Title

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VH2+ antigen-experienced B cells in the CSF are expanded and enriched in pediatric anti-NMDA receptor encephalitis

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Abstract

Pediatric and adult autoimmune encephalitis (AE) are often associated with antibodies to the NR1 subunit of the N-methyl-D-aspartate receptor (NMDAR). Very little is known regarding the cerebrospinal fluid (CSF) humoral immune profile and antibody genetics associated with pediatric αNMDAR-AE. Using a combination of cellular, molecular and immunogenetics tools, we collected CSF from pediatric subjects and generated 1) flow cytometry data to calculate the frequency of B cell subtypes in the CSF of pediatric subjects with αNMDAR-AE and controls, 2) a panel of recombinant human antibodies from a pediatric case of αNMDAR-AE that was refractory to treatment and 3) a detailed analysis of the antibody genes that bound the NR1 subunit of the NMDAR. Antigen-experienced B cells including memory, plasmablast and antibody-secreting cells were expanded in the pediatric αNMDAR-AE cohort, but not in the controls. These antigen-experienced B cells in the CSF of a pediatric case of NMDAR-AE that was refractory to treatment had expanded use of VH2 heavy chain genes with high somatic hypermutation that all bound to the NR1 subunit of the NMDAR. A CDR3 motif was identified in this refractory case that likely drove early-stage activation and expansion of naïve B cells to antibody-secreting cells, facilitating autoimmunity associated with pediatric αNMDAR-AE through the production of antibodies that bind NR1. These features of humoral immune responses in the CSF of pediatric αNMDAR-AE patients may be relevant for clinical diagnosis and treatment.

INTRODUCTION

Encephalitis is a potentially devastating inflammatory disease of the brain caused by infection or autoimmune conditions.(1–4) The most common form of autoimmune

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encephalitis (AE) in adults and children is associated with antibodies against *N*-methyl-D-aspartate glutamate receptors (NMDARs), termed αNMDAR-AE (anti-NMDAR-autoimmune encephalitis).(5, 6) The NR1 subunit of the NMDAR (also known as GluN1) is the primary target of antibodies against the NMDAR.(7–9) Anti-NMDAR antibodies can be detected in the serum and CSF, albeit misdiagnosis based in part on overinterpretation of serum positivity has been reported.(10) Prognosis in αNMDAR-AE is dependent on early recognition, prompt reduction of αNMDAR antibodies, and removal of associated tumors (when identified).(11, 12) Even with these treatments, relapses occur in up to 25% of patients.(13, 14) and outcomes range from full/partial recovery to death.(15, 16)

IgG purified from CSF of adult and pediatric αNMDAR-AE patients(17) and monoclonal αNMDAR antibodies expressed by B cells from the cerebrospinal fluid of adult NMDAR-AE patients(18, 19) bind, cross-link, and internalize the NR1 subunit of the NMDAR, leading to altered neuronal synaptic function. In a separate case, a human monoclonal antibody cloned from an 18-year old patient with αNMDAR-AE was shown to rapidly localize to synaptic NMDAR's of primary rat neurons but did not alter synaptic calcium flux.(20) The mechanism of αNMDAR antibody emergence has recently been clarified with evidence that the NR1 subunit of the NMDAR is present in germinal centers of patients with αNMDAR-AE.(21, 22) In addition, cervical lymph nodes and ovarian teratomas in patients with NMDAR-AE harbor both the NR1 protein and germinal centers where B cells are activated to produce IgA and IgG antibody against the NR1 subunit of the NMDAR.(21) This data substantiates the observation of B cell removal as an effective second-line treatment for pediatric αNMDAR-AE(23) and αNMDAR-AE in general.(24, 25)

There have been no focused studies to understand humoral immune response and associated antibody genetics among pediatric αNMDAR-AE patients. Thus, our lab focused on profiling the humoral immune response to NR1, the obligatory subunit of NMDA receptors in pediatric αNMDAR-AE patients and pediatric OND controls.(26) To do this, we used flow cytometry to determine the frequency of B cell subsets including ASCs in the CSF of pediatric patients diagnosed with αNMDAR-AE and pediatric OND controls. Next, we used electrophysiology to evaluate whether antibody pools from a pediatric patient with severe αNMDAR-AE impacted neuron function. Finally, we isolated ASCs and used single cell PCR technology to determine the frequency of CSF derived B cells expressing antibodies that bind to the NR1 subunit of the NMDA receptor. Profiling the humoral immune response could aid in our understanding of why attacks on the nervous system occur among pediatric patients diagnosed αNMDAR-AE. Study samples came from multiple pediatric patients, including one severe case that resulted in death. This patient's sample was included to understand the immunologic profile of severe disease. Our hope is that these data will aid clinicians' ability to prognosticate and treat patients in the future.

METHODS

Subjects

All pediatric subjects and/or their legally authorized study partners signed the written informed consent approved by the Institutional Review Board of the UT Southwestern Medical Center (UTSW), in accordance with the Federal-wide Assurance on file with the

Department of Health and Human Services (USA). Samples were collected and processed through the Neuroscience Biorepository at UTSW(27, 28) under authorization by the IRB. This cohort includes 7 pediatric patients diagnosed with autoimmune encephalitis with detectable αNMDAR antibodies in either the cerebrospinal fluid (CSF) or serum and met criteria for αNMDAR-AE.(29) An additional 13 pediatric patients with no detectable anti-NMDAR antibodies were included as an Other Neurological Disease (OND) control population. See Table 1 for a summary of the cohort.

Cell-based assay for NMDAR autoreactivity by serum and CSF supernatant

Serum and CSF supernatant were used to determine binding of IgG antibodies to the NR1 subunit of the NMDAR(30, 31) using the commercially available cell based assay (CBA) from EurImmun (https://www.euroimmun.com/products/indications/autoantikrper-diagnostik/neurology/autoimmune-encephalitis/aak-gegen-nmdar.html) and following manufacturer recommendations. Images were acquired using an inverted Zeiss LSM780 microscope.

Electrophysiology

Acute brain slice preparation: Four to seven days following the injection procedure, mice were anesthetized with the inhalation anesthetic, isoflurane, then were rapidly decapitated and their brains removed into ice-cold dissection artificial cerebral spinal fluid (ACSF). Acute coronal slices 300–350μm thick containing hippocampus were made on a vibrating microtome (Leica Biosystems, Wetzlar, Germany) in ice-cold dissection ACSF. Immediately after slicing, a cut was made between CA3 and CA1 to prevent recurrent excitation, and a second cut was made between the left and right hemispheres. Slices from each hemisphere were held for 30–45 minutes at 35°C in separate recovery chambers, each containing ACSF with 2mM $MgCl_2$ and 1mM $CaCl_2$. Slices were cooled to room temperature (RT, 24°C) and maintained at RT prior to recording.

Extracellular “field” electrophysiology: All recordings were performed at 32–33°C in Warner Instruments (Hamden, CT) low-profile PH-1 recording chambers continuously perfused with ACSF containing 2.5mM $CaCl_2$ and 1mM $MgCl_2$. 0.1ms current pulses were delivered via A-M Systems (Carlsborg, WA) Model 2100 stimulators through concentric bipolar stimulating electrodes (Model #CBARB75, FHC, Bowdoin, ME). Stimulating and recording electrodes (1–2 MΩ) were placed laterally in the *stratum radiatum* 400–500μm apart. Postsynaptic responses were measured with A-M Systems Model 1800 dual-channel amplifiers, highpass filtered at 1–5kHz, and digitized at 10kHz with a Molecular Devices (Sunnyvale, CA) Digidata 1440 digitizer. Signals were acquired with Clampex (v 10.7, Molecular Devices). All experiments were preceded by a stable baseline for at least 20-minutes.

Input/Output (I/O) curves: Stimulus intensity was increased from 0–10mA in 2mA steps at a rate of 0.05Hz. The fEPSP slope and fiber volley amplitude of 5 consecutive traces is averaged at each stimulus intensity per slice.

Analysis and statistics: fEPSP slope fitting and fiber volley measurements were made using Clampfit (v 10.7, Molecular Devices). Data cleaning and descriptive statistics were determined using custom Python 3.0 scripts, and hypothesis testing and posthoc analysis was performed using Statistica (Dell, Round Rock, TX).

Solutions:

Dissection ACSF contained (in mM): 87 NaCl, 3 KCl, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 20 dextrose, 75 sucrose, 7 $MgSO_4$, 0.5 $CaCl_2$ and saturated with 5%CO_2/95%O_2 at pH 7.3. Recording (standard) ACSF contained (in mM): 126 NaCl, 3 KCl, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 10 dextrose, 1 $MgCl_2$, 2.5 $CaCl_2$ and saturated with 5%CO_2/95%O_2 at pH 7.3. Slice maintenance ACSF contained (in mM): 126 NaCl, 3 KCl, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 10 dextrose, 2 $MgCl_2$, 1 $CaCl_2$ and saturated with 5%CO_2/95%O_2 at pH 7.3.

Flow Cytometry

CSF was centrifuged and cells were stained with a panel of antibodies that identify B cell subsets including total CD19+ B cells, naïve B cells, memory B cells, plasmablasts (PBs) and antibody-secreting cells (ASCs). Individual antigen-experienced CD19+CD27+ B cells (which include memory B cells, plasmablast B cells and antibody-secreting B cells) from the CSF of Subject 1547 were isolated by fluorescence-activated cell-sorting into 96-well plates with 4 μL of 40 mM DTT and 14 U RNasin Ribonuclease Inhibitors in 0.5x PBS. Cells were flash frozen on dry ice after sorting and stored at −80°C.

Single-cell Antibody PCR

Individually sorted antigen experienced CD19+CD27+ B cells from the CSF were lysed, mRNA was reverse-transcribed, and immunoglobulin variable regions were amplified with multiple rounds of PCR as previously described.(32) Sanger sequencing was performed at the UTSWMC (University of Texas Southwestern Medical Center) sequencing core to generate the antibody variable domain reads.

Sequence Analysis

Sequences were analyzed using the VDJServer analysis portal.(33) V, D, J gene calls, FR and CDR regions were annotated using IgBlast v1.14.1(34) with VDJServer IMGT 2019.01.23 germline database. 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. CDR3 charge properties and mutational analysis was performed using Alakazam in the Immcantation suite v4.2.0.(35) GraphPad Prism software was used to determine the statistical significance of differences between groups and build graphs for figures. 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.(36) Replacement frequency in CDR:FR was analyzed excluding near-zero ratios.

VH2+ Clone Cluster Identification

Of the 31 VH2–70 rearrangements, 28 were identified with a CDR3 motif of VH2–70-N-DH6-N-DH2-N-JH6. Clustering of these rearrangements was defined by the CDR3 amino acid sequence having the same CDR3 length and discordance in 2 or fewer amino acids.

Recombinant human antibody cloning, expression, and isolation

HEK293T cells (ATCC, Manassas, VA) were maintained in HyClone Dulbeccos Modified Eagles Medium (DMEM) (GE Healthcare Life Sciences). All recombinant human antibodies (rhAbs) were transiently transfected into HEK293T cells with the lipid transfection reagent JetPEI (PolyPlus Transfection) as done previously.(37, 38) Supernatants from these cultures were collected on days 3, 5, 7 and 10. The cell pellets were spun down and supernatants were passed through 0.2um filters and subjected to antibody purification on the NGC QUEST 10 system. The concentration of the antibodies were determined by sandwich ELISA as done previously.(37, 38)

Cell-based assay for NMDAR and AQP4 autoreactivity by rhAbs

Autoreactivity for the NMDA receptor (the NR1 subunit)(30, 31) and AQP4 was detected using a commercially-available cell-based assay (Euroimmun, FA 112d-1010–51 and FA 1128-1010-50). Cells were incubated with 20 μg/mL rhAbs in PBS + 0.1% Tween-20 (Sigma-Aldrich) + 1% Goat Serum (Life Technologies) for 30 min in a humidified chamber. After washing with PBS + 0.2% Tween-20, cells were incubated with the provided secondary antibody for 30 min in a humidified chamber. The wash was repeated, then cells were incubated with 1 μg/mL DAPI (Invitrogen) for 5 min. After washing, a coverslip was added to each slide and visualized on a Zeiss LSM780 confocal microscope using a 20x/0.80NA air objective lens. AQP-4 slides were visualized on a Zeiss Axioscope.A1 widefield microscope using a 20x/0.45NA air objective lens. Fluorescence intensity was measured using FIJI (https://fiji.sc/).

Cell-based assay for Hep2 autoreactivity by rhAbs

Hep2 autoreactivity was detected using a commercially-available cell-based assay (Bion Enterprises, ANK-120). Cells were incubated with 20 μg/mL rhAbs in PBS + 0.1% Tween-20 + 1% Goat Serum for 30 min in a humidified chamber. After washing twice with PBS, cells were incubated with the provided secondary antibody for 30 min in a humidified chamber. The washes were repeated, and cells were incubated with 1 μg/mL DAPI in PBS for 5 min. After washing, the slide was coverslipped and visualized on a Zeiss Axioscope.A1 widefield microscope using a 20x/0.45NA air objective lens. Fluorescence intensity was measured through FIJI. Positive staining of a rhAb was defined as fluorescence intensity greater than or equal to the mean fluorescent intensity of the negative control plus 6 standard deviations.

Quantification of autoreactivity by rhAbs

Autoreactivity was quantified using Fiji.(39) Cells were identified by using the Threshold function to detect nuclei and were added to the Region of Interest (ROI) manager. The mean fluorescence intensity (MFI) of positively-stained cells was measured, and the MFI of the

background (regions where no cells were detected) was subtracted from this value. The MFI of NR1-expressing cells were normalized to that of control cells that do not express NR1.

RESULTS

We had previously utilized a panel of flow cytometry markers to immune-profile B cell subsets in the CSF of patients with neurodegenerative diseases.(37, 38, 40) Because αNMDAR-AE patients present with high titers of αNMDAR IgG1 antibody in the CSF or blood, we asked whether the B cell subset profile in pediatric αNMDAR-AE patients was different from pediatric OND controls. Indeed, while 43% of B cells in the CSF of pediatric OND controls were antigen-naïve B cells (Figure 1A), 75% of B cells in the CSF of pediatric αNMDAR-AE patients were antigen-experienced B cells including memory B cells, plasmablasts (PBs) and antibody-secreting B cells (Figure 1B). One of the pediatric αNMDAR-AE patients was refractory to treatment and displayed features typical of this diagnosis including serum and CSF binding to NR1 of the NMDAR and reduction of stimulus-evoked NMDAR-mediated synaptic responses in area CA1 of mouse hippocampal slices using extracellular “field” recordings. (Supplemental Figure 1). This treatment refractory pediatric αNMDAR-AE patient case also had a significant expansion of antibody-secreting B cells in the CSF compared to pediatric OND controls (42% vs19%, Figure 1A and 1C) and the pediatric αNMDAR-AE patient cohort (42% vs 15%, Figure 1B and 1C). In addition, the ASC:PB ratio was 14.00 in this treatment refractory pediatric αNMDAR-AE patient case compared to 1.12 in the pediatric OND control cohort and 1.50 in the pediatric αNMDAR-AE patient cohort (Figure 1D).

To investigate this treatment refractory pediatric αNMDAR-AE patient’s humoral immune response to the NMDAR, we used single-cell PCR to amplify heavy chain genes from antigen-experienced CD19+CD27+ B cells in the CSF. Heavy chain antibody gene usage was analyzed (Figure 2A) and revealed that this subject had an expansion of antibody-experienced B cells in the CSF using variable heavy chain family 2 genes (VH2+) to 39.7% (expected frequency of 9.8%). In fact, 83.3% of VH2+ B cells in the CSF of this patient displayed heavy somatic hypermutation accumulation with an average of 3.6% mutation frequency overall (Figure 2B) and 2.3% replacement frequency overall (Figure 2C) indicating an antigen-driven response. Of note, VH3+ and VH4+ B cells in the CSF of this same patient accumulate codon replacement mutations in the CDRs as a mechanism of increasing antigen-affinity with CDR:FR ratios >2.0 (VH3; 2.6 and VH4; 2.3). VH2+ B cells in the CSF of this patient, however, had a replacement frequency CDR:FR ratio of 0.8 (Figure 2D).

Further investigation of these thirty-one VH2+ B cells revealed that all 31 used the VH2–70 gene, two DH segments (DH6–13 and DH2–2) and the JH6 gene with a total CDR3 length of sixty-three nucleotides. Clonal clusters were identified for fifteen of these sequences. Six of these clones had three sequences per clone, and four clone clusters had two sequences. We cloned five of the antibodies from CSF-derived B cells of this pediatric αNMDAR-AE patient using the VH2–70/DH6–13/DH2–2/JH6 CDR3 motif. The CDR3 motif of all five VH2+ B cells that we cloned was: AR-X-RG-XQQX-ED-PNXY-XXDV and all five

used the VL1–51 light chain. We also cloned five antibodies expressed by non-VH2+ CSF-derived B cells of this same pediatric αNMDAR-AE patient (Table 2).

Binding of these 10 recombinant human antibodies (rhAbs) to the NR1 subunit of the NMDAR is presented in Figure 3 (Panel A, images; Panel B, quantification). Of the 10 rhAbs produced, 5 of them bound to the NR1 subunit of the NMDAR (AG06, AG07, AG08, AG09 and AG10). Of note, all 5 that bound NR1 utilized the VH2–70 heavy chain antibody gene with the AR-X-RG-XQQX-ED-PNXY-XXDV CDR3 motif. None of the 10 rhAbs bound to the control antigen, AQP4 (Supplemental Figure 2) or the control cell line, Hep2 (Supplemental Figure 3). Of note, both the heavy and light chains of the 5 rhAbs that bound NR1 were heavily mutated (Table 2), indicating an antigen-driven response.

Finally, we sought to determine if the germline configuration of the antibody rearrangement prior to encounter with the driving antigen could also bind the NR1 subunit of the NMDAR. To do this, we identified one clonally related cluster (AG06 and AG09, Figure 4A) and reverted the heavy chain antibody variable genes of these two rhAbs to their germline configuration. AG08 was also reverted to germline configuration because this rearrangement maintained the AR-X-RG-XQQX-ED-PNXY-XXDV CDR3 motif, but did not meet criteria to cluster with any other VH2+ rearrangements due to 4 AA substitutions at the DH2–2 to JH6 junction (Figure 4A). The three reverted rhAbs were designated AG06gL, AG08gL and AG09gL to indicate that the antibody heavy chain no longer contained the somatic hypermutations of their highly mutated counterparts (Figure 4B). These new germline rhAbs (AG06gL, AG08gL and AG09gL) and their highly mutated counterparts (AG06, AG08 and AG09) were tested for binding to a cell line expressing the NR1 subunit of the NMDAR (Figure 5A). The frequency of cells that were positively stained with AG06gL and AG09gL were reduced compared to their highly mutated counterparts, while no cells were positively stained with AG08gL (Figure 5B). The mean fluorescence intensity of AG06gL binding to NR1, however, was similar to AG06, while the mean fluorescence intensity of AG09gL binding to NR1 was 4.4-fold less than AG09 (Figure 5C). AG08gL binding to NR1 was below the detection threshold. Thus, accumulation of somatic hypermutations increases NR1 binding intensity, but NR1 binding by the original antibody rearrangements prior to somatic hypermutation accumulation is frequently detectable. Of note, exact matches of the CDR3 amino acid sequences from these 3 rhAbs are not in the AIRR Data Commons,(41) Immune Epitope Database,(42) or Genbank(43) and CDR3 motif queries are not accommodated on these platforms.

DISCUSSION

This study investigates binding to the NR1 subunit of the NMDAR by highly expanded antigen-experienced B cells using the VH2–70 gene isolated from the CSF of a pediatric αNMDAR-AE patient refractory to treatment. Indeed, here we show that only those B cells utilizing the VH2–70 gene bind NR1. This is a novel finding that may be subject-specific but may also be pediatric-specific, as others have not detected any VH2+ antigen-experienced B cells in the CSF of adult αNMDAR-AE cases.(18, 19) In fact, it is evident that certain sub-motifs within the CDR3 gene-sequence of these VH2+ antigen-experienced B cells drive continued NR1 binding in the absence of somatic hypermutation and have not been

previously reported in other databases including the current AIRR Data Commons which contains ~1 billion CDR3 AIRR-seq derived sequences.(41)

The first indication of this unexpected finding of VH2+ enrichment was discordance in CSF-derived B cell subtypes between a cohort of pediatric αNMDAR-AE patients and OND controls. Antigen-experienced B cells in the CSF of the pediatric αNMDAR-AE patients were 3-fold greater than what was observed in the pediatric OND controls. In fact, the pediatric OND cohort maintained an expected balance of naïve B cells and antigen-experienced B cells (memory, plasmablasts and antibody-secreting cells). Within the three subtypes of antigen-experienced B cells, we expected an expansion of memory B cells in the pediatric αNMDAR-AE patients since they constitute the B cell pool from which ASCs emerge. Indeed, the memory B cell frequency was increased in the pediatric αNMDAR-AE patients. Expansion of memory B cells, however, did not result in a subsequent increase in the frequency of plasmablasts or ASCs in the pediatric αNMDAR-AE patients, despite sampling in the acute phase of disease in these cases. Of note, others have reported expansion of antibody secreting cells (“plasma cells”) in the CSF of four adult αNMDAR-AE patients compared to 25 adult controls,(19) but this is the first report focused on pediatric αNMDAR-AE patients.

The pediatric αNMDAR-AE case that was refractory to treatment displayed an extensive enrichment of long-lived antibody-secreting cells (ASC’s) in the CSF, such that 42% of all B cells in the CSF were ASCs. This particular B cell subtype (ASC’s) is incredibly difficult to target by anti-CD20 B cell depletion therapy because they do not express CD20.(44, 45) Furthermore, as ASCs are long-lived,(46) they do not require replenishment from the memory B cell pool, and thus depletion of CD20+ memory B cells would not impact ASC frequency. Even steroids would not be a successful therapeutic as ASCs are less focused on proliferation and more focused on antibody production.(46) Antibodies that target CD19,(47, 48) which is expressed at low levels on ASCs,(47, 48) might be successful, if penetrance into CNS could be overcome. Proteasome inhibitors target protein production,(49) and may be useful in the treatment of αNMDAR-AE(50) but one side effect is the development of peripheral neuropathy.(51) Future efforts must be made to identify patients with αNMDAR-AE who demonstrate expansion of ASCs in the CSF and develop therapeutic options targeting this antigen-experienced B cell subtype with careful consideration of current recommendations.(23–25)

Immunogenetic studies revealed an unexpected expansion of VH2+ antigen-experienced B cells in the CSF of the pediatric αNMDAR-AE case that was refractory to treatment. There are only 2 germline genes within the VH2 family(52) and it is seldom used by B cells even at the expected frequency of 9.8%.(53–59) Yet in this treatment-refractory pediatric αNMDAR-AE case, there was a 4-fold increase in VH2 usage over the expected frequency. In the adult αNMDAR-AE studies reported by others,(18, 19) no VH2+ CSF-derived B cells were detected. This raises the possibility that the immunogenetics of pediatric αNMDAR-AE are discordant from adult αNMDAR-AE since essentially the same approaches were used to generate the repertoires. We used somatic hypermutation analyses to investigate whether this expansion of VH2+ B cells was an antigen-driven response.(60, 61) To do this, we calculated the overall mutation-frequency (MF) and replacement-frequency (RF) of the

VH2+ antigen-experienced B cells. The data showed that while well above baseline, the MF and RF were significantly reduced compared to VH3+ antigen-experienced B cells in the CSF of this same patient. It should be noted, however, that all of the VH2+ B cells were using the same VH, DH(2) and JH segments; thus, there could be skewing of the MF and RF due to low diversity. Within the VH2+ B cells, there was also a lack of targeting of replacement mutations to the CDRs as evidenced by the low percentage of replacements in CDR versus FR. This is in contrast to VH3+ and VH4+ B cells from this same αNMDAR-AE case, which demonstrated high skewing of replacement mutations towards CDR1 and CDR2 as evidenced by the high percentage of replacements in CDR versus FR. It is thought that this bias towards replacement mutation accumulation in the CDRs generates greater antigen binding diversity and affinity in the CDRs of the antibody while bias against replacement mutation accumulation in the FRs facilitates structural integrity in the FRs of the antibody.(62–65) However, none of these studies featured analysis of human VH2+ antigen-experienced B cells.

Since antigen-experienced B cells from the CSF of the αNMDAR-AE case refractory to treatment were expanded as evaluated by flow cytometry and were highly enriched for VH2 usage with unusual replacement mutation features, we asked whether they bound to the NR1 subunit of the NMDAR. We included 5 VH2+ antigen-experienced B cells, 2 using VH3, 2 using VH4 and 1 using VH5. Others have shown that 5.9% of ASC's from the CSF of adult αNMDAR-AE patients bind the NR1 subunit of the NMDAR using VH3, VH4 or VH5 antibody genes, but they did not have any candidates to test using VH2.(18, 19) We found that 5 of 5 VH2+ B cells bound the NR1 subunit, but none of those using VH3, VH4 or VH5 genes bound NR1 despite compelling evidence of antigen-driven selection. Since others have not detected VH2+ ASCs in the CSF of adult αNMDAR-AE patients, we suspect that this could be a phenotype of pediatric αNMDAR-AE refractory to treatment. Furthermore, VH2–70+ antigen-experienced B cells in the CSF may be grossly enriched for binding to the NR1 subunit of the NMDAR, which may also explain the near non-existent detection (<1%) of the VH2–70 gene in large healthy human antibody rearrangement datasets.(41, 66, 67) Whether these NR1-binding VH2+ B cells in the CSF matriculated from germinal centers or extrafollicular sites remains unknown, although others have provided evidence that B cell activation in response to NR1 occurs in the cervical lymph nodes.(21) Of note, affinity maturation occurs at both sites.(68) Understanding the role of NR1-binding B cells in refractory cases of pediatric αNMDAR-AE, and the tissue structures from which they matriculate is of critical importance.

We further suspect that this propensity towards NR1-binding by VH2+ antibodies is primarily driven by a CDR3 motif we identified in the NR1-binding VH2+ antibodies. Indeed, it has been well-established that long CDR3 lengths, use of tryptophan and tyrosines, and positively charged residues (arginine, lysine and histidine) can infer autoreactivity.(69–75) The NR1-binding VH2+ antibodies we investigated here all have these features. For example, the CDR3 length of the VH2+ antibodies is 63 nucleotides due to use of the longest J-segment (JH6) and an unusual incorporation of two D-segments in tandem (DH6–13 and DH2–2). All NR1-binding VH2+ antibodies had arginine (R) at positions 2 and 4, and tyrosine (Y) at position 15. AG06, AG09, AG07 and AG08 also had a tyrosine at position 14, and AG10, AG07 and AG08 had a tyrosine at position 17. In

addition, the majority of SHM within the NR1-binding VH2+ antibodies were focused in frameworks and by convention do not contribute to antigen binding. Due to these features, we expected that removing the SHM from the NR1-binding VH2+ antibodies would not completely abrogate binding. Indeed, of the 3 NR1-binding VH2+ antibodies we reduced to germline configurations, only AG08gL displayed complete abrogation of positively stained NR1-expressing cells. Of note, AG08 had a considerable deviation from AG06 and AG09 in the DH2–2 to JH6 junction which may have required compensation by SHM to maintain NR1 binding. SHM accumulation in AG06 and AG09 may also support higher affinity binding to NR1 or may be carry over mutations due to high B cell proliferation.(68) It is also possible that NR1 is not the driving antigen as we suggest. For example, others have demonstrated that NR1 antibodies emerge following neurotropic virus infection.(76–79)

We have demonstrated that pediatric αNMDAR-AE is characterized by an expansion of antigen-experienced B cells in the CSF by flow cytometry. Immunogenetics support the notion that VH2 expansion within the antigen-experienced B cell repertoire of the CSF is driven by a gross enrichment of NR1 binding. These VH2+ antibodies also display features of a unique CDR3 motif that may drive initial NR1 reactivity prior to SHM incorporation. These results are limited by the inclusion and sequence data of only one pediatric case of αNMDAR-AE refractory to treatment and examination of antibodies from 5 VH2+ antigen-experienced B cells. Nevertheless, expansion of VH2+ antigen-experienced B cells in the CSF of pediatric αNMDAR-AE may be an indicator of NR1-associated autoimmunity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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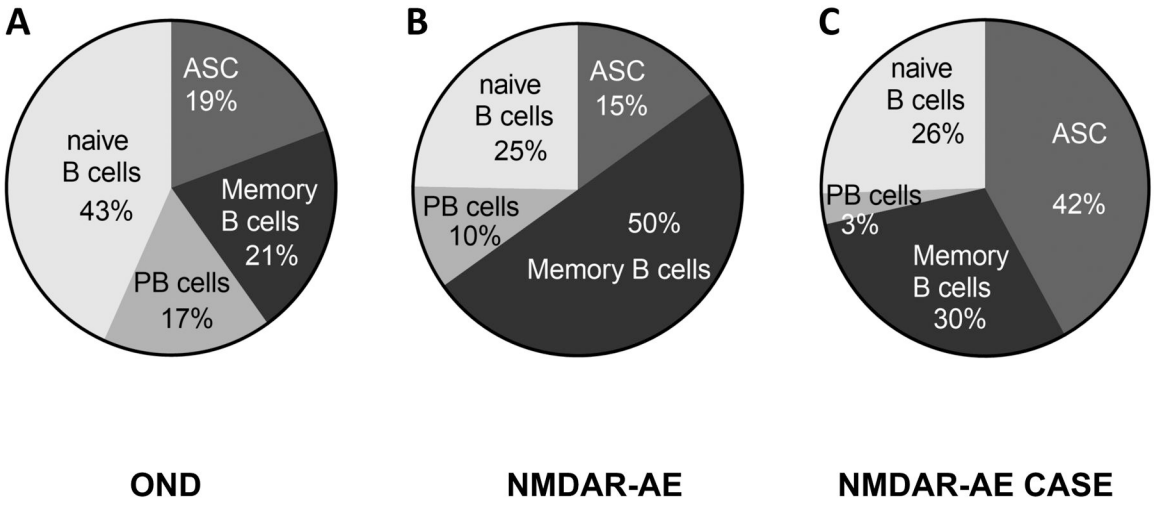
KEY POINTS

1. Antibody Secreting Cell expansion in the CSF of pediatric NMDAR-AE
2. VH2 overuse in treatment refractory pediatric NMDAR-AE case
3. Identification of a CDR3 motif of NR1-binding VH2–70+ B cells



D

	OND cohort	NMDAR-AE cohort	NMDAR-AE CASE
AntigenE: Naïve	1.33	3.00	2.88
ASC:naïve	0.44	0.60	1.62
ASC:MEM	0.9	0.30	1.40
ASC:PB	1.12	1.50	14.00

FIGURE 1. B cell profile in the CSF of pediatric Autoimmune Encephalitis.

Cerebrospinal fluid (CSF) was collected from pediatric patients with autoimmune encephalomyelitis (AE). Shown are B cell subset frequencies by flow cytometry for (A) pediatric patients who tested negative for antibodies against the NMDAR (OND), (B) pediatric patients who tested positive for antibodies against the NMDA receptor and met diagnostic criteria for NMDAR-AE (NMDAR-AE) and (C) one pediatric NMDAR-AE case refractory to treatment (NMDAR-AE CASE). (D) Ratios of B cell subsets in each group. In D, abbreviations are as follows: AntigenE, Antigen Experienced; ASC, antibody secreting cell; MEM, memory; PB, plasmablast.

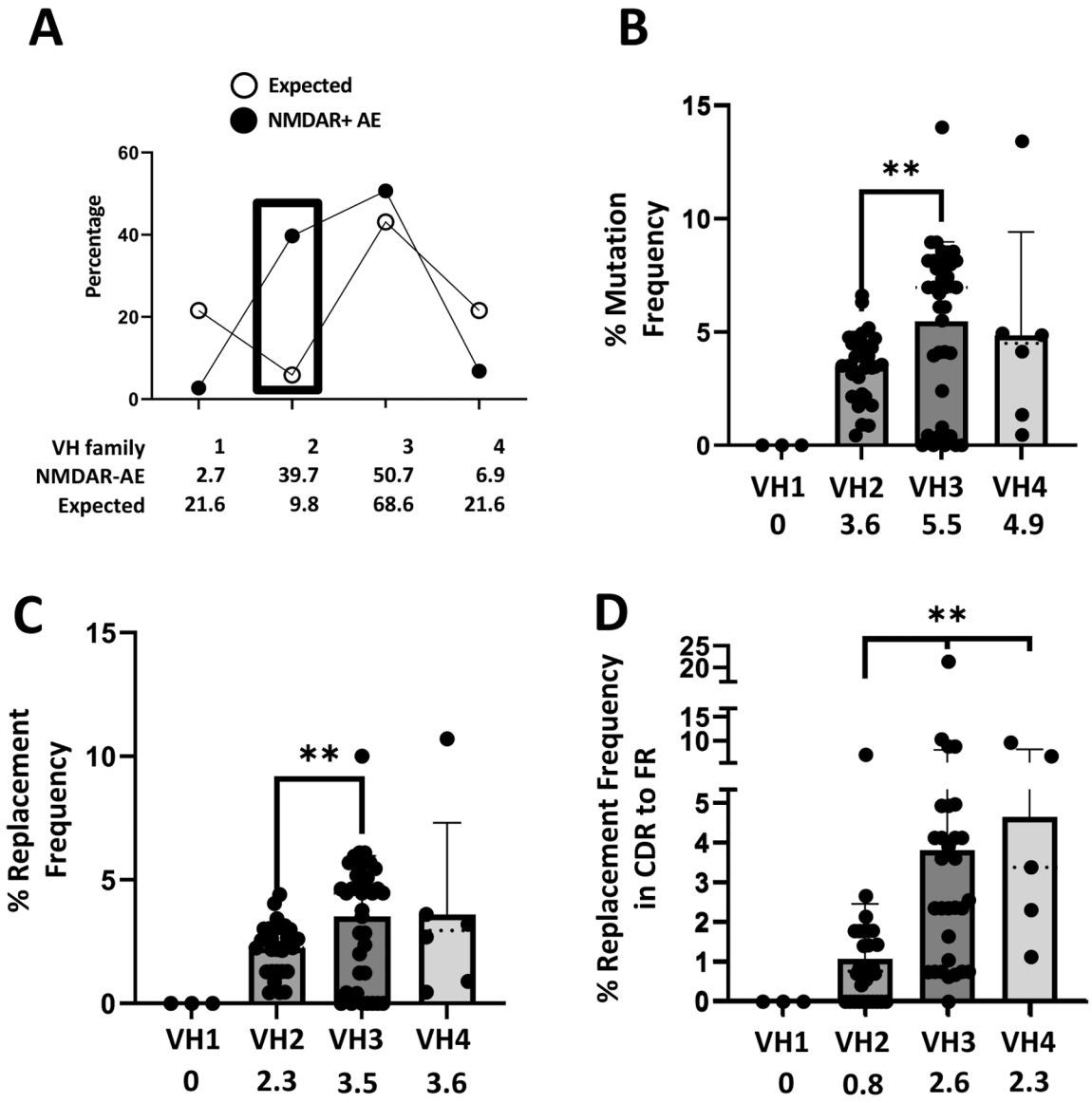


FIGURE 2. Antibody variable heavy chain use dysregulated in pediatric NMDAR-AE.

(A) Single B cells from the cerebrospinal fluid of a pediatric NMDAR-AE patient were queried for their antibody variable heavy chain usage and compared to the expected frequency in healthy controls. (B) Mutational frequencies within each variable antibody heavy chain family. (C) Replacement frequencies within each variable antibody heavy chain family. (D) CDR:FR ratio of replacement frequency within each variable antibody heavy chain family.

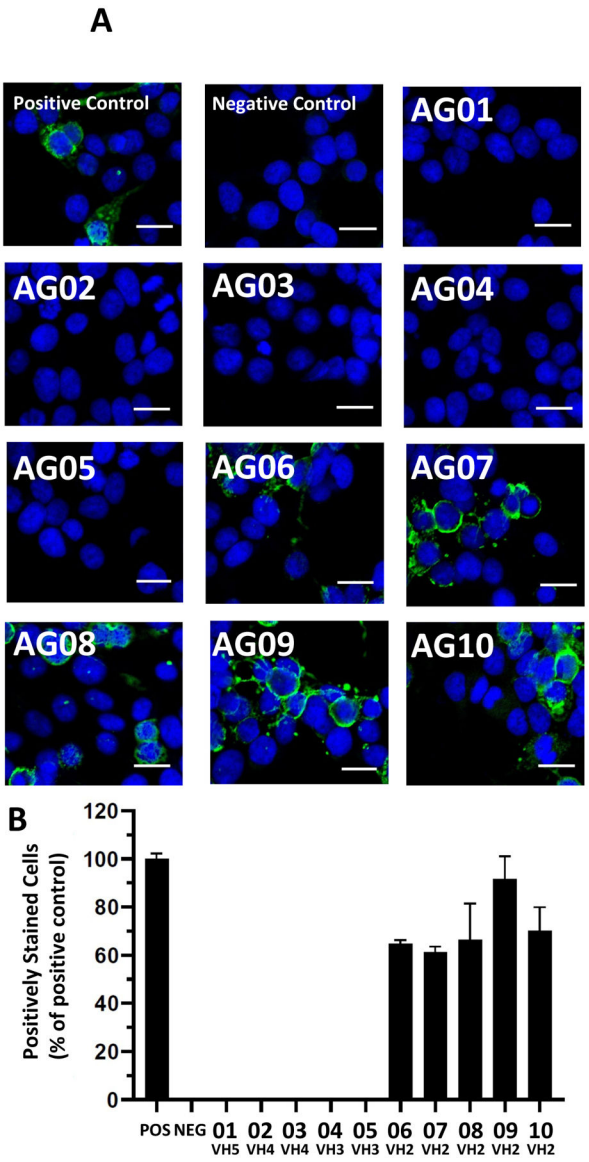


FIGURE 3. Binding of recombinant human antibodies to the NR1 subunit of the NMDAR.
(A) Antibodies expressed by individual B cells from the CSF of a pediatric NMDAR-AE patient refractory to treatment were expressed and tested for binding to the NR1 subunit of the NMDAR using a commercially available kit as described in the methods. Blue: DAPI staining. Green: NMDAR. Scale bar: 20 μm. (B) Frequency of positively-stained cells.

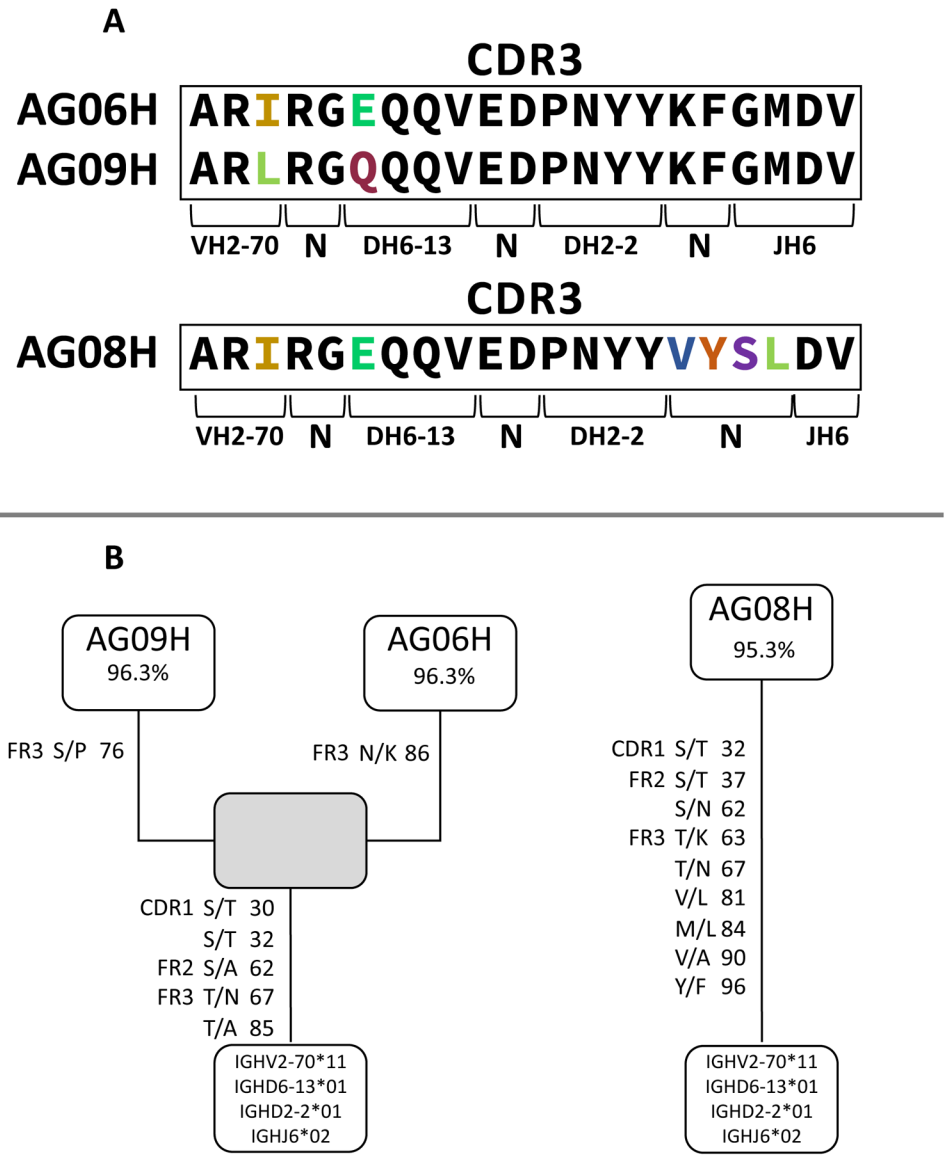


FIGURE 4. Clonal Expansion of VH2+ antigen experienced B cells in the CSF of a pediatric NMDAR-AE patient refractory to treatment.

(A) The CDR3 amino acid sequences of AG06, AG08 and AG09 are in the boxes with differences indicated in color. (B) Clonal relationship of AG06, AG08 and AG09 using a Hamming distance of 2. Replacement mutations are indicated by region, codon number and the amino acid replacement. The gray box indicates a common clone member that was not detected in the repertoire. Germline V, D and J genes are indicated at the root of the clone.

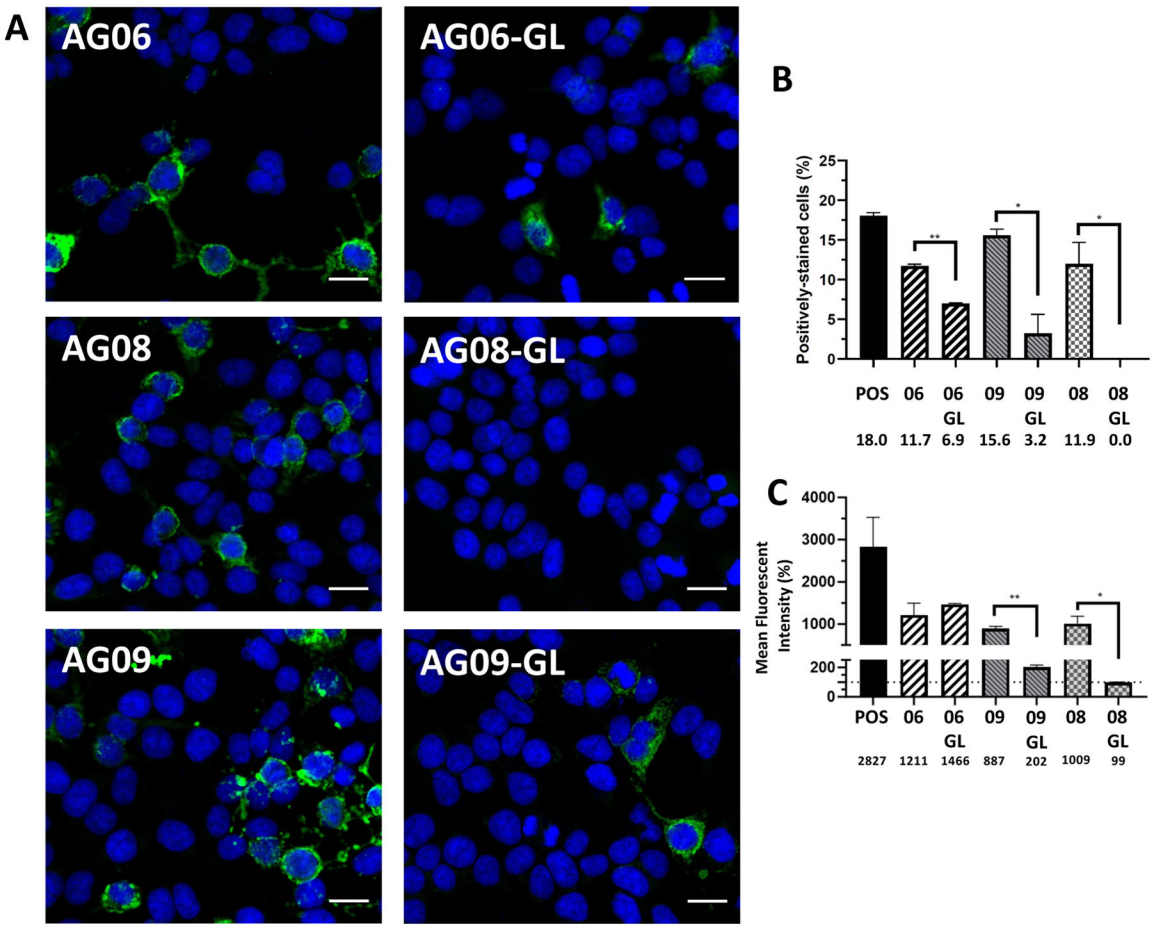


FIGURE 5: Comparison of rhAbs to germline configuration.

(A) Antibodies expressed by individual B cells from the CSF of a pediatric NMDAR-AE patient refractory to treatment were reverted to their germline configuration and tested for binding to the NR1 subunit of the NMDAR using a commercially available kit as described in the methods. Blue: DAPI staining. Green: NMDAR. Scale bar: 20 μm. (B) Frequency of positively-stained cells. (C) Mean fluorescence intensity of cells. The horizontal dotted line represents the MFI of control cells as the threshold of detection.

TABLE 1.

Subject information

NMDAR-AE Subject Code	Age	Sex
0972	8	F
1306	15	F
1463	2	F
0978	8	M
1547	3	M
1712	13	F
1783	17	M
OND Subject Code[1]		
1282	5	F
1295	16	M
1506	2	M
1515	15	M
1470	18	M
1739	14	M
1792	4	M
1819	15	F
1839	13	F
0800	8	M
1904	2	F

[1]OND category included subjects diagnosed with demyelinating disease, psychosis, cancer, or physical trauma

TABLE 2.

rhAb gene information

rhAb Name	VH gene	JH gene	CDR3 AA length	#RM	VL gene	#RM
AG01	5–51	4	30	8	VL2–14	8
AG02	4–31	3	54	24	VL2–14	8
AG03	4–39	4	57	2	VK2–30	1
AG04	3–74	4	42	14	VL1–51	2
AG05	3–49	4	30	12	VL2–14	8
AG06	2–70	6	63	1	VL1–51	2
AG07	2–70	6	63	10	VL1–51	3
AG08	2–70	6	63	9	VL1–51	1
AG09	2–70	6	63	7	VL1–51	5
AG10	2–70	6	63	6	VL1–51	1

Abbreviations: VH, variable heavy; JH, junction heavy; CDR3, complementarity determining region 3; AA, amino acid; #RM, number of replacement mutations; VL, variable lambda light chain; VK, variable kappa light chain