Human Aquaporin 4281-300 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 spot assay. Quantitative immunofluorescence microscopy was performed to determine whether hAQP4281-300 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 hAQP4281-300 generated a significantly (P<.01) greater T11 and T17 immune response than any of the other linear peptides screened. This 20mer peptide contains 2 dominant immunogenic 15mer peptides. hAQP4284-298 induced predominantly an IL-17 and granulocyte-macrophage colony-stimulating factor T11 cell phenotype, whereas hAQP4285-299 resulted in a higher frequency of T11 cells. hAQP4281-300 did not interfere with recombinant AQP4 autoantibody binding.

Conclusions: hAQP4281-300 is the dominant linear immunogenic determinant of hAQP4 in the context of HLA-DRB1*03:01. Within hAQP4281-300 are 2 dominant immunogenic determinants that induce differential T11 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.


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 M2 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).\textsuperscript{10,11} 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\textsuperscript{+} T cells,\textsuperscript{12} in some patient cohorts, NMO-IgG is undetectable in a substantial number of patients with NMO.\textsuperscript{3} In patients with NMO-IgG, antibody isotype switching from IgM to IgG could not occur without CD4\textsuperscript{+} T cell involvement.\textsuperscript{13,14} The response to B cell–depleting therapies is not consistently beneficial in patients with NMO,\textsuperscript{7-9} and CD3\textsuperscript{+} T cells are abundantly present in NMO lesions.\textsuperscript{15} We hypothesize that hAQP4-specific CD4\textsuperscript{+} 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\textsuperscript{+} T\textsubscript{H} cells were further defined by their expression of interferon \(\gamma\) (IFN-\(\gamma\)), 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\textsubscript{286-301} (Table 2) were generated by JPT Innovative Peptide Solutions.

#### MICE

Generation of transgenic mice expressing HLA-DRB1*03:01 was previously described.\textsuperscript{16} Briefly, DRBI*0301 (DR3) transgenic mice were generated by coinjection of an HLADRoL genomic fragment and a DRBI*030113 gene fragment into (C57BL/6 \(\times\) DBA/2) F1 C57BL/6 embryos and backcrossed to B10 mice.\textsuperscript{15} Subsequently, the DR3 gene was introduced into the class II\textsuperscript{+} negative H2b\textsuperscript{+} strain\textsuperscript{19} by mating the B10.M-DRBI*0301 line with the B10.MHCII\textsuperscript{+} 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

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Figure 1. The M1 isoform of human aquaporin 4 (hAQP4). hAQP4 consists of 6 transmembrane \(\alpha\)-helices. There are 2 isoforms of hAQP4, M1 and M23, which differ in their \(N\) terminus start site. Three immunogenic linear determinants in the context of HLA-DRB1*03:01 are described in this study.
ping 15mers within that peptide. Spots were counted with an automated ELISpot plate reader (Bioreader 5000; Biosys).

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

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. 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. 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.

**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. Next, cells (2.5-5.0 × 10^6 cells per well) were incubated with a single overlapping hAQP4 peptide (50 µg/mL), while length hAQP4 (50 µg/mL), media only, or concanavalin A (1 µg/mL) for 48 hours in 96-well ELISpot plates (MultiScreen 96-Well Plates; Millipore). Capture and detection of cytokines were accomplished using monoclonal antibodies (eBiosciences) specific for mouse IFN-γ (clone AN-18 [capture] and R4-6A2 [detection]), IL-17 (clone eBio17CK15A5 [capture] and eBio17B7 [detection]), GM-CSF (clone MP1-22E9 [capture] and MP1-2231G6 [detection]), or IL-5 (clone TRFK5 [capture] and JS1-5A10 [detection]). All experiments were repeated at least twice. To identify dominant 15mer determinants within immunodominant 20mer determinants, mice were immunized with the 20mer peptide, and immune recall was performed with overlapping 15mers within that peptide. Spots were counted with an automated ELISpot plate reader (Bioreader 5000; Biosys).

**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 (hAQP4_281-300), 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.
RESULTS

AQP4281-300 is the immunodominant linear determinant of hAQP4 in the context of HLA-DRB1*03:01. ELISpot assays were used to characterize the T-cell repertoire of HLA-DRB1*03:01 mice immunized with whole-protein hAQP4. The IFN-γ and IL-17 ELISpot assays identified hAQP4281-300 (peptide 29) as the immunodominant linear determinant in lymph node cells and splenocytes (Figure 1 and Figure 2A and B). T_{H17} cellular immune responses by splenocytes against hAQP4281-300 were not significantly different from those against full-length hAQP4 (Figure 1 and Figure 2B). None of the overlapping hAQP4 or full-length hAQP4 peptides induced an IL-5–driven T_{H2} response (data not shown).

THE DOMINANT IMMUNOGENIC REGIONS WITHIN hAQP4281-300

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+ T cells are ideally 12 to 15 amino acids in length. Thus, the immunodominant determinants within hAQP4281-300 were identified by performing IFN-γ, IL-17, and GM-CSF ELISpot assays with 15mer peptides spanning hAQP4281-300 (Table 2).

In lymph node cells and splenocytes from HLA-DRB1*03:01 mice immunized with AQP4281-300, AQP4281-300 induced a significantly higher T_{H17} response than other 15mers (Figure 1 and Figure 3A). AQP4284-298 also induced the strongest GM-CSF–driven T_{H1} 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. AQP4285-298 resulted in a significantly higher T_{H1} 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 AQP4285-298.

BINDING OF NMO-rAb TO SURFACE FULL-LENGTH hAQP4

We subsequently examined whether hAQP4281-300 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 hAQP4281-300.

Figure 2. Aquaporin 4281-300 is the immunodominant linear determinant of human aquaporin 4 (hAQP4). HLA-DRB1*03:01 transgenic mice were immunized with a full-length hAQP4 complete Freund adjuvant emulsion, resulting in each mouse receiving 100 µg of antigen. On day 10, cells taken from the lymph nodes (A) and spleens (B) were collected from mice to generate single-cell suspensions. Thereafter, cells (0.5 × 10^6 cells) were incubated for 48 hours in 96-well enzyme-linked immunosorbent spot assay plates coated with anti–interferon-γ (IFN-γ) or anti–interleukin-17 (IL-17) with single overlapping hAQP4 peptides (50 µg/mL) (Table 1), media only, full-length hAQP4 (50 µg/mL), or concanavalin A (ConA) (1 µg/mL). Spot-forming units represent the absolute number of cells that are secreting a specific cytokine in response to antigen in the well (*P < .001). Error bars indicate SE.
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-Ii, 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 encephalogenicity of T cells in another autoimmune disorder of the CNS, multiple sclerosis, is largely defined by cytokine phenotype.

T11 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+ T11 cells was characterized by the production of IL-17. T1117 cells appear to facilitate the initiation and perpetuation of CNS autoimmune diseases and mediate proinflammatory and allergic responses. IL-17 mediates the localization of neutrophils to the sites of infection. It is now recognized that T1117 cells possess substantial plasticity compared with other T11 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 T1117 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 poten-
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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