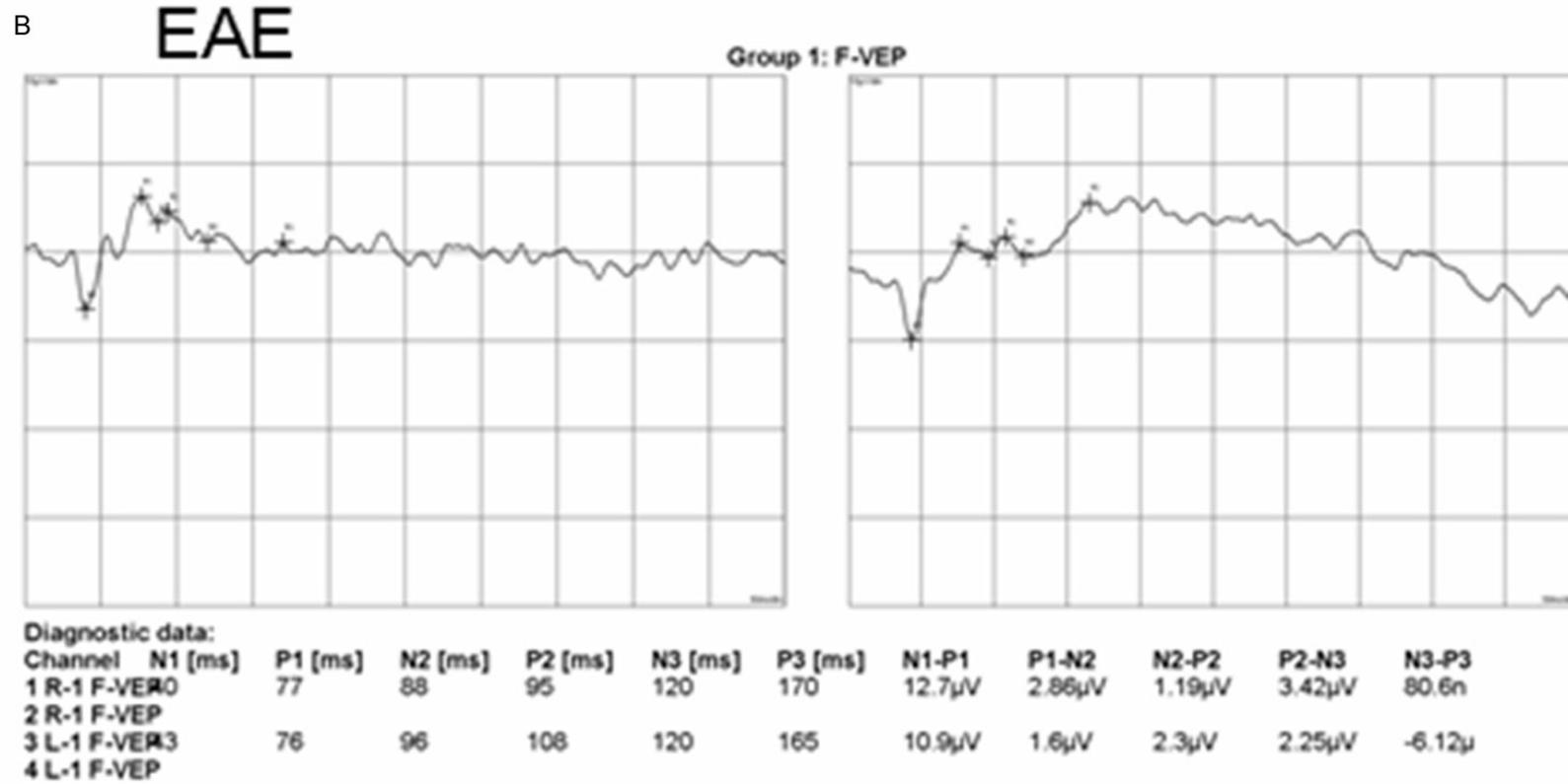
### *Visual-evoked potentials (VEP)*

To permit VEP recording, control and EAE mice were anesthetized and stainless steel screws were implanted to place electrodes in visual cortex. At least 5 days were allowed for recovery before electrophysiological testing. The visual stimulator was Ganzfeld 450 to deliver flash stimuli for Flash VEP, flash was used for 100 times with 1 sec interval, at the stimulation frequency GF LED Flash 1.299 Hz [19, 20].

### *Histology and HE staining*

Mice were perfused with 4% paraformaldehyde. Optic nerve and spinal cord tissues were





**Figure 1.** EAE mice are not able to respond efficiently to visual stimuli in VEP examination compared to control mice. A. Representative traces of VEP from control mice. B. Representative traces of VEP from EAE mice.

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prepared separately in 4% paraformaldehyde for 48 hours followed by dehydration in 50%, 70%, 85%, 95% and 100% ethanol (2 hours each) and embedded in paraffin for sections. 4-7  $\mu$ m sections were made for optic nerve and spinal cord tissues. Sections were stained with hematoxylin and eosin. Images were taken by Olympus (CX41) microscope.

### ELISA

The serum was collected and drawn from orbit from each mouse. The concentration of cytokines was determined by multiplex ELISA (JRDUN biotechnology, Shanghai) according to manufacturer's instructions.

### Real-time PCR

RNA isolation: RNA was isolated from freshly dissected optic nerve tissues of 4 control mice and 4 EAE mice with Trizol. The cDNA samples were prepared using random primers at 37C for 60 min, then 85C for 5 min, 4C for 5 min and stored at -20C. Reverse transcription and qRT-PCR was performed using ABI Prism machine and Sybr-Green with parameters (95C 10 min; 95C 15 sec, 60C 45 sec; repeat 40x; 60C 1 min). Data were calculated with respect to GAPDH for each sample within an assay. Interleukin 6 receptor, alpha (IL6R) primers: GACAACAGCAACAGAGACTAC and TTCCTTCC-TTCAGAGCCTATG; aquaporin 4 (AQP4) F 5' GGG-TCTATTGCCTTGTGGATG 3' and R 5' GATCTCT-GTGC GG TGTATCTG 3'; interleukin 23 receptor (IL23R) F 5' GAACACTGGGAAGCCTAC 3' Primer R 5' GACAGCTTGGACCCATAC 3'; glyceraldehyde-3-phosphate dehydrogenase (GAPDH) Primer F 5' ATCACTGCCACCCAGAAG 3' Primer R 5' TCCACGACGGACACATTG 3'.

### Western blot analysis

For analysis of protein levels, freshly dissected optic nerve tissues (3 control mice and 3 EAE mice) were sonicated in RIPA lysis buffer in the presence of protease inhibitor cocktail and centrifuged at 12000 g for 15 min. The information of the antibodies was listed in **Table 1**.

## Results

### Establishment and verification of EAE model

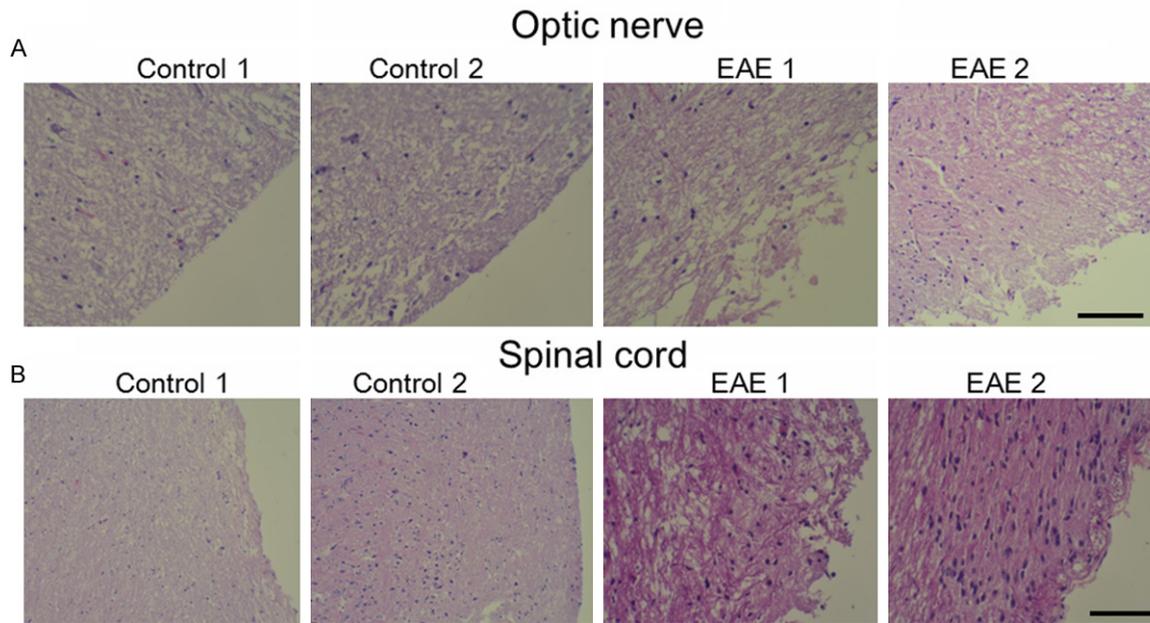
We employed a standard EAE model of optic neuritis in which C57B/6 mice were immunized

with an emulsion of MOG35-55 peptide and complete Freund's adjuvant (CFA) to examine chemokine and cytokine changes. Optic nerve function was evaluated through visual evoked potential (VEP). Typical flash VEP waveforms could be recorded in control animals. Generally, the light stimulus causes an increase in responses. A higher intensities negative component (N1) was preceded by a positive deflection (P1) in control group (**Figure 1A**). In sharp contrast, in EAE mice, there was no responses detected following similar N1 stimuli, and the latency was significantly prolonged (**Figure 1B**). The lack of responses in flash VEP in EAE mice suggested that the optic nerves were dysfunctional, confirming that our EAE model was validated to study the pathology in optic nerve. To determine demyelinating progression in the EAE model, we performed histological examination on optic nerve and spinal cord tissues. In control mice, both optic nerve and spinal cord tissues exhibited intact and smooth cell membranes; there was no demyelination and no immune cell infiltration in optic nerve and spinal cord (**Figure 2A, 2B**). However, in EAE mice, demyelinating lesion was detected in both optic nerve and spinal cord tissues: myelins are broken and disorganized; immune cell infiltration occurred at the wrapping membrane and tiny blood vessels (**Figure 2A, 2B**). In agreement with VEP results, our histological experiments demonstrated the demyelinating lesion in the EAE mice, which allows us to EAE model to study biological changes during demyelination

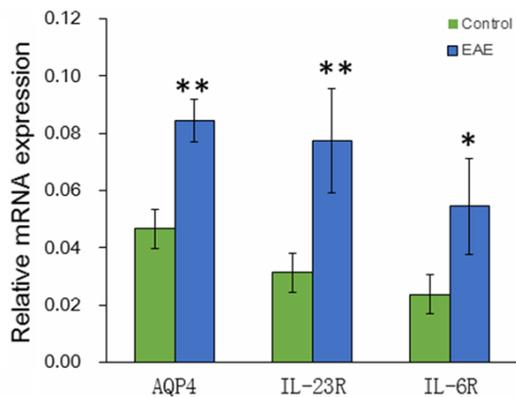
### AQP4, IR-6R and IR-23R mRNA levels are increased in EAE mice

The development of EAE in the C57BL/6 mouse background is dependent on the activation of CD4<sup>+</sup>T cells and CD11b<sup>+</sup>macrophages/microglia in the CNS [21]. These cells secrete and produce different cytokines. To investigate whether EAE will increase these cytokines, we first chose to compare AQP4, IR-6R and IR-24R between EAE and control animals.

We examined whether demyelinating process is able to alter AQP4, IR-6R and IR-23R at transcriptional level using real-time quantitative RT-PCR. qRT-PCR analysis of RNA isolated from optic nerves in EAE mice revealed a significant elevation of AQP4, IR-6R, and IR-23R mRNA expression (**Figure 3**). Hence, we determined that EAE model had a strong effect on increas-



**Figure 2.** HE staining results confirmed immune cell filtration and demyelination occurrence in EAE mice. A. Representative images of optic nerve from control and EAE mice. B. Representative images of spinal cord from control and EAE mice. Scale bar 100  $\mu$ m.



**Figure 3.** EAE mice exhibit increased mRNA levels of AQP4, IL-6R and IL-23R. Quantitative real-time RT-PCR analysis of the representative genes from 4 animals from control or EAE mice. \*\*P<0.01; \*P<0.05.

ing transcriptional levels of AQP4, IR-6R and IR-23R compared to WT controls. These results suggested that the EAE-induced demyelinating procession regulates inflammatory cytokines and AQP4 expression at the transcription level.

*EAE models increase the expression of AQP4, IL-6R and IL-23R protein levels*

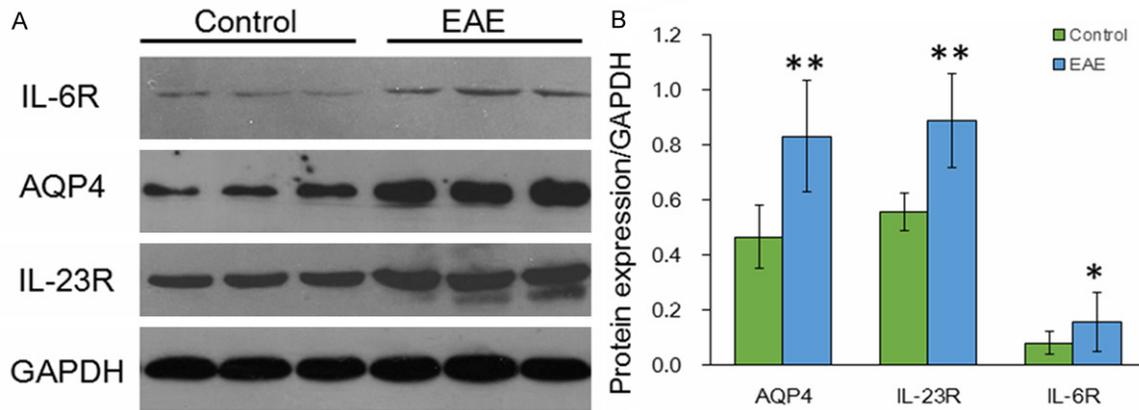
Since AQP4, IL-6R and IL-23R mRNA levels are altered in EAE mice, we evaluated the protein

expression in optic nerve tissues by Western blot analysis. EAE and control mice optic nerves were harvested. Consistent with mRNA results, EAE mice showed a significant increase in AQP4, IR-6R and IL-23R protein levels compared to control mice (**Figure 4**), confirming that the demyelinating lesion in EAE model elicits elevation in cytokines (IL-6R and IR-23R) and AQP4. These qRT-PCR and Western blot results suggest that inflammatory cytokines are regulated in demyelinating progression and their function may be involved in demyelinating optic neuritis development.

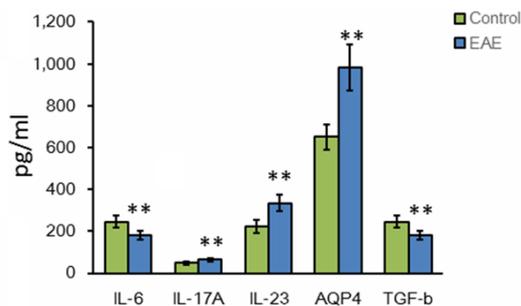
*Cytokines and AQP4 levels were elevated in EAE mice serum.*

The qRT-PCR and Western blot were analyzed using optic nerve tissues. Thus, we decided to measure different cytokines in serum from EAE and control mice. We measured IL-6, IL-17A, IL-23, and transforming growth factor beta (TGF- $\beta$ ) production by ELISA assay to determine whether EAE model modulates production of these cytokines. We determined that IL-6, IL-17A, and IL-23 are significantly elevated in EAE mice (**Figure 5**). However, TGF- $\beta$  was significantly reduced in EAE mice (**Figure 5**). These results suggest that EAE model selectively increases certain cytokines, including IL-6,

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**Figure 4.** EAE mice exhibit an increase in IL-6R, AQP4, and IL-23R protein levels. Protein levels were examined by western blot analysis. A. Representative western blots of IL-6R, AQP4 and IL-23R from control and EAE mice. B. Quantification of IL-6R, AQP4 and IL-23R expression in control and EAE mice. \*\* $P < 0.01$ ; \* $P < 0.05$ .



**Figure 5.** EAE model induces elevation of several cytokines in serum. Certain cytokines were significantly higher in EAE mice serum. ELISA analysis from 10 control animals and 15 EAE animals. \*\* $P < 0.01$ .

IL-17A, and IL-13, which is consistent with the elevation of mRNA and protein levels in EAE mice. In NMO patients, AQP4 antibody level has been shown to correlate with some cytokines, such as IL-6 and IL-8 [22]. Since we found selected cytokines are increased in EAE mice, including IL-6, we next examined AQP4 level in EAE mice serum using ELISA assay. We found AQP4 is elevated in EAE mice serum (**Figure 5**).

### Discussion

In this study, we provided compelling evidence that immunological responses occur in optic nerve tissues using EAE as a mice model of optic neuritis. Using real-time qRT-PCR, Western blots and ELISA assays, we have demonstrated that IL-6R, IL-23R and AQP4 are increased at both transcriptional and translational levels in optic nerves, IL-6, IL-17A, IL-23

and AQP4 levels are selectively elevated in EAE mice serum. To our knowledge, AQP4 was not examined together with selected cytokines in EAE mice model. Our results indicated that the subgroup of cytokines and AQP4 correlate during the pathogenetic mechanisms of optic neuritis, thus the future therapies may focus on them for repurposing in optic neuritis.

We first utilized VEP and histological analysis to validate the EAE model, in order to study immune cell infiltration during optic neuritis progression. Our observations are consistent with human optic neuritis caused acute inflammation and inflammation induced devastating responses such as axonal degeneration [4, 5].

AQP4 has been lined to demyelinating lesions in neuromyelitis optica (NMO) [4, 23] as a separate disease entity from MS. Interestingly, pattern-specific loss of AQP4 immunoreactivity distinguishes NMO from demyelinating disorders such as multiple sclerosis, thus the anti-AQP4 antibody is a sensitive diagnostic biomarker for NMO [11, 12]. Our qRT-PCR, Western blots and ELISA data are consistent with patient serum reactivity, AQP4 levels are increased in EAE mice. Additional studies are needed to further distinguish the role of AQP4 in optic neuritis and NMO progression in other animal models. Future studies will focus on the underlying immune responses associated with AQP4 in EAE model.

In addition to AQP4, a number of studied molecular biomarkers might be helpful to elucidate

immunopathogenesis of optic neuritis. The discovery of the specific anti-AQP4 IgG1 biomarker indicates the immune reaction against AQP4 occurrence at the NMO lesions, thus NMO can be distinguished from MS [4]. Despite the recent sensitive assays, AQP4-specific antibodies are not detected in 10-40% of NMO patients [24]. It is possible that antibodies in some NMO patients cross-react with other antigens. For example, anti-myelin oligodendrocyte glycoprotein (MOG) antibodies have been identified in some NMO patients [25]. MOG antibodies may serve as a prognostic tool in patients with an AQP4 seronegative NMO phenotypes and can be a potential biomarker to test in this subgroup of patients. Since EAE model is usually considered as a mouse model for MS, one future direction is to examine MOG antibodies levels to further distinguish these IIDD. Many studies have illustrated that some cytokines and chemokines are elevated in cerebrospinal fluid (CSF) of NMO patients. For example, IL-6 is significantly higher in NMO patients CSF compared to MS patients or non-inflammatory neurological disorders [4, 22]. AQP4-reactive B and T cells are required to produce the IgG1 biomarker against AQP4, especially for immunoglobulin class switching. There are different rodent models to raise pathogenic AQP4-reactive T cells to the T-helper-17 to further characterize demyelination and T cell infiltration into the optic nerves and CNS. The key roles of immunopathogenic of Th17 polarized AQP4-reactive T cells in NMO can trigger and localize CNS AQP4 inflammation [26, 27].

Among these biomarkers, several pieces of evidence strongly suggest T helper (Th) 17 cells are involved in optic neuritis, especially in NMO [28]. Th17 cells are proinflammatory effector T cells that are characterized by their ability to product high amount of IL-8, IL-17, IL-21 and IL-23 [28]. These cytokines exhibit various biological roles and may induce chronic inflammatory and autoimmune diseases as well as affecting B-cell proliferation and stimulating antibody production [17, 28]. Specifically, NMO patients peripheral blood T cells show greater proliferation to AQP4, and these AQP4-specific T cells exhibit Th17 polarization, and monocytes from NMO generate more IL-6, an important Th17-polarizing cytokine [29]. IL-6 levels in peripheral blood and cerebrospinal fluid (CSF) both show a strong clinical correlation in NMO,

and IL-6 enhances survival and anti-AQP4 antibody production in NMO [27]. The roles of IL-17, a signature cytokine of Th17, in the pathogenic process in EAE have been debating in inducing inflammatory tissue damage. IL-17 is specifically increase in NMO patients compared to MS patients, as well as IL-23 [30]. The elevation of IL-17 and IL-23 in EAE mice in this study is consistent with the idea that immunopathology in optic neuritis is dependent on IL-17 mediated pathways [5, 30]. Th17 mediated immunopathology is dominant in optic nerve and brain.

In summary, we have provided novel evidence that the increase of IL-6R, IL-23R and AQP4 at both transcriptional and translational level in EAE mice, a model of IIDDs that initiates optic neuritis. As we gain a better understanding of this demyelinating disease, it may a platform to serve a future drug design and potential therapeutic effectiveness. Thus these results may serve a deeper understanding of immunological responses in optic neuritis. Therapeutic strategies target the IL-6 receptor and complement proteins are ongoing clinical evaluation for NMO [4].

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### Disclosure of conflict of interest

None.

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