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Immune Repertoire Profiling Reveals Its Clinical Application Potential and Triggers for Neuromyelitis Optica Spectrum Disorders

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Abstract

Background and Objectives

Neuromyelitis optica spectrum disorders (NMOSD) is widely recognized as a CNS demyelinating disease associated with AQP4-IgG (T cell–dependent antibody), and its trigger is still unclear. In addition, although the treatment of NMOSD currently can rely on traditional immunosuppressive and modulating agents, effective methods to predict the efficacy of these therapeutics are lacking.

Methods

In this study, high-throughput T-cell receptor (TCR) sequencing was performed on peripheral blood from 151 pretreatment patients with AQP4-IgG$^+$ NMOSD and 151 healthy individuals. We compared the TCR repertoire of those with NMOSD with that of healthy individuals and identified TCR clones that were significantly enriched in NMOSD. In addition, we treated 28 patients with AQP4-IgG$^+$ NMOSD with immunosuppressants and followed up for 6 months to compare changes in NMOSD-specific TCRs (NMOSD-TCRs) before and after treatment. Moreover, we analyzed transcriptome and single-cell B-cell receptor (BCR) data from public databases and performed T-cell activation experiments using antigenic epitopes of cytomegalovirus (CMV) to further explore the triggers of AQP4-IgG$^+$ NMOSD.

Results

Compared with healthy controls, patients with AQP4-IgG$^+$ NMOSD had significantly reduced diversity and shorter CDR3 length of TCRβ repertoire. Furthermore, we identified 597 NMOSD-TCRs with a high sequence similarity that have the potential to be used in the diagnosis and prognosis of NMOSD. The characterization of NMOSD-TCRs and pathology-associated clonotype annotation indicated that the occurrence of AQP4-IgG$^+$ NMOSD may be associated with CMV infection, which was further corroborated by transcriptome and single-cell BCR analysis results from public databases and T-cell activation experiments.

Discussion

Our findings suggest that the occurrence of AQP4-IgG$^+$ NMOSD may be associated with CMV infection. In conclusion, our study provides new clues to uncover the causative factors of AQP4-IgG$^+$ NMOSD and provides a theoretical foundation for treating and monitoring the disease.

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Glossary

AQP4 = aquaporin-4; **AQP4-abs** = AQP4-specific antibodies; **AQP4-IgG** = AQP4 immunoglobulin G; **BCR** = B-cell receptor; **CMV** = cytomegalovirus; **CTL** = cytotoxic T lymphocyte; **DEG** = differentially expressed genes; **DJ** = D-J; **FDR** = false discovery rate; **GO** = gene ontology; **GSEA** = Gene Set Enrichment Analysis; **HC** = healthy control; **HLA** = human leukocyte antigen; **IFN-I** = type-I interferons; **IL-6** = interleukin 6; **IMGT** = the international ImMunoGeneTics information system; **ISG** = interferon-stimulated gene; **KEGG** = Kyoto Encyclopedia of Genes and Genomes; **McPAS** = manually curated catalogue of TCR sequences; **MIRA** = Multiplexed Identification of TCR Antigen; **MM** = module membership; **NMOSD** = neuromyelitis optica spectrum disorders; **NOD** = nucleotide-binding oligomerization domain; **PBMCs** = peripheral blood mononuclear cells; **PIRD** = Pan immune repertoire database; **SLE** = systemic lupus erythematosus; **TCR** = T-cell receptor; **VD** = V-D; **VDJ** = V-D-J; **VDJdb** = a curated database of T-cell receptor sequences with known antigen specificity; **VDMV** = Valine-Asparticacid-Methionine-Valine.

Introduction

Neuromyelitis optica spectrum disorder (NMOSD) is an inflammatory demyelinating disease of the CNS with acute optic neuritis and transverse myelitis as the main clinical features, which is caused by serum pathogenic antibodies attacking aquaporin-4 (AQP4) in the optic nerve and spinal cord astrocytes in most patients.[1] NMOSD has a high disability rate, and prognostic modeling studies suggest that patients with NMOSD have a 58%–83% risk of motor disability within 5 years without treatment.[2,3] Current NMOSD treatment relies on traditional immunosuppressive and immunomodulating agents. However, despite standard immunotherapy, more than one-third of patients with NMOSD have recurrent episodes and disability progression, and there is still a lack of effective monitoring indicators and treatments for patients.[4]

Because AQP4 immunoglobulin G (AQP4-IgG) antibodies were reported in 2004,[5] NMOSD has been considered a humoral autoimmune disease. Approximately 80% of patients with NMOSD are seropositive for AQP4-IgG (AQP4-IgG[+]),[6] and its titer in the blood is 1,000-fold higher than that in the CSF, indicating that most AQP4-IgG is produced in the periphery and subsequently enters the CNS.[7] AQP4-IgG binds to AQP4 expressed on foot processes of astrocytes in the CNS, leading to astrocyte membrane damage and damage to neurons through complement-dependent cytotoxicity and antibody-dependent cytotoxicity. However, the specific mechanism that triggers peripheral blood B cells secreting AQP4-IgG in patients with AQP4-IgG[+] NMOSD is unclear.

In addition, there is growing evidence implicating that T cells are associated with the development of NMOSD.[8,9] The AQP4-specific antibodies (AQP4-abs) in the serum of patients with NMOSD are mainly IgG1, a T cell–dependent IgG subclass.[10] However, the presence of AQP4-abs alone is insufficient to trigger CNS inflammation.[11] When AQP4-specific T cells were transferred simultaneously with AQP4-IgG to rats, inflammatory tissue damage similar to NMOSD was produced,[12] suggesting the vital role of AQP4-specific T cells in NMOSD. Moreover, AQP4-specific T cells have now been identified in patients who exhibit Th17 polarization.[13] T-cell receptor (TCR) repertoire has the potential as a biomarker for the diagnosis and treatment of autoimmune diseases and cancers in several studies.[14,15] However, there are scarce studies on the TCR characteristics of patients with NMOSD and their diagnostic and prognostic potential for this disease.

In this study, we performed high-throughput sequencing of the TCRβ chain variable region in the peripheral blood samples of patients with NMOSD and healthy controls (HCs) to comprehensively characterize the TCR repertoire of NMOSD and dissected the differences of NMOSD-specific clones before and after treatment. Meanwhile, we combined single-cell B-cell receptor (BCR) and transcriptome data from public databases to explore potential triggers of NMOSD based on the characteristics of NMOSD-TCR, BCR, and differentially expressed genes (DEGs) and further performed experimental validation. The characteristics of the immune repertoire in the peripheral blood of patients with NMOSD that we demonstrated and its triggers provided clinical implications for the treatment of this disease.

Methods

Participants

Patients with NMOSD (n = 151) were recruited from the outpatient clinic and wards of West China Hospital between October 2014 and March 2017. All patients with NMOSD were seropositive for anti-AQP4 antibodies (cell-based assay, EUROIMMUN AG, Luebeck, Germany) and were untreated with immunosuppressants or immunomodulators before blood collection.[2,16] In addition, we also performed a second blood collection for patients who received immunotherapy after the first blood collection to observe the changes in TCR characteristics pretreatment and posttreatment. At the same time, we recruited healthy individuals with matched sex and age to patients with NMOSD as controls (HCs, n = 151). All HC samples were without a history of cancer and autoimmune diseases. The ethics committee and institutional review board of West China Hospital approved this study, and all participants signed a written informed consent document.

TCR Repertoire Sequencing

Five milliliter of peripheral blood was collected from each participant into ethylendiaminetetraacetic acid tubes. The genomic DNA was extracted and purified using the AxyPrep

Table 1 Clinical Profiles of Patients With AQP4-IgG⁺ NMOSD and HC Samples

Clinical	HC (N = 151)	NMOSD (N = 151)	*p* Value
Gender			NS
Male	18 (11.9%)	20 (13.2%)	
Female	133 (88.1%)	131 (86.8%)	
Collecting age; median (range), y	44 (20–81)	45.3 (16.7–81.2)	NS
Age at onset; median (range), y	—	39.46 (9–79)	
Disease duration; median (range), y	—	2 (0.01–32.6)	
EDSS scores; median (range)	—	3 (0–9)	
AAR; median (range)	—	1.02 (0.12–9.03)	
Immunosuppressant[a]			
AZA	—	11 (39.3%)	
MMF	—	17 (60.7%)	
Curative effect[b]			
1 & 2	—	21 (71.4%)	
3 & 4	—	7 (28.6%)	

Abbreviations: AAR = annual recurrence rate, Immunosuppressant; AZA = azathioprine; EDSS = expanded disability status scale; HC = health controls; MMF = mycophenolate mofetil; NMOSD = neuromyelitis optica spectrum disorders; NS = not significant.
[a] Details of the medication used in 28 patients with NMOSD with AQP4-IgG⁺ before and after treatment are provided in eTable 1, links.lww.com/NXI/A873.
[b] Curative effect, 1 indicates significantly effective, with no relapse or only 1 mild relapse during the treatment period, 2 indicates effective, with 1–2 relapses and the ARR is significantly lower than pretreatment ARR, 3 means noneffective with posttreatment ARR greater than or equal to the pretreatment ARR, and 4 indicates relapse after discontinuation of the drug.

Blood Genomic DNA Midprep kit and stored at −80°C. Rearranged TCR sequences were amplified from gDNA using a multiplex PCR (using a mixture of specific forward primers for the V gene and specific reverse primers for the J gene) according to the previous protocol.[17] The amplified PCR products were purified, and libraries were constructed and sequenced by single-end 100 bp on the genetic sequencer produced by the MGI platforms.

TCR Data Analysis

We filtered the low-quality reads and aligned them to the IMGT (the international ImMunoGeneTics information system) database to obtain the counts and percentages of CDR3 sequences and *V, D,* and *J* genes for each sample using IMonitor.[18]

By counting the number of supports for each clone in the disease and control groups separately and performing the Fisher 1-tailed test[19] and false discovery rate (FDR) correction on clones contained in samples with more than 5% of patients with NMOSD, clones with FDR <0.05 were identified as NMOSD-TCRs.

We annotated CDR3 sequences of NMOSD-TCRs using self-written Perl code based on 3 existing public databases: PIRD (Pan immune repertoire database),[20] VDJdb (a curated database of T-cell receptor sequences with known antigen specificity),[21] and McPAS (manually curated catalogue of TCR sequences).[22] We grouped all relevant diseases in the 3 databases into 4 major categories: cancer, autoimmune diseases, foreign microbes, and others, and the method used to calculate the proportion of pathology-related clones in the sample was described in a previous study.[15]

CMV Peptides Stimulation In Vitro

Blood samples were obtained from the serum of patients with AQP4-IgG⁺ NMOSD without immunotherapies and healthy volunteers. Peripheral blood mononuclear cells (PBMCs) were separated by density gradient centrifugation (Ficoll-Paque PLUS, GE). PBMCs were stimulated with cytomegalovirus (CMV) peptides (5, 10, and 20 μg/mL) in the 1640 complete medium, including 1 μg/mL of antihuman CD40 (W17212H, UltraLEAF), 1 μg/mL of antihuman CD28 (CD28.2, Biolegend), 10% fetal bovine serum, 1% non-essential amino acids, 1% penicillin, and 1% streptomycin for 12 hours. T-cell activation was evaluated by CD154 expression.

Statistics

All comparisons between unpaired samples in the article were performed using the Wilcox test and the paired *t* test between paired samples. All graphs were plotted based on R 4.0.4.

Data Availability

The data reported in this paper have been deposited in the OMIX, China National Center for Bioinformation / Beijing Institute of Genomics, Chinese Academy of Sciences (https://ngdc.cncb.ac.cn/omix: accession no.OMIX003989).

Results

Abnormal TCR Repertoire in Patients With NMOSD

We collected whole blood from 151 patients with AQP4-IgG seropositive NMOSD and 151 age-matched and sex-matched HC samples (eTable 1, links.lww.com/NXI/A873) before treatment for TCR characterization. Clinical information of patients with AQP4-IgG⁺ NMOSD and HC samples is summarized in Table 1. The study design and the analysis performed during the process are shown in the flowchart (Figure 1A).

We started by comparing the diversity of the immune repertoire of NMOSD and HC groups. A single clone was defined as the amino acid sequence of CDR3 and the *V, J* gene, and statistics on the number of total, unique, and public clones (clones shared by 2 or more samples) showed 5,456,144, 4,818,067, and 242,124, respectively, in all patients with NMOSD. By contrast, 9,847,689 clones were obtained in the TCR repertoire in all HC samples, of which 8,252,145 were unique clones and 570,771 were public clones (eFigure 1A, links.lww.com/NXI/A872), indicating that the TCR

Figure 1 TCR Repertoire Characteristics of NMOSD and HCs Are Significantly Distinct
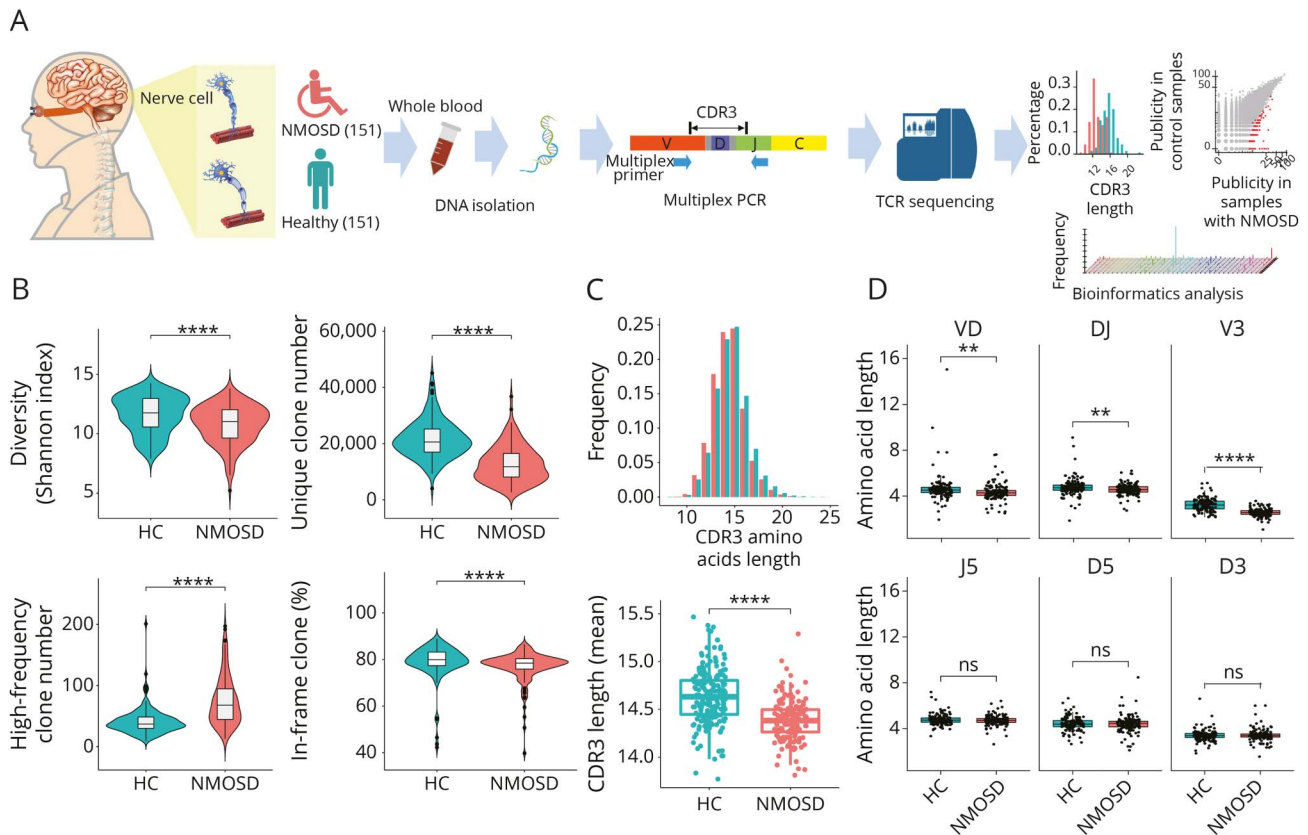
(A) Study design and analysis workflow. (B) The violin diagram shows the comparison of the Shannon index, unique clone numbers, high-frequency clone numbers, and the percentage of in-frame clones between NMOSD and HC (Wilcox test; ****: *p* value <0.0001), respectively. (C) The distribution of CDR3 amino acid sequence lengths for all clones of NMOSD and HCs, and the box plot shows the comparison of the average length of CDR3 amino acid sequences between the 2 groups. (D) The box plot demonstrates the differences in VD, DJ insertion, and V3, J5, D5, and J3 deletion lengths of all clones between NMOSD and HCs. HC = healthy controls; NMOSD = neuromyelitis optica spectrum disorders; TCR = T-cell receptor.

repertoire of different trait groups has specific characteristics, which led to the difference in public clones.

To characterize the level of sequence similarity of TCR repertoire within and between NMOSD and HCs, we calculated the proportion of shared clones between samples, including unique (eFigure 1B left, links.lww.com/NXI/A872) and total clones (eFigure 1B right, links.lww.com/NXI/A872). The results showed that while there was some heterogeneity in the TCR repertoire within NMOSD, which were similar to the results of a previous study on systemic lupus erythematosus,[15] there were more significant differences in TCR repertoire between patients with NMOSD and HCs.

Next, we introduced the Shannon H index of diversity, unique clone number, and high-frequency clone number to compare the differences in TCR repertoire between the NMOSD and HC groups (Figure 1B). The results showed that the diversities were restricted, and the number of unique clones and the proportion of in-frame clones of patients with NMOSD were significantly lower than those of HCs. By contrast, the number of high-frequency clones of NMOSD was considerably higher than that of HCs.

The length and composition of CDR3, mainly influenced by the V, (D), and J gene usage and the corresponding differences of junctional diversity, usually play a critical role in adaptive immune responses to various nonself-antigens and are associated with the risk of recognizing autoantigens in autoimmune diseases.[23] We compared the difference of the distribution and average length of CDR3 amino acid sequences between NMOSD and HC groups (Figure 1C), and the results revealed that the mean length of CDR3 sequences in NMOSD was significantly shorter than that in HCs, which is consistent with findings in other autoimmune diseases.[24]

To investigate the reason for this shortened CDR3 sequence, we further compared the length changes of inserted and deleted amino acid sequences in the NMOSD and HC groups (Figure 1D), which are shown in the schematic diagram of CDR3 composition (eFigure 1C, links.lww.com/NXI/A872). Compared with HCs, the lengths of V-D and D-J insertions of TCR clones in patients with NMOSD were significantly shorter compared with those in HCs, whereas the length of V3 deletions was substantially greater (Figure 1D). These findings suggest that the reduced insertion length, rather than

the increased deletion length, predominantly contributes to the overall decrease in CDR3 length in NMOSD.

Next, we compared the differences between patients with NMOSD and HCs in the usage of the *V* gene (eFigure 2A, links.lww.com/NXI/A872), *J* gene (eFigure 2B, links.lww.com/NXI/A872), and *VJ* pairing (eFigure 2C and eFigure 2D, links.lww.com/NXI/A872) from TCR clones. The results showed that patients with NMOSD and HCs differed significantly in the *V, J* gene usage and *VJ* pairing.

Occurrence of NMOSD May Be Associated With CMV Infection

To further explore the relationship between NMOSD and the TCR repertoire, we used the Fisher exact test for comparison of TCRs in patients with NMOSD and HCs and screened for disease-specific TCRs in NMOSD. We found 597 TCRs that were significantly enriched in NMOSD (eTable 2, links.lww.com/NXI/A874, FDR <0.05) (Figure 2A). We then investigated the characteristics of 533 unique CDR3 sequences among the 597 NMOSD-TCRs and randomly sampled an equal number of CDR3 sequences from the total TCR repertoire of NMOSD as controls. Compared with the randomly sampled sequences, the CDR3 sequences of NMOSD-TCRs were significantly shorter (eFigure 3A, links.lww.com/NXI/A872).

Clonal expansion of antigen-specific T cells occurs by activating major histocompatibility complex-antigen epitopes presented by antigen-presenting cells. During this process, activated T cells proliferate rapidly, generating many T cells with the same TCR, and these clonally amplified TCRs can bind to the corresponding antigenic epitopes.[25]

To explore the binding capacity of NMOSD-TCRs and AQP4 autoantigens, we searched the literature for reported T-cell epitopes of AQP4[11,26-28] and predicted the affinity of NMOSD-TCRs and randomly sampled CDR3 sequences to these antigenic epitopes using ERGO (peptide tcr matching prediction software). The results revealed that the average affinity of NMOSD-TCRs and AQP4 autoantigenic peptides was significantly higher than randomly sampled TCRs, implying that these NMOSD-TCRs may bind to the antigenic epitopes of AQP4 (eFigure 3B, links.lww.com/NXI/A872).

To explore the function of NMOSD-TCRs, we performed pathology-associated clonotype annotation on the 533 obtained unique CDR3 sequences. According to the annotation results, we found that 86.1% (459/533) of these unique CDR3 sequences of NMOSD-TCRs were associated with foreign microbial infections (eFigure 4A left, links.lww.com/NXI/A872); it was remarkably (*p* value <2.2e-16, Fisher exact test) higher than the proportion (7.9%, 42/533) in the randomly selected 533 TCRs (eFigure 4A right, links.lww.com/NXI/A872), implying that NMOSD may be associated with foreign microbes.

We further clustered the disease-specific and randomly sampled CDR3 amino acid sequences separately using the Levenshtein distance.[15] Most of the CDR3 sequences of NMOSD-TCRs were perfectly clustered into multiple clusters, and each cluster was associated with the corresponding foreign microbial infection (Figure 2B left). By contrast, the randomly sampled clones were loosely clustered and did not form larger clusters (Figure 2B right). This may be due to T cells of patients with NMOSD exhibiting clonal amplification of multiple antigenic epitopes against AQP4 and a certain similarity between clonally amplified CDR3 sequences, resulting in a distinctly different clonotypic profile from the randomly sampled CDR3.

Further fine annotation of specific foreign microbial-associated CDR3 revealed that CDR3 sequences of NMOSD-TCRs were mainly associated with viral infections, most notably CMV (66.8%, 356/533) and influenza (58.5%, 312/533) (Figure 2C), while only 3% (16/533) of both in randomly selected CDR3 sequences (eFigure 4B, links.lww.com/NXI/A872).

Infectious diseases are involved in autoimmune pathogenesis through molecular mimicry, epitope spreading, superantigen production, and other pathogenic mechanisms.[29] We hypothesized that NMOSD might result from viral infection forming molecular mimicry. Therefore, we used the TCRMatch tool[30] (tools.iedb.org/tcrmatch/) to predict the antigenic epitopes of all NMOSD-TCRs using the unique CDR3 sequences of NMOSD-TCRs and aligned all of them with the NMOSD autoantigen AQP4. Intriguingly, we found that the T-cell antigenic epitope of one of the CMVs was identical to amino acid sequences 68–71 of AQP4 (Figure 2D left). The CDR3 sequence corresponding to this antigenic epitope closely resembles one of the NMOSD-TCR sequences (Figure 2D right). Furthermore, we found that the shared core peptide (VDMV; Valine-Asparticacid-Methionine-Valine) partially overlaps with the antigenic epitope of *Clostridium perfringens*, which has been reported in the literature to exhibit cross-reactivity with AQP4.[31]

To exclude the potential effect from antigenic preferences of the annotation database, we used the protein sequences of all currently known human viruses aligned to AQP4 and screened for viral proteins with at least 5 contiguous amino acid identities (eFigure 4C, links.lww.com/NXI/A872). Of interest, the viral proteins of CMV stood out again. In addition, there was a case report of a patient presenting with NMOSD after CMV infection, the latter being suspected to be the trigger for the former.[32] In a recent study, a patient with AQP4-IgG[+] NMOSD in the acute phase of CMV infection showed significant symptomatic relief with antiviral medication after ineffective by using methylprednisolone, immunoglobulin, and plasma replacement.[33] This may be due to that CMV proteins molecularly mimic the antigenic epitopes of AQP4 and lead to NMOSD, and therefore, antiviral drugs may have a positive impact on the treatment of this disease.

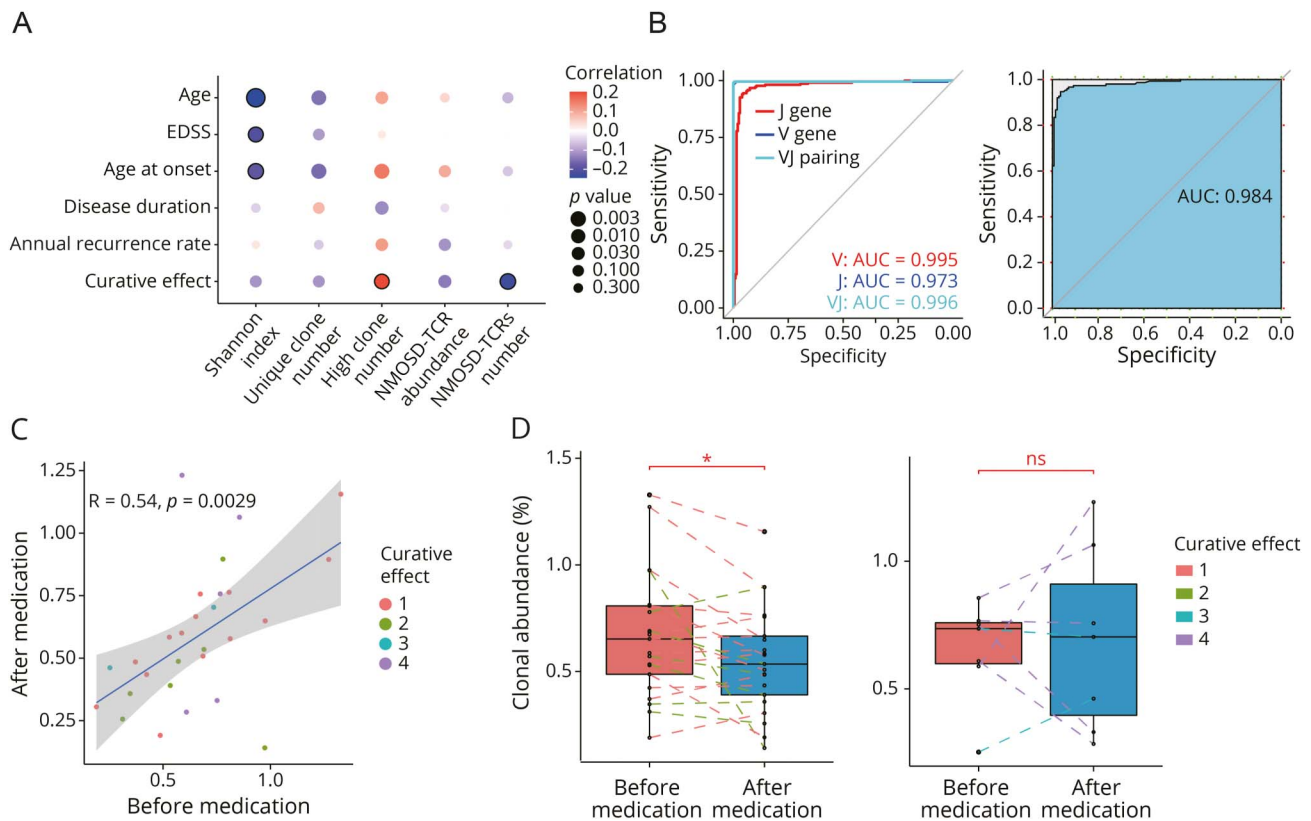**Figure 2** Identification of NMOSD-Associated TCRs and Their Characterization
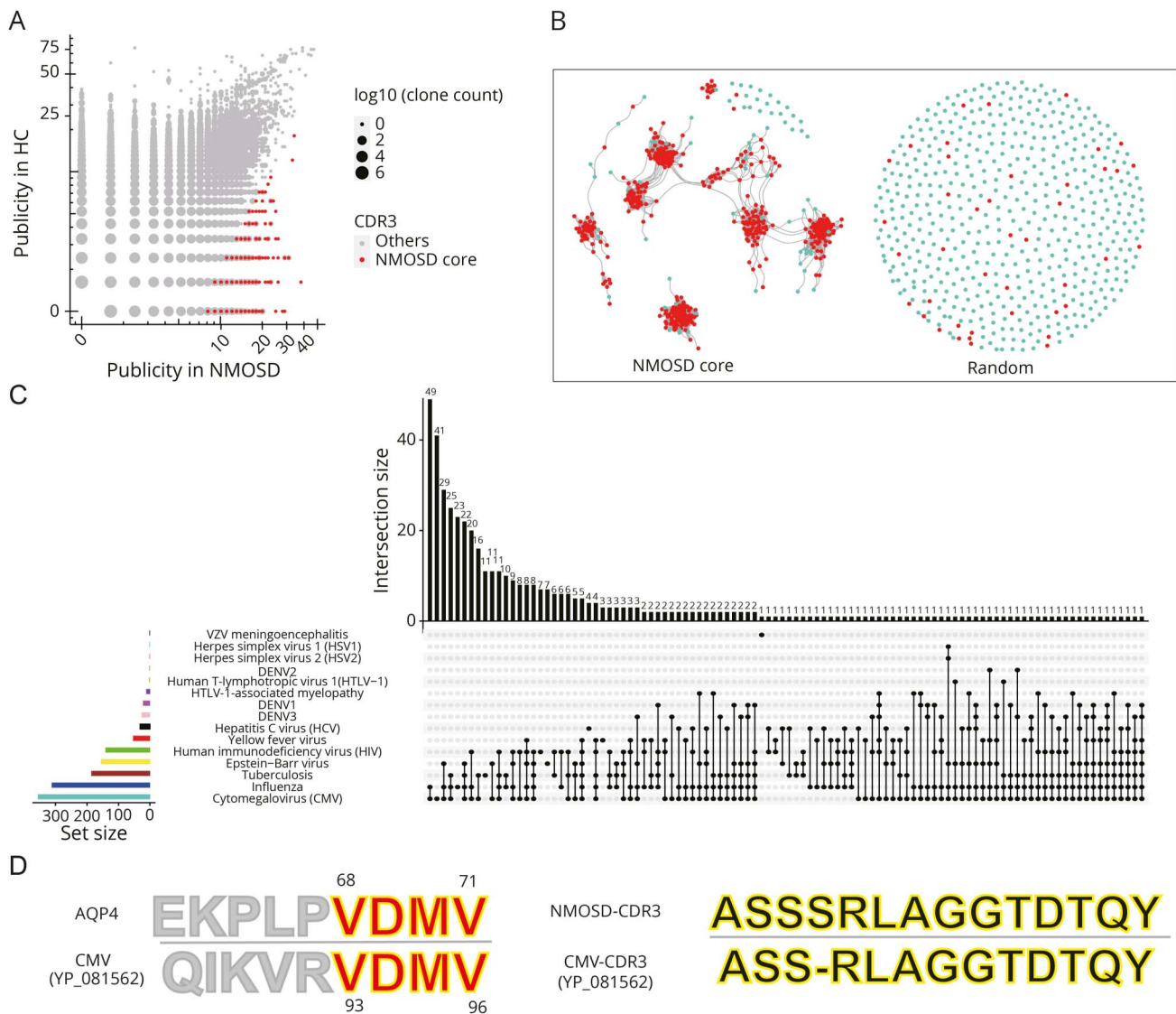
(A) Scatterplot demonstrates the publicity of TCRβ clones in patients with NMOSD and HCs, the size of the dots indicates the clone abundance, and the red dots represent the NMOSD-TCRs identified by the Fisher exact test. (B) Scatterplots show the sequence similarity of the unique CDR3 of NMOSD-TCRs (left) and randomly sampled CDR3 (right) separately. Levenshtein distance was used to calculate the similarity between clonotypes. (C) The annotation results reveal clones associated with different foreign microbes among NMOSD-TCRs. (D) The left plot illustrates the alignment results between a specific CMV viral protein T-cell antigenic epitope and AQP4. The right plot demonstrates the sequence similarity between the target NMOSD-TCR and the corresponding CMV infection–associated CDR3 on the left panel. AQP4 = aquaporin-4; HC = healthy control; NMOSD = neuromyelitis optica spectrum disorders; TCR = T-cell receptor.

Application of TCR Repertoire Indicators in the Diagnosis and Prognosis of NMOSD

The current diagnosis of NMOSD is based on clinical presentation, imaging, and the detection of serum AQP4-IgG.[34] Although the specificity of AQP4-IgG–based assays is almost 100%, the sensitivity is only approximately 60%–80%, which means that 20%–40% of patients with clinical presentation of NMOSD fail to detect AQP4-IgG.[35,36] It is probably due to the inability to detect at the serologic level or the existence of non-AQP4 antigens (e.g., myelin oligodendrocyte glycoprotein antigens).[37] Therefore, new methods are still needed to improve assay sensitivity.

The correlation analysis showed a decrease in TCR diversity with increasing disease severity of NMOSD patients, indicating that the clinical characteristics of NMOSD could be reflected at the TCR level (Figure 3A). To explore whether NMOSD can be diagnosed by *V, J* gene usage, *VJ* pairing, and abundance of NMOSD-TCRs, we used these indicators as features to classify NMOSD and HCs, respectively, with excellent classification results (area under curve scores achieved 0.97 or more, Figure 3B), indicating that the *V, J* gene usage, *VJ* pairing, and NMOSD-TCRs have great potential as biomarkers for disease diagnosis.

(A) Correlation between TCR repertoire and clinical indicators. The color represents the Pearson correlation coefficient, the size of the circle indicates the p value, and the black circle indicates whether the p value is significant. (B) Performance of classifying patients with NMOSD and HCs using V, J gene usage, VJ pairing (left) and NMOSD-TCRs as features, respectively. (C) Scatterplot demonstrates the correlation of CDR3 abundance of NMOSD-TCRs pretreatment and posttreatment; blue line is fitted curve, 1–4 indicates the efficacy of immunosuppressants or immunomodulators, where 1 indicates significantly effective, with no relapse or only 1 mild relapse during the treatment period, 2 indicates effective, with 1–2 relapses, and the annual recurrence rate (ARR) is significantly lower than pretreatment ARR, 3 means noneffective, with posttreatment ARR greater than or equal to the pretreatment ARR, and 4 indicates relapse after discontinuation of the drug. (D) Boxplots show the difference in the abundance of CDR3 of NMOSD-TCRs pretreatment and posttreatment in paired samples with good (left) and poor (right) efficacy, respectively (good efficacy: 1 and 2; poor efficacy: 3 and 4; t test; **, p value <0.01; ns, p value >0.05). NMOSD = neuromyelitis optica spectrum disorders; TCR = T-cell receptor.

The clinical treatment for NMOSD mainly uses immunosuppressive and modulating drugs such as azathioprine and mycophenolate mofetil or some emerging monoclonal drugs such as rituximab, satralizumab, inebilizumab, and eculizumab.[38] However, there is great heterogeneity in the response of different patients to different drugs, making it difficult to predict their efficacy. There is an urgent need to find markers to predict the efficacy of NMOSD drugs.

We compared the TCR repertoire of 28 pretreatment and posttreatment pairs of patients with AQP4-IgG$^+$ NMOSD. The proportion of high-frequency clones in the pretreatment patients was significantly reduced after treatment (eFigure 5A, links.lww.com/NXI/A872), while there was no significant difference in CDR3 length (eFigure 5B, links.lww.com/NXI/A872). When comparing the abundance of the V gene (eFigure 5C up, links.lww.com/NXI/A872), J gene (eFigure 5C down, links.lww.com/NXI/A872), and VJ pairing (eFigure 5D, links.lww.com/NXI/A872) in paired samples before and after treatment, only a few genes or VJ pairing were significantly changed after treatment. It suggested that the abundance of high-frequency clones may have changed after treatment, and no novel clones were produced.

In addition, correlation analysis revealed that the abundance of NMOSD-TCR before and after treatment had a significant positive correlation in paired samples, and we found that patients with poor efficacy seemed to deviate from the fitted curve, implying a tendency for NMOSD-TCR disorder in the poorly efficacious samples (Figure 3C). Next, we differentiated patients according to efficacy and compared the difference of NMOSD-TCRs between pretreatment and posttreatment samples. Of interest, the abundance of NMOSD-TCRs decreased significantly after treatment in patients with good outcomes (Figure 3D left), but there was no significant change in patients with poor outcomes and even showed an increasing trend in some patients (Figure 3D right), suggesting that the NMOSD-TCRs have potential as predictors of drug efficacy.

Gene Expression Profile of NMOSD Indicates Its Association With Viral Infection

To explore the changes of gene expression profiles in patients with NMOSD, we performed DEG analysis (eFigure 6A left, links.lww.com/NXI/A872) using RNA-seq data from a previous study on 7 patients with NMOSD and 18 HCs,[9] and the DEGs are summarized in eTable 3, links.lww.com/NXI/A875.

Figure 4 Identification of Coexpression Modules and Hub Genes in NMOSD

(A) Line chart illustrates the GSEA enrichment analysis results; different colors indicate different gene ontology terms. (B) Heatmap shows the correlation of all modules and traits, with numbers in the modules indicating correlation coefficients and *p* values. (C) KEGG enrichment results of genes in cyan modules. (D) The network diagram displays the top 20 hub genes reidentified using cytohubba; 4 genes with a green background are transcription factors predicted by iRegulon. GSEA = Gene Set Enrichment Analysis; NMOSD = neuromyelitis optica spectrum disorders.

The Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis showed that the upregulated genes were mainly related to viral infection and innate immune–related pathways, such as Epstein-Barr virus infection, Herpes simplex virus 1 infection, nucleotide-binding oligomerization domain (NOD)-like receptor, and retinoic acid-inducible gene I (RIG-I)–like receptor signaling pathway (eFigure 6A right, links.lww.com/NXI/A872). Gene Set Enrichment Analysis (GSEA) demonstrated that interferon-related and T-cell receptor signaling pathways were significantly activated (Figure 4A). These results suggested that NMOSD may be due to the activation of innate immune pathways and TCR

signaling pathways after viral infection, resulting in antiviral response.

Next, we identified coexpression modules in NMOSD by weighted correlation network analysis with a soft threshold of 8 for the scale-free network, as shown in eFigure 6B, links.lww.com/NXI/A872 and Figure 4B, and a total of 24 coexpression modules were obtained. The correlation coefficient between the cyan module and NMOSD reached 0.53 (p value = 2.1e-11) (Figure 4B), and further KEGG enrichment analysis indicated that this module was associated with viral infections and innate immunity (Figure 4C).

We screened 47 hub genes in the cyan module (eFigure 6C, links.lww.com/NXI/A872) using gene significance > 0.2 and module membership (MM) > 0.8 as thresholds, and protein–protein interactions network analysis revealed strong protein interactions between these hub genes (eFigure 6D, links.lww.com/NXI/A872). We further screened the top 20 hub genes from them using cytoHubba (Figure 4D) and found that most of the hub genes are viral infection–related and interferon-related genes. Transcription factor enrichment analysis showed that most of the top 20 hub genes were regulated by *STAT1*, *STAT2*, *MYB*, and *IRF1*. It is suggested that these hub genes and transcription factors could potentially be used as therapeutic targets for NMOSD.

Characterization of AQP4-IgG Suggested That CMV Infection May Lead to NMOSD

The bulk RNA data analysis results indicated high interferon-related gene expression in NMOSD. Previous studies have shown that type-I interferons (IFN-1) could promote the proliferation of TH17 cells and B-cell maturation in NMOSD.[9,39] To investigate the changes in cellular levels of patients with NMOSD, we compared the differences in scRNA-seq results between whole blood samples from 1 patient with NMOSD and 3 HCs from the Gene Expression Omnibus database.[40] We first removed batch effects (eFigure 7A left, links.lww.com/NXI/A872) using harmony for 10X single-cell data of them and performed dimensionality reduction and clustering using Seurat.

We identified 16 distinct cell subpopulations (eFigure 7A right, links.lww.com/NXI/A872) by using classical marker genes (eFigure 7B, links.lww.com/NXI/A872), and the highly expressed genes of each subpopulation are shown in eFigure 7C, links.lww.com/NXI/A872. There were significant differences in cell composition between patients with NMOSD and HCs, where patients with NMOSD had a relatively high proportion of NK CD56^{dim} and CD8$^+$ cytotoxic T lymphocyte (CD8 CTL) cells and a lower proportion of Naive CD8+ T (CD8 Tn) cells (eFigure 7D, links.lww.com/NXI/A872), which is consistent with those observed in previous studies.[8,41] Then, we compared DEGs in all subpopulations of patients with NMOSD with those in HCs (eFigure 8A and eFigure 8B, links.lww.com/NXI/A872).

Enrichment analysis of DEGs in each cluster revealed that consistent with the results of bulk RNA, highly expressed genes in multiple clusters were concentrated in Th17, BCRs, antigen processing and presentation, viral infection, and NOD-like related pathways (eFigure 8C, links.lww.com/NXI/A872).

Because the peripheral blood of patients with NMOSD may contain B cells that secrete AQP4-IgG, we next analyzed the BCR data at the single-cell level. Unlike HCs, we found the most significantly amplified BCRs (clonotype1) in patients with NMOSD belonged to IgG class (eFigure 9A, links.lww.com/NXI/A872). Antibody modeling and affinity prediction showed that clonotype1 had the highest affinity with AQP4, which was presumed to be an AQP4-IgG (eFigure 9B, links.lww.com/NXI/A872). Then, we aligned the CDR3 amino acid sequences of clonotype1 to the SAbDab antibody database,[42] revealing similarities to CMV-related antibodies (eFigure 9C, links.lww.com/NXI/A872). The structural similarity between the light chain of clonotype1 and the CMV-associated antibody 7kbb further suggests the possibility of correlation between NMOSD and CMV infection (root mean square deviation = 0.7 Å) (eFigure 9D, links.lww.com/NXI/A872).

T-Cell Activation Experiments Demonstrated the Association Between AQP4-IgG$^+$ NMOSD and CMV Infection

To explore the human leukocyte antigen (HLA) genes associated with the risk of NMOSD, we collected blood samples from 20 patients with AQP4-IgG$^+$ NMOSD for HLA typing and used Han Chinese samples from 1000 Genomes Project as controls (eTable 4, links.lww.com/NXI/A876). The results of the association analysis of the HLA regions revealed that *HLA-DPB1*05:01*, *DPB1*04:01*, *DQB1*02:01*, and *DRB1*03:01* were associated with disease susceptibility in our AQP4-IgG$^+$ NMOSD cohort (eFigure 10, links.lww.com/NXI/A872). Notably, *DRB1*05:01* was identified as the major risk factor (OR = 4.4, p value = 0.016), and some susceptibility genes (*DRB1*03:01* and *DPB1*05:01*) were consistent with findings from previous studies.[43,44]

To further validate the correlation between AQP4-IgG$^+$ NMOSD and CMV infection, we synthesized all T-cell epitopes containing shared core peptides (VDMV), combined them to form a peptide pool (eAppendix 1, eTable 4, links.lww.com/NXI/A871, links.lww.com/NXI/A876), and performed T-cell activation experiments on PBMCs from 5 untreated patients with AQP4-IgG$^+$ NMOSD and 5 healthy donors. The flow cytometry results demonstrated significant activation of CD4$^+$ T cells in PBMCs from patients with NMOSD after stimulation with 10 and 20 μg/mL of CMV peptide pools compared with those unstimulated (Figure 5A). By contrast, HCs exhibited no significant change in T-cell activation levels (Figure 5B left). Furthermore, we observed significantly higher levels of CD4$^+$ T-cell activation in NMOSD blood samples compared with HCs after

Figure 5 CMV Peptides Activate T Cells From Patients With NMOSD
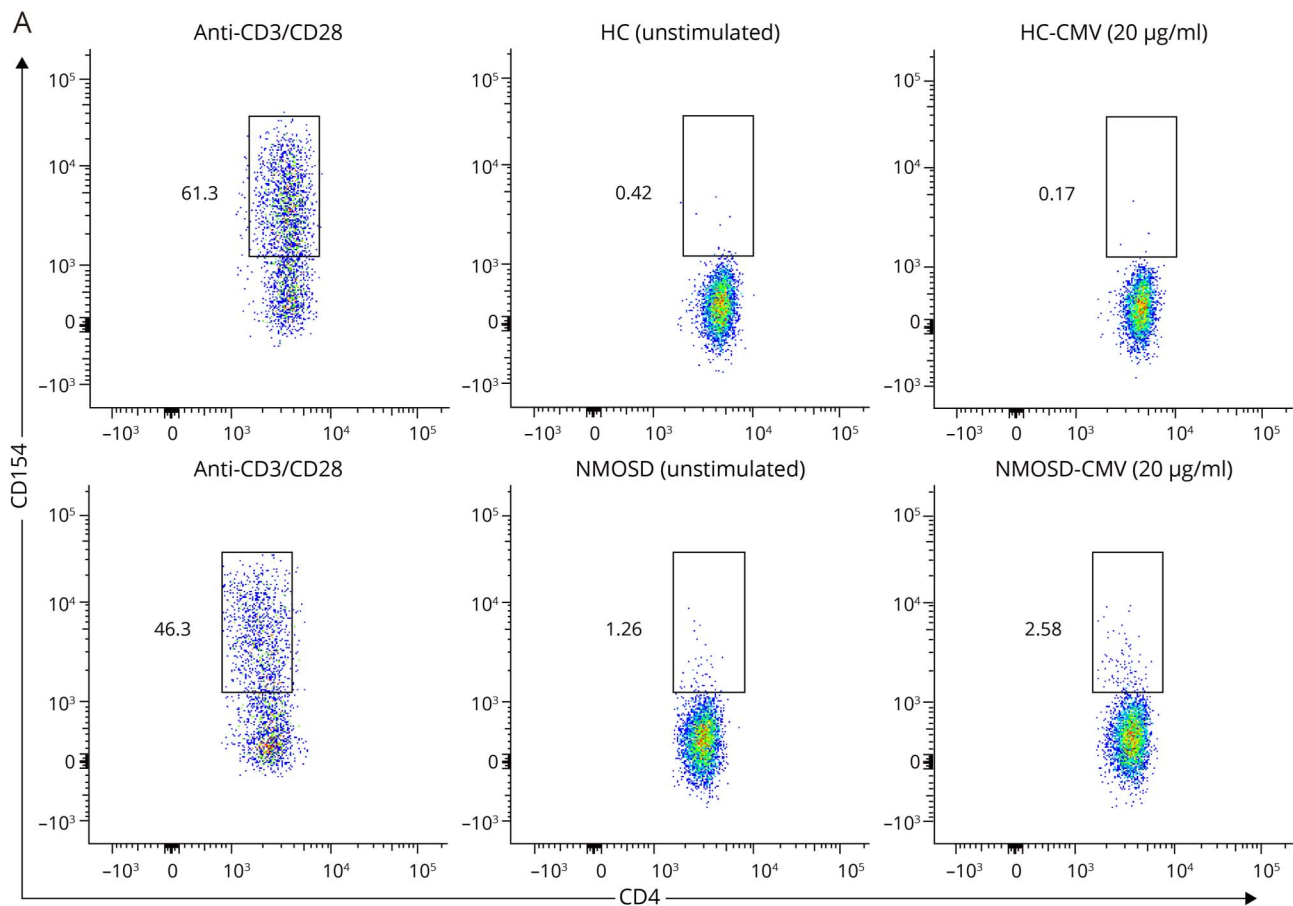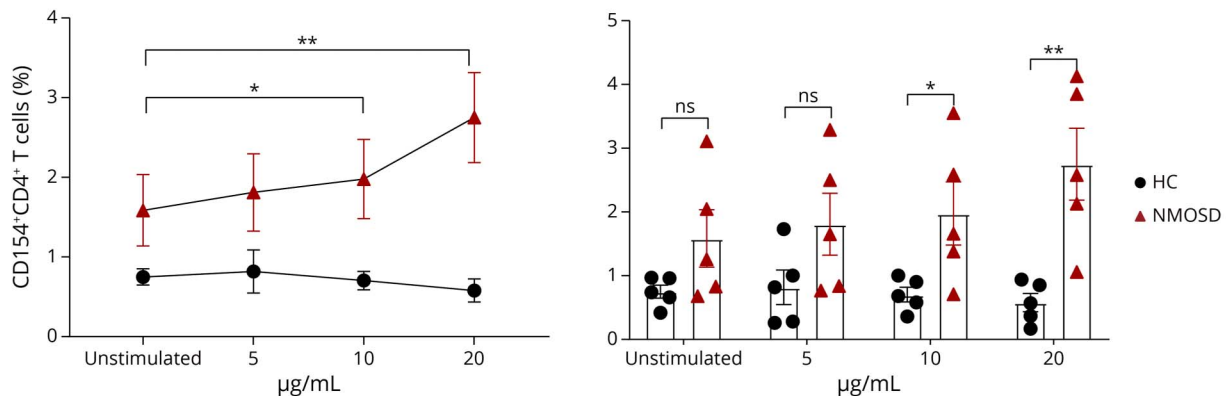
Peripheral blood mononuclear cells (PBMCs) were collected from patients with AQP4-IgG⁺ NMOSD without immunotherapies (n = 5) and healthy controls (HCs) (n = 5) and tested for activation to CMV peptides. (A) PBMCs were stimulated with CMV peptides for 12 hours in the presence of anti-CD40 and anti-CD28 (both 1 μg/mL). Extracellular CD154 expression was used to assess CMV antigen-specific CD4⁺ T-cell levels after stimulation by flow cytometry (Flowjo v10). Unstimulated samples as negative controls, while samples with the addition of anti-CD3 and anti-CD28 (both 1 μg/mL) as positive controls. (B) The line chart and bar plot depict the changes in the proportion of CD154⁺CD4⁺ T cells after stimulation with varying concentrations of CMV peptides (5, 10, and 20 μg/mL) (paired *t* test; *: *p* value <0.05, **: *p* value <0.01) and the differences between the 2 groups of healthy individuals and patients with NMOSD (Wilcox test; *: *p* value <0.05, **: *p* value <0.01, ns = not significant), respectively. AQP4-IgG = AQP4 immunoglobulin G; NMOSD = neuromyelitis optica spectrum disorders.

stimulation by a high concentration of CMV peptide pools (Figure 5B right). In summary, our findings suggest that CMV may trigger the production of AQP4-specific autoreactive T cells in patients with specific HLA alleles by sharing a core peptide (VDMV) with AQP4.

Discussion

Approximately two-thirds of patients with NMOSD are caused by AQP4-IgG–mediated damage to the CNS. Although there is speculation that a history of infection or

inflammation may be involved in the pathogenesis of NMOSD, specific cases have only been reported sporadically.[33,45] In this study, we performed high-throughput TCR sequencing on patients with AQP4-IgG⁺ NMOSD with pretreatment and posttreatment and normal individuals. By identifying NMOSD-TCRs and integrating the public database of transcriptome expression changes of patients with NMOSD and characterization of clonally amplified BCR structures analyzed by scRNA-seq data, viral infection, especially CMV, was found to be a possible pathogenic trigger of NMOSD. This study deepens the understanding of the pathogenic mechanism of NMOSD and may provide new clues for the treatment of the disease.

By characterizing the immune repertoire, we found that the TCRβ diversity is restricted in NMOSD. The number of unique clones and the proportion of in-frame clones of patients with NMOSD were significantly lower than those in HCs, suggesting that some clones were significantly amplified into high-frequency clones possibly due to the presence of autoantigens in NMOSD. Differences in the proportion of in-frame clones suggest that NMOSD is more prone to abnormal *V-D-J* recombination, resulting in a higher proportion of nonfunctional TCR clones. Among these identified NMOSD-TCRs, we found that their CDR3 amino acid sequences were significantly shorter and had a higher sequence similarity than randomly selected CDR3 sequences. The shorter CDR3 may increase the likelihood of self-recognition and thus increase the risk of autoimmune disease.

In one of our previous studies,[15] to identify disease-specific clones that were significantly enriched in the disease group with high specificity, we raised the filtering criteria and ignored oligoclonal TCRs that were less abundant in the sample (198 systemic lupus erythematosus-associated TCR clones with p value < 5e-4 and 53 RA-associated clones with p value < 1e-3 were identified using IMisc). However, several studies have shown that specific antigenic epitopes in an antigenic molecule are generally targeted by multiple TCRs.[19,46] In a recent study, to directly characterize the CD8 T-cell response to SARS-CoV-2, the researchers identified 23,179 unique SARS-CoV-2–specific CD8 TCRs across Multiplexed Identification of TCR Antigen (MIRA) experiments.[46] The identified TCRs mapped to almost all antigenic epitopes of SARS-CoV-2 predicted by NetMHCIIpan (545 peptides). In another study, the investigators discovered 69 PP65 (one of the CMV antigenic epitopes) associated TCRβ chains in the MIRA experiment.[19] Given the high heterogeneity of TCRs in the population and the possible presence of oligoclonal TCRs in some patients, we performed the Fisher 1-tailed test[19] for all clones in this study and finally identified 597 NMOSD-TCRs.

Pathology-associated clonotype annotation and antigenic epitope prediction revealed that the occurrence of NMOSD might be associated with viral infection. Furthermore, transcriptome data at the bulk and single-cell levels also showed that patients with NMOSD were significantly enriched for viral infections

and innate immunity–related pathways. In addition, high interferon-related and viral infection–related gene expression occurred in NMOSD, and the high-frequency clonally amplified antibody clonotype1 (AQP4-IgG1) in NMOSD had a significantly higher affinity for AQP4 than all other antibodies. The high sequence and structural similarity between the CDR3 sequence of the clonotype1 light chain and CMV-associated antibodies further support the possibility that the occurrence of NMOSD is associated with CMV infection.

In addition, we compared the changes in TCR characteristics of patients with AQP4-IgG⁺ NMOSD pretreatment and posttreatment, and we found that the CDR3 amino acid sequence abundance of NMOSD-TCRs was significantly lower in patients with good efficacy but not in patients with poor efficacy, suggesting the potential of NMOSD-TCRs as predictors of prognosis.

Based on these findings, we hypothesized that the CMV induces clonal expansion of human self-reactive CD8⁺ T cells and Th17 polarization of autoreactive CD4⁺ T cells because of antigenic mimicry with AQP4 in patients with AQP4-IgG⁺ NMOSD. At the same time, viral infection contributed to the activation of innate immune signaling pathways such as interferon, retinoic acid-inducible gene I–like receptor, and NOD-like receptor signaling pathways. Clonally amplified autoreactive T cells mediated the activation of B cells to produce autoreactive plasma cells that secrete high-affinity antibodies. These plasma cells and the antibody AQP4-IgG enter the CNS and bind to AQP4 on the surface of astrocytes resulting in neural demyelination, which contributes to the pathogenesis of NMOSD (Figure 6).

Similar to CMV infection, the proportion of neutrophils and effector CD8⁺ T cells and the levels of IFN-I, interferon-stimulated genes (ISGs) and Interleukin 6 (IL-6) in the blood of patients with AQP4-IgG⁺ NMOSD were significantly higher than in normal individuals.[8,47-51] Epidemiologically, people of Asian and African ancestry have a higher risk of AQP4-IgG⁺ NMOSD than Caucasians,[6,52] and it is more prevalent in female individuals,[53] which is also consistent with CMV infection.[54] In addition, in a 2011 study evaluating 24 bacterial and viral infections in 19 patients with NMOSD, serologic evidence of recent viral infections was found to be significantly higher in the acute phase of NMOSD than in controls. The investigators speculated that multiple viral infections might be associated with the development of AQP4-IgG⁺ NMOSD in the early stages of the disease.[55] Of interest, another study observed a relatively higher IgG response to CMV-related peptides in patients with AQP4-IgG⁺ NMOSD based on analysis of the customized peptide microarray.[56] Combined with the findings of this study, we hypothesize that the occurrence of AQP4-IgG⁺ NMOSD may be associated with CMV infection.

However, CMV infection is highly prevalent in the population,[57] but the prevalence of NMOSD is relatively low. Our

AQP4-IgG = AQP4 immunoglobulin G; NMOSD = neuromyelitis optica spectrum disorders.
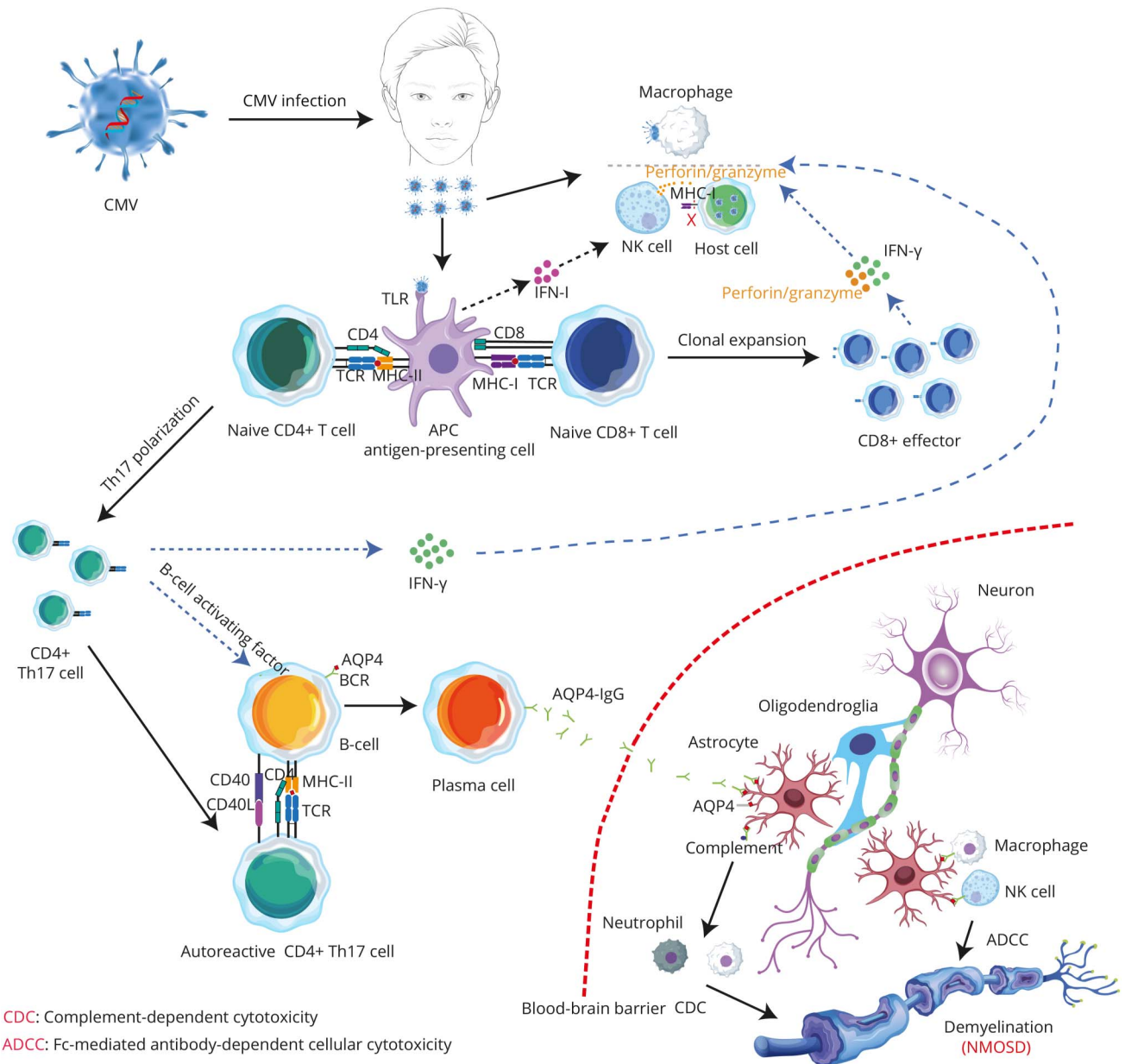
findings further confirmed that genetic factors such as HLA genes play an important role in the pathogenesis of NMOSD, and some of these HLA genes (e.g., *HLA-DPB1*05:01 and HLA-DRB1*03:01*) are responsible for increasing the genetic risk of NMOSD, which is consistent with the previous studies.[43,44] Considering the low prevalence of NMOSD and these additional risk factors together, we speculated that CMV infection may be necessary but not sufficient to trigger NMOSD when present alone.

To verify that CMV infection is associated with NMOSD, we performed antigen peptide stimulation experiments in vitro and initially indicated that CMV antigen peptides could activate

antigen-specific CD4⁺ T cells from the periphery of patients with NMOSD, which was not found in healthy individuals. It is suggested that CMV may be involved in NMOSD pathogenesis through the activation of self-reactive T cells.

Last, it is important to acknowledge that while our study primarily focused on the viral reactivity of clonally expanded T cells in NMOSD, other nonself-antigens, such as those from *C perfringens*, may also contribute to the development of NMOSD.[58] In a previous report by Cree et al it was demonstrated that *C perfringens* peptide has been demonstrated to cross-react with AQP4 antigenic peptide and can activate autoreactive T cells.[31] This study was conducted in the North

American population, whereas NMOSD was not found to be associated with *C perfringens* in our previous cohort and another study investigating gut microbiology in a Chinese population.[59,60] Nonetheless, we found that the antigenic epitopes of *C perfringens*, which cross-react with AQP4, share a core sequence overlap with the antigenic epitopes of CMV identified in our study. These shared core peptides may serve as the basis for cross-reactivity of different foreign microbes with AQP4. This finding suggests that differences in genetic background may result in distinct responses to pathogens across populations and that multiple external pathogens capable of cross-reacting with the AQP4 antigen might be involved in the pathogenesis of NMOSD.

However, it should be noted that there are several limitations to this work. First, the pathology clonotype annotation of NMOSD-TCRs in this study may be influenced by the antigen preference of the database. Based on this concern, we used all currently known human viruses to align with AQP4 protein sequences, and CMV proteins were uncovered among pentapeptides with consistent sequences. Second, whereas we found the possibility that NMOSD is associated with CMV infection, further confirmation at the serologic level is needed in the future. In addition, although our study provides evidence for the possibility that CMV infection is associated with the pathogenesis of NMOSD and preliminarily demonstrated through in vitro experiments that CMV antigenic peptides can activate peripheral antigen-specific T cells from patients with NMOSD, further antigen cross-reactivity experiments and validation in animal models are needed to confirm whether CMV activates self-reactive T cells through molecular mimetic mechanisms and thus participates in the pathogenesis of NMOSD.

In summary, our findings expand the understanding of potential triggers for the occurrence of AQP4-IgG⁺ NMOSD and provide clues to the potential of NMOSD-TCRs as markers for predicting the efficacy of immunosuppression, and these findings have clinical implications for the treatment of AQP4-IgG⁺ NMOSD.

Disclosure
H. Zhou: 2021YFS0173 and 2022YFS0315; Z. Shi: 21HXFH041 and 2021M692295; The other authors report no relevant disclosures. Go to Neurology.org/NN for full disclosures.

Appendix Authors

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Ziyan Shi, PhD	Department of Neurology, West China Hospital, Sichuan University, Chengdu, China	Drafting/revision of the article for content, including medical writing for content; study concept or design
Wei Zhang, PhD	City University of Hong Kong, Shenzhen Research Institute, Shenzhen, China	Drafting/revision of the article for content, including medical writing for content; study concept or design
Lin Zhu, PhD	Department of Neurology, West China Hospital, Sichuan University, Chengdu, China	Drafting/revision of the article for content, including medical writing for content
Shanshan Tang, PhD	Department of Neurology, West China Hospital, Sichuan University, Chengdu, China	Drafting/revision of the article for content, including medical writing for content
Hongxi Chen, PhD	Department of Neurology, West China Hospital, Sichuan University, Chengdu, China	Major role in the acquisition of data
Xiaofei Wang, PhD	Department of Neurology, West China Hospital, Sichuan University, Chengdu, China	Major role in the acquisition of data
Qin Du, PhD	Department of Neurology, West China Hospital, Sichuan University, Chengdu, China	Major role in the acquisition of data
Shuaicheng Li, PhD	City University of Hong Kong, Shenzhen Research Institute, Shenzhen, China	Study concept or design
Ying Zhang, PhD	Department of Neurology, West China Hospital, Sichuan University, Chengdu, China	Major role in the acquisition of data
Wenqin Luo, PhD	Department of Neurology, West China Hospital, Sichuan University, Chengdu, China	Major role in the acquisition of data
Xin Jin, PhD	College of Life Sciences, University of Chinese Academy of Sciences, Beijing, China	Study concept or design
Mingyan Fang, PhD	Department of Neurology, West China Hospital, Sichuan University, Chengdu, China	Drafting/revision of the article for content, including medical writing for content; major role in the acquisition of data; study concept or design
Hongyu Zhou, PhD	Department of Neurology, West China Hospital, Sichuan University, Chengdu, China	Drafting/revision of the article for content, including medical writing for content; major role in the acquisition of data; and study concept or design

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