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## Peripheral VH4+ Plasmablasts Demonstrate Autoreactive B Cell Expansion Toward Brain Antigens in Early Multiple Sclerosis Patients

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### Abstract

Plasmablasts are a highly differentiated, antibody secreting B cell subset whose prevalence correlates with disease activity in Multiple Sclerosis (MS). For most patients experiencing partial transverse myelitis (PTM), plasmablasts are elevated in the blood at the first clinical presentation of disease (known as clinically isolated syndrome or CIS). In this study we found that many of these peripheral plasmablasts are autoreactive and recognize primarily gray matter targets in brain tissue. These plasmablasts express antibodies that over-utilize immunoglobulin heavy chain V-region subgroup 4 (VH4) genes, and the highly mutated VH4+ plasmablast antibodies recognize intracellular antigens of neurons and astrocytes. Most of the autoreactive, highly mutated VH4+ plasmablast antibodies recognize only a portion of cortical neurons, indicating that the response may be specific to neuronal subgroups or layers. Furthermore, CIS-PTM patients with this plasmablast response also exhibit modest reactivity toward neuroantigens in the plasma IgG antibody pool. Taken together, these data indicate that expanded VH4+ peripheral plasmablasts in early MS patients recognize brain gray matter antigens. Peripheral plasmablasts may be participating in the autoimmune response associated with MS, and provide an interesting avenue for investigating the expansion of autoreactive B cells at the time of the first documented clinical event.

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**Conflicts of Interest:** Nancy Monson reports patent US 8,394,583 B2 on MSPrecise™, a diagnostic tool for predicting conversion to MS.

## Keywords

Plasmablast; Multiple Sclerosis; autoantibody; B cell; antigen receptor genetics

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## Introduction

Multiple Sclerosis (MS) is an autoimmune disorder of the central nervous system (CNS) that results in a loss of neurological function [39, 60]. Throughout disease the adaptive immune system mediates brain inflammation and lesion formation in these patients [7, 43, 61]. Although MS was historically thought to be driven by T cells [44], evidence for the importance of B cells in MS produced a shift in this perspective [25, 26]. The efficacy of B cell depletion therapy (BCDT) [40, 50], the prevalence of B cells in type II MS lesions [61], and the inverse correlation between memory B cell repopulation and benefit from BCDT [1] all support the hypothesis that B cells play a central role in pathogenesis [25, 26].

One B cell subtype of particular interest is the plasmablast, which normally develops in the blood as a part of the antigen-stimulated memory B cell response to pathogens [30, 32, 55, 69]. Plasmablasts are identified by the upregulation of CD27 and simultaneous expression of CD38 and CD95, and secrete large amounts of antibody [3, 32, 34, 48, 71]. They also produce IL-10, which positively feeds back to promote the differentiation and expansion of IgG and IgM antibody secreting cells [41]. After 2-3 weeks, plasmablasts either undergo apoptosis or differentiate into long-lived plasma cells [30, 32, 55, 69], thus the presence of plasmablasts indicates an active B cell response and represents the fraction of B cells that are currently responding to stimulus. Plasmablasts also represent a large proportion (5-40%) of the B cell pool in the cerebrospinal fluid (CSF) of untreated early and established MS patients [58] and their frequency correlates with increased inflammation as assessed by MRI [18]. They are also elevated in the blood of patients experiencing their first clinical attack, and when left untreated, their frequency continues to rise [58].

Despite these observations, the role of plasmablasts and secreted antibodies in MS has been difficult to explicate [25, 54]. The function of secreted antibody was particularly questioned since BCDT begins to take effect before antibody titers are appreciably altered [40]. However the importance of the antibody goes beyond its secreted form, as membrane bound antibody is the primary factor in issuing B cell development and effector function [35, 64]. For example, B cells that lack cognate antigen recognition in germinal centers are removed from the B cell pool while those that engage cognate antigen are prompted towards activation, clonal expansion and affinity maturation [64, 101]. Thus, there is a critical relationship between the antibody expressed by B cells, the antigen recognized, and the B cell's potential to participate in an immune response.

Still, autoreactive antibodies secreted by plasmablasts or plasma cells may contribute to the disease by binding to self-antigens and mediating cell and tissue damage. Previous data from our laboratory demonstrated that B cells in the CSF of early and established MS patients express antibodies with a particular mutational pattern that bind gray matter targets in brain tissue, such as neurons and astrocytes [57]. Others have demonstrated that antibodies from clonally expanded MS CSF B cells that bind gray matter targets mediate complement

deposition and damage to neurons *in vitro*, and pooled CSF antibodies from MS patients can mediate neurological dysfunction *in vivo* [12, 24].

Since we found that clinically isolated syndrome (CIS) patients display an expansion of plasmablasts during their first attack of partial transverse myelitis (PTM), we asked whether the peripheral plasmablasts from these patients harbor autoreactivity to CNS antigens. CIS-PTM patients are of particular interest as they display an expansion of plasmablasts [58], and focusing on this group increases homogeneity of the patients in the study. To this end, we isolated single peripheral plasmablasts from our CIS-PTM patients, cloned the expressed antibodies, and investigated the antibody's reactivity to brain antigens using a panel of methodologies. We found that antibodies expressed by plasmablasts from these early MS patients display high levels of reactivity for cellular and protein targets in the brain. Remarkably, only those antibodies that utilized variable heavy chain family 4 (VH4) genes bound strongly to brain antigens. Elevated levels of CNS reactive antibodies were detected in the plasma pool of many patients for whom CNS-reactive plasmablasts were detected. To our knowledge this is the first evidence for reactivity of peripheral plasmablasts from CIS-PTM patients to brain antigens, demonstrating their autoreactive nature.

## Methods

### Patient Sample Processing

Persons recruited for this study gave informed consent for the collection and utilization of blood according to the guidelines provided by the institutional review board at UTSWMC. Treatment naïve clinically isolated syndrome (CIS) patients with partial transverse myelitis symptoms (PTM) at high risk for developing MS, age and gender matched treatment naïve Neuromyelitis Optica (NMO) patients with established disease (used in the genetic analysis, cloning, and plasma antibody experiments), age and gender matched NMO patients on Cellcept therapy (used in the plasma antibody ELISA experiments), and age and gender matched healthy donors were included in this study (Table 1). CIS-PTM patients were defined as high risk for MS because the patients presented with at least one non-enhancing brain white matter lesion by MRI and the CSF was positive for oligoclonal banding or had a high IgG index. Average time to MS evolution was 12 months. NMO patients were diagnosed by the 2006 criteria and either ELISA or a cell-based assay was used to detect aquaporin-4 (AQP4) reactive antibodies in patient serum (Table 1). Only treatment naïve NMO patients were used as comparators for immunoglobulin gene analysis and antibody cloning. Peripheral blood mononuclear cells (PBMCs) were isolated from the blood by Ficoll separation and stained with fluorescent antibodies as previously described [58]. B cells were gated from PBMCs as CD45<sup>+</sup>CD19<sup>+</sup> cells, then memory B cells (CD19<sup>+</sup>CD27<sup>+</sup>) and plasmablasts (CD19<sup>+</sup>CD27<sup>hi</sup>), as defined by others [34, 48]) were sorted individually into 96-well plates using the BD FACSAria flow cytometer (BD Biosciences, San Jose, CA).

### Single Cell Polymerase Chain Reaction and Immunoglobulin Gene Analysis

Individually sorted B cell subpopulations were flash frozen and lysed. Upon thawing, mRNA was reverse transcribed and immunoglobulin variable regions were amplified with multiple rounds of PCR as previously described [58]. Sanger sequencing was used at the

UTSWMC sequencing core to generate the antibody variable domain reads. Sequence data was analyzed using the VDJserver online repertoire analysis tool (<https://vdjserver.org/>). Unproductive antibody rearrangements and truncated sequence reads (did not extend from the beginning of CDR1 to the first two codons of the J gene) were filtered out of the database. CIS-PTM and NMO sequence data was compared to healthy control CD19+ B cells provided by Peter Lipsky at UTSWMC [37, 67] and influenza responding plasmablasts from otherwise healthy donors as previously described [106]. GraphPad Prism software was used to determine the statistical significance of differences between groups and build graphs for figures. Frequencies were first subject to an arcsine transformation, as is appropriate for comparisons of frequencies, and non-parametric ANOVA was used with a post-hoc analysis to do pairwise comparison of patient groups with the healthy controls using the Dunnett multiple comparison method [108].

### Antibody Cloning and Production

Plasmablasts from CIS-PTM and NMO patients expressing highly mutated VH4 or VH3 heavy chains were selected for production. The variable domains were synthesized (Integrated DNA Technologies, Corallville, IA) and bi-directionally cloned into an IgG1 backbone provided by Michel Nussenzweig at the Rockefeller University as previously described [93]. The 6 IgG1 antibodies cloned from one healthy donor used as controls were also previously described [51]. Betty Diamond at the Hofstra Northwell School of Medicine provided the DNA for two control IgG1 antibodies, B1 and G11, which serve as isotype negative and positive controls, respectively [110]. Protocol details are provided in the supplemental methods.

### Tissue and Antigen ELISAs

Mouse tissues were used in this study to avoid epitope degradation that may occur when tissues are not immediately processed post mortem. Mouse brain and kidney lysates were made as described elsewhere [51] and the protocol was adapted from this reference. Protocol details are provided in the supplemental methods. In brief, plates were coated with 10 µg/mL of lysate or 1 µg/mL of purified antigen in bicarbonate buffer overnight, then blocked. Dilutions of rhAbs or patient plasma were added overnight, followed by incubation with biotinylated anti-human IgG (eBioscience, San Diego, CA) then streptavidin-HRP (BD Pharmingen, San Jose, CA) and detection by TMB substrate according to manufacturer recommendations (eBioscience, San Diego, CA). Background signal (typically 0.05 to 0.09, defined as wells without primary antibody) was subtracted from each measured well. Patient rhAbs were defined as positive by ELISA if the average absorbance of the rhAb at 20 and 10 µg/mL was more than two standard deviations higher than the average absorbance of the healthy donor rhAbs at 20 µg/mL.

### Immunohistochemistry (IHC)

Healthy and diseased (experimental autoimmune encephalomyelitis [EAE] or stroke) mouse brains were preserved, cryosectioned, and used for immunohistochemistry as described previously [57, 86]. Protocol details are provided in the supplemental methods. Blinded experts in histology and pathology (co-authors RC, DR and AS) assessed staining of the

rhAbs. Images for publication were prepared using Zeiss ZEN lite software (Zeiss, Oberkochen, Germany).

### Immunocytochemistry

Hep2 ICC was performed with a Hep-2 Substrate Slide antinuclear antibody kit according to the manufacturer's instructions (MBL International, Woburn, MA). For SH-Sy5y staining, glass coverslips were coated with laminin, and SH-Sy5y were plated overnight. The following day, cells were fixed and blocked before adding primary rhAbs overnight. The next day, secondary antibodies were added followed by DAPI staining and imaging. Protocol details are provided in the Supplemental Methods.

### Supplemental Methods

A separate document includes detailed methodology for the following: Antibody Cloning and Production, Tissue Antigen ELISAs, Immunohistochemistry (IHC), Stained Cell Enumeration of Mouse Cortical Sections, IHC Signal Affinity Verification, Immunocytochemistry (ICC), Cell Based Assay for AQP4 Binding, Cellular Fractionation and Western Blotting, and rhAb Binding by Flow Cytometry.

## Results

### Expansion of peripheral PBs is common to patients experiencing transverse myelitis symptoms

Previously, our laboratory demonstrated that peripheral plasmablasts are expanded in clinically isolated syndrome (CIS) patients experiencing their first partial transverse myelitis (PTM) attack [58]. To ascertain the extent of this increase, we determined the frequency of CD19+CD27<sup>high</sup> plasmablasts in PBMCs from CIS-PTM patients as compared to Neuromyelitis Optica (NMO) patients and healthy donors (Fig. 1a). Plasmablasts are typically expanded in NMO, a demyelinating neurological disease where patients similarly experience PTM, but do not classically exhibit the same pattern of brain inflammation as MS patients [78]. Therefore, these patients provided a relatively homogenous comparator for our CIS-PTM cohort, rather than patients with a variety of other neurological diseases. As expected, peripheral plasmablasts were expanded in NMO patients over steady state production found in healthy donors (2.12% vs 0.71%,  $p=0.01$ ), and the frequency was similarly elevated in CIS-PTM patients (2.10% vs 0.71%,  $p=0.005$ ) (Fig.1b).

### Expanded peripheral PBs in CIS-PTM patients over-utilize VH4 antibody genes

Considering the importance of the B cell receptor in the development and function of B cells, we then investigated the antibody gene repertoire of CIS-PTM plasmablasts for features of dysregulation. B cells generate their unique antigen receptor by the processes of immunoglobulin gene segment recombination, light and heavy chain pairing, and somatic hypermutation [56]. Previous data from our laboratory [16] demonstrated that CSF B cells of CIS-PTM patients at high risk to convert to MS tend to over-utilize one family of immunoglobulin gene segments, immunoglobulin heavy chain V-region subgroup 4 (VH4). To determine whether CIS-PTM peripheral plasmablasts also tend to utilize VH4 genes, we isolated and sequenced the variable domain of immunoglobulins from individual peripheral

plasmablasts of CIS-PTM and NMO patients. Antibody repertoire data from these peripheral plasmablasts were compared to the ones deposited in published control databases of healthy total peripheral B cells [67] and flu-responding peripheral plasmablasts from otherwise healthy donors [106].

Approximately 19% of peripheral plasmablasts from healthy donors responding to influenza infection utilize VH4 genes (Fig.1c), which is a similar proportion to that of healthy donor total peripheral B cells (20%, Supplemental Fig. 1b) [37]. In contrast, 32% of peripheral plasmablasts from CIS-PTM patients utilize VH4 genes, which was statistically higher than flu-responding peripheral plasmablasts (CIS-PTM vs HD, 32% vs 19%,  $p=0.04$ ) (Fig.1c). Plasmablasts from NMO patients demonstrated a similar trend toward expansion of VH4 usage, although this did not reach statistical significance (33%,  $p=0.13$ ). When individual genes within the VH4 family are assessed, no single gene was expanded in CIS-PTM patients compared to controls, nor were there expanded genes in the other heavy chain variable domain families (Supplemental Fig. 1a). Similarly, no particular kappa light chain V gene or V gene family was overrepresented (Supplemental Fig. 2a, 2c). However, CIS-PTM plasmablasts more commonly expressed antibodies that utilized downstream light chain J gene segments in comparison to flu-responding plasmablasts (Supplemental Fig. 2d). JK5 was expanded in CIS-PTM plasmablasts in comparison to flu-responding plasmablasts (40% vs 11%,  $p=0.0065$ ), while JK1 was under-utilized (4.5% vs 38%,  $p=0.0072$ ). Common indicators of autoreactivity such as increased heavy chain CDR3 length and an over-representation of positively charged heavy chain CDR3 regions [102] were not present (Supplemental Fig. 1d, 1e). Additional antibody genetic analysis is provided in Supplemental Figures 1 and 2.

### **CIS-PTM peripheral plasmablasts bind strongly to brain antigens**

We then sought to examine whether the antibodies expressed by the peripheral plasmablasts of CIS-PTM patients are autoreactive. To do this, recombinant human antibodies (rhAbs) from 38 peripheral plasmablasts were generated from seven treatment naive CIS-PTM patients (designated as CIS with a two-digit number). 10 rhAbs from four treatment naive NMO patients were also generated (designated as NMO with a two-digit number) and 6 rhAbs from one healthy donor were used as controls (designated as HD10 with a one- or two-digit number). All of the antibodies chosen for cloning were from the two most commonly represented heavy chain V-gene families (in both healthy donors and patients), VH3 and VH4, and had significant mutation accumulation that indicated they had undergone affinity maturation (Supplemental Table 1). Of the selected CIS-PTM plasmablasts from which the isotype could be determined, 85% belonged to the IgG isotype (Supplemental Table 1).

We first tested these rhAbs for binding to brain antigens using a mouse brain lysate ELISA. The six rhAbs from healthy donor peripheral B cells were not reactive in this assay (Fig. 2). In contrast, 29% of the CIS-PTM plasmablast rhAbs (11 out of 38) were highly reactive to the brain lysate, and 22% of the NMO rhAbs (2 out of 9) were similarly reactive. Of the seven CIS-PTM patients tested, six had one or two rhAbs that demonstrated strong reactivity



to the lysate (Fig.2a), demonstrating that these antibodies can be found in many CIS-PTM patients.

The results of the mouse brain lysate ELISA were then confirmed using a commercially produced human brain lysate (Supplemental Fig. 3a, 3b). Of the 37 rhAbs from CIS-PTM patients tested in this ELISA, 19 of them (51 %) displayed positive reactivity to human brain lysate (Supplemental Fig. 3b). Nine of these bound both mouse and human brain lysate, while the remaining ten bound human brain lysate, but not mouse brain lysate. Of the nine rhAbs from the NMO patients, 3 of them (33 %) displayed positive reactivity to human brain lysate. Two of these bound both mouse and human brain lysate while one additional rhAb bound human brain lysate, but not mouse lysate (Supplemental Fig. 3b). This may be due to differences between human and mouse proteins, or it may be that the commercial human brain lysate contains a wider variety of antigens.

To investigate the specificity of the rhAbs, we tested 17 of the 19 rhAbs that were positive in the human brain lysate ELISA and 19 of the rhAbs that were negative in the human brain lysate ELISA (n = 36) for recognition of an in-house prepared mouse kidney lysate (Supplemental Fig. 3c, 3d). As expected, none of the healthy donor rhAbs bound to mouse kidney lysate. One of the NMO rhAbs displayed positive reactivity to mouse kidney lysate (Supplemental Fig. 3b). None of the brain-negative rhAbs from CIS-PTM patients (n = 19) displayed reactivity to mouse kidney lysate, but 5 of the 17 CIS-PTM rhAbs that displayed positive reactivity to brain lysate also displayed reactivity to mouse kidney lysate. Thus, 14 % of the CISPTM rhAbs (5 out of 36) and 11 % of the NMO rhAbs (1 out of 9) are polyreactive.

We also investigated 36 rhAbs from CIS-PTM patients for reactivity to whole native H1N1 influenza antigen (FLU), and ovalbumin (OVA) (Supplemental Fig. 4). Of the 12 CIS-PTM rhAbs we tested that demonstrated positive binding to human brain lysate but not kidney lysate in the ELISA, only one displayed positive reactivity to FLU and OVA (CIS59). Of the 5 CIS-PTM rhAbs we tested that demonstrated positive binding to both human brain lysate and kidney lysate in the ELISA, 3 displayed positive reactivity to FLU and OVA (CIS56, CIS57, CIS28). The remaining two polyreactive rhAbs (CIS48 and CIS42) were not reactive to either FLU or OVA. Thus, 92 % of the brain lysate specific rhAbs from CIS-PTM patients (11 out of 12) maintained their exclusivity for brain antigens while 60 % (3 out of 5) of the polyreactive rhAbs from CIS-PTM patients maintained their binding promiscuity. One of the NMO rhAbs that bound to kidney lysate also bound FLU, but none of the NMO antibodies bound to OVA.

Interestingly, when the CIS-PTM rhAbs are categorized by VH family, only VH4 utilizing rhAbs react strongly to brain lysate (Fig.3a, 3b). None of the rhAbs utilizing VH3 genes met our criteria for strong binding (Fig.3a, 3b) despite having similar mutation frequencies as the VH4+ rhAbs used in this study (Supplemental Table 1). Reactivity to brain lysate by ELISA was noted for rhAbs using six of the nine represented VH4 genes (Fig.3c), suggesting that the autoreactive plasmablast response occurs with multiple antibody genes, and possibly also many antibody specificities. Four of the nine VH4 genes were employed by rhAbs in both the positive and negative category (VH4-30, 4-39, 4-59, and 4-61), suggesting that other

factors such as light chain pairing, CDR3 length and charge, or mutation accumulation govern whether antibodies with these antibodies will be autoreactive. Two of the nine genes (VH4-b and 4-38) were exclusively represented in the rhAbs that bound to brain lysate, and three of the nine genes (VH4-4, 4-31, and 4-34) were only utilized by rhAbs that were not reactive in this assay (Fig.3c). However, it should be noted that the relative representation of antigens in the lysate might encourage false negatives.

### **CIS-PTM peripheral plasmablasts bind primarily to neurons and astrocytes**

Next, we tested the binding of CIS-PTM rhAbs to brain tissues by fluorescent immunohistochemistry. We used brain tissues from healthy, experimental autoimmune encephalomyelitis (EAE) and stroke mice, which represent normal and inflamed brain tissues (Fig. 4, Supplemental Figures 4-11). In addition to the CIS-PTM rhAbs, NMO rhAbs (n=3) and two antibodies from lupus patients (B1 as a negative control and G11 as a positive control) were utilized as controls in this part of the study [110]. Though the specificities of B1 and G11 have not been fully characterized, B1 does not bind to multiple types of brain tissue [57], NMDA receptors [19], DNA, LPS, or recombinant human insulin [110]. In contrast, G11 is a polyreactive antibody that binds to DNA [110] as well as NMDA receptors [19]. As expected, B1 showed no reactivity to these brain tissues and G11 showed positive reactivity (Fig. 4 and data not shown). We found that the two structures most commonly recognized are astrocytes in the corpus callosum (marked by expression of GFAP) and neuronal bodies in the cortex (marked by NeuN). The rhAb CIS19 is presented in Fig. 4 as an example of binding to neuronal bodies, and rhAb CIS07 is presented in Fig. 4 as an example of binding to astrocytes. Overall, the majority of highly mutated VH4 expressing CIS-PTM plasmablast rhAbs recognized both neurons and astrocytes in multiple brain tissue types (Fig.4e, Supplemental Table 1, Supplemental Fig.6-12, 14a), with a smaller portion recognizing only astrocytes (Fig.4e, Supplemental Fig. 12, 14a).

Of those rhAbs that recognized neuronal cell bodies, the majority bound to the cytoplasm in a ring-like structure around the nucleus as illustrated by rhAb CIS19 (Fig.4a-d and 4f, top row and left-hand images). When viewed in the z-direction as in Fig. 4f and Supplemental Fig. 14c, it becomes clear that the green staining of these example rhAbs does not overlap with the blue staining of DAPI in the nucleus. Of those rhAbs that recognized astrocytes, the majority bound cell processes in the corpus callosum as illustrated by rhAb CIS07 (Fig.4a-d, middle images). Additionally the rhAbs were tested for binding to oligodendrocytes, but only CIS68 displayed co-localization with the oligodendrocyte marker PDGFR (Supplemental Fig. 13). In some of the cortical images there appeared to be recognition of neuropil, the areas of unmyelinated axons, dendrites and glial cells in parts of the tissue where cell bodies are less dense (Supplemental Fig. 7, 9, 11). However, lambda scan analysis indicated that the signal of the neuropil binding was no different than background autofluorescence, and is likely a staining artifact. Additional data from lambda scans verified that the staining observed is true positive signal (Supplemental Figure 14b).

### **Enumeration of Cortical Cells Stained by rhAbs**

For the rhAbs that recognized neurons, rhAb binding was not observed in every NeuN stained neuron in the cortex. To quantify this effect we counted the number of cells stained



by a particular rhAb as compared to the cells stained by NeuN (Supplemental Fig.15). By defining regions of interest (ROIs, Supplemental Fig.15a), measuring the signal intensity in that ROI, and setting a threshold for positive staining as compared to autofluorescence, the number of cells positive for rhAb or NeuN staining was determined. These values were used to create histogram plots of maximal signal intensity in each counted ROI (Supplemental Fig.15c) and obtain the percentage of cells that were stained by only the rhAb, only NeuN or both (Supplemental Fig.15b). Each rhAb shows a slightly different binding pattern, but the majority of all counted ROIs in the cortex were double positive for NeuN and rhAb staining (range: 56%—70%, Supplemental Fig.15d). For each rhAb there was also a substantial portion of neurons that do not stain with the rhAb (range: 19—37%, Supplemental Fig.15d), indicating that these rhAbs could be specific for neuronal groups or layers.

### **Peripheral plasmablasts from CIS-PTM patients recognize cytoplasmic and nuclear targets on human neurons**

From the immunofluorescent staining of brain tissue, it appeared that the binding of CIS-PTM rhAbs was primarily located outside of the nucleus of these cells (Fig. 4, Supplemental Fig. 7, 9, 11). Indeed, orthogonal views demonstrated that rhAb binding did not overlap with the nuclear stain DAPI in cortical neurons stained by NeuN (Fig. 4f and Supplemental Fig. 14c). To further test this finding, we utilized immunocytochemistry (ICC) on a human neuroblastoma cell line (SH-Sy5y) as well as the human epithelial cell line (Hep-2) that is commonly used for detecting anti-nuclear antibodies in lupus patients (Fig.5, Supplemental Fig. 16-18) [88]. For both our CIS-PTM and NMO cohorts we found anti-nuclear rhAbs as well as rhAbs that bound outside of the nucleus of these cell lines (Fig.5a). As expected, negative control antibody B1 does not bind to the fixed and permeabilized SH-Sy5y cells (Fig. 5a) while G11, the positive control antibody, demonstrates strong binding across the entire body of the cell. Among 38 CIS-PTM rhAbs, 21 (55%) bound SH-Sy5y, including 10 of the 11 CIS-PTM rhAbs that demonstrated strong binding by the brain lysate ELISA. Of these 21 CIS-PTM rhAbs that bound to targets outside the nuclei of these cells, only one bound to nuclear targets alone (Fig. 5b, Supplemental Fig. 16, 17). Of the 10 NMO rhAbs, 3 of them bound SH-Sy5y; one to nuclear targets alone and two to the cytoplasm. Only one of 6 healthy donor rhAbs showed cytoplasmic staining of SH-Sy5y (Supplemental Fig. 17), and none displayed positive binding of Hep-2 cells (Supplemental Fig. 18). One NMO plasmablast rhAb and 7 (20%) of the CIS-PTM plasmablast rhAbs bound the Hep-2 cells, but again most were not anti-nuclear in nature (Supplemental Fig. 18). Performing ICC on both SH-Sy5y and Hep-2 also permitted us to evaluate polyreactivity by this method. In so doing, we observed that 7 of the 21 CIS-PTM rhAbs bound Hep-2 cells (4 to cytoplasmic targets and 3 to nuclear targets) and were thus polyreactive (SH-Sy5y+Hep-2+). Of the 20 CIS-PTM rhAbs that displayed cytoplasmic binding to SH-Sy5y by ICC, 4 of them (20%) were polyreactive to cytoplasmic targets only (SH-Sy5y<sub>cytoplasmic</sub>+Hep-2<sub>cytoplasmic</sub>). since they also displayed cytoplasmic binding to Hep-2 by ICC.

To confirm the binding observed here, a western blot was run with select rhAbs with cellular fractionated lysate made from SH-Sy5y and Hep-2 cells (Supplemental Fig. 19). The positive control antibody G11 bound both the cytosol and nuclear/membrane fraction of both SH-Sy5y and Hep-2 cells, as was expected. The negative control rhAb R1 did not bind the

lysates, as expected, as well as CIS05 which did not bind these cells in any other test. The only CIS-PTM rhAb that showed nuclear specificity by ICC, CIS10, also showed specificity for nuclear antigens by western blot. RhAbs CIS34, CIS42, and CIS46 bound both the cytoplasmic and the nuclear/membrane fractions of both SH-Sy5y and Hep-2 cells. These rhAbs may bind extracellular proteins, which would be included in the nuclear fraction cell lysate, or may bind some non-physiological linearized epitopes. NMO06, though reactive to SH-Sy5y by ICC, did not bind to the lysates, possibly due to the loss of conformational epitopes. Even so, the cytosolic CIS-PTM rhAb binding seen by ICC mirrors the previous result of cytosolic recognition of cortical neurons in mouse brain tissue (Fig. 4, Supplemental Fig. 14c), confirming the peripheral plasmablast response toward neuronal antigens in CIS-PTM patients.

Autoantibodies to AQP4 are detected in ~75% of NMO patients [11, 109]. By standard clinical testing, all of the untreated NMO patients from whom rhAbs were cloned were positive for serum AQP4 antibodies (Table 1). However, none of the peripheral plasmablast rhAbs bound to AQP4 when tested on a transfected cell line (Supplemental Fig. 20). The expected frequency of AQP4 binding by peripheral B cells of NMO patients is unknown, but in the CSF, about half of clonally expanded B cells bind to AQP4 [10, 53]. This same frequency was observed in our two CSF rhAbs (R1 and R2) cloned from a CIS-PTM patient who later converted to NMO. AQP4 binding antibodies can utilize VH4, VH3, and VH2 family genes [11], so a larger study is needed to identify B cells that express AQP4 binding antibodies in the periphery of NMO patients.

### **CIS-PTM peripheral plasmablasts bind intracellular and extracellular antigens**

The ICC methodology included the use of Triton-X, which could interfere with conformational epitopes of intracellular antigens. Thus, to evaluate whether the intracellular staining observed by ICC was towards conformational epitopes, we performed intracellular and cell surface staining of the human neuroblastoma cell line SH-Sy5y with select rhAbs and acquired data by flow cytometry. We chose to test one rhAb from each patient in the CIS-PTM cohort that displayed strong binding by ICC. As shown in Supplemental Fig. 21a, all 6 of the HD rhAbs, the CIS05 rhAb, and the negative control rhAb B1 showed no reactivity to intracellular and intact conformational antigens. In contrast, CIS19, CIS28, CIS42, CIS56 and the positive control rhAb G11 displayed reactivity to intracellular intact conformational antigens, while CIS49, CIS68 and CIS71 did not. Of these 7 CIS-PTM rhAbs that displayed strong binding by ICC, only one polyreactive rhAb (CIS56) showed reactivity to the cell surface of the SH-Sy5y neuroblastoma cell line (Supplemental Fig. 21b). A scatter plot of the geometric MFIs for both the intracellular and cell surface staining are presented in Supplemental Fig. 21c.

### **CIS-PTM plasmablast antibodies are well represented in the plasma pool**

Even when expanded, plasmablasts are only a small portion of the peripheral B cell pool, and thus may have limited impact on the ongoing auto-reactivity associated with MS. To assess whether plasmablast expansion and detection of autoreactive antibodies in the blood occurred in the same patients, we tested plasma samples from 16 treatment naïve CIS-PTM patients, 7 NMO patients, and 8 healthy donors with plasmablasts responding to recent

influenza vaccination for reactivity to brain lysate by ELISA (Fig.6). For comparison, the plasma antibodies were also tested for binding to native influenza antigen. Plasma from 7 of the 16 treatment naïve CIS-PTM patients displayed antibody reactivity to the brain at least 2 standard deviations above the mean of healthy donors at 20 µg/mL (Fig.6b). Of the 7 CIS-PTM patients whose plasma displayed antibody reactivity to the brain lysate, 4 of them were CIS-PTM patients from which autoreactive rhAbs from single peripheral plasmablasts were identified (Table 1, Supplemental Table 1). Only one NMO patient's plasma displayed similar reactivity, and notably we were able to clone plasmablast rhAbs from this patient with high affinity for brain lysate (Supplemental Table 1, Fig. 2). Long-lived plasma cells in the bone marrow contribute substantially to the plasma antibody pools, and in the case of MS patients, modest affinity of the plasma antibody pools towards brain lysate may be due to a low frequency of autoreactive long-lived plasma cells in the bone marrow. Conceivably, plasmablasts in the CSF could also contribute to this reactivity as well. However, the fact that this affinity is modest may also be due to under-representation of antigens in the whole brain lysate pool. Thus, we also tested for binding to whole cell lysate made from the SH-Sy5y human neuroblastoma cells, since the majority of the autoreactive plasmablast rhAbs bind neurons. Here, 10 of the 16 CIS-PTM patients display plasma antibody reactivity at least 2 standard deviations above the mean of healthy donors at 20 µg/mL, with an apparent increase in affinity.

## Discussion

In this study, we discovered that expanded and highly mutated VH4+ peripheral plasmablasts from CIS-PTM patients experiencing their first documented clinical attack express antibodies that bind neurons and astrocytes. We and others previously demonstrated plasmablast expansion in the CSF is a common feature to many CIS-PTM, NMO and MS patients [18, 20, 58]. Here we extend that observation to demonstrate that the frequency of peripheral plasmablasts in our CIS-PTM cohort was similar to that of NMO patients, a CNS autoimmune disease in which patients also experience PTM, but classically without brain inflammation [78]. Peripheral plasmablast expansion is not a common feature reported by others, although our cohort displayed a high occurrence of plasmablast expansion in the periphery. This discrepancy may be partially explained by heterogeneity in the study group of the previous reports, as patients with both partial transverse myelitis (PTM) and optic neuritis (ON) symptoms are included, as well as patients at various stages of disease. Our study focuses only on patients experiencing their first clinical attack of PTM because we previously determined these patients are more likely to have a plasmablast expansion [58]. Interestingly, the four patients in our previous study who later displayed additional MRI activity had elevated frequencies of CD27<sup>high</sup> plasmablasts at the time of their initial clinical event, which recapitulated the finding that plasmablast frequency correlates with parenchymal inflammatory disease activity [18].

The antibody genetics of a B cell population can have a profound impact on our understanding of disease, as the development and function of a B cell is dependent on the antibody it expresses [14, 17, 64, 65, 70, 102]. In fact, antibody genetic studies have led to several key discoveries in MS that show expansion of particular genes, excessive receptor editing, dysregulation in B cell selection [6, 16, 22, 23, 37, 38, 54, 58, 67, 75-77, 79, 80, 83,

84, 96-98, 105], and even a mutational biomarker that identifies patients who will convert to MS with 86-92% accuracy [16, 84, 85]. Here, we demonstrate that VH4 genes are used more extensively by peripheral plasmablasts from CIS-PTM patients in comparison to a previously published healthy donor plasmablasts responding to influenza infection (Fig. 1b). However, the specific genes within the VH4 family were utilized at frequencies similar to these two control populations. This indicates that most VH4 family genes are slightly over-utilized in CIS-PTM patients, rather than particular VH4 genes driving the over-representation of the entire family. This data also suggests that VH4 expansion may be a generalized feature of patients with CNS diseases who experience PTM symptoms. Others have demonstrated VH4 family expansion in the CSF of MS patients [9, 76], which may suggest that VH4 expansion is an early and prolonged feature of particular CNS diseases. Interestingly, B cells from the CSF of NMO patients are dominated by an expansion of VH2 genes, and rarely VH4 genes [11].

In this study, we demonstrate that the expansion of VH4 utilization in peripheral plasmablasts translates to an increase in autoreactivity toward brain antigens. We used a brain lysate ELISA to initially screen rhAbs cloned from plasmablasts for binding to brain targets, but discovered that this assay could lead to false negatives. For example, several rhAbs that were not reactive in the brain lysate ELISA exhibited binding to neuronal cell bodies and astrocyte processes in the brain (Supplemental Table 1). However, it should be noted that the lysate preparation consists mainly of cytosolic and easily soluble proteins, such as the abundant myelin proteins [36]. Thus, the probability of identifying primary targets under-represented in the brain lysate such as non-myelin and hydrophobic targets is diminished. Indeed, more than half of all the CIS-PTM rhAbs displayed positive binding by the SH-Sy5y cell-based assays (Fig. 5, Supplemental Figs. 16, 17), while only a third displayed positive reactivity by SH-Sy5y lysate ELISA (Supplemental Fig. 5). Additionally many antibodies recognize conformational epitopes, and the degree to which a technique may alter these epitopes can profoundly affect the outcome. For example, tissue staining done with MS CSF rhAbs on formalin fixed paraffin embedded brain tissue yielded almost no positive staining [74], but positive staining is detected with similar rhAbs when tested on paraformaldehyde fixed and gently unmasked tissue [12, 57]. However this unmasking may denature some conformational epitopes. For these reasons, we agree that a multi-tiered pipeline for characterizing the autoreactive potential of antibodies is necessary, as suggested by others [109].

Autoantibodies to extracellular neuronal antigens are known to contribute to cognitive dysfunction in a variety of CNS disorders, including nerve hyper-excitability, limbic encephalitis, encephalopathy, and autoimmune disorders with neurological involvement such as systemic lupus erythematosus (SLE) and myasthenia gravis [42, 46, 47, 52, 72, 95]. Others have identified a wide variety of neuron, astrocyte and oligodendrocyte B cell autoantigens in MS that are expressed both extracellularly and intracellularly [4, 5, 15, 21, 27-29, 31, 33, 62, 68, 73, 87, 90-92, 94, 99, 103]. However, the majority of the autoreactive CIS-PTM plasmablast antibodies in our study recognized intracellular antigens expressed by neurons. Antibody recognition of intracellular antigens is only beginning to be understood as a means to drive autoimmunity [49, 59, 81, 89, 100, 107]. Most studies in SLE have focused on anti-nuclear antibodies since their presence correlates well with exacerbation of disease

[81]. One leading thought is that anti-nuclear autoantibodies can enter the cell and exert cytotoxic effect there [49, 107]. Perhaps a similar effect could be expected from antibodies that bind cytoplasmic proteins in MS, although there are certainly other scenarios that should be considered [104]. As evidenced with orthogonal images, binding of our CIS-PTM plasmablast rhAbs within the cell was largely excluded from the nucleus.

Of note, many of our rhAbs were polyreactive and recognized targets independent of tissue origin. Recent work by others [13] demonstrated that antibodies contributing to the oligoclonal banding observed in MS patients are directed against ubiquitous intracellular proteins. However, eleven of the rhAbs we tested are brain-specific by ELISA (Brain+Kidney-Flu-OVA-) and fourteen of the rhAbs we tested are brain-specific by ICC (Sy5y+Hep-2-). Of the three NMO rhAbs we tested, only NMO03 was brain specific by ICC (Sy5y+Hep-2-). Future experiments on the ability of polyreactive and intracellular brain specific binding CIS-PTM plasmablast rhAbs to participate in a pathogenic response against primary human cells are needed to determine the role of these antibodies in MS.

When rhAb reactivity is considered in aggregate (Supplemental Table 1), it is interesting to note that while the CSF-derived rhAbs from these patients were largely reactive to neurons and astrocytes in the gray matter of the brain [57], many of the rhAbs generated from the peripheral plasmablasts were directed towards both gray and white matter targets. These data suggest that peripheral plasmablasts have a wider array of autoreactive specificities compared to CSF-derived B cells, although their specificity and significance to MS pathology remains unknown. Nevertheless, this scenario is consistent with an underlying dysregulation in tolerance of peripheral B cells in these CIS-PTM patients, and indeed others have demonstrated that there is a break in the peripheral tolerance checkpoint in MS patients [51]. The exact mechanism of CNS-reactive effector B cell development in the blood is still unknown, particularly as it relates to the importance of specific antigens driving the autoreactive plasmablast expansion. Indeed, this break in tolerance could involve both B cell intrinsic and extrinsic mechanisms. Furthermore the rapid return of memory B cells in the periphery following B cell depletion is a strong indicator of poor response to therapy [1], and further emphasizes the importance of studying the development of autoreactive B cells in the blood.

In MS patients, the blood brain barrier is often altered [63], allowing increased exchange of antigen-stimulated cells between the CNS and periphery [45, 66]. Although in our studies we did not detect clonal relatedness between peripheral blood and CSF B cells, others have found clonal overlap between these compartments in MS [8, 98]. Thus, one might predict that, as we observed, peripheral plasmablasts display autoreactivity towards CNS antigens. In MS and CIS patients, there may be an underlying open access to gray matter targets throughout the disease course, and access to white matter targets primarily during distinct points in time. It is also possible that there is less reactivity to white matter targets at later stages of disease due to immune response exhaustion to those targets [2, 82]. Alternatively, gray matter targets may be more immunogenic, considering the more extensive clonal expansion of CSF B cells that bind to these targets as compared to the peripheral plasmablasts [58, 75, 98]. Interestingly, others have demonstrated that antibodies targeting neurons from clonally expanded CSF B cells from MS patients mediate demyelination of

axons *in vitro*, highlighting their potential to participate in the pathogenesis of disease [12]. Delineating the pathway by which autoreactive plasmablasts develop, persist and mediate pathogenesis in MS patients will greatly improve our understanding of the disease, and is particularly important given that the frequency of plasmablasts increases the longer that CIS-PTM patients are left untreated [58]. Of note, clonal overlap between the CSF and periphery has also been observed in NMO patients [20, 53], suggesting CNS autoreactivity should be evident among peripheral antigen experienced B cells from NMO patients. However, we observed that CNS autoreactivity, including AQP4, among the peripheral plasmablast rhAbs is less extensive in NMO patients. Although other studies demonstrated that approximately 50 % of all clonally expanded CSF B cells from NMO patients bind AQP4 (range 3–97 %) [11, 53], the frequency of AQP4 binding by peripheral B cells from NMO patients is lower (19 % on average, range 0–40 %) [53]. Our data would support the concept that AQP4-reactive B cells may be enriched in the CNS of NMO patients rather than the periphery. Indeed, others have demonstrated that NMO plasmablasts express higher levels of the chemokine receptor CXCR3 during relapse, which may aid in retention of affinity-matured clones in the CNS [20] and subsequent lack of detection in the periphery. Further studies are warranted to investigate this discordance in CNS autoreactivity by peripheral plasmablasts of PTM and NMO patients.

In summary, plasmablasts may be either direct perpetrators of autoimmunity or simply biomarkers of disease severity in MS [18], but they are certainly the footprints of previously activated B cells. The increased representation of VH4-utilizing autoreactive plasmablasts in the periphery of CIS-PTM patients demonstrates a B cell autoimmune response directed toward neurons and astrocytes early in disease. Their presence in the periphery proposes intriguing questions about the origin and function of autoreactive plasmablasts in CIS-PTM patients, and provides a unique avenue to explore the autoreactive B cell response in MS.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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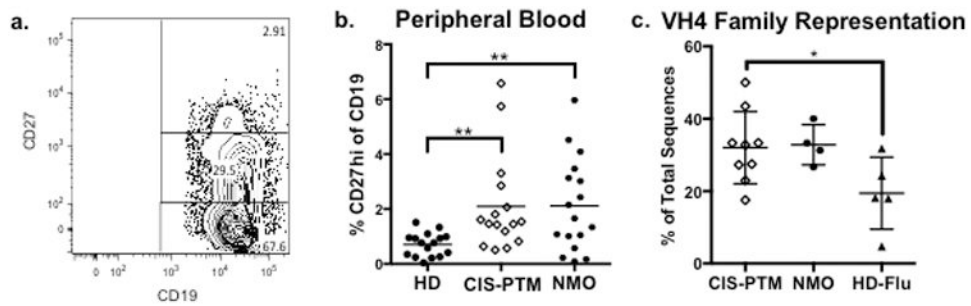
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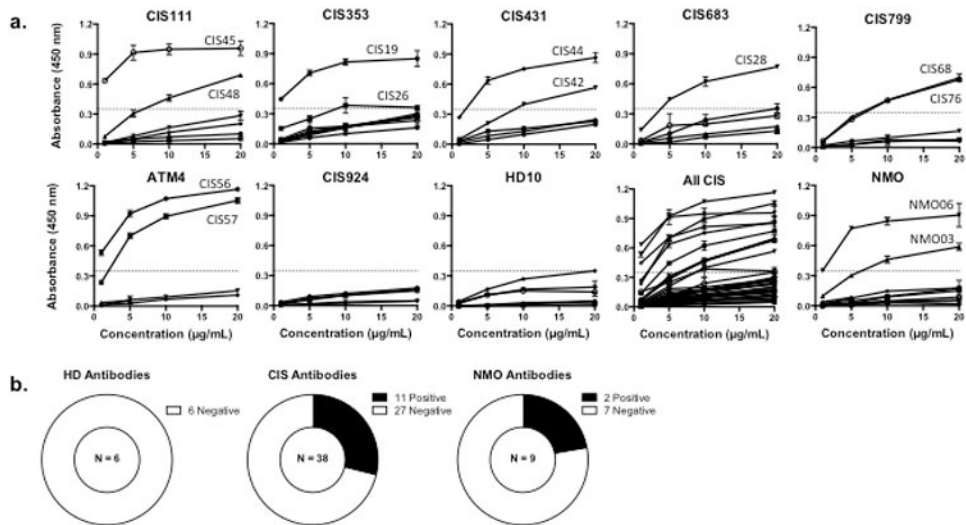
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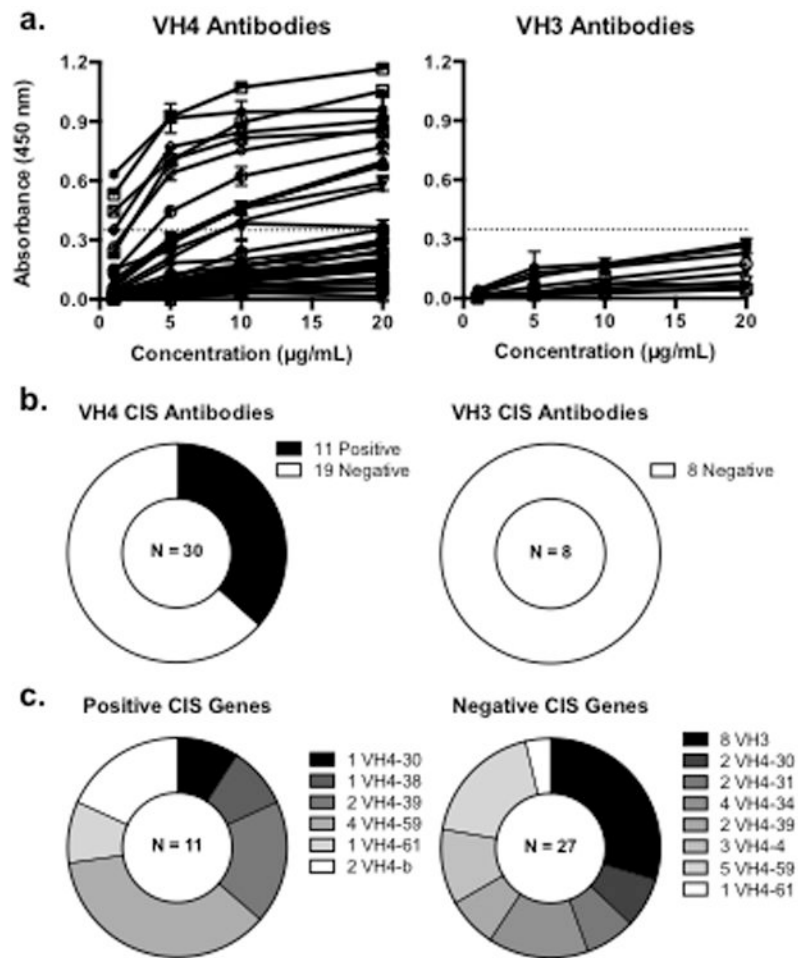
**Fig. 1. plasmablast expansion and genetics**

(a.) Representative flow cytometry plot for detecting CD19+CD27<sup>high</sup> plasmablasts from the peripheral blood of a CIS-PTM patient. (b.) Percentage of plasmablasts (CD19+ CD27<sup>high</sup>) of CD19+ B cells in blood samples from patient groups as measured by flow cytometry. Levels are elevated for CIS-PTM patients as compared to healthy donors, much like that of NMO patients. (c.) Representation of VH4 family genes in single cell PCR plasmablast repertoires of CIS-PTM and NMO patients as compared to plasmablasts responding to influenza infection in otherwise healthy donors. VH4 family gene usage is increased in CIS-PTM patients as compared to plasmablasts responding to influenza infection. NMO patients have a similar trend toward VH4 expansion, although it did not reach statistical significance. \*p<0.05, \*\*p<0.01



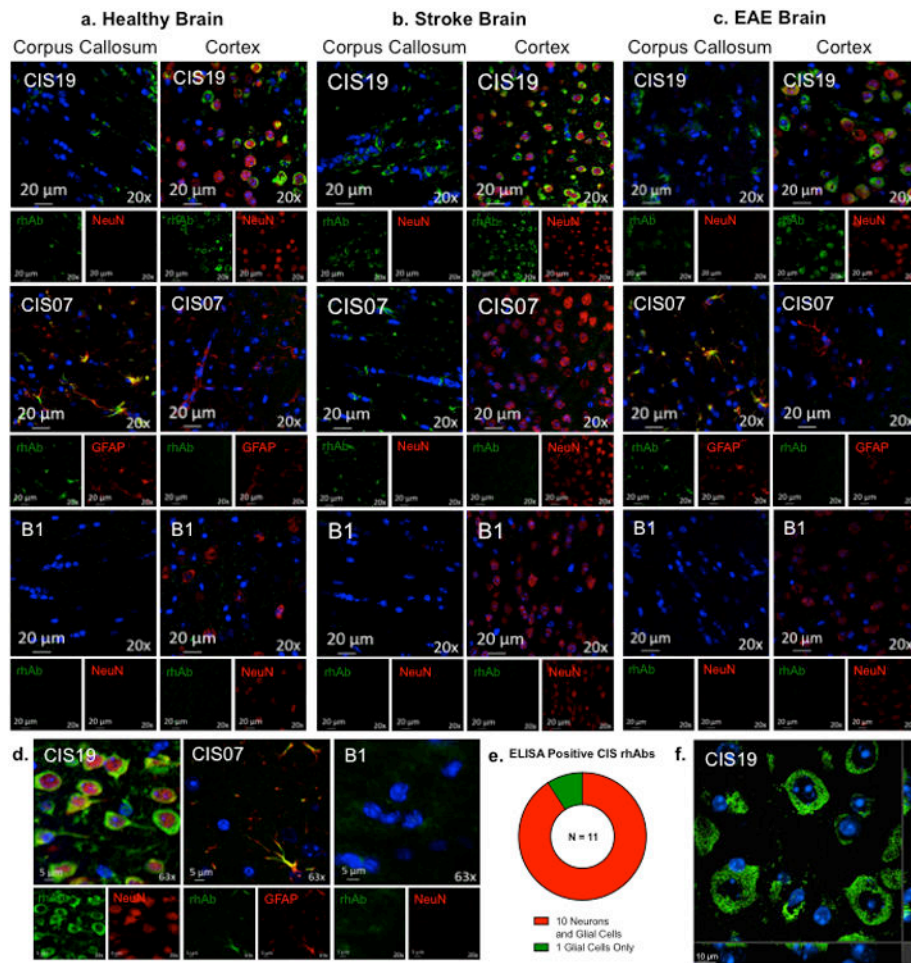
**Fig. 2. Brain lysate reactivity of plasmablast antibodies by ELISA**

(a.) Brain ELISA results grouped by CIS-PTM patient, then by patient diagnosis (“All CIS” or “NMO”). Six of the seven CIS-PTM patients each contained two highly reactive rhAbs (33-50%), meaning the  $OD_{450}$  of the rhAb at 20 and 10  $\mu\text{g}/\text{mL}$  was more than 2 standard deviations above the mean  $OD_{450}$  of healthy donor (HD) rhAbs at 20  $\mu\text{g}/\text{mL}$ . The names of the positive rhAbs are designated for each titration curve. A dashed line in each graph represents the threshold for positive binding. (b.) Summary of ELISA results grouped by patient type. 11 of the 38 CIS-PTM rhAbs and 2 of the 9 NMO rhAbs bound strongly to the brain lysate.



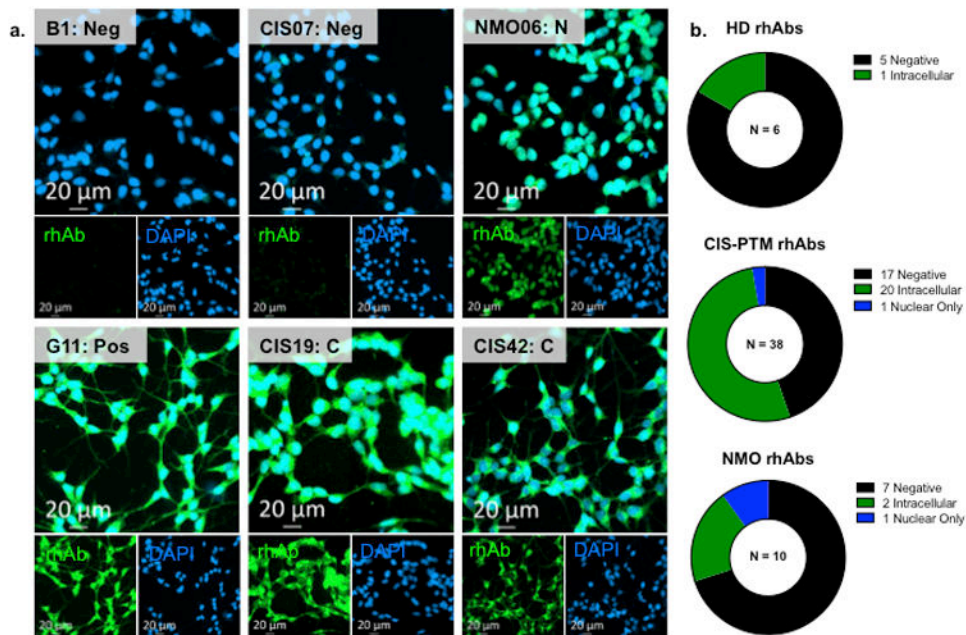
**Fig. 3. Comparison of VH4 and VH3 rhAbs**

(a.) Brain ELISA results for CIS-PTM rhAbs grouped by heavy chain V gene family. A dashed line in each graph represents the threshold for positive binding. (b.) Summary of ELISA results for CIS-PTM rhAbs grouped by heavy chain V gene family. All VH3 rhAbs showed little reactivity of brain lysate, but many (37%) of the VH4 family rhAbs bound strongly to the lysate. (c.) V gene segments utilized by rhAbs that did and did not recognize brain lysate. Only VH4 family genes recognized whole brain lysate above the level of healthy donor antibodies, but only two of the VH4 genes were specifically found in the positive group. Four of the VH4 family genes represented were utilized by rhAbs in both the positive and negative group, and three others were used only by rhAbs that did not bind brain lysate.



**Fig. 4. Plasmablast rhAbs react to neurons and glial cells in mouse brain**

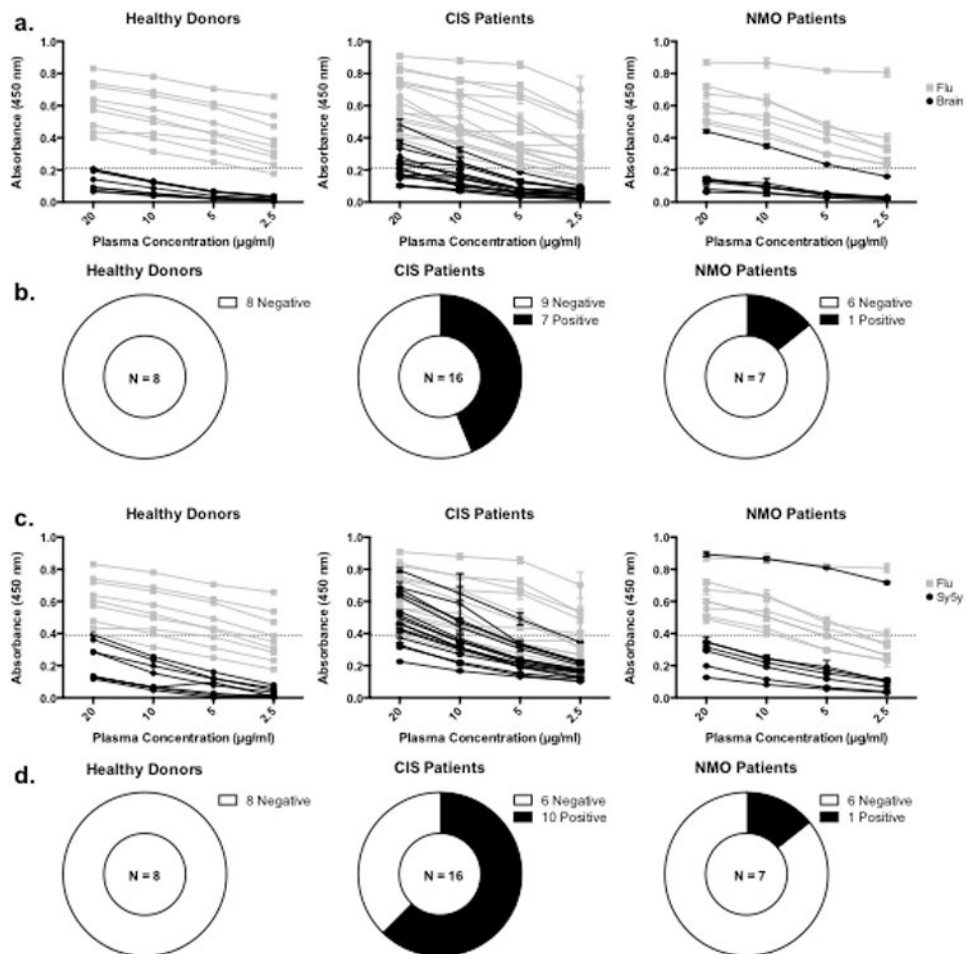
Example 20 $\times$  images of example CIS-PTM rhAbs CIS19 and CIS07 binding neurons and astrocytes in the white (corpus callosum, left) and gray (cortex, right) matter of healthy mouse brain (a.), stroke brain (b.) and EAE brain (c.) compared to the negative control antibody B1. Remaining images are in Supplemental Figures 6-13. RhAb staining is shown in green, DAPI is shown in blue, and NeuN is shown in red. Scale bar represents 20  $\mu$ m. (d.) 63 $\times$  images of example rhAbs CIS19 and CIS07 compared to the negative control antibody B1. RhAbs that recognized neurons did so in a ring-like pattern around the nucleus (as demonstrated by CIS19) and rhAbs that recognized astrocytes bound to cell body processes (as demonstrated by CIS07). Scale bar represents 5  $\mu$ m. (e.) Of the 11 CIS-PTM rhAbs that were positive by mouse brain ELISA, one recognized exclusively glia and the remaining ten recognized both neurons and astrocytes. (f.) Orthogonal view of CIS19 staining in the cortex of healthy mouse brain, with only rhAb and DAPI overlay shown. Scale bar represents 10  $\mu$ m.



**Fig.5. Reactivity to Sy5y by ICC**

Immunocytochemistry (ICC) of rhAbs on the human neuroblastoma line, SH-Sy5y. (a.) Representative images of CIS-PTM rhAbs, one NMO rhAb and the control rhAbs B1 and G11 not binding or binding to SH-Sy5y neurons by ICC. B1 is an isotype negative control, and G11 is a positive control. Remaining images are in Supplemental Figs. 16 and 17. (b.) Summary of ICC staining of rhAbs on SH-Sy5y cells. Most healthy antibodies did not recognize SH-Sy5y, while over half of the CIS-PTM rhAbs and almost one third of NMO rhAbs bound to this cell line. Intracellular binding that included more than just the nucleus was most common, while a subset of rhAbs demonstrated nuclear binding.





**Fig.6. ELISAs with patient plasma**

ELISA results with plasma taken from 8 healthy donors responding to influenza vaccination, 16 treatment naïve CIS-PTM patients, and 7 NMO patients. (a.) Absorbance data from plasma ELISAs on brain lysate and influenza antigen grouped by patient classification. Each black line represents ELISA data from an individual plasma antibody sample tested on brain lysate while each gray line represents ELISA data from an individual plasma antibody sample tested on influenza antigen. The dashed line on each graph represents the cutoff for positive binding (the average absorbance from healthy donor plasma on brain lysate at 20 µg/mL plus two standard deviations). (b.) Summary of brain lysate ELISAs with plasma antibodies. Plasma antibody samples from 1 NMO and 7 CIS-PTM patients were two standard deviations above the mean of healthy plasma antibody samples at 20 µg/mL. (c.) Absorbance data from plasma ELISAs on SH-Sy5y lysate and influenza antigen grouped by patient classification. Data are presented as in a, except that SH-Sy5y data is displayed in black. (d.) Summary of SH-Sy5y lysate ELISAs with plasma antibodies. Plasma antibody samples from 1 NMO and 10 CIS-PTM patients were two standard deviations above the mean of healthy plasma samples at 20 µg/mL.



Patient information. Patients are grouped by diagnosis and whether they were further investigated by genetic analysis. Final columns list results of plasma ELISAs (Fig.6). Patients who were included in previous studies are denoted by <sup>a</sup>, <sup>b</sup>, or <sup>c</sup>. PB: plasmablast, CBA: cell based assay for aquaporin-4 reactivity, AZT: azathioprine

**Table 1**

Patient	Sex	Age	Diagnosis at Draw	Current Diagnosis	Relapses	MRI Changes	Treatment at Draw	Current Treatment	Percent of PBs in Blood	Percent of PBs in CSF	PBs Sorted from Blood	Number of Productive PB Sequences	Plasma Brain ELISA	Plasma Sy5y ELISA
CIS Patients Included in Genetic Analysis														
CIS924 <sup>a</sup>	M	69	TM	TM	0	0	None	None	7.1	33.3	144	47	+	+
CIS799 <sup>a</sup>	F	28	CIS	RRMS	0	0	None	Avonex	5.74	13.3	190	68	+	+
CIS991 <sup>a</sup>	F	34	CIS	TM	0	0	None	steroids	3.3	15.4	73 <sup>d</sup>	25	+	+
CIS111 <sup>abc</sup>	F	62	CIS	PPMS	0	0	None	Avonex	3.04	5.77	190 <sup>d</sup>	58	-	+
CIS663 <sup>a</sup>	F	32	TM	TM	0	0	None	None	2.85	10.8	192	121	-	+
CIS431 <sup>abc</sup>	F	27	CIS	RRMS	1	1	None	Giftanya	2.07	35.9	190 <sup>d</sup>	81	+	+
ATM4	M	24	CIS	RRMS	0	0	Prednisone	None	1.54	16.1	176	28	+	-
CIS353 <sup>abc</sup>	F	58	CIS	RRMS	1	1	None	Copaxone	1.46	11.4	190 <sup>d</sup>	51	+	+
CIS683 <sup>abc</sup>	F	39	CIS	RRMS	0	0	None	Tecfidera	0.82	26.6	190 <sup>d</sup>	63	-	-
CIS Patients Not Included in Genetic Analysis														
CIS287 <sup>bc</sup>	F	45	TM	TM	0	0	None	Betaseron	6.58	37.9			-	-
CIS873 <sup>dc</sup>	F	19	RRMS	RRMS	0	0	None	Avonex	2.98	26			+	+
CIS527 <sup>dc</sup>	F	43	CIS	RRMS	0	0	None	Copaxone	1.81	14.1			-	-
CIS699 <sup>a</sup>	F	37	CIS	RRMS	0	0	None	Avonex	1.61	7.87			-	+
CIS787 <sup>dc</sup>	M	33	CIS	RRMS	0	0	None	Copaxone	1.45	36.9			-	+
CIS251 <sup>dc</sup>	F	53	TM	Sarcoidosis	0	0	None	None	1.43	2.57			-	-
CIS942 <sup>a</sup>	F	52	CIS	CIS	0	0	None	Copaxone	1.19	12.2			-	-
CIS528 <sup>dc</sup>	M	32	CIS	RRMS	0	0	None	Avonex	0.64	2.41			-	-
CIS371 <sup>dc</sup>	F	56	CIS	CIS	0	0	None	Copaxone	0.51	13.9			-	-
NMO Patients in Genetic Analysis														
NM0.1	F	55	NMO	NMO	+	+	None	Cellcept	3.47	n/a	95	31	+	+
NM0.2	F	36	NMO	NMO	+	+	None	None	0.22	n/a	83	15		

Patient	Sex	Age	Diagnosis at Draw	Current Diagnosis	Relapses	MRI Changes	Treatment at Draw	Current Treatment	Percent of PBs in Blood	Percent of PBs in CSF	PBs Sorted from Blood	Number of Productive PB Sequences	Plasma Brain ELISA	Plasma Sysy ELISA	
NM0.7	F	54	NMO	NMO	+	CBA	None	Cellcept	4.09	n/a	94	32	-	-	
NM0.8	F	64	NMO	NMO	+	CBA	None	Rituxan	2.43	n/a	94	25	-	-	
NMO Patients Not Included in Genetic Analysis															
NM0.3	F	39	NMO	NMO	-	CBA	Cellcept	Cellcept	1.05	n/a	-	-	-	-	
NM0.4	F	61	NMO	NMO	+	ELISA	Cellcept	Cellcept	0.09	n/a	-	-	-	-	
NM0.5	F	41	NMO	NMO	+	ELISA	Cellcept	Rituxan	1.08	n/a	-	-	-	-	
NM0.6	M	47	NMO	NMO	-	CBA	Cellcept	Rituxan	1.01	n/a	-	-	-	-	
NM0.9	F	53	NMO	NMO	-	CBA	Cellcept	Cellcept	1.34	n/a	-	-	-	-	
NM0.10	F	61	NMO	NMO	+	ELISA	Cellcept	Cellcept	0.17	n/a	-	-	-	-	
NM0.31	F	46	NMO	NMO	+	Unknown	AZT	Unknown	0	n/a	-	-	-	-	
NM0.33	M	38	NMO	NMO	+	Unknown	Cellcept	Unknown	5.97	n/a	-	-	-	-	
NM0.70	F	45	NMO	NMO	+	Unknown	AZT	Unknown	4.52	n/a	-	-	-	-	
NM0.260	F	47	NMO	NMO	-	Unknown	Cellcept	Unknown	3.02	n/a	-	-	-	-	
NM0.626	M	36	NMO	NMO	-	Unknown	AZT	Unknown	2.15	n/a	-	-	-	-	
NM0.740	F	29	NMO	NMO	-	Unknown	AZT	Unknown	3.13	n/a	-	-	-	-	
NM0.745	F	50	NMO	NMO	Unknown	Unknown	Cellcept	Unknown	1.66	n/a	-	-	-	-	

<sup>a</sup> CSF and peripheral B cells previously studied by flow cytometry.

<sup>b</sup> Peripheral B cells previously studied by genetic analysis.

<sup>c</sup> CSF B cell previously studied by genetic analysis.

<sup>d</sup> Memory B cells also sorted (productive/total sorted): CIS991: (49/95) CIS111: (14/94) CIS431: (71/188) CIS353: (54/190) CIS683: (61/188)