Increased T cell immunity against aquaporin-4 and proteolipid protein in neuromyelitis optica

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Abstract

In neuromyelitis optica (NMO), B cell autoimmunity to aquaporin-4 (AQP4) has been shown to be essential. However, the role of T cells remains ambiguous. Here, we first showed an increase in CD69+ activated T cells in peripheral blood mononuclear cells (PBMC) during NMO relapses. Next, T cell responses to AQP4 and myelin peptides were studied in 12 NM0 patients, 10 multiple sclerosis (MS) patients and 10 healthy subjects (HS). Four hours after adding one of 28 overlapping AQP4 peptides, a mixture of AQP4 peptides (AQP4-M) or one of six distinct myelin peptides to two day-cultured PBMC, CD69 expression on CD4+ T cells was examined. Data were analyzed by paired t-test, frequency of samples with three-fold increase of CD69 on CD4+ cells (fSI3), and mean stimulation index (mSI). The T cell response to AQP4-M was significantly increased in NMO (fSI3=10/12, mSI=5.50), with AQP4(11-30) and AQP4(91-110) representing the two major epitopes (AQP4(11-30), fSI3=11/12, mSI=16.0; AQP4(91-110), fSI3=11/12, mSI=13). Significant but less extensive responses to these two epitopes were also observed in MS and HS. Significant reactivities against AQP4(21-40), AQP4(61-80), AQP4(101-120), AQP4(171-190) and AQP4(211-230) were exclusively found in NMO. In addition, responses to AQP4(81-100) were higher and more frequently detected in NMO, without reaching statistical significance. Interestingly, among the six myelin peptides studied, PLP(95-116) induced a significant T cell response in NMO (fSI3=7/12, mSI=4.60). Our study suggests that cellular as well as humoral responses to AQP4 are necessary for NMO development, and that the immune response to myelin protein may contribute to disease pathogenesis.

Introduction

Neuromyelitis optica (NMO) was originally characterized as a variant of multiple sclerosis (MS) that mainly attacks the optic nerves and spinal cord. The disease concept of NMO is now being refined by the discovery of serum autoantibodies to aquaporin-4 (NMO-IgG or anti-AQP4 IgG) that are absent in MS (1-2). Accumulating evidence has indicated that NMO-IgG is not merely a biomarker for NMO but acts as a key player in the pathogenesis of NMO (3-4). Distribution of NMO lesions, where immunoglobulin is deposited, can be partly explained by localization of AQP4 (5). Loss of AQP4 and of glial fibrillary acidic protein (GFAP) was observed in early NMO lesions (6-7). Effects of therapeutic plasmapheresis (8), which is clinically applied to remove potentially detrimental humoral components, also support the assumption that antibodies contribute to the disease process.

Investigations into the function and character of NMO-IgG have further clarified that NMO has a distinct pathogenesis from MS. As a result, a new concept proposes the existence of a clinical spectrum that is defined by the presence of NMO-IgG and shares NMO pathogenesis, even though the diagnostic criteria for NMO may not be fulfilled (8). This new strategy has led to the opticospinal type of multiple sclerosis (OSMS), prevalent in East Asia, to be identified as NMO disorder spectrum (1,8).

The underlying molecular mechanisms of NMO remain to be fully elucidated. While the role of NMO-IgG has been well recognized, that of T cells in NMO has not yet been established. However, T cell help is necessary for B cells to mount an antibody responses against AQP4, a protein antigen. T cells and antibodies are reported to work together, and in conjunction can induce or enhance the disease process in some myelin oligodendrocyte glycoprotein (MOG)-induced experimental autoimmune encephalomyelitis (EAE) models (9). Eventually, T cell-induced EAE in addition to immunoglobulin at the induction phase generated lesions similar to NMO in structure and distribution (4).

In this study we provide evidence that T cells are activated during NMO relapses and that T cell reactivity to AQP4 and to PLP in NMO is increased. Hence, we propose that T cell autoimmunity and autoantibodies are important for the development of NMO.

Materials and methods

Subjects

Twelve NMO patients seropositive for anti-AQP4 antibody, 12 relapsing-remitting MS patients and 10 healthy subjects (HS) were enrolled for this study. Subjects with NMO fulfilled the proposed NMO criteria (10). Subjects with MS fitted the McDonald MS criteria (11), and NMO-IgG was absent from their serum. Three subjects with NMO (NM01, 2 and 3) were monitored for T cell activation *in vivo* between relapses and remissions. Findings from the first analysis of the antigenic T cell response during NMO relapse led us to initiate analysis of the T cell antigenic response in other samples. However, the analysis for all remaining enrolled individuals was carried out during the remission stage only.

The present study was approved by the Medical Ethics Committee of Nagasaki Kawatana Medical Center, and all of the subjects provided written informed consent.

HLA typing

Genomic DNA for HLA-genotyping was prepared using the QIAamp DNA mini kit (Qiagen, Germany). HLA-DRB1 alleles were determined by the Dynal RELI SSO HLA-DRB1 typing kit (Invitrogen, USA). HLA-DPB1 typing was performed with the SSP All Set DPB1 kit (Invitrogen, USA).

Stimulating antigens (Table 1)

Peptides were commercially synthesized by Invitrogen (purity>90%) (Japan). Sequences of 32 AQP4 overlapping peptides were based on deduced AQP4 sequences (12). Among the original set of 32, AQP4(41-60), AQP4(121-140), AQP4(161-180), and AQP4(191-210) were found to be insoluble and were excluded from further study. The remaining 28 AQP4 peptides were used as single peptides or as a mixture of all the peptides (AQP4-M). Two myelin MOG peptides, three proteolipid protein (PLP) peptides and one myelin basic protein (MBP) peptide, namely MOG(13-28), MOG(145-160), PLP(95-116), PLP(139-154), PLP(185-209) and MBP(83-99), were also used in this study. These peptides were selected on the basis of reported T cell epitopes in MS

(13-15). The single peptides were used as stimulatory antigens at a final concentration of 10 µg/ml. AQP4-M was adjusted to a final concentration of 2 µg/ml.

Stimulation of peripheral blood mononuclear cells (PBMC)

Freshly isolated PBMC were seeded at 5×10^5/well and cultured in the wells of 96-well flat-bottom culture plates in AIM-V media (Invitrogen, USA). Stimulating antigen was added to the wells either immediately or after two days of culture to stimulate the resting state; thereafter, samples were incubated with antigen for four hours. A three-fold increase in CD69+CD4+ cells was determined as positive.

Flow cytometry

Samples were stained with fluorescently labeled antibodies for CD3-fluorescein isothiocyanate (FITC), CD69-phycoerythrin (PE), and CD4-phycoerythrin-cyanin (PE-Cy5) (all from BD Bioscience, USA). Then the cells were analyzed by FACS Calibur (BD Bioscience).

Statistics

Statistical analysis was performed using paired *t*-test with GraphPad PRISM.

Results

T cell activation in relapses

We longitudinally analyzed CD4+ T cells isolated from NMO1 in states of remission and relapse. As shown in Figure1a, CD69, an early activation marker, tended to be upregulated on both the CD4+ T cells and the CD4- T cells in relapses. At the relapse with the largest lesions among those considered in the current analysis, MRI demonstrated that the area of total lesions extended from supratentorial to medulla. Samples taken during that severe relapse exhibited the most robust CD69 expression with levels reaching 31.7% on total T cells, 42.6% on CD4+ T cells, and 18.0% on CD4- T cells.

This expression pattern was also observed in two other NMO patients (Fig.1b-c). CD4+ CD69+ and CD4- CD69+ T cells were found to be upregulated from 0.5% to 11.9%, and from 1.1% to 2.8%, respectively, at a relapse of NMO2 in comparison to NMO2 remission. CD4+ CD69+ and CD4- CD69+ T cells were upregulated from 0.2% to 21.9% and from 0.1% to 4.4%, respectively, at a relapse of NMO3. The ratio of CD4+ CD69+ T cells to CD4- CD69+ T cells indicated that dominance of CD4+ T cell activation over CD4- T cell activation was usually, but not always, observed in the relapse phases of the three NMO patients examined (Fig. 1d).

Antigenic T cell response in the most severe relapse of NMO1

During the most severe relapse of subject NMO1, 42.6% of CD4+ T cells expressed CD69 during the most severe relapse of NMO1. Immediate stimulation by AQP4-M or any of the myelin peptides caused CD69 expression on CD4+ T cells to decrease to about one third (Fig. 2a).

When CD4+ T cells were cultured for two days in medium alone, CD69 expression was down-regulated (Fig. 2b). Subsequent stimulation with AQP4-M or PLP(95-116) induced 13.9% of and 7.8% of CD4+ T cells, respectively. Stimulation with either PLP(139-154), MBP(83-99) or MOG(145-160) were able to up-regulate CD69, but to a much lesser extent.

Two days culture for resting cells

Based on the fact that two days of unstimulated culture caused down-regulation

of CD69 on CD4+ T cells, we introduced a two-day incubation period for resting cells when we analyzed the T cell antigenic response derived from NMO and MS in remission. As a result, two days of culture in most cases led to sufficient downregulation of CD69 to provide optimal conditions for our assay (Fig. 3). However, frequencies of CD4+CD69+ T cells remained over 0.8% in a few cases. In such cases, addition of antigenic peptide decreased the CD69+ population (data not shown). Therefore, we only included cases with less than 0.8% of CD4+ T cells expressing CD69 after the resting step in our study.

Antigenic responses to AQP4 in NMO remission and specificity of AQP4

Data from one representative NMO sample is shown in Figure 4. In this patient CD69 expression in CD4+ T cells was sufficiently downregulated to 0.04% after two days of culture. While the sample exceeded SI > 3 in response to AQP-M, AQP4(11-30), AQP4(91-110), it did not significantly react to the other AQP4 peptides. Among myelin peptides, the highest reactivity was seen in response to PLP(95-16), with a SI slightly below 3 .

When AQP4–M was applied as antigen, CD69 expression was increased in all of the NMO samples (Figure 3). Paired *t*-test confirmed the statistical significance of the T cell response to AQP4-M in NMO (p=0.0009). In tissue culture-based techniques such as the conventional proliferation assay, stimulation index (SI) is usually calculated to determine if the antigenic response is positive. Accordingly, positivity of each response in our study was determined by a more than three-fold increase in CD69+ T cells with antigenic stimulation in comparison to those cultured in the absence of antigen. The frequency of SI>3 denoted as fSI3 was 10/12 in NMO (Table 2, Fig. 3). With these standards, the MS and HS samples exhibited a weaker response to AQP4-M that did not reach statistical significance. Four of the 12 MS samples and none of the HS-derived T cells showed a positive response to AQP4-M. The mean SI (mSI) was 5.50 in NMO, 2.60 in MS and 1.50 in HS.

Although the remaining two NMO samples did not exceed SI>3, they did appear to respond to AQP4-M. In one sample, CD4+CD69+ T cells were increased from 0.06% to 0.17%. In the other sample, the frequency of CD4+CD69+ T cells increased from 0.63% to 1.10% in response to AQP4-M. The observed 0.37%

increase in frequency is not less than that for the 10 other samples, which also showed more than three-fold SI and thus satisfied our criteria.

We developed a set of overlapping peptides of AQP4 to determine the T cell epitopes of AQP4（Table 1 ）. When we evaluated upregulation of CD69 in response to stimulation by each AQP4 peptide using paired t-test, the responses to AQP4(11-30), AQP4(21-40), AQP4(61-80), AQP4(91-110), AQP4(101-120), AQP4(171-190) and AQP4(211-230) were found to have reached significance in NMO ($P<0.05$). In particular, those to AQP4(11-30), AQP4(91-110) and AQP4(101-120) were very confirmative ($p<0.01$). Intriguingly, the responses to AQP4(11-30) and AQP4(91-110) also reached significance in samples from MS patients and HS, although the responses were weaker when compared to those in NMO patients (Table 2 and Fig. 3) as also indicated by mSI and fSI3. Eleven of the 12 NMO samples responded to AQP4(11-30) and AQP4(91-110) (SI>3). In the one sample with a response below SI 3, stimulation by AQP4(11-30) increased CD69+CD4+ T cells from 0.63% to 1.08%. The increase in frequency was not regarded as small when compared to the other positive cases. Seven of the 12 NMO samples also reacted positively to AQP4(171-190). Six of the 12 subjects reacted to AQP4(21-40), 8 to AQP4(101-120), and three to AQP4(181-200). With regard to other peptides to which more than one NMO samples responded positively, AQP4(31-50) induced a positive response in two of the 12 samples, and AQP4(211-230) in three of the 11 NMO samples. Five of the 12 NMO samples reacted positively to AQP4(81-100) and had a high mSI although paired t-test failed to show significance.

Positive response to the two major epitopes, AQP4(11-30) and AQP4(91-110), also prevailed among MS patients and HS, as confirmed by paired t-test. AQP4(91-110) induced activation of T cells derived from eight of the 12 MS patients and from five of the nine HS, meanwhile AQP4(11-30) upregulated CD69 expression in CD4+ T cells derived from three of the 11 MS patients and two of the nine HS.

T cell response against PLP(95-116) in NMO remission

Paired t-test revealed a significant T cell response to PLP(95-116) in NMO, but not to any of the other myelin peptides. The fSI3 was 7/12 NMO samples.

Although the paired *t*-test confirmed significance of the T cell response to PLP(95-116) in HS samples, only one exceeded SI>3. The statistical significance of the T cell response to PLP(95-116) was not confirmed in MS samples.

HLA association with T cell responses

We attempted to discover an HLA association with the observed antigenic response (Table 3). There was little difference found between the T cell epitopes of HLA-DR15 carriers and non-carriers and HLA-DP5 carriers and non-carriers (Table 3b). Although the number of analyzed samples was too small to obtain a solid conclusion, no HLA association with T cell epitopes of AQP4 was detected here.

Discussion

Although the role of T cells in NMO has begun to attract the interest of researchers, the body of knowledge on this matter remains substantially smaller than that for humoral factors. Moreover, specific human T cell epitopes associated with NMO pathogenesis have yet to be reported.

In this study we demonstrated that T cell activation is related to disease activity, as evidenced by up-regulated CD69 expression in freshly isolated peripheral blood T cells of NMO patients. One can argue that other factors contribute to T cell activation. Especially infection can be a candidate for inducing T cell activetion because infection can precede NMO relapses. However, apparent infection was not associated with the relapses analyzed here. Although only NMO1 had chronic urinary infection, exacerbations of infection were not observed. We also found that in relapse states of NMO activation of CD4+ T cells tends to dominate over that of CD4- T cells, a population composed mainly of CD8+ T cells. Although the study sample size was underpowered, it is likely that clinical monitoring T cells present in peripheral blood of NMO patients will help to discriminate relapse from remission phases.

In peripheral blood from one subject examined in this study during relapse, 42.6% of CD4+ T cells were activated. This frequency was extraordinarily high in comparison to other relapses experienced by the same patient and those experienced by other patients. The area of total lesions observed during this relapse represented the largest of all examined. This observation indicates that the degree of T cell activation may be one of the determining factors for lesion size, although this theory remains uninvestigated and it is likely that many other factors contribute to the lesion feature. It is also important to note that the sampling in this study was carried out on the first day of clinically observable manifestation of the relapse.   Hence, T cell activation may have preceded the relapse or occurred in the early phase of relapse. Considering these findings along with the results from T cell monitoring for the three subjects with NMO, we theorize that T cell activation, especially that of CD4+ T cells, was necessary to induce attacks of NMO. Hence, further study was focused on antigen reactivity of CD4+ T cells. However, it is likely that CD8+ T cells are also correlated to relapses of NMO, since CD4-CD69+ T cells were also increased during the

relapses analyzed in this study. The roles of CD8+ T cells and HLA class I association in NMO, which have not yet been established, need to be investigated further .

In the analysis of the antigen reactive T cell response, we cultured PBMC in medium alone for two days in order to induce a resting state. When we started this study, we isolated PBMC and immediately added antigen, carrying out the analysis of the frequency of CD69+ T cells four hours later. However, immediate stimulation by antigen led to an unexpected decrease in the CD69+ T cell population (Fig. 2a). A previous study of MS demonstrated that activated autoreactive T cells underwent Fas-mediated cell death upon antigen stimulation (16). Likewise, the CD69+ T cell population was probably decreased due to activation induced cell death (AICD), although there remains a theoretical possibility that CD69 expression was downregulated in single cells. Regardless, a considerable portion of the in-vivo activated T cells were missed in our analysis when the antigen was added immediately to freshly isolated PBMC. On the other hand, ex-activated T cells, once rested, are capable of re-activation by relevant antigen, and can be used in antigen-reactive T cell analysis. We found that in most cases, two days of culture was enough to rest the cells. However, there were a few cases in which more than 0.8% of the T cells maintained expression of CD69 after two days of culture. In such cases, AICD or the down-regulating effect of CD69 on cells was not negligible noise for the analysis. The frequencies of autoreactive T cells in neuroimmunological disorders such as MS have been previously assessed by limiting dilution technique, enzyme-linked immunospot assays (ELISPOT) for detection of IFN-γ-secreting cells and flow cytometry-based technique using peptide-MHC tetramers as reviewed by (13). The results differ greatly depending on the methodology used. While old tissue culture-based technique showed about 1 autoantigen-specific T cell per 10^6-10^7, earlier reports showed frequencies higher than than 1 per 10^4 cells. It was described that MHC tetramer-based assays do not work for autoreactive HLA class II-restricted T cells, probably due to low-affinity TCR recognition of autoantigens that fall below the threshold of assay sensitivity (13). Our method has been shown to be as easy and sensitive as any other previous method to detect autoreactive response. Although reactive CD69 expression was not

guaranteed to proceed the steps for cell proliferation or cytokine production and it remains unkown how two days of culture induced population change, the data obtained in this study are reasonable and persuading as discussed below.

The potential population change in the unstimulated two-day culture tempers our presumption that 13.9% of CD4+ T cells can be reactivated by AQP4 *in vivo* during the relapse stage of NMO1 with largest lesions observed in this study. However, we do believe that the finding that a considerable portion of the activated T cells in the relapse state had the ability to respond to AQP4 likely reflects a certain biological relevance. Interestingly, T cell responses against some of the myelin peptides, MOG(145-160), PLP(95-116), PLP(139-154), and MBP(83-99) were also shown to be involved in the relapse of NMO1. In particular, PLP(95-116) induced CD69 expression in 7.8% of CD4+ T cells.

To study how these antigen-specific responses prevail in NMO, further study was carried out. Although analysis of relapses would be the best way to answer this question, sampling difficulty limited our study to only the remission state. Increased T cell immunity to AQP4-M was clearly demonstrated in NMO samples. Four of the 12 MS and none of HS samples were positive, as determined by SI > 3. Paired *t*-test did not confer significance to the response to AQP4-M in either MS or HS.

Although the increased T cell response to AQP4 in NMO is clear, AQP4-reactive T cells were present in the natural T cell repertoire. AQP4(11-30) and AQP4(91-110), the two major epitopes, induced positive responses even in HS samples, as confirmed by paired *t*-test. fSI3 also showed that the T cell response to the two major epitopes, especially to AQP4(91-110), prevailed. The prevalence of the response to AQP4 may be due to past infection with common pathogens, which conferred immunological memory to a part of AQP4 via molecular mimicry (17), or to other, as yet unknown mechanisms (13). Although the presence of AQP4-specific T cells in the natural repertoire may be independent of NMO pathogenesis, another possibility is that the breakdown of tolerance for AQP4 and subsequent induction of AQP4-specific T cells is a step in the early disease process, but one that is insufficient to induce NMO without additional factors. Responses to AQP4(21-40), AQP4(101-120), AQP4(171-190) and AQP4(211-230) were weaker than those to AQP4(11-30) and AQP4(91-110)

with regard to mSI and fSI3, but reached significance by paired *t*-test in NMO samples. Among these, the mSI of reactions to AQP4(21-40), AQP4(101-120) and AQP4(171-190) exceeded the threshold value of 3. Response to AQP4(81-100) in NMO appeared strong with regard to mSI. Hence, the response to AQP4(81-100) may be important for disease progression in a particular set of NMO patients, even though paired *t*-test failed to show significance in this study cohort. The six epitopes satisfying mSI > 3 are all located in the intracellular domains of AQP4. These T cell epitopes are highly consistent with T cell epitopes in C57BL/6 mice and in SJL/J mice as determined by a different approach (17). More interestingly, recently reported B cell epitopes of NMO reported recently (18) are quite similar to our identified T cell epitopes, suggesting that T cell assistance is needed to mount an antibody response against an intracellular portion of AQP4.

It is currently hypothesized that astrocytes expressing AQP4 are a main and primary target of autoimmunity in NMO pathogenesis (6-7). The increase in T cell immunity to AQP4 that was shown here supports this hypothesis. However, it is also an intriguing question to consider what roles autoimmunity to myelin proteins can play in NMO. AQP4 is expressed in tissues outside the CNS such as in the kidney and retina, yet no lesions produced by autoimmunity to AQP4 have been described outside of the CNS. In other words, no lesions occur in the absence of myelin proteins. Recent reports have shown that AQP4 is preserved in NMO demyelinating lesions (20) and that animal models of NMO can be produced using myelin-specific T cells and patient serum (3-4). In the present study PLP(95-116) exhibited increased potential to stimulate T cells derived from NMO. T cell immunity to PLP as observed here may indicate that immunity to myelin proteins plays at least some role in NMO, although there remains a possibility that the observed T cell response to PLP is an epiphenomenon that is not fully associated with disease pathogenesis. There is also a possibility that this phenomenon may be due to epitope spreading as disease progresses, and can be primarily absent. However, since T cell immunity to PLP in NMO samples was demonstrated to be more robust than in MS samples, it is unlikely that PLP(95-116) is a mere dispersed epitope without any function after primary self-destruction.

In conclusion, this is the first demonstration of increased T cell immunity to AQP4 and PLP. The current study provides circumstantial, but strong evidence that antigenic T cell reactivity to AQP4 proteins is indispensable in NMO pathogenesis and that the immune response to myelin protein may participate in the development of NMO lesions, although further studies are needed to fully clarify the role of T cells in this nervous system disorder.

Abbreviations

NMO	neuromyelitis optica
MS	multiple sclerosis
HS	healthy subjects
AQP4	aquaporin-4
Stimulation index	SI
Mean SI	mSI
Frequency of SI >3	mSI3
PBMC	peripheral blood mononuclear cells
MOG	myelin oligodendrocyte glycoprotein
PLP	proteolipid protein
MBP	myelin basic protein
GFAP	glial fibrillary acidic protein
EAE	experimental autoimmune encephalomyelitis
AICD	activation induced cell death

Reference

1   Lennon, V. A., Wingerchuk, D. M., Kryzer, T. J., Pittock, S. J., Lucchinetti, C. F., Fujihara, K., Nakashima, I., and Weinshenker, B. G. 2004. A serum autoantibody marker of neuromyelitis optica: distinction from multiple sclerosis. *Lancet* 364:2106.

2   Lennon, V. A., Kryzer, T. J., Pittock, S. J., Verkman, A. S., and Hinson, S. R. 2005. IgG marker of optic-spinal multiple sclerosis binds to the aquaporin-4 water channel. *J Exp Med* 202:473.

3   Saadoun, S., Waters, P., Bell, B. A., Vincent, A., Verkman, A. S., and Papadopoulos, M. C. 2010. Intra-cerebral injection of neuromyelitis optica immunoglobulin G and human complement produces neuromyelitis optica lesions in mice. *Brain* 133:349.

4   Bradl, M., Misu, T., Takahashi, T., Watanabe, M., Mader, S., Reindl, M., Adzemovic, M., Bauer, J., Berger, T., Fujihara, K., Itoyama, Y., and Lassmann, H. 2009. Neuromyelitis optica: Pathogenicity of patient immunoglobulin in vivo. *Ann Neurol* 66:630.

5   Pittock, S. J., Weinshenker, B. G., Lucchinetti, C. F., Wingerchuk, D. M., Corboy, J. R., and Lennon, V. A. 2006. Neuromyelitis optica brain lesions localized at sites of high aquaporin 4 expression. *Arch Neurol* 63:964.

6   Misu, T., Fujihara, K., Nakamura, M., Murakami, K., Endo, M., Konno, H., and Itoyama, Y. 2006. Loss of aquaporin-4 in active perivascular lesions in neuromyelitis optica: a case report. *Tohoku J Exp Med* 209:269.

7   Roemer, S. F., Parisi, J. E., Lennon, V. A., Benarroch, E. E., Lassmann, H., Bruck, W., Mandler, R. N., Weinshenker, B. G., Pittock, S. J., Wingerchuk, D. M., and Lucchinetti, C. F. 2007. Pattern-specific loss of aquaporin-4 immunoreactivity distinguishes neuromyelitis optica from multiple sclerosis. *Brain*.

8   Wingerchuk, D. M., Lennon, V. A., Lucchinetti, C. F., Pittock, S. J., and Weinshenker, B. G. 2007. The spectrum of neuromyelitis optica. *Lancet Neurol* 6:805.

9   Krishnamoorthy, G., Lassmann, H., Wekerle, H., and Holz, A. 2006. Spontaneous opticospinal encephalomyelitis in a double-transgenic mouse

model of autoimmune T cell/B cell cooperation. *J Clin Invest* 116:2385.

10   Wingerchuk, D. M., Lennon, V. A., Pittock, S. J., Lucchinetti, C. F., and Weinshenker, B. G. 2006. Revised diagnostic criteria for neuromyelitis optica. *Neurology* 66:1485.

11   Polman, C. H., Reingold, S. C., Edan, G., Filippi, M., Hartung, H. P., Kappos, L., Lublin, F. D., Metz, L. M., McFarland, H. F., O'Connor, P. W., Sandberg-Wollheim, M., Thompson, A. J., Weinshenker, B. G., and Wolinsky, J. S. 2005. Diagnostic criteria for multiple sclerosis: 2005 revisions to the "McDonald Criteria". *Ann Neurol* 58:840.

12   Lu, M., Lee, M. D., Smith, B. L., Jung, J. S., Agre, P., Verdijk, M. A., Merkx, G., Rijss, J. P., and Deen, P. M. 1996. The human AQP4 gene: definition of the locus encoding two water channel polypeptides in brain. *Proc Natl Acad Sci U S A* 93:10908.

13   Sospedra, M. and Martin, R. 2005. Immunology of multiple sclerosis. *Annu Rev Immunol* 23:683.

14   Ohashi, T., Yamamura, T., Inobe, J., Kondo, T., Kunishita, T., and Tabira, T. 1995. Analysis of proteolipid protein (PLP)-specific T cells in multiple sclerosis: identification of PLP 95-116 as an HLA-DR2,w15-associated determinant. *Int Immunol* 7:1771.

15   Illes, Z., Kondo, T., Yokoyama, K., Ohashi, T., Tabira, T., and Yamamura, T. 1999. Identification of autoimmune T cells among in vivo expanded CD25+ T cells in multiple sclerosis. *J Immunol* 162:1811.

16   Bieganowska, K. D., Ausubel, L. J., Modabber, Y., Slovik, E., Messersmith, W., and Hafler, D. A. 1997. Direct ex vivo analysis of activated, Fas-sensitive autoreactive T cells in human autoimmune disease. *J Exp Med* 185:1585.

17   Nelson, P. A., Khodadoust, M., Prodhomme, T., Spencer, C., Patarrouyo, J., C., Varrin-Doyer, M., Ho, J. D., Stroud, R. M., and Zamvil, S. S. 2010. Immunodominant T cell determinats of aquaporin-4, the autoantigen associated with neuromyelitis optica. *PLoS One* 5:e15050.

18   Kampylafka, E. I., Routsias, J. G., Alexopoulos, H., Dalakas, M. C., Moutsopoulos, H. M., and Tzioufas, A. G. 2011. Fine specificities of antibodies against AQP4: Epitope mapping reveals intracellular epitopes. *J Autoimmun*

36:221.

19   Benoist, C. and Mathis, D. 2001. Autoimmunity provoked by infection: how good is the case for T cell epitope mimicry? *Nat Immunol* 2:797.

20   Matsuoka, T., S., Suzuki, S. O., Suenaga, T., Iwaki, T., and Kira, J. I. 2011. Reappraisal of aquqporin-4 astrocytopathy in Asian neuromyelitis optoca and multiple sclerosis patients. *Brain Pathol* (Epub ahead of print)

Figure Legends
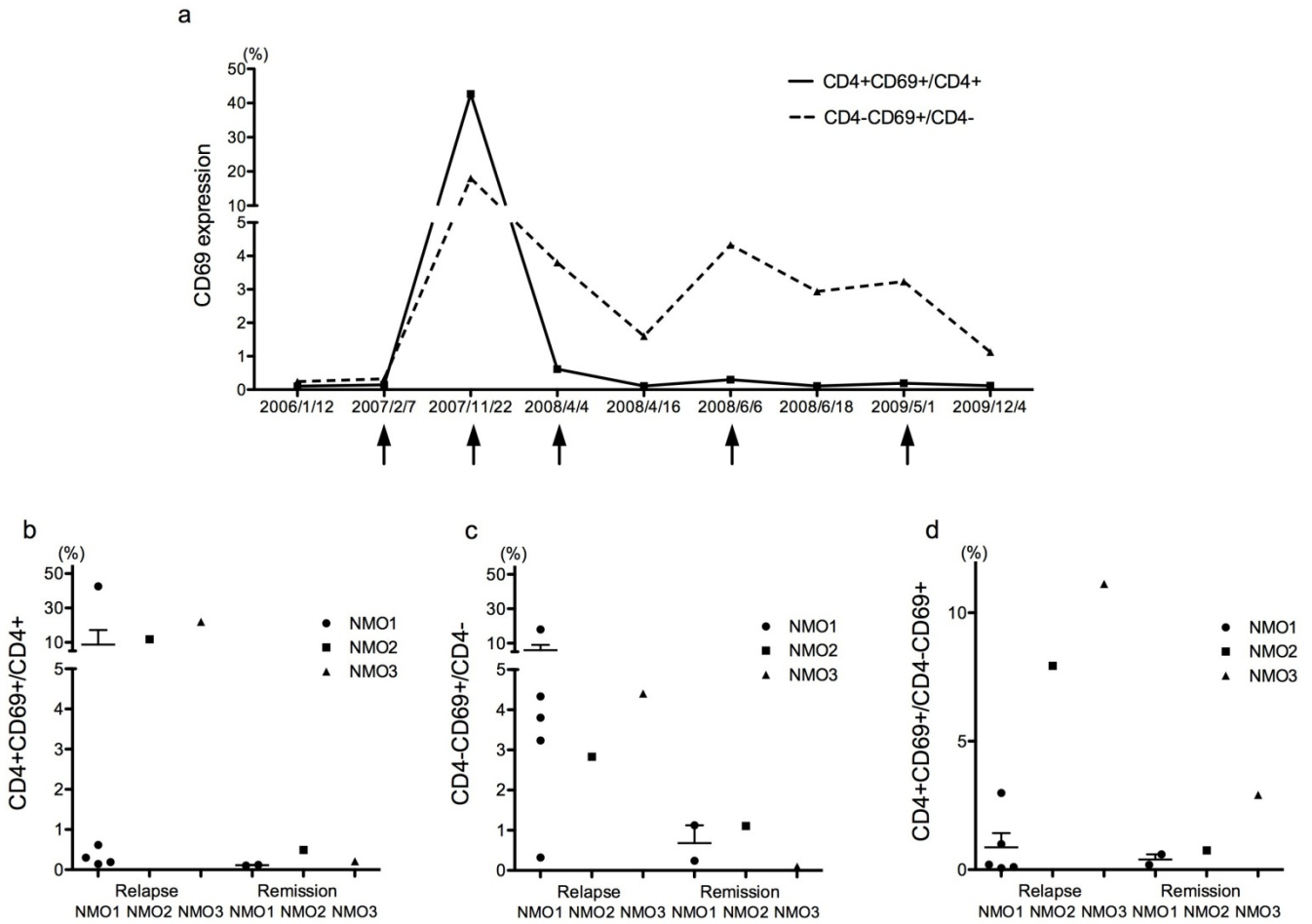
Figure 1. (a) Frequencies of CD69+CD4+ and CD69+CD4- T cells were longitudinally monitored in NMO1 from January 1, 2006 to December 4, 2009. Arrows indicated relapses. (b) Frequencies of CD69+CD4+ T cells are shown in remissions and relapses of NMO1-3. (c) Frequencies of CD69+CD4- T cells are shown in remissions and relapses of NMO1-3. (d) Ratio of CD69+CD4+ cells to CD69+CD4- cells in the CD3 fraction are shown.

a
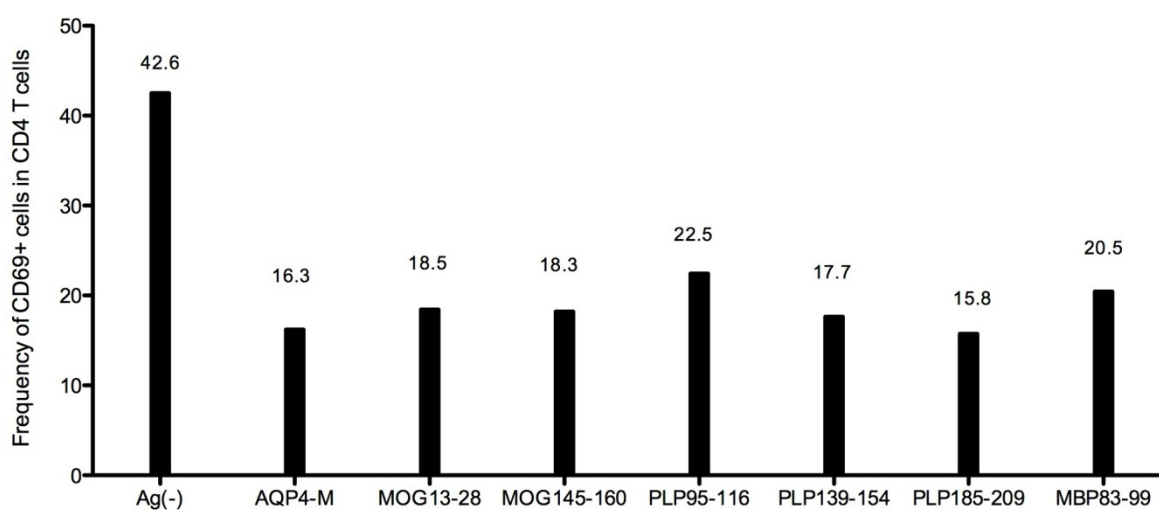
b
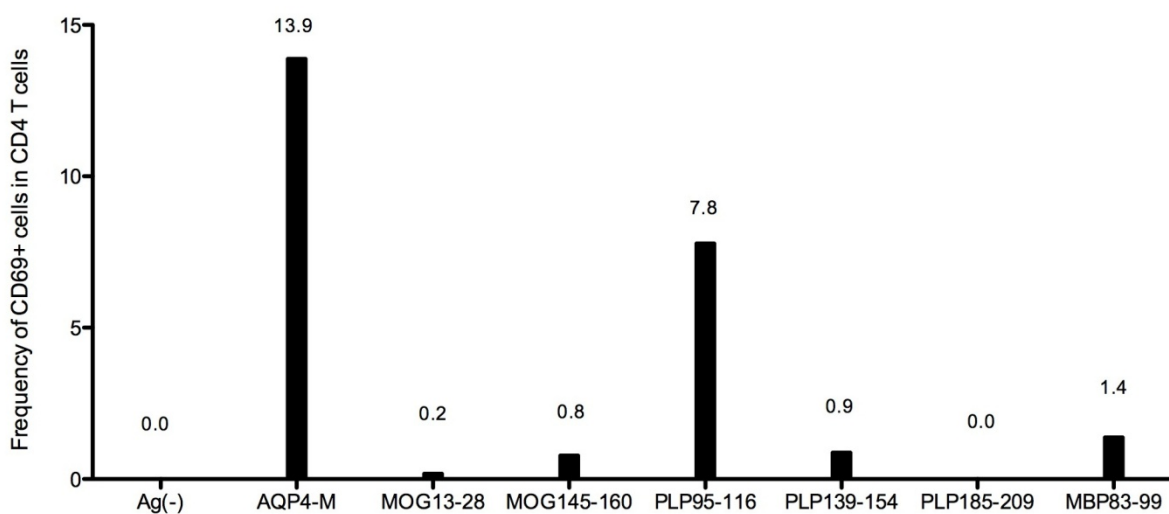
Figure 2. Frequencies of NMO1 CD69+ cells in CD4+ T cells in response to antigenic stimulation. (a) The indicated antigen was added to freshly isolated PBMCs. This stimulation non-specifically down-regulated CD69 expression on CD4+ T cells or decreased CD69+CD4+ T cells. (b) PBMC were rested for two days before the indicated antigen was added.
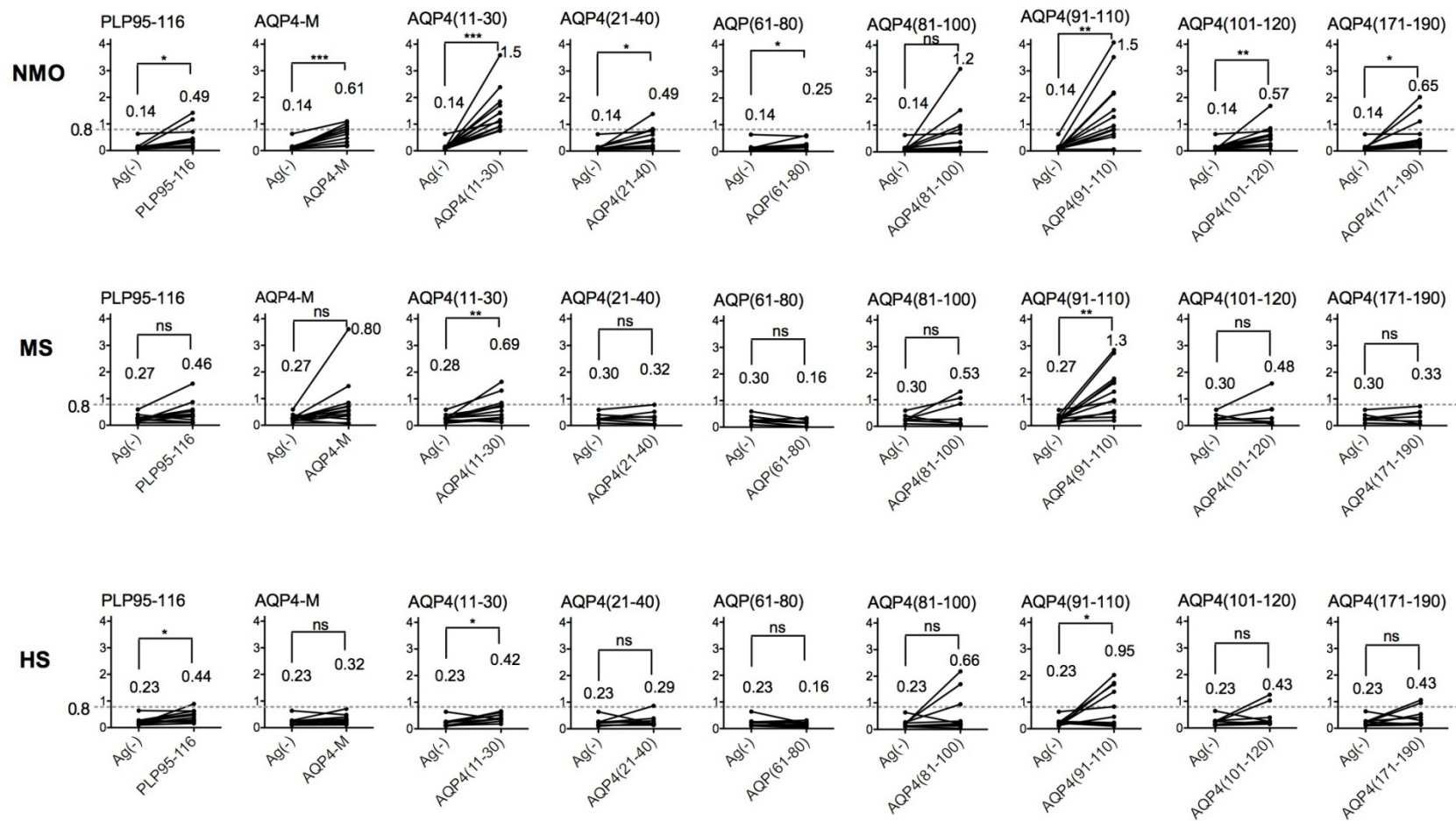
Figure 3. Frequencies of CD69+ cells in CD4+ T cells in response to AQP4 and PLP(95-116). PBMCs were isolated from NMO, MS, and HS. The cells were rested for two days. AQP4-M, a single AQP4 peptide or PLP(95-116) was added. Four hours later, frequencies of CD69+ CD4+ T cells were analyzed. * indicates statistically significant T cell activation ($p<0.05$).
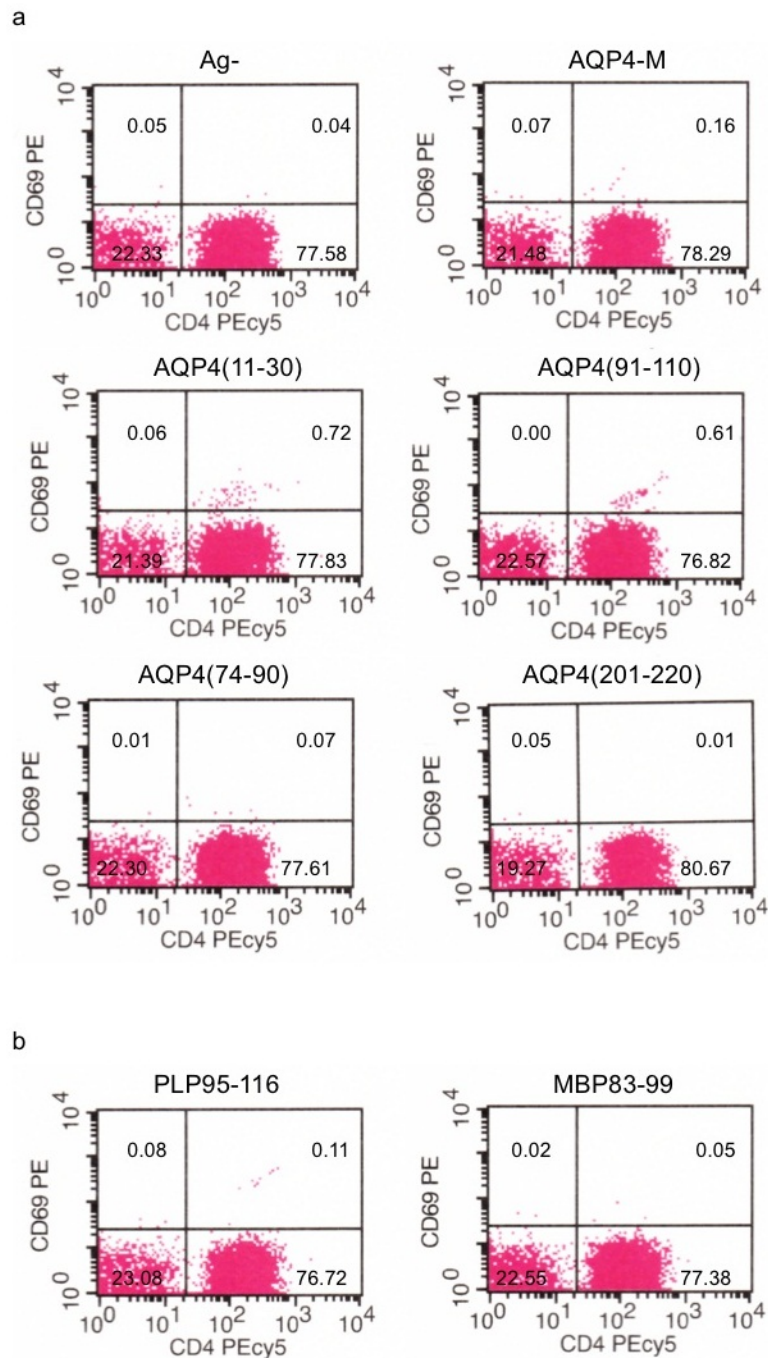
Figure 4. Representive data derived from one of the NMO samples. In this patient two days culture sufficiently downregulated CD69 expression in CD4+ T cells to 0.04%. While the sample exceeded SI > 3 in response to AQP-M, AQP4(11-30), AQP4(91-110), it did not significantly react to AQP4(74-90) and (201-220) like the other AQP4 peptiedes not shown here. The highest response was available in response to PLP(95-16) among myelin peptides, but SI was slightly below 3 .

Table 1. Amino acid sequences and solubility of AQP-4 overlapping peptides and myelin peptides.

Peptides		Amino acid sequences	Solubility
AQP4	(1-20)	MSDRPTARRWGKCGPLCTRE	soluble
	(11-30)	GKCGPLCTRENIMVAFKGVW	soluble
	(21-40)	NIMVAFKGVWTQAFWKAVTA	soluble
	(31-50)	TQAFWKAVTAEFLAMLIFVL	soluble
	(41-60)	EFLAMLIFVLLSLGSTINWG	insoluble
	(51-70)	LSLGSTINWGGTEKPLPVDM	soluble
	(61-80)	GTEKPLPVDMVLISLCFGLS	soluble
	(74-90)	SLCFGLSIATMVQCFGH	soluble
	(81-100)	IATMVQCFGHISGGHINPAV	soluble
	(91-110)	ISGGHINPAVTVAMVCTRKI	soluble
	(101-120)	TVAMVCTRKISIAKSVFYIA	soluble
	(111-130)	SIAKSVFYIAAQCLGAIIGA	soluble
	(121-140)	AQCLGAIIGAGILYLVTPPS	insoluble
	(131-150)	GILYLVTPPSVVGGLGVTMV	soluble
	(141-160)	VVGGLGVTMVHGNLTAGHGL	soluble
	(151-170)	HGNLTAGHGLLVELIITFQL	soluble
	(161-180)	LVELIITFQLVFTIFASCDS	insoluble
	(171-190)	VFTIFASCDSKRTDVTGSIA	soluble
	(181-200)	KRTDVTGSIALAIGFSVAIG	soluble
	(191-210)	LAIGFSVAIGHLFAINYTGA	insoluble
	(201-220)	HLFAINYTGASMNPARSFGP	soluble
	(211-230)	SMNPARSFGPAVIMGNWENH	soluble
	(221-240)	AVIMGNWENHWIYWVGPIIG	soluble
	(231-250)	WIYWVGPIIGAVLAGGLYEY	soluble
	(241-260)	AVLAGGLYEYVFCPDVEFKR	soluble
	(251-270)	VFCPDVEFKRRFKEAFSKAA	soluble
	(261-280)	RFKEAFSKAAQQTKGSYMEV	soluble
	(271-290)	QQTKGSYMEVEDNRSQVETD	soluble
	(281-300)	EDNRSQVETDDLILKPGVVH	soluble
	(291-310)	DLILKPGVVHVIDVDRGEEK	soluble
	(301-318)	VIDVDRGEEKKGKDQSGE	soluble
	(309-323)	EKKGKDQSGEVLSSV	soluble
MOG	13-28	RALVGDEVELPCRISP	soluble
MOG	145-160	VFLCLQYRLRGKLRAE	soluble
PLP	95-116	AVRQIFGDYKTTICGKGLSATV	soluble
	139-154	HCLGKWLGHPDKFVGI	soluble
	185-209	SIAFPSKTSASIGSLCADARMYGVL	soluble
MBP	83-99	ENPVVHFFKNIVTPRTP	soluble

Table 2. T cell response to AQP4 and myelin antigens. The frequency of CD69+ cells in CD4+ T cells was determined four hours after antigenic stimulation after by flow cytometry. SI was calculated in comparioson to non-stimuated CD4+ T cells.

Peptides	NMO		MS		HS	
	Mean SI	SI>=3	Mean SI	SI>=3	Mean SI	SI>=3
AQP4-M	**5.50±3.40 (1.70-15.00)**	10/12 (83.3%)	2.60±1.80 (0.24-6.10)	4/12 (33.3%)	1.50±0.64 (0.75-2.60)	0/10 (0.0%)
AQP4(1-20)	1.10±1.00 (0.00-3.40)	1/12 (8.3%)	0.65±0.40 (0.00-1.50)	0/11 (0.0%)	0.53±0.20 (0.19-0.85)	0/9 (0.0%)
(11-30)	**16.00±12.00 (1.70-40.00)**	11/12 (91.7%)	**2.50±1.50 (0.58-6.30)**	3/11 (27.3%)	**2.20±0.91 (0.55-3.20)**	2/9 (22.2%)
(21-40)	**4.80±5.20 (0.75-20.00)**	6/12 (50.0%)	0.96±0.65 (0.25-2.10)	0/7 (0.0%)	1.50±0.92 (0.30-3.50)	1/9 (11.1%)
(31-50)	2.10±2.60 (0.25-9.60)	2/12 (16.7%)	0.71±0.41 (0.29-1.40)	0/7 (0.0%)	0.97±0.67 (0.30-2.40)	0/9 (0.0%)
(51-70)	1.00±0.64 (0.00-2.50)	0/12 (0.0%)	0.43±0.21 (0.14-0.78)	0/7 (0.0%)	0.85±0.65 (0.30-2.30)	0/9 (0.0%)
(61-80)	**2.10±1.20 (0.65-5.00)**	1/12 (8.3%)	0.54±0.42 (0.08-1.30)	0/7 (0.0%)	0.81±0.39 (0.30-1.20)	0/9 (0.0%)
(74-90)	1.10±0.78 (0.00-2.90)	0/12 (0.0%)	0.75±0.46 (0.25-1.50)	0/7 (0.0%)	0.93±0.88 (0.14-3.10)	1/9 (11.1%)
(81-100)	14.00±28.00 (0.51-101.00)	5/12 (41.7%)	1.80±1.80 (0.18-5.20)	2/7 (28.6%)	3.00±3.40 (0.34-10.00)	3/9 (33.3%)
(91-110)	**13.00±9.80 (1.00-36.00)**	11/12 (91.7%)	**5.30±3.50 (1.10-11.00)**	8/12 (66.7%)	**6.30±6.60 (0.48-17.00)**	5/9 (55.6%)
(101-120)	**6.00±7.30 (1.00-28.00)**	8/12 (66.7%)	1.50±0.99 (0.14-2.70)	0/7 (0.0%)	2.10±1.70 (0.38-5.00)	2/9 (22.2%)
(111-130)	1.60±1.50 (0.13-5.40)	2/12 (16.7%)	0.97±0.61 (0.25-2.50)	0/11 (0.0%)	0.62±0.30 (0.27-1.30)	0/9 (0.0%)
(131-150)	1.40±0.64 (0.35-2.60)	0/12 (0.0%)	0.38±0.27 (0.00-0.77)	0/7 (0.0%)	0.66±0.47 (0.30-1.80)	0/9 (0.0%)
(141-160)	1.20±0.89 (0.00-3.10)	1/12 (8.3%)	0.71±0.24 (0.36-1.10)	0/7 (0.0%)	0.81±0.50 (0.19-1.70)	0/9 (0.0%)
(151-170)	1.40±0.80 (0.35-2.60)	0/12 (0.0%)	0.49±0.28 (0.00-0.77)	0/7 (0.0%)	1.00±0.90 (0.26-3.30)	1/9 (11.1%)
(171-190)	**7.00±9.10 (1.00-34.00)**	7/12 (58.3%)	1.10±0.68 (0.15-2.00)	0/7 (0.0%)	2.20±1.60 (0.47-4.50)	3/9 (33.3%)
(181-200)	2.20±1.70 (0.33-5.90)	3/12 (25.0%)	0.75±0.27 (0.32-1.10)	0/7 (0.0%)	1.40±0.89 (0.20-3.10)	1/9 (11.1%)
(201-220)	1.60±1.50 (0.24-5.50)	1/12 (8.3%)	0.57±0.37 (0.14-1.20)	0/7 (0.0%)	1.10±0.86 (0.41-3.10)	1/9 (11.1%)
(211-230)	**2.20±1.00 (0.70-3.80)**	3/11 (27.3%)	1.00±0.89 (0.43-3.00)	0/7 (0.0%)	0.91±0.60 (0.30-2.30)	0/9 (0.0%)
(221-240)	0.97±0.55 (0.24-2.00)	0/11 (0.0%)	0.49±0.15 (0.28-0.66)	0/7 (0.0%)	0.79±0.49 (0.27-1.60)	0/9 (0.0%)
(231-250)	1.20±0.59 (0.32-2.00)	0/11 (0.0%)	0.43±0.24 (0.14-0.78)	0/7 (0.0%)	0.88±0.69 (0.23-2.30)	0/9 (0.0%)
(241-260)	1.60±0.80 (0.75-3.30)	1/11 (9.1%)	0.55±0.23 (0.25-0.96)	0/7 (0.0%)	0.82±0.40 (0.22-1.50)	0/9 (0.0%)
(251-270)	1.10±0.52 (0.46-2.10)	0/11 (0.0%)	0.42±0.26 (0.00-0.77)	0/7 (0.0%)	0.53±0.29 (0.14-1.00)	0/9 (0.0%)
(261-280)	1.40±1.20 (0.00-4.30)	1/11 (9.1%)	0.73±0.77 (0.00-2.40)	0/7 (0.0%)	0.74±0.43 (0.22-1.40)	0/9 (0.0%)
(271-290)	1.00±0.71 (0.34-2.60)	0/12 (0.0%)	0.52±0.52 (0.00-1.60)	0/7 (0.0%)	0.69±0.35 (0.30-1.20)	0/9 (0.0%)
(281-300)	1.10±0.44 (0.25-2.00)	0/12 (0.0%)	0.46±0.21 (0.18-0.71)	0/7 (0.0%)	0.64±0.39 (0.20-1.50)	0/9 (0.0%)
(291-310)	1.40±1.40 (0.00-4.30)	2/12 (16.7%)	0.50±0.34 (0.08-1.10)	0/7 (0.0%)	0.64±0.35 (0.19-1.40)	0/9 (0.0%)
(301-318)	1.10±0.80 (0.24-2.60)	0/12 (0.0%)	0.45±0.29 (0.08-1.00)	0/7 (0.0%)	0.61±0.24 (0.25-1.10)	0/9 (0.0%)
(309-323)	1.10±0.81 (0.00-2.90)	0/11 (0.0%)	0.48±0.21 (0.19-0.83)	0/7 (0.0%)	0.58±0.23 (0.20-0.85)	0/9 (0.0%)
MOG(13-28)	1.60±1.10 (0.37-3.80)	1/12 (8.3%)	0.74±0.51 (0.07-1.60)	0/9 (0.0%)	0.97±0.88 (0.20-3.40)	1/10 (10.0%)
(145-160)	1.60±0.79 (0.75-3.30)	1/12 (8.3%)	0.72±0.25 (0.33-1.00)	0/9 (0.0%)	0.94±0.68 (0.29-2.60)	0/10 (0.0%)
PLP(95-116)	**4.60±4.40 (1.10-17.00)**	7/12 (58.3%)	1.80±1.40 (0.44-5.40)	1/12 (8.3%)	**2.30±1.70 (0.97-6.80)**	1/10 (10.0%)
(139-154)	1.20±0.61 (0.37-2.70)	0/12 (0.0%)	0.70±0.56 (0.00-2.00)	0/9 (0.0%)	0.87±0.57 (0.26-1.90)	0/10 (0.0%)
(185-209)	1.30±1.00 (0.29-3.80)	1/12 (8.3%)	0.74±0.41 (0.32-1.60)	0/9 (0.0%)	0.83±0.70 (0.16-2.70)	0/10 (0.0%)
MBP(83-99)	1.20±0.72 (0.00-2.40)	0/12 (0.0%)	0.64±0.27 (0.08-0.93)	0/9 (0.0%)	0.81±0.59 (0.16-2.20)	0/10 (0.0%)

Table 3. (a) HLA allele and T cell epitope. (b) Comparison of T cell epitope between HLA-DR15 carrier and non- carrier and between HLA-DP5 carriers and non-carrier.

(a)

Total

HLA		AQP4(11-30)	AQP4(21-40)	AQP4(91-110)	AQP4(101-120)	AQP4(171-190)
HLA-DRB1	0101	0/2 (0.0%)	0/1 (0.0%)	1/2 (50.0%)	0/1 (0.0%)	0/1 (0.0%)
	0401	1/1 (100.0%)	0/1 (0.0%)	1/1 (100.0%)	1/1 (100.0%)	1/1 (100.0%)
	0405or0410	5/8 (62.5%)	2/7 (28.6%)	5/8 (62.5%)	3/7 (42.9%)	3/7 (42.9%)
	0802	3/5 (60.0%)	0/5 (0.0%)	3/5 (60.0%)	1/5 (20.0%)	1/5 (20.0%)
	0803	3/3 (100.0%)	1/3 (33.3%)	3/3 (100.0%)	3/3 (100.0%)	2/3 (66.7%)
	0901	2/3 (66.7%)	0/2 (0.0%)	1/3 (33.3%)	0/2 (0.0%)	0/2 (0.0%)
	1302	1/4 (25.0%)	0/3 (0.0%)	3/4 (75.0%)	1/3 (33.3%)	0/3 (0.0%)
	1401	0/1 (0.0%)	0/1 (0.0%)	1/1 (100.0%)	0/1 (0.0%)	1/1 (100.0%)
	1405	0/1 (0.0%)	1/1 (100.0%)	0/1 (0.0%)	1/1 (100.0%)	1/1 (100.0%)
	1406	1/1 (100.0%)	0/1 (0.0%)	1/1 (100.0%)	1/1 (100.0%)	1/1 (100.0%)
	1501	7/11 (63.6%)	4/10 (40.0%)	8/11 (72.7%)	4/10 (40.0%)	4/10 (40.0%)
	1502	3/6 (50.0%)	1/6 (16.7%)	5/6 (83.3%)	1/6 (50.0%)	3/6 (50.0%)
	unidentified	2/5 (40.0%)	1/4 (25.0%)	5/6 (83.3%)	1/4 (25.0%)	0/4 (0.0%)
HLA-DPB1	0201	6/9 (66.7%)	4/8 (50.0%)	6/9 (66.7%)	4/8 (50.0%)	6/8 (75.0%)
	0202	1/2 (50.0%)	2/2 (100.0%)	1/2 (50.0%)	2/2 (100.0%)	2/2 (100.0%)
	0401	2/5 (40.0%)	0/5 (0.0%)	3/5 (60.0%)	2/5 (40.0%)	2/5 (40.0%)
	0402	0/4 (0.0%)	0/3 (0.0%)	2/4 (50.0%)	0/3 (0.0%)	0/3 (0.0%)
	0501	7/13 (53.8%)	2/10 (20.0%)	10/13 (76.9%)	3/10 (30.0%)	4/10 (40.0%)
	0901	2/5 (40.0%)	1/5 (20.0%)	4/5 (80.0%)	1/5 (20.0%)	2/5 (40.0%)
	1401	1/1 (100.0%)	0/1 (0.0%)	1/1 (100.0%)	1/1 (100.0%)	1/1 (100.0%)
	unidentified	5/10 (50.0%)	2/9 (22.2%)	9/11 (81.8%)	3/9 (33.3%)	1/9 (11.1%)

NMO

HLA		AQP4(11-30)	AQP4(21-40)	AQP4(91-110)	AQP4(101-120)	AQP4(171-190)
HLA-DRB1	0401	1/1 (100.0%)	0/1 (0.0%)	1/1 (100.0%)	1/1 (100.0%)	1/1 (100.0%)
	0405or0410	2/2 (100.0%)	2/2 (100.0%)	1/2 (50.0%)	2/2 (100.0%)	2/2 (100.0%)
	0802	1/1 (100.0%)	0/1 (0.0%)	1/1 (100.0%)	1/1 (100.0%)	1/1 (100.0%)
	0803	3/3 (100.0%)	1/3 (33.3%)	3/3 (100.0%)	3/3 (100.0%)	2/3 (66.7%)
	1302	1/1 (100.0%)	0/1 (0.0%)	1/1 (100.0%)	1/1 (100.0%)	0/1 (0.0%)
	1406	1/1 (100.0%)	0/1 (0.0%)	1/1 (100.0%)	1/1 (100.0%)	1/1 (100.0%)
	1501	5/5 (100.0%)	4/5 (80.0%)	4/5 (80.0%)	4/5 (80.0%)	4/5 (80.0%)
	1502	2/2 (100.0%)	1/2 (50.0%)	2/2 (100.0%)	0/2 (0.0%)	1/2 (50.0%)
	unidentified	2/3 (66.7%)	1/3 (33.3%)	3/3 (100.0%)	1/3 (33.3%)	0/3 (0.0%)
HLA-DPB1	0201	5/5 (100.0%)	3/5 (60.0%)	4/5 (80.0%)	3/5 (60.0%)	4/5 (80.0%)
	0202	1/1 (100.0%)	1/1 (100.0%)	1/1 (100.0%)	1/1 (100.0%)	1/1 (100.0%)
	0401	1/1 (100.0%)	0/1 (0.0%)	1/1 (100.0%)	1/1 (100.0%)	1/1 (100.0%)
	0501	4/4 (100.0%)	2/4 (50.0%)	4/4 (100.0%)	3/4 (75.0%)	3/4 (75.0%)
	0901	1/1 (100.0%)	1/1 (100.0%)	1/1 (100.0%)	0/1 (0.0%)	1/1 (100.0%)
	1401	1/1 (100.0%)	0/1 (0.0%)	1/1 (100.0%)	1/1 (100.0%)	1/1 (100.0%)
	unidentified	4/5 (80.0%)	2/5 (40.0%)	5/5 (100.0%)	3/5 (60.0%)	1/5 (20.0%)

(b)

Total

HLA		AQP4(11-30)	AQP4(21-40)	AQP4(91-110)	AQP4(101-120)	AQP4(171-190)
HLA-DRB1	15 carrier	9/16 (56.3%)	5/15 (33.3%)	12/16 (75.0%)	5/15 (33.3%)	7/15 (46.7%)
	15 negative	5/11 (45.5%)	1/9 (11.1%)	7/11 (63.6%)	4/9 (44.4%)	3/9 (33.3%)
	unidentified	2/5 (40.0%)	1/4 (25.0%)	5/6 (83.3%)	1/4 (25.0%)	0/4 (0.0%)
HLA-DPB1	05 carrier	7/13 (53.8%)	2/10 (20.0%)	10/13 (76.9%)	3/10 (30.0%)	4/10 (40.0%)
	05 negative	4/9 (44.4%)	3/9 (33.3%)	5/9 (55.6%)	4/9 (44.4%)	5/9 (55.6%)
	unidentified	5/10 (50.0%)	2/9 (22.2%)	9/11 (81.8%)	3/9 (33.3%)	1/9 (11.1%)

NMO

HLA		AQP4(11-30)	AQP4(21-40)	AQP4(91-110)	AQP4(101-120)	AQP4(171-190)
HLA-DRB1	15 carrier	6/6 (100.0%)	5/6 (83.3%)	5/6 (83.3%)	4/6 (66.7%)	5/6 (83.3%)
	15 negative	3/3 (100.0%)	0/3 (0.0%)	3/3 (100.0%)	3/3 (100.0%)	2/3 (66.7%)
	unidentified	2/3 (66.7%)	1/3 (33.3%)	3/3 (100.0%)	1/3 (33.3%)	0/3 (0.0%)
HLA-DPB1	05 carrier	4/4 (100.0%)	2/4 (50.0%)	4/4 (100.0%)	3/4 (75.0%)	3/4 (75.0%)
	05 negative	3/3 (100.0%)	2/3 (66.7%)	2/3 (66.7%)	2/3 (66.7%)	3/3 (100.0%)
	unidentified	4/5 (80.0%)	2/5 (40.0%)	5/5 (100.0%)	3/5 (60.0%)	1/5 (20.0%)