Human Aquaporin 4_{281-300} Is the Immunodominant Linear Determinant in the Context of HLA-DRB1*03:01

Relevance for Diagnosing and Monitoring Patients With Neuromyelitis Optica

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Objective: To identify linear determinants of human aquaporin 4 (hAQP4) in the context of HLA-DRB1*03:01.

Design: In this controlled study with humanized experimental animals, HLA-DRB1*03:01 transgenic mice were immunized with whole-protein hAQP4 emulsified in complete Freund adjuvant. To test T-cell responses, lymph node cells and splenocytes were cultured in vitro with synthetic peptides 20 amino acids long that overlap by 10 amino acids across the entirety of hAQP4. The frequency of interferon γ, interleukin (IL) 17, granulocyte-macrophage colony-stimulating factor, and IL-5–secreting CD4+ T cells was determined by the enzyme-linked immunosorbent sport assay. Quantitative immunofluorescence microscopy was performed to determine whether hAQP4_{281-300} inhibits the binding of anti-hAQP4 recombinant antibody to surface full-length hAQP4.

Setting: Academic neuroimmunology laboratories.

Subjects: Humanized HLA-DRB1*03:01/+ H-2b−/− transgenic mice on a B10 background.

Results: Peptide hAQP4_{281-300} generated a significantly (P<.01) greater T_{H1} and T_{H17} immune response than any of the other linear peptides screened. This 20mer peptide contains 2 dominant immunogenic 15mer peptides. hAQP4_{284-298} induced predominantly an IL-17 and granulocyte-macrophage colony-stimulating factor T_{H1} cell phenotype, whereas hAQP4_{285-299} resulted in a higher frequency of T_{H1} cells. hAQP4_{281-300} did not interfere with recombinant AQP4 autoantibody binding.

Conclusions: hAQP4_{281-300} is the dominant linear immunogenic determinant of hAQP4 in the context of HLA-DRB1*03:01. Within hAQP4_{281-300} are 2 dominant immunogenic determinants that induce differential T_{H1} phenotypes. hAQP4 determinants identified in this study can serve as diagnostic biomarkers in patients with neuromyelitis optica and may facilitate the monitoring of treatment responses to pharmacotherapies.


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EUROMYELITIS OPTICA (NMO) is a demyelinating inflammatory disorder of the central nervous system (CNS) that is clinically and pathologically defined as the co-occurrence of optic neuritis and myelitis.1 NMO-IgG, an autoantibody that binds to human aquaporin 4 (hAQP4), is detectable in the serum of most patients with NMO.2,3 AQP4 plays an important role in the transportation of water across the cell membrane of multiple cell types. Within the CNS, it is highly expressed in the foot processes of astrocytes.4,5 There are 2 isoforms of hAQP4: M1 and the shorter M23 isoform, which lacks the first 22 amino acids but is otherwise identical in sequence.6 Possibly because of the identification of the NMO-IgG antibody in patients with NMO, neurologists have focused on pharmacotherapies that predominantly target the humoral immune system.7,8 There is evidence to suggest a cellular immune response in NMO. Recently, HLA
haplotype analyses of patients with NMO suggest a positive association with HLA-DRB1*03:01 (HLA-DR17), a gene that codes for a major histocompatibility class (MHC) II molecule that presents linear antigens 12 to 15 amino acids in length to CD4+ T cells. In some patient cohorts, NMO-IgG is undetectable in a substantial number of patients with NMO. In patients with NMO-IgG, antibody isotype switching from IgM to IgG could not occur without CD4+ T cell involvement. The response to B cell–depleting therapies is not consistently beneficial in patients with NMO, and CD3+ T cells play an important role in the pathogenesis of NMO.

To test our hypothesis, we screened 32 peptides of 20 amino acid length that overlap by 10 amino acids and span the entirety of hAQP4 in HLA-DRB1*03:01 transgenic mice. This process led us to identify the immunodominant linear determinants that stimulate cellular immune response in the context of HLA-DRB1*03:01. After identification of 1 immunodominant 20mer peptide, we determined dominant immunogenic 15mer peptides within. Proliferating CD4+ T cells were further defined by their expression of interferon γ (IFN-γ), interleukin (IL) 17, and granulocyte-macrophage colony-stimulating factor (GM-CSF).

### METHODS

#### PEPTIDES AND PROTEIN

Whole-protein AQP4 M1 was donated by William Harries, PhD, of the Membrane Protein Expression Center & Center for Structures of Membrane Proteins Macromolecular Structure Group (University of California, San Francisco) (Figure 1). Synthetic peptides 20 amino acids long that overlapped by 10 amino acids across the entirety of hAQP4 (Table 1) and synthetic peptides 15 amino acids long that overlapped by a single amino acid spanning the immunodominant 20mer AQP4*286-301 (Table 2) were generated by JPT Innovative Peptide Solutions.

#### MICE

Generation of transgenic mice expressing HLA-DRB1*03:01 was previously described. Briefly, DRB1*0301 (DR3) transgenic mice were generated by coinjection of an HLA-DRL genomic fragment and a DRB1*030113 gene fragment into (C57BL/6 × DBA/2) F1 C57BL/6 embryos and backcrossed to B10 mice. Subsequently, the DR3 gene was introduced into the class II−negative H2b− line by mating the B10.M-DRBI*0301 line with the B10.MHCII− line. All mice were bred and maintained in a pathogen-free mouse colony at The University of Texas Southwestern Medical Center at Dallas according to the
guidelines set forth by the National Institutes of Health and the institution. All experiments were approved by the Institutional Animal Care and Use Committee at The University of Texas Southwestern Medical Center at Dallas.

**ENZYME-LINKED IMMUNOSORBENT SPOT ASSAY**

The frequency of IFN-γ, IL-17, GM-CSF, and IL-5-secreting CD4+ T cells was determined by the enzyme-linked immunosorbent spot (ELISpot) assay. Groups of 3 male HLA-DRB1\(^*\)03:01 mice were inoculated in the inguinal and axillary regions with 100 µg of whole-protein hAQP4 emulsified in complete Freund adjuvant in a 1:1 ratio. On day 10, lymph nodes and spleens were collected to generate single-cell suspensions.

**STATISTICAL ANALYSIS**

A 1-way analysis of variance test was used to compare the 32 different treatment groups. If the analysis of variance was found to be significant, the Bonferroni test, a pairwise post hoc test, was performed to determine which pairs of treatments were significantly different. After reviewing the graphic results for these data, only 1 peptide in this group of 32, peptide 29 (hAQP4574-587), was compared with all antigen recalls. SPSS statistical software, version 19 (SPSS Inc), was used in these statistical analyses; all statistical tests were 2-sided, and \( P < .05 \) indicated significance.

Recombinant monoclonal anti-hAQP4 antibody (NMO-rAb) and isotype control were generated from clonally expanded plasmablasts recovered from the cerebrospinal fluid of a seropositive patient with NMO as described previously. U87MG cells stably transfected with M23 hAQP4 were grown on coverslips and fixed with 4% paraformaldehyde for 15 minutes and then rinsed with 1× phosphate-buffered saline. Coverslips were subsequently blocked with 10% normal goat serum and then incubated with recombinant antibody (10 µg/mL) with or without T-cell peptide (5 µg/mL; 40-fold molar excess) in 5% normal goat serum overnight at 4°C. Coverslips were washed 5 times with 1× phosphate-buffered saline and then incubated with rabbit polyclonal anti-AQP4 (sc-20812; Santa Cruz Biotechnology) (4 µg/mL) in 2% goat serum and 0.1% Triton X100 for 1 hour at room temperature.

Images were obtained using a spinning disc confocal microscope (Olympus IX81; Olympus), and the amount of red and green fluorescence was quantified using Image J software (National Institutes of Health). The ratio of green to red fluorescence was measured in multiple independent fields, and the binding percentage was subsequently calculated by comparing the green to red fluorescence ratio in the presence and absence of AQP4 peptide.

**GENEATION OF NMO RECOMBINANT ANTIBODY AND QUANTITATIVE IMMUNOFLUORESCENCE MICROSCOPY**

†Recipient of National Institutes of Health grant AI11001.
RESULTS

AQP4<sub>281-300</sub> is the immunodominant linear determinant of hAQP4 in the context of HLA-DRB1<sup>*</sup>03:01. ELISPOT assays were used to characterize the T-cell repertoire of HLA-DRB1<sup>*</sup>03:01 mice immunized with whole-protein hAQP4. The IFN-γ and IL-17 ELISPOT assays identified hAQP4<sub>281-300</sub> (peptide 29) as the immunodominant linear determinant in lymph node cells and splenocytes (Figure 1 and Figure 2A and B). TH17 cellular immune responses by splenocytes against hAQP4<sub>281-300</sub> were not significantly different from those against full-length hAQP4 (Figure 1 and Figure 2B). There was a trend toward higher IFN-γ secretion in splenocytes after recall with AQP4<sub>285-298</sub> (data not shown). Because of the insufficient number of lymph node cells, GM-CSF ELISPOT assays could not be performed.

THE DOMINANT IMMUNOGENIC REGIONS WITHIN hAQP4<sub>281-300</sub>

Because of their biophysical properties, linear peptides that are bound in the antigen-binding groove of the MHC class II molecule to be presented to CD4<sup>+</sup> T cells are ideally 12 to 15 amino acids in length. Thus, the immunodominant determinants within hAQP4<sub>281-300</sub> were identified by performing IFN-γ, IL-17, and GM-CSF ELISPOT assays with 15mer peptides spanning hAQP4<sub>281-300</sub> (Table 2). In lymph node cells and splenocytes from HLA-DRB1<sup>*</sup>03:01 mice immunized with AQP4<sub>281-300</sub>-AQP4<sub>284-298</sub> induced a significantly higher TH17 response than other 15mers (Figure 1 and Figure 3A). AQP4<sub>284-298</sub> also induced the strongest GM-CSF–driven TH response in splenocytes significant from other 15mers (data not shown). Because of the insufficient number of lymph node cells, GM-CSF ELISPOT assays could not be performed. AQP4<sub>285-298</sub> resulted in a significantly higher TH1 response than other 15mers in lymph nodes cells (Figure 1 and Figure 3B). There was also a trend toward higher IFN-γ secretion in splenocytes after recall with AQP4<sub>285-298</sub>.

BINDING OF NMO-rAb TO SURFACE FULL-LENGTH hAQP4

We subsequently examined whether hAQP4<sub>281-300</sub> could inhibit the binding of NMO-rAb to surface AQP4 (Figure 4). Using a quantitative immunofluorescence-binding assay, we observed no significant inhibition of the binding of 2 NMO-rAbs in the presence of a 40-fold molar excess of hAQP4<sub>281-300</sub>.
The identification of immunodominant determinants of hAQP4 may have important implications for understanding the origin of NMO and monitoring disease activity in patients with this disorder. As previously stated, there is accumulating evidence to suggest a cellular immune response against hAQP4 in NMO. Other investigators recently identified dominant determinants of hAQP4 in different wild-type mouse strains, including C57BL/6 (H-2b) and SJL/J (H-2s). One group of investigators found a dominant determinant that overlaps with hAQP4281-300, namely, hAQP4289-306, in C57BL/6 mice. However, it is difficult to compare this observation with ours for 2 reasons: (1) hAQP4 was obtained from different sources and (2) the C57BL/6 genetic background does not express H-2-Lea, the equivalent gene of the human class II MHC molecule HLA-DRα.

This study specifically aimed to identify immunodominant linear determinants of hAQP4 in the context of HLA-DRB1*03:01 because this HLA haplotype was recently associated with NMO in several patient cohorts. ELISPOT assays allowed us to determine the frequency of antigen-specific T cells specific for hAQP4 peptide determinants and to characterize their cytokine profiles. This is relevant because encephalitogenicity of T cells in another autoimmune disorder of the CNS, multiple sclerosis, is largely defined by cytokine phenotype.

T cells, defined by the signature cytokine IFN-γ, were initially implicated in CNS autoimmunity. Perhaps the most convincing evidence to support a pathogenic role of IFN-γ in patients with multiple sclerosis was generated in a clinical study in which 7 of 18 patients who received recombinant IFN-γ therapy experienced a disease exacerbation. In the last decade, another subclass of pathogenic CD4+ T cells was characterized by the production of IL-17. These T cells appear to facilitate the initiation and perpetuation of CNS autoimmune diseases and mediate proinflammatory and allergic responses. IL-17 mediates the presence of neutrophils at sites of tissue damage. It is now recognized that TIL cells possess substantial plasticity compared with other T cells. In the setting of NMO, however, the increased levels of IL-6 found in the cerebrospinal fluid of patients with NMO may allow for the survival of hAQP4-specific TIL cells while inhibiting FOXP3+ T-regulatory cells. In addition, in a Chinese patient cohort, a polymorphism in the IL-17 gene was recently associated with anti-AQP4 antibody–positive NMO. Uzawa et al did not find elevated GM-CSF levels in the cerebrospinal fluid of patients with NMO and active clinical disease. However, the accumulation of eosinophils and granulocytes in the NMO lesion may suggest that this cytokine also plays a pathogenic role.

An animal model of NMO with the hAQP4 determinants identified in this study is currently under development in our laboratory. Perhaps more important, our observations may have immediate human applications. We are developing assays to determine a potential...
tially low frequency of hAQP4284-298- and hAQP4285-299- specific CD4\(^+\) T cells in patients with NMO and controls together with other investigators. The biological relevance of linear hAQP4 determinants identified in this study in NMO disease activity and in response of patients with NMO to pharmacotherapies will ultimately have to be evaluated in controlled clinical trials.

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