Human C5-specific single-chain variable fragment ameliorates brain injury in a model of NMOSD

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Neurol Neuroimmunol Neuroinflamm 2019;6:e561. doi:10.1212/NXI.0000000000000561

Abstract

Objective
Using phage display, we sought to screen single-chain variable fragments (scFvs) against complement C5 to treat neuromyelitis optica spectrum disorder (NMOSD).

Methods
After 5 rounds of phage display, we isolated individual clones and identified phage clones specifically binding to C5 using ELISA. Using aquaporin-4 (AQP4)-transfected cells in vitro, we confirmed whether these scFvs prevented complement-dependent cytotoxicity (CDC) caused by the serum of patients with NMOSD and human complement (hC). We selected an NMOSD mouse model, in which intracerebral NMOSD immunoglobulin G (IgG) and hC injections induce NMOSD-like lesions in vivo.

Results
We obtained scFvs to test specificity and blocking efficiency. The scFv C5B3 neutralized C5 in the complement activation pathway, which prevented AQP4-IgG-mediated CDC in AQP4-transfected cells. In an NMOSD mouse model, C5B3 prevented AQP4 and astrocyte loss, decreased demyelination, and reduced inflammatory infiltration and membrane attack complex formation in lesions.

Conclusions
We used phage display to screen C5B3 against C5, which was effective in inhibiting cytotoxicity in vitro and preventing CNS pathology in vivo.
Neuromyelitis optica spectrum disorder (NMOSD) is a disabling autoimmune disease of the CNS, which is characterized by optic neuritis and transverse myelitis.\textsuperscript{1,2} The discovery of autoantibodies against astrocytic aquaporin-4 immunoglobulin G (AQP4-IgG) distinguished NMOSD from MS based on its immunologic pathogenesis.\textsuperscript{3–5} Current NMOSD therapies, including general immunosuppressive agents, plasma exchange, and B-cell depletion, target AQP4-IgG and inflammatory reactions.\textsuperscript{6–8}

Complement plays a fundamental role in the pathogenesis of NMOSD.\textsuperscript{3} Complement can attack astrocytes through complement cascade activation and membrane attack complex (MAC; C5b-9) formation in astrocytic membranes.\textsuperscript{9,10} Moreover, secondary inflammatory cascades involving granulocytes and macrophage infiltration lead to demyelination and neuron death.\textsuperscript{11–14} Increasingly, NMOSD treatment studies have focused on complement C5. C5 can initiate the terminal complement cascade.\textsuperscript{15} Moreover, C5 inhibitor can prevent MAC formation by blocking complement-dependent cytotoxicity (CDC).\textsuperscript{16} Eculizumab, which is a therapeutic humanized monoclonal IgG that inhibits C5 convertase in the classical complement pathway, showed benefits in relapsing NMOSD.\textsuperscript{17–19}

Monoclonal antibody therapy is a new treatment strategy with high efficacy and tolerability in autoimmune diseases.\textsuperscript{6,20,21} However, most monoclonal antibodies are of a murine origin. The clinical application of murine monoclonal antibody-induced human anti-mouse antibody (HAMA) is slightly limited.\textsuperscript{22} Replacing murine with homologous human sequences through genetic engineering can produce “chimeric” or “humanized” antibodies.\textsuperscript{23} Phage display is an important method for obtaining fully human antibodies against a given antigen.\textsuperscript{24} In this study, we explored human single-chain variable fragments (scFvs) to extend NMOSD complement therapy. Using phage display techniques, we developed a fully human C5-targeted scFv (C5-scFv) as a therapy for NMOSD.\textsuperscript{25}

The collection of serum from the participants. We obtained informed consent from all the participants.

**Phage display procedure and C5-scFv expression and purification**

We performed the phage display selection process as previously described with some adjustments.\textsuperscript{26} The semisynthetic scFv phage library (Creative Biolabs, Shirley, NY) for *Escherichia coli* TG1 bacteria was cultured and infected with M13KO7 helper phage (NEB, Ipswich, MA). We concentrated the phage library with 20% polyethylene glycol precipitation. After preparing the scFv phage library, we performed 5 rounds of panning (adsorption-elution-amplification).\textsuperscript{25} After 5 rounds of selection, we isolated individual clones and identified phage clones specifically binding to antigen with ELISA.\textsuperscript{25} C5-scFv expression and purification were based on a previous study with some adjustments.\textsuperscript{27} To obtain a 40-mg/mL C5-scFv concentration, we concentrated the C5-scFv using Amicon Ultra centrifugal filter units (Millipore, Billerica, MA).

**Surface plasmon resonance**

We performed surface plasmon resonance (SPR) measurements on a Biacore T200 instrument (GE Healthcare, Chicago, IL). We used 10 mM NaAC (pH 5.5) to dilute C5. Following this, we immobilized C5 onto the surface of a carboxymethyl dextran matrix 5 sensor chip. We measured binding with a 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer (pH 7.4) at 25°C as the running buffer (20 μL/min). We injected the C5-scFv for 90 seconds over the sensor chip, followed by a 180-second washout period with the same flow rate. For each injection, we regenerated the flow cells for 30 seconds using an injection of 10 mM glycine-hydrochloric acid (pH 1.5). We calculated the antibody-antigen binding constants using Biacore evaluation software (GE Healthcare).

**ELISA**

We coated C5 on a 96-well streptavidin-coated plate overnight at 4°C, blocked it with 2% bovine serum albumin (BSA), and incubated it with phage supernatant as the primary antibody. We detected bound phages by adding rabbit horseradish peroxidase/anti-M13 conjugate antibody (1:1,000; Creative Diagnostics, New York City, NY) for 40 minutes at room temperature with continuous gentle shaking. We stained the wells with 100 μL of 3,3′,5,5′-Tetramethylbenzidine (MYbiotech, Xi’an, China) and stopped the peroxidase reaction with 50 μL of 2.5 M H2SO4. We used a microplate reader (Bio-Rad Laboratories, Hemel Hempstead, UK) to measure the absorbance at 450 nm.

**Methods**

**Standard protocol approval, registration, and patient consent**

The Committee on the Ethics of Animal Experiments of Shaanxi Normal University approved all the animal experiments. Moreover, all the animal experiments followed the NIH Guide for the Care and Use of Laboratory Animals. The Ethics Committees of Tianjin Medical University approved the collection of serum from the participants. We obtained informed consent from all the participants.
NMOSD antibodies
We obtained AQP4-IgG serum (serumNMOSD) from 5 AQP4-IgG–positive patients with high AQP4-IgG titers. Shi et al.28 have previously described these patients’ detailed clinical information. We purified the total IgG with protein-A resin (GeneScript, Piscataway, NJ) according to a previously described protocol.29 After undergoing concentration using Amicon Ultra centrifugal filter units (Millipore), the final concentration of IgG (IgGNMOSD) was 15 mg/mL. We collected control serum (serumCtrl) from healthy volunteers.

NMOSD animal model induction
We purchased 8- to 12-week-old weight-matched female C57BL/6 mice from the Experimental Animal Laboratories of the Academy of Military Medical Sciences. We normally fed and maintained the mice in a suitable air-filtered environment under a 12-hour light-dark cycle. We randomly assigned the mice to different groups as follows: (1) 6 μL of IgGNMOSD and 4 μL of human complement (hC) as the control group (n = 5); (2) 6 μL of IgGNMOSD, 4 μL of hC, and 5 μL of CSB3 (n = 5); and (3) 4 μL of hC and 5 μL of CSB3 (n = 5). We intraperitoneally anesthetized the mice with 10% chloral hydrate (3 mL/kg) and mounted a stereotactic frame (RWD Life Science, Shenzhen, China). We made a midline scalp incision to expose the bregma and lambda. For intra-parenchymal injections at a skull position 2.5 mm to the right of the bregma, we made a 0.7-mm diameter burr hole with a high-speed drill (RWD Life science). We inserted a 33-G needle attached to a 25-μL gas-tight glass syringe (Hamilton, Reno, NV) 3 mm deep to infuse 6 μL of IgGNMOSD and 4 μL of hC with or without 5 μL of CSB3 (total volume, 15 μL at 1 μL/min). We injected another group of mice with 4 μL of hC and 5 μL of CSB3 (total volume, 15 μL). We maintained rectal temperature at 37°C during anesthesia. After disinfecting the scalp surface, we sutured the scalp. Seven days later and immediately before euthanasia, we anesthetized (10% chloral hydrate) the mice and perfused them through the left cardiac ventricle with phosphate-buffered saline (PBS), followed by 4% paraformaldehyde (PFA). Following this, we processed them for frozen sectioning.

Complement-dependent cytotoxicity
We stably transfected Chinese hamster ovary (CHO) cells with human M23-AQP4 as previously described.30 We cultured M23-AQP4–overexpressing CHO cells in 1640 Nutrient Mix Medium supplemented with 10% fetal bovine serum, 100 μg/mL streptomycin, and 100 U/mL penicillin (Gibco, Grand Island, NY). In a humidified atmosphere of 5% CO2 at 37°C, we cultured the cells. When the cells were confluent, we washed them with PBS. Following this, we added 2.5% hC to the wells with 10% serumNMOSD with or without 500 μg/mL C5-scFv (CSB3, CSB35, and CSA6) for 30 minutes. We incubated some wells with serumCtrl or different dilutions of serumNMOSD with 2.5% hC and other wells with different dilutions of hC with 10% serumNMOSD with or without different dilutions of CSB3. We assayed cytotoxicity using a LIVE/DEAD Viability/Cytotoxicity Kit (Invitrogen, Carlsbad, CA).

Immunocytochemistry
We incubated M23-AQP4–overexpressing CHO cells with 10% serumNMOSD and 2.5% hC with or without CSB3. We washed the wells with PBS, fixed them with 4% PFA, and blocked them with 5% BSA. We incubated the cells with rabbit polyclonal anti-CSb-9 (1:100; Abcam, Cambridge, UK) primary antibodies in 5% BSA at 4°C overnight, followed by incubation with goat anti-rabbit IgG-conjugated Alexa Fluor 594 (1:1,000; Invitrogen, Carlsbad, CA) in 5% BSA. After secondary antibody incubation, we washed them again with PBS and examined them using a fluorescence microscope (Leica DM6000B; Leica, Wetzlar, Germany). The investigators were blinded to the experimental groups during pathology staining and analysis.

Immunohistochemistry
We selected the frozen sections (thickness, 6 μm) through the needle tract. We permeabilized the frozen sections with cold acetone, blocked them (5% BSA), and immunostained them with the following primary antibodies at 4°C overnight: rabbit polyclonal anti-AQP4 (1:100; Santa Cruz Biotechnology, Dallas, TX), goat polyclonal anti–glial fibrillary acidic protein (GFAP) (1:1,000; Abcam), rat anti–myelin basic protein (MBP, 1:200; Millipore), rat anti–CD45 (1:100; BD Biosciences, San Jose, CA), rabbit polyclonal anti–ionized calcium-binding adaptor molecule 1 (IBA1, 1:1,000; Wako, Osaka, Japan), and rabbit polyclonal anti–CSb-9 (1:100; Abcam). Following this, we immunostained the frozen sections with the appropriate Alexa Fluor-conjugated secondary antibody (1:1,000; Invitrogen). We examined the tissue sections with a Leica DM 6000B microscope and defined the areas using ImageJ software (ImageJ 1.51j8; NIH).29 The investigators were blinded to the experimental groups during pathology staining and analysis.

Statistical analyses
We present the data as mean ± standard error of the mean. We used a 2-tailed unpaired t test to compare 2 groups, and we used a 1-way analysis of variance for comparisons between 3 groups. We considered a p value <0.05 to be significant. For our analysis, we used SPSS for Windows software (version 17.0; SPSS, Inc, Chicago, IL).

Data availability
We will share the study data upon request from any qualified investigator.

Results
Phage display screened human scFv clones against C5
We performed 5 rounds of panning (adsorption-elution-amplification) of the semisynthetic scFv phage library. As shown by ELISA, the ability of the phage library to bind to C5 gradually increased (figure 1A). Following this, we tested 239 randomly chosen individual colonies from the fifth round of selection for reactivity with C5 by ELISA (figure 1B). We sequenced 32 scFvs with high-level binding activities and
a low binding background. The analysis of the sequences led to the identification of 8 unique sequences (C5B3, C5B35, C5B98, C5B106, C5A6, C5A102, C5A121, and C5A152). Of the 32 scFvs, 9 scFvs had the same complementarity determining region (i.e., C5B3). C5B35 and C5A6 were represented 5 and 8 times, respectively (table). We evaluated C5B3 antibody specificity with different concentrations of C5 and control proteins. Our results showed that C5B3 did not bind to the control protein and had good specificity (figure 1C). In figure 1D, the SPR sensogram shows the concentration-dependent binding of C5B3 to C5. The affinity of C5B3 binding to C5 ($K_D$) was 4.395 μM.

**C5B3-scFv inhibited AQP4-IgG–mediated CDC in vitro**

We cultured M23-AQP4-overexpressing CHO cells to confluence and incubated them with different dilutions of serumNMOSD or serumCtrl with different dilutions of hC for 30 minutes. We treated some wells with different dilutions of C5-scFv (C5B3, C5B35, and C5A6). As shown in figure 2, we

| Table | Amino acid sequence and repeated sequences number |
|-------|-----------------------------------------------|
| CSB3  | AKTTRFFDY QQARTSPST 9                        |
| CSA35 | AKNNRMFDY QQNAYFPTT 5                        |
| CSA6  | AKVSTPFDY QQPHYRPQT 8                        |

Abbreviations: C5-scFv = human C5-targeted single-chain variable fragment; CDR = complementarity determining region; HV = heavy chain variable region; LV = light chain variable region.
added 2.5% hC to the wells with 10% serumNMOSD with or without 500 μg/mL of C5-scFv. Figure 2A displays representative LIVE/DEAD staining, and figure 2B summarizes the statistical results. Compared with serumCtrl, serumNMOSD caused marked cytotoxicity, which was rescued by C5B3 (<0.01), but not C5B35 or CSA6. This finding indicates that C5B3, but not C5B35 or CSA6, inhibits cytotoxicity; however, all the sequences specifically bind to C5. To further clarify whether the preventive effect of C5B3 is dependent on complement or AQP4-IgG, we fixed the CHO cells and incubated them with 10% serumNMOSD with and without 800 μg/mL of C5B3 for immunostaining of AQP4 (red) and human IgG (green). The scale bar size for both A and D stains are all 200 μm. ***p < 0.001. AQP4 = aquaporin-4; CDC = complement-dependent cytotoxicity; hC = human complement; IgG = immunoglobulin G.

Figure 2 C5B3 prevented AQP4-IgG-mediated CDC without affecting AQP4-IgG binding to AQP4

(A) After incubation with 10% serumNMOSD and 2.5% hC with or without C5B3, C3A6, and C5B35, we measured CHO cells cytotoxicity with LIVE/DEAD staining. (B) The percentage of dead cells from the experiments shown in Fig A. (C.a) CDC in M23-AQP4–overexpressing CHO cells incubated with 2.5% hC and different concentrations of serumNMOSD and C5B3. (C.b) CDC in M23-AQP4–overexpressing CHO cells incubated with 10% serumNMOSD and different concentrations of hC and C5B3. (D) We fixed the CHO cells and incubated them with 10% serumNMOSD with and without 800 μg/mL of C5B3 for immunostaining of AQP4 (red) and human IgG (green). The scale bar size for both A and D stains are all 200 μm. ***p < 0.001. AQP4 = aquaporin-4; CDC = complement-dependent cytotoxicity; hC = human complement; IgG = immunoglobulin G.

In the control studies, C5B3 did not affect the binding of serumNMOSD and AQP4. Immunofluorescence showed that C5B3 at a high concentration (800 μg/mL) had no effect on AQP4-IgG binding to AQP4 (figure 2D). Collectively, these results indicate that the protective role of C5B3 is dependent on hC, but not AQP4-IgG.
To confirm the protective effect of C5B3 on MAC formation, we immunostained the cells to detect C5b-9 as shown in figure 3. There was apparent C5b-9–positive particle deposition on the plasma membranes of CHO cells treated with serumNMOsD and hC; however, deposition markedly decreased in cells treated with serumNMOsD hC, and C5B3 (figure 3A). Figure 3B shows the percentage of C5b-9–positive cells. We found a significant reduction in C5b-9–positive cells in the group treated with serumNMOsD hC, and C5B3 (20.23 ± 2.65 vs 80.88 ± 3.54%, p < 0.001).

### C5B3 decreased NMOSD mouse model lesions in vivo

To explore whether the inhibitory effect of C5B3 on AQP4-IgG–mediated CDC in vitro could be translated to an NMOSD mouse model in vivo, we intracerebrally injected 6 μL of IgGNMOSD and 4 μL of hC with or without 5 μL of C5B3 into mouse brains as described in our previous reports.28,29,32 The mice were weight matched and in good health. At 7 days after surgery, 5 animals in each experimental group survived at the end of the experiments with a survival rate of 100%. The extensive loss of AQP4, GFAP, and MBP in the group treated with IgGNMOSD and hC indicated that the mouse model can mimic the pathology of patients with NMOSD (figure 4A; see figure 4B for high magnification). In figure 4C, we quantified the areas of AQP4, GFAP, and myelin loss shown in figure 4A. Compared with the group treated with IgGNMOSD and hC, the group treated with IgGNMOSD hC, and C5B3 showed greatly reduced loss of AQP4 (1.813 ± 0.1858 vs 2.669 ± 0.126 mm², n = 5, p < 0.01), GFAP (0.9202 ± 0.1067 vs 1.461 ± 0.092 mm², n = 5, p < 0.05), and MBP (1.034 ± 0.08372 vs 1.450 ± 0.04557 mm², n = 5, p < 0.05; figure 4C). In the control group, we injected 4 μL of hC and 5 μL of C5B3 and found minimal loss of AQP4, GFAP, and MBP.

CNS inflammatory infiltration is an important pathologic feature of patients with NMOSD and NMOSD animal models. In the mouse brain following intracranial injections of IgGNMOSD and hC, we found pronounced leukocyte infiltration, including infiltration of macrophages and active microglia. Figure 5A depicts the infiltration of CD45+ and IBA1+ cells and C5b-9 formation, and figure 5B shows the extent of infiltration. We detected a mass of CD45+ leukocytes, IBA1+ macrophages, and activated microglia in mice injected with IgGNMOSD and hC. In contrast, C5B3 greatly decreased the areas of CD45+ leukocytes (1.449 ± 0.150 vs 2.201 ± 0.062 mm², n = 5, p < 0.01), IBA1+ macrophages and activated microglia (1.429 ± 0.155 vs 2.068 ± 0.036 mm², n = 5, p < 0.01), and the area of C5b-9 formation (0.889 ± 0.094 vs 1.433 ± 0.0545 mm², n = 5, p < 0.01) in the mouse brains.

### Discussion

In this study, we obtained complement C5-scFvs from the human phage display library and selected the best 3 clones to test C5 specificity with ELISA. Furthermore, we investigated the efficiency of C5-scFvs in blocking AQP4-IgG– and hC–mediated CDC using M23-AQP4–transfected CHO cells and found the best sequence, C5B3. We found that C5B3 inhibited MAC formation and decreased the death rate of M23-AQP4–transfected CHO cells in vitro. We also explored the therapeutic effect of C5B3 in an NMOSD mouse model in vivo. C5B3 prevented AQP4 and astrocyte loss, decreased demyelination, and reduced inflammatory infiltration in...
lesions. In the future, investigators should perform a comparison of the therapeutic effect of C5B3 with other monoclonal inhibitors of C5.

Monoclonal antibody therapy is a promising strategy to treat NMOSD. Several monoclonal antibody therapies are under evaluation for the treatment of NMOSD.\textsuperscript{18,21} Rituximab, a chimeric monoclonal antibody against CD20, has been reported to be efficacious and safe in controlling NMOSD, resulting in relapse rate reduction and disability stabilization.\textsuperscript{6,31} Aquaporumab (mAb-53), a recombinant nonpathogenic human monoclonal antibody to AQP4, can bind to AQP4, but cannot activate the complement response. Although mAb-53 has not been clinically applied to patients, it has had a therapeutic effect in an NMOSD mouse model.\textsuperscript{21} In some case reports of patients with NMOSD, tocilizumab, a humanized monoclonal antibody to the IL6 receptor, has significantly reduced relapse rates and possibly stabilized disability levels and is currently under development.\textsuperscript{32,33}

Many studies have investigated several monoclonal antibodies against complement components. Complement C1-targeted monoclonal antibodies were efficient in an NMOSD mouse model.\textsuperscript{19} Eculizumab is an approved treatment for atypical hemolytic uremic syndrome and paroxysmal nocturnal hemoglobinuria.\textsuperscript{19} An open-label trial reported that eculizumab could significantly reduce relapse frequency in patients with NMOSD.\textsuperscript{35} Most murine-derived monoclonal antibodies for NMOSD treatment have the potential to generate HAMA-neutralizing antibodies. The HAMA response can decrease therapeutic effectiveness and limit long-term application. In this regard, a fully human scFv would be necessary for

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**Figure 4 C5B3 decreased NMOSD mouse model lesions in vivo**

We injected the mouse brains with 6 μL of IgG\textsubscript{NMOSD} and 4 μL of hC with or without 5 μL of C5B3. (A) GFAP, AQP4, and MBP immunofluorescence at 7 days after injection. The white lines delimit the lesion with AQP4, GFAP, and MBP loss. The yellow lines represent the needle tract. Scale bar is 500 μm. (B) High magnification of AQP4, GFAP, and MBP in injected hemispheres and noninjected contralateral hemispheres. Scale bar is 100 μm. The white dashed lines delimit the lesion. (C) Lesion size quantification from experiments. We used 1-way analysis of variance (ANOVA) to compare between the 3 groups; n = 5/ group; *p < 0.05, **p < 0.01. AQP4 = aquaporin-4; GFAP = glial fibrillary acidic protein; MBP = myelin basic protein; NMOSD = neuromyelitis optica spectrum disorder.
NMOSD treatment. Pexelizumab, which is a recombinant humanized scFv that binds to C5, was the first scFv to enter clinical trials.\textsuperscript{36,37} Researchers designed pexelizumab to inhibit complement-mediated tissue damage in patients after coronary artery surgery.\textsuperscript{38} Compared with humanized monoclonal antibodies, C5B3 may have lower immunogenicity, which will require further research for confirmation.

The microenvironment of the lesion site after NMOSD induction is crucial for NMOSD treatment, especially concerning the control of inflammatory cells and complement reactions within the brain. In our experiment, C5B3 was effective for the treatment of AQP4-IgG–mediated lesions in an NMOSD model. C5B3 inhibited lesion formation by blocking MAC, reducing inflammation, improving the lesion site microenvironment, and reducing astrocyte damage, which protected the myelin and neurons.

There are some limitations to this study. The NMOSD model cannot fully mimic the pathogenesis of patients with NMOSD. We encourage researchers to use caution if directly extrapolating animal data to humans. Future preclinical or clinical studies should investigate the efficacy and pharmacokinetics of C5B3 in patients with NMOSD.

Using phage display technology, we obtained a fully human scFv against C5 and provided a proof-of-concept for this scFv in NMOSD treatment. C5B3 inhibited AQP4-IgG–mediated CDC in vitro and ameliorated NMOSD mouse model lesions in vivo. Moreover, C5B3 may have low immunogenicity, which indicates that it has a high potential for future clinical translation. In the future, C5B3 may serve as a promising treatment for NMOSD.

Acknowledgment
The authors thank the patients for participating in this study and the neuroimmunology team for their experimental and technical support.

Study funding
The National Natural Science Foundation (81571596, 81771279, and 81601044) and Fundamental Research Funds for the Central Universities (GK201701009) supported this work.
Disclosure
Y.P. Yan received research support from the National Natural Science Foundation (81571596, 81771279, and 81601044) and Fundamental Research Funds for the Central Universities (GK201701009). W.L. Zhu, Z. Wang, S.Y. Hu, Y. Gong, Y.C. Liu, H.H. Song, X.L. Ding, and Y. Fu report no disclosures. Disclosures available: Neurology.org/NN.

Publication history
Received by Neurology: Neuroimmunology & Neuroinflammation September 17, 2018. Accepted in final form February 5, 2019.

Appendix Author contributions

| Name               | Location               | Role                  | Contribution                                      |
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