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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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Abstract

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 antih-AQP4 recombinant antibody to surface full-length hAQP4.

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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_H 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$_{281–330}$ is the dominant linear immunogenic determinant of hAQP4 in the context of *HLA-DRB1*03:01*. Within hAQP4$_{281–330}$ are 2 dominant immunogenic determinants that induce differential T_H 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.

Neuromyelitis 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.[7–9]

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),[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$^+$ T cells.[12] In some patient cohorts, NMO-IgG is undetectable in a substantial number of patients with NMO.[2] In patients with NMO-IgG, antibody isotype switching from IgM to IgG could not occur without CD4$^+$ T_H cell involvement.[13,14] The response to B cell–depleting therapies is not consistently beneficial in patients with NMO,[7–9] and CD3$^+$ T cells are abundantly present in NMO lesions.[15] We hypothesize that hAQP4-specific CD4$^+$ 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_H cells were further defined by their expression of interferon γ (IFN-γ), interleukin (IL) 17, and granulocyte-macrophage colony-stimulating factor (GM-CSF).

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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 $AQP4_{298–301}$ (Table 2) were generated by JPT Innovative Peptide Solutions.

MICE

Generation of transgenic mice expressing *HLA-DRB1* 03:01* was previously described.[16] Briefly, DRBI*0301 (*DR3*) transgenic mice were generated by coinjection of an HLADRoL genomic fragment and a DRB1*030113 gene fragment into (C57BL/6 × DBA/2) F1 C57BL/6 embryos and backcrossed to B10 mice.[15] Subsequently, the *DR3* gene was introduced into the class II–negative $H2b^{-/-}$ strain[16] 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. Next, cells ($2.5–5.0 \times 10^5$ cells per well) were incubated with a single overlapping hAQP4 peptide (50 μg/mL), whole-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 by 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 JES1-5A10 [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).

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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.[17] 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[18] (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. Coverslips were subsequently washed and then incubated with goat antihuman AlexaFluor 488 (A-11013; Invitrogen) and goat antirabbit AlexaFluor 594 (DI-1594; Vector) in 2% goat serum for 1.5 hours at room temperature in the dark. Samples were then washed, fixed, and mounted with mounting media containing DAPI (H-1500; Vector).

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.

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

$AQP4_{281–300}$ 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 $hAQP4_{281–300}$ (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 $hAQP4_{281–300}$ 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).

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THE DOMINANT IMMUNOGENIC REGIONS WITHIN hAQP4$_{281–300}$

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.[12] Thus, the immunodominant determinants within hAQP4$_{281–300}$ were identified by performing IFN-γ, IL-17, and GM-CSF ELISpot assays with 15mer peptides spanning hAQP4$_{281–300}$ (Table 2).

In lymph node cells and splenocytes from *HLA-DRB1* 03:01* mice immunized with AQP4$_{281–300}$, AQP4$_{284–298}$ induced a significantly higher T_H17 response than other 15mers (Figure 1 and Figure 3A). AQP4$_{284–298}$ also induced the strongest GM-CSF–driven T_H 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$_{285–298}$ 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 AQP4$_{285–298}$.

BINDING OF NMO-rAb TO SURFACE FULL-LENGTH hAQP4

We subsequently examined whether hAQP4$_{281–300}$ could inhibit the binding of NMO-rAb to surface AQP4 (Figure 4).[17] 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$_{281–300}$.

COMMENT

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).[19,20] One group of investigators found a dominant determinant that overlaps with hAQP4$_{281–300}$, namely, hAQP4$_{289–306}$, in C57BL/6 mice.[20] 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-IE*α, 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.[10,11] 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,[21] is largely defined by cytokine phenotype.

T_H1 cells, defined by the signature cytokine IFN-γ, were initially implicated in CNS autoimmunity.[22] 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

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patients who received recombinant IFN-γ therapy experienced a disease exacerbation.[23,24] In the last decade, another subclass of pathogenic $CD4^+$ T_H cells was characterized by the production of IL-17. T_H17 cells appear to facilitate the initiation and perpetuation of CNS autoimmune diseases[25] and mediate proinflammatory and allergic responses. IL-17 mediates the localization of neutrophils to the sites of infection.[26] T_H17 cells have also been shown to play a critical role in the production of GM-CSF in the periphery and CNS.[27,28] Our group and other investigators recently found that GM-CSF may play a critical role in different models of active and passive experimental autoimmune encephalomyelitis.[28–31] Our own results indicated that GM-CSF is secreted by lymph node cells and splenocytes after antigen restimulation in the presence of IL-12. In an adoptive transfer model of experimental autoimmune encephalomyelitis, we also found that GM-CSF is highly expressed by encephalitogenic T cells.

The identification of $AQP4_{284–298}$ infers that the cellular immune response may play a critical role in NMO disease development and progression because of its ability to stimulate a pronounced T_H17 immune response in the context of *HLA-DRB1*03:01*. The level of IL-17 is increased in patients with NMO during disease relapses.[32,33] This finding would explain the presence of neutrophils at sites of tissue damage.[15] It is now recognized that T_H17 cells possess substantial plasticity compared with other T_H cells.[34] 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 T_H17 cells while inhibiting $FOXp3^+$ T-regulatory cells.[35–37] In addition, in a Chinese patient cohort, a polymorphism in the IL-17 gene was recently associated with anti-AQP4 antibody–positive NMO.[33] Uzawa et al[35] 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.[15]

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 potentially low frequency of $hAQP4_{284–298}$– and $hAQP4_{285–299}$–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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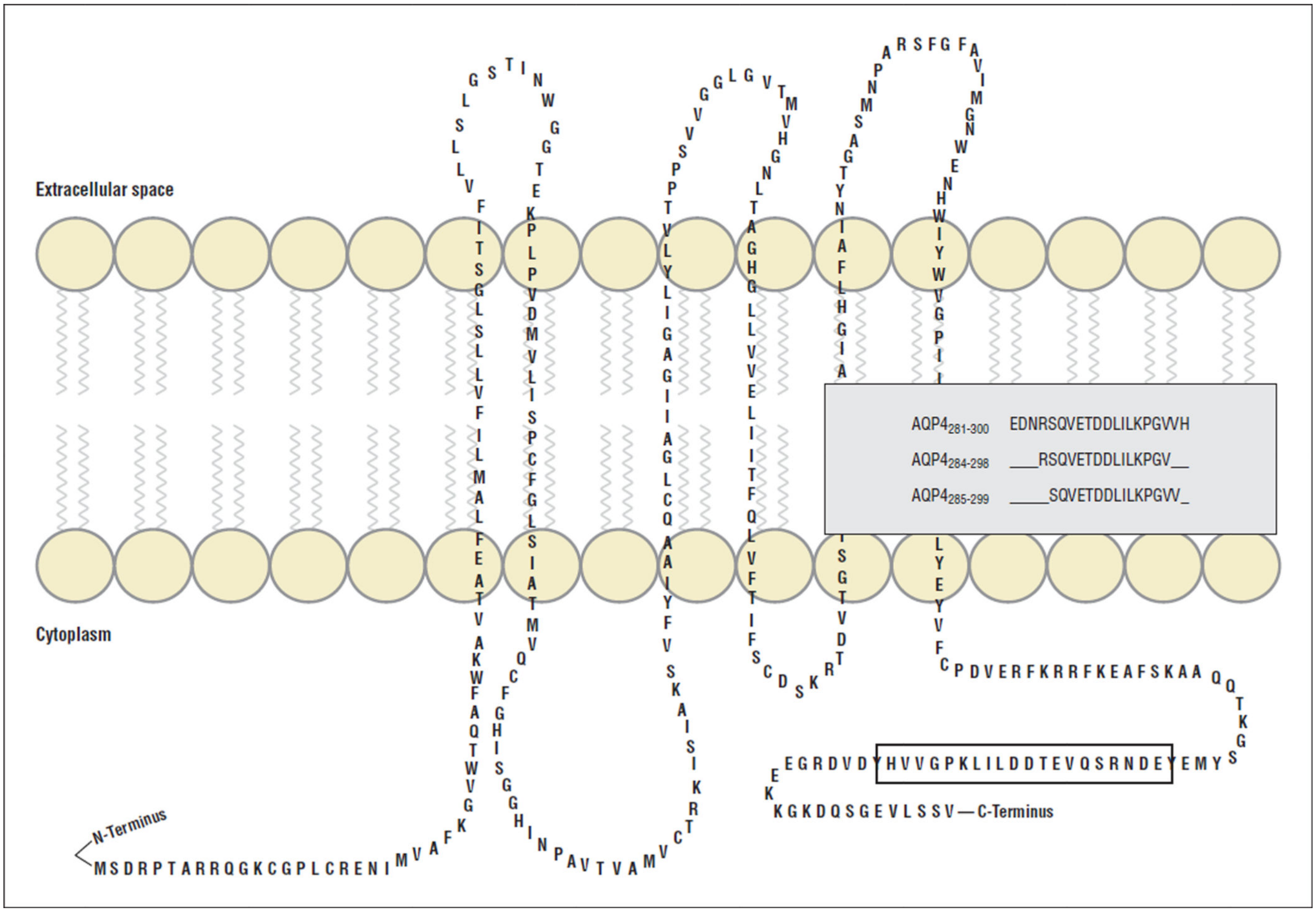

Figure 1.

The M1 isoform of human aquaporin 4 (hAQP4). hAQP4 consists of 6 transmembrane α-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.

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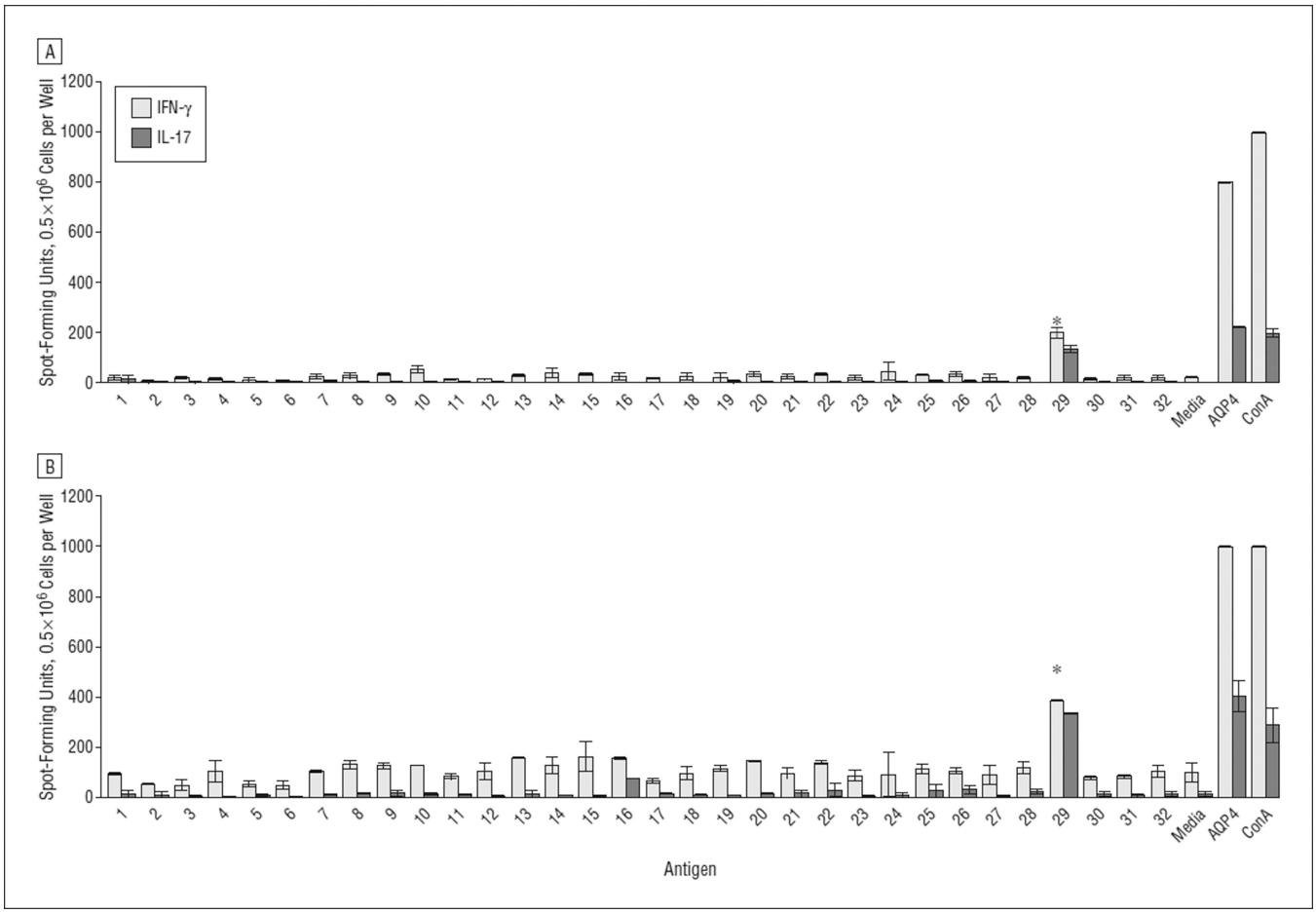

Figure 2.

Aquaporin $4_{281–300}$ 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.

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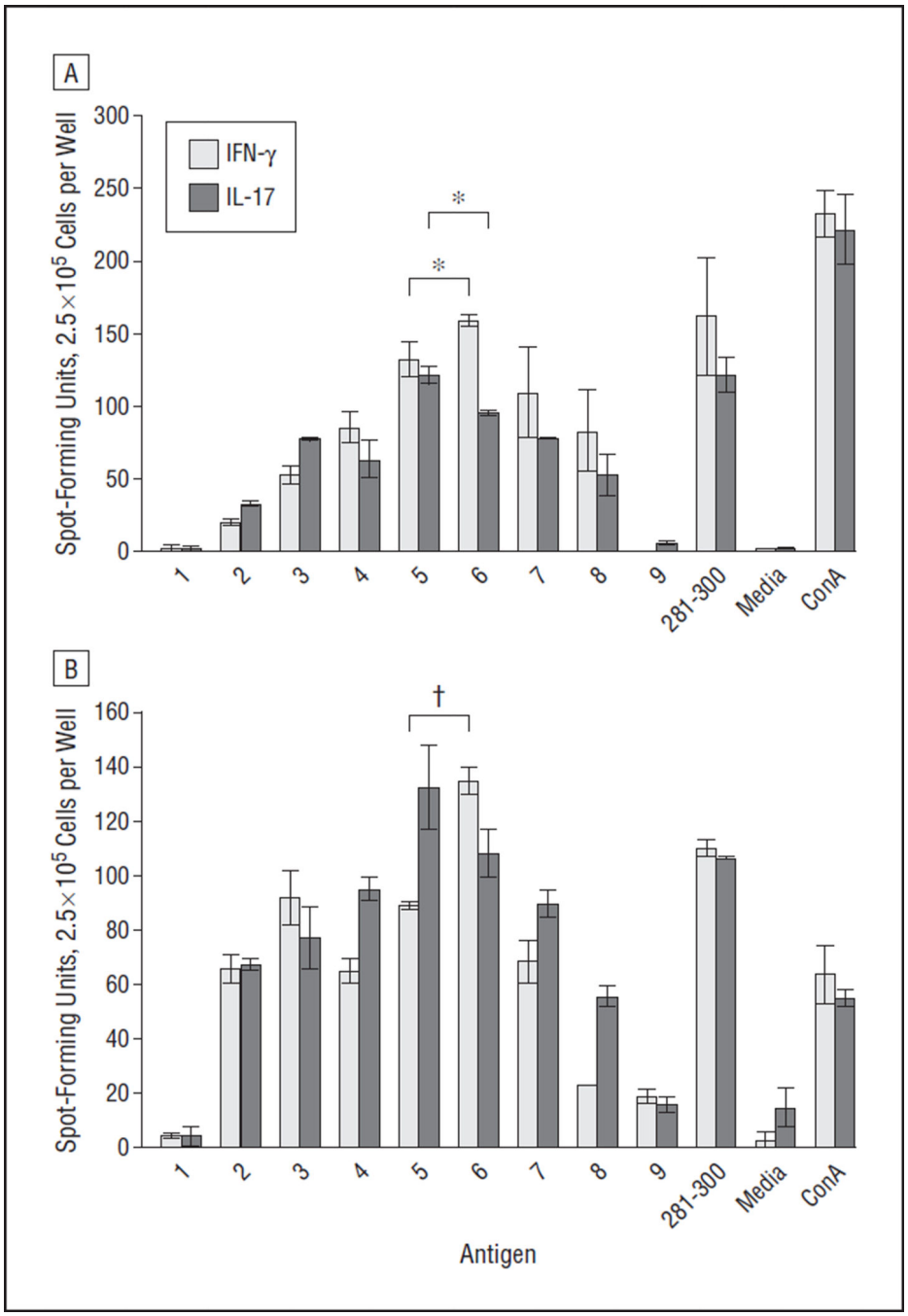

Figure 3.

Aquaporin $4_{284–298}$ ($AQP4_{284–298}$) and $AQP4_{285–299}$ are the immunogenic regions of $AQP4_{281–300}$. *HLA-DRB1*03:01* transgenic mice were immunized with $AQP4_{281–300}$. On day 10, cells (2.5×10^5 cells) taken from the lymph nodes (A) and spleens (B) were incubated for 48 hours in enzyme-linked immunosorbent spot assay plates coated with anti–interferon-γ (IFN-γ) or anti–interleukin-17 (IL-17) with a single overlapping peptide of AQP4 (50 μg/mL) (Table 2), human $AQP4_{281–300}$ 50 μg/mL), media only, 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 (*$P < .05$ and †$P < .01$). Error bars indicate SE.

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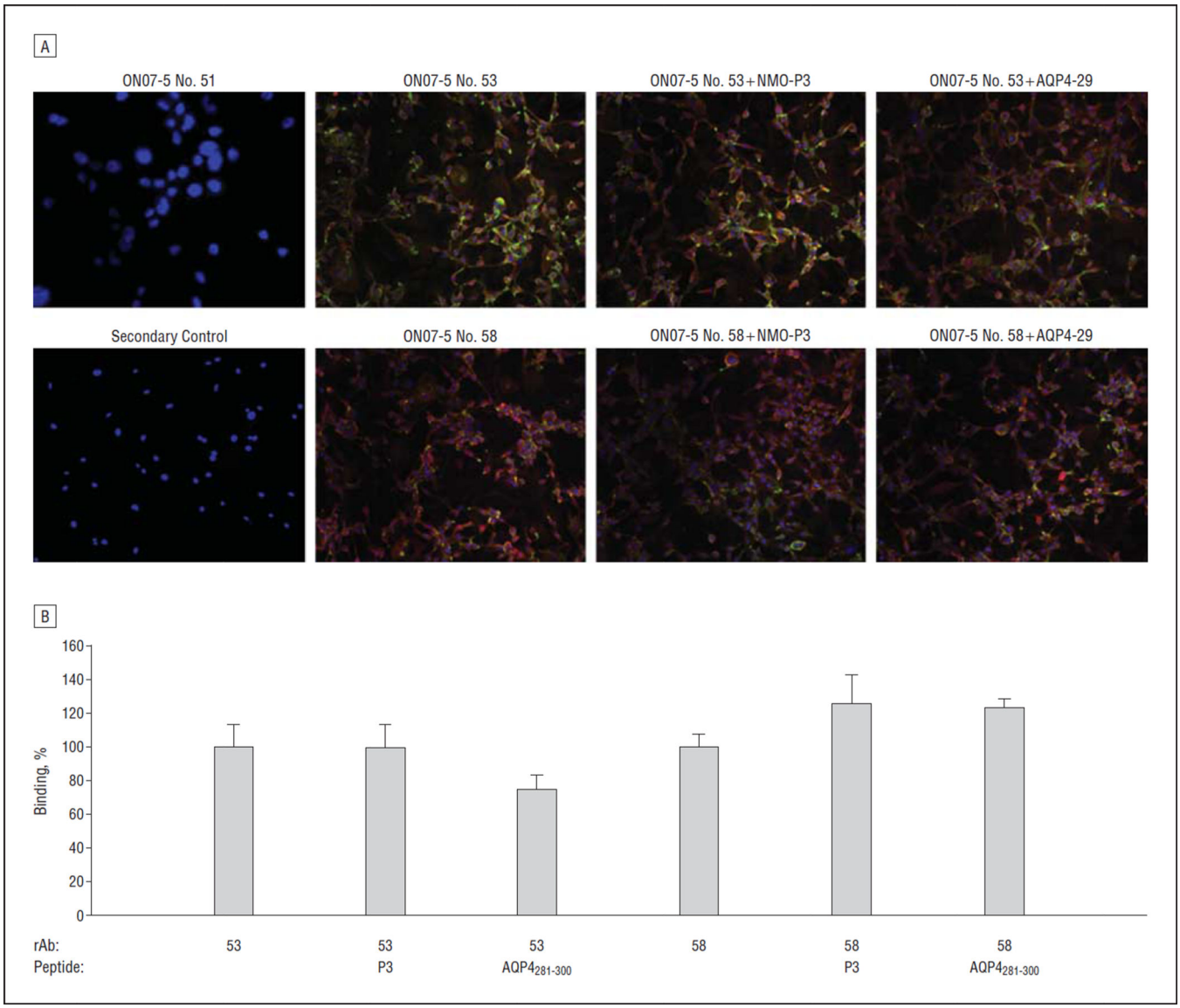

Figure 4.

Aquaporin $4_{281–300}$ ($AQP_{281–300}$) does not inhibit AQP4 antibody binding. AQP4-specific and isotype control recombinant antibodies (rAbs) were incubated with a U87MG glial cell line stably transfected with the M23 isoform of human AQP4 in the presence or absence of AQP4 peptide. A, Fluorescence micrographs demonstrate robust binding of AQP4-specific rAbs 53 and 58 (green) to the transfected cell line. No staining was observed in the absence of rAb (secondary control) or when using an isotype control rAb (ON7-5 No. 51). A rabbit polyclonal anti-AQP4 antibody against the intracellular C-terminal portion of AQP4 was used as an internal control for AQP4 expression. B, Percentage binding of AQP4-specific rAbs 53 and 58 in the presence and absence of AQP4 peptides. NMO indicates neuromyelitis optica. Error bars indicate SE.

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Table 1

Synthetic Peptides 20 Amino Acids Long That Overlap by 10 Amino Acids Across the Entirety of Human Aquaporin 4 (AQP4)

Peptide No.	Sequence	AQP4
1	MSDRPTARRWGKCGPLCTRE	1–20
2	GKCGPLCTRENIMVAFKGVW	11–30
3	NIMVAFKGVWTQAFWKAVTA	21–40
4	TQAFWKAVTAEFLAMLIFVL	31–50
5	EFLAMLIFVLLSLGSTINWG	41–60
6	LSLGSTINWGGTEKPLPVDM	51–70
7	GTEKPLPVDMVLISLCFGLS	61–80
8	VLISLCFGLSIATMVQCFGH	71–90
9	IATMVQCFGHISGGHINPAV	81–100
10	ISGGHINPAVTVAMVCTRKI	91–110
11	TVAMVCTRKISIAKSVFYIA	101–120
12	SIAKSVFYIAAQCLGAIIGA	111–130
13	AQCLGAIIGAGILYLVTPPS	121–140
14	GILYLVTPPSVVGGLGVTMV	131–150
15	VVGGLGVTMVHGNLTAGHGL	141–160
16	HGNLTAGHGLLVELIITFQL	151–170
17	LVELIITFQLVFTIFASCDS	161–180
18	VFTIFASCDSKRTDVTGSIA	171–190
19	KRTDVTGSIALAIGFSVAIG	181–200
20	LAIGFSVAIGHLFAINYTGA	191–210
21	HLFAINYTGASMNPARSFGP	201–220
22	SMNPARSFGPAVIMGNWENH	211–230
23	AVIMGNWENHWIYWVGPIIG	221–240
24	WIYWVGPIIGAVLAGGLYEY	231–250
25	AVLAGGLYEYVFCPDVEFKR	241–260
26	VFCPDVEFKRRFKEAFSKAA	251–270
27	RFKEAFSKAAQQTKGSYMEV	261–280
28	QQTKGSYMEVEDNRSQVETD	271–290
29	EDNRSQVETDDLILKPGVVH	281–300
30	DLILKPGVVHVIDVDRGEEK	291–310
31	VIDVDRGEEKKGKDQSGEVL	301–320
32	VDRGEEKKGKDQSGEVLSSV	304–323

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Table 2

Aquaporin $4_{281–300}$ Overlapping Peptides

Peptide No.	Amino Acids	Sequence
1	280–294	VEDNRSQVETDDLIL
2	281–295	EDNRSQVETDDLILK
3	282–296	DNRSQVETDDLILKP
4	283–297	NRSQVETDDLILKPG
5	284–298	RSQVETDDLILKPGV
6	285–299	SQVETDDLILKPGVV
7	286–300	QVETDDLILKPGVVH
8	287–301	VETDDLILKPGVVHV