

Antibodies Against Glutamic Acid Decarboxylase 65 Are Locally Produced in the CSF and Arise During Affinity Maturation

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Abstract

Background and Objectives

Antibodies (Abs) against the cytoplasmic protein glutamic acid decarboxylase 65 (GAD65) are detected in patients with neurologic syndromes together referred to as GAD65-Ab spectrum disorders. The response of some of these patients to plasma exchange or immunoglobulins indicates that GAD65-Abs could contribute to disease pathogenesis at least at some stages of disease. However, the involvement of GAD65-reactive B cells in the CNS is incompletely understood.

Methods

We studied 7 patients with high levels of GAD65-Abs and generated monoclonal Abs (mAbs) derived from single cells in the CSF. Sequence characteristics, reactivity to GAD65, and the role of somatic hypermutations of the mAbs were analyzed.

Results

Twelve CSF-derived mAbs were generated originating from 3 patients with short disease duration, and 7/12 of these mAbs (58%) were GAD65 reactive in at least 1 detection assay. Four of 12 (33%) were definitely positive in all 3 detection assays. The intrathecal anti-GAD65 response was polyclonal. GAD65-Abs were mostly of the IgG1 subtype and had undergone affinity maturation. Reversion of 2 GAD65-reactive mAbs to their corresponding germline-encoded unmutated common ancestors abolished GAD65 reactivity.

Discussion

GAD65-specific B cells are present in the CNS and represent a sizable fraction of CSF B cells early in the disease course. The anti-GAD65 response in the CSF is polyclonal and shows evidence of antigen-driven affinity maturation required for GAD65 recognition. Our data support the hypothesis that the accumulation of GAD65-specific B cells and plasma cells in the CSF is an important feature of early disease stages.

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Glossary

Ab = antibody; **AI** = Ab index; **CA** = cerebellar ataxia; **GABA** = γ -aminobutyric acid; **GAD** = glutamic acid decarboxylase; **GAD65-Ab SD** = GAD65 antibody spectrum disorder; **Ig** = immunoglobulin; **LE** = limbic encephalitis; **LGII** = leucine-rich glioma-inactivated 1; **mAb** = monoclonal antibody; **NNO** = neuritis nervi optici; **OCB** = oligoclonal band; **SHM** = somatic hypermutation; **SPS** = stiff-person syndrome; **TLE** = temporal lobe epilepsy; **UCA** = unmutated common ancestor.

The cytoplasmic protein glutamic acid decarboxylase (GAD) is the rate-limiting enzyme in the synthesis of the major inhibitory neurotransmitter γ -aminobutyric acid (GABA).¹ High serum levels of antibodies (Abs) against the isoform GAD65 have been associated with neurologic syndromes like stiff-person syndrome (SPS), cerebellar ataxia (CA), limbic encephalitis (LE), and temporal lobe epilepsy (TLE)²⁻⁶ together referred to as GAD65 antibody spectrum disorders (GAD65-Ab SDs) and circulating GAD-reactive B cells in peripheral blood are abundant in these patients.⁷ However, low levels of GAD65-Abs are also detectable in around 1%–8% of patients with diverse neurologic disorders and healthy controls.⁸⁻¹⁰ This finding complicates the evaluation of serum GAD65-Abs in patients presenting with neurologic symptoms and questions the relevance of these peripheral GAD65-Abs in disease pathogenesis. Currently, the detection of GAD65-Abs in CSF in SPS and an intrathecal production of GAD65-Abs in CA and TLE are therefore proposed as diagnostic criteria for GAD65-Ab-associated neurologic disorders,¹¹ highlighting the relevance of an intrathecal anti-GAD65 response in these disorders. Still, the intrathecal B-cell repertoire and the abundance of GAD65-specific B cells in the CNS are largely unknown. A more refined understanding of the immune response in these patients would however be of dire need, as an effective treatment in patients with GAD65-Ab SD remains challenging. We previously found that immune therapy including B-cell depletion with rituximab did not result in an improvement of outcome in patients with long-standing disease.¹² In contrast, prompt initiation of treatment was associated with good outcome in a cohort of patients with GAD65-Ab-associated CA.¹³ Therefore, early initiation of immune therapy seems to be critical, and understanding the involved immune reactions is crucial to facilitate therapeutic decisions. We now sought to analyze autoimmunity to GAD65 in the CNS by generating a panel of monoclonal antibodies (mAbs) derived from B cells in the CSF of 7 patients with GAD65-Ab SD. We could detect a sizeable fraction of GAD65-reactive B cells in the CNS in 3 patients at early stages of disease and provide evidence that GAD65-reactive B cells experienced antigen-driven affinity maturation. Overall, these findings support a role of CNS B-cell responses against GAD65 in early disease pathogenesis.

Methods

Study Approval

The study was performed according to the Declaration of Helsinki after approval by the Institutional Review Board of

the Ludwig-Maximilians-Universität, Munich, and the University of Ulm. All patients or their legal representatives gave written informed consent before study enrollment.

Patients

We included 7 patients with GAD65-Ab SD, all of which exhibited high GAD65-Ab titers both in serum and CSF and evidence of intrathecal GAD65-Ab production. Patient recruitment and CSF sampling were performed between September 2017 and April 2022. Intrathecal GAD65-Ab synthesis was determined by calculating the Ab index (AI) using the following formula: $(\text{CSF GAD65-specific IgG}) \times (\text{serum total IgG}) / ([\text{CSF total IgG}] \times [\text{serum GAD65-specific IgG}])$, with an AI above 1.4 indicating intrathecal synthesis.¹⁴ Clinical information and CSF characteristics are provided in Table 1. Fresh CSF was centrifuged at 300g for 15 minutes at 4°C. Cell pellets were either immediately used for single-cell sorting (patient no. 1) or directly frozen in 500 μ L freezing medium (90% fetal calf serum and 10% dimethyl sulfoxide), stored at –80°C for 3 days at –196°C afterward, and used later for single-cell sorting (patient nos. 2–7). All CSF samples were processed within 20 minutes after lumbar puncture.

Generation of Monoclonal Abs

After pre-enrichment of B cells by negative selection with magnetic cell sorting using anti-CD3, anti-CD14, anti-CD56, the enriched B cells were stained with the following Abs: anti-CD3, anti-CD14, anti-CD56, anti-CD19, anti-CD27, anti-CD20, anti-CD24, anti-CD138, and anti-CD38. After single-cell sorting, reverse transcription-PCR was performed, followed by cDNA amplification and a nested PCR approach as described previously.¹⁵ PCR products were sequenced and cloned into the expression vector pTT5. Immunoglobulin (Ig) G subclasses were determined as described previously.¹⁶ Ig vector pairs were transiently transfected into HEK293A cells, and Ab purification was performed by immobilized metal affinity chromatography as described previously.¹⁷ A detailed description of CSF single-cell isolation, Ig sequence analysis, and production of mAbs is provided in the supplementary material as eMethods, [links.lww.com/NXI/A808](https://www.lww.com/NXI/A808).

Screening for GAD65 Reactivity

ELISA

GAD65-Abs were detected and quantified by human anti-GAD65 ELISA (IgG) (Euroimmun).

Staining of Brain Tissue

In a tissue-based assay, primate cerebellar tissue (Euroimmun, FB 1111-1010-17) was used. Patient-derived mAbs (50 μ g/mL),

Table 1 Characterization of the Study Cohort and CSF Samples

Pt. no.	Clinical presentation	Age at onset (y)	Disease duration (mo) ^a	Sex	Symptoms constellation	Relevant comorbidities	Trt. at sampling	Previous Trt.	CSF	GAD65-AI	GAD65 mAbs ^b	CSF	No. of sorted cells	No. of readable sequences ^c
1	LE	57	81	F	Focal seizures	Autoimmune thyroiditis, LADA, vitiligo, and type A gastritis	—	IVGC with oral tapering	<1 cell/μL, no OCBs	3.8	—	Fresh	18	2 (2)
2	LE	28	2	F	Generalized and focal seizures and cognitive impairment	—	—	—	3 cells/μL, OCBs	4.1	+	Frozen	24	6 (6)
3	LE	56	122	M	Recurrent status epilepticus, cognitive impairment, and organic affective disorder	Type 1 diabetes, Hashimoto thyroiditis, and type A gastritis	—	IVIG	1 cell/μL, no OCBs	3.1	—	Frozen	30	1 (0)
4	CA	59	3	F	Limb ataxia, gait ataxia, and dysarthria	CLL and Hashimoto thyroiditis	—	IVGC and IVIG	<1 cell/μL, no OCBs	5.6	+	Frozen	72	16 (12)
5	NNO	43	172	F	Recurrent NNO	—	AZA	AZA	<1 cell/μL, OCBs	3.4	—	Frozen	31	0
6	LE	36	5	F	Generalized seizure, cognitive impairment, and cerebellar signs	—	—	—	4 cells/μL, no OCBs	1.5	—	Frozen	30	6 (2)
7	CA	53	10	F	Limb ataxia and gait ataxia	—	—	—	3 cells/μL, OCBs	1.7	+	Frozen	24	10 (8)

Abbreviations: AI = antibody index; AZA = azathioprine; CA = cerebellar ataxia; CLL = chronic lymphocytic leukemia; IVGC = IV glucocorticosteroid; IVIG = IV immunoglobulin; LADA = latent autoimmune diabetes in adults; LE = limbic encephalitis; NNO = neuritis nervi optici; OCBs = specific oligoclonal bands in CSF; Pt. = patient; Trt. = treatment.

^a Reflects time from disease onset to CSF sampling.

^b Patients in whom GAD65-reactive mAbs were generated from CSF cells.

^c Readable sequences include all heavy and light chains; number of sequences from matching heavy and light chains in brackets.

sera (1:100), and CSF supernatant (1:1) were analyzed for brain reactivity following the manufacturer's instructions. For detection, the goat anti-human IgG Alexa Fluor 488 Ab (Southern Biotech 2049-30; 5 μg/mL) was applied.

Cell-Based Assay

In addition, mAbs tested positive in the assays described above were tested by indirect immunofluorescence test in a cell-based assay on GAD65-transfected HEK293 cells and control transfected cells (Euroimmun, FA 1022-1005-50). MABs were used at a concentration of 50 μg/mL and in a first step diluted 1:10 followed by dilutions of 1:100/1:400/1:1,600/1:6,400/1:25,600 if positive, and applied undiluted if negative.

Generation of Germline Abs

To identify somatic hypermutations (SHMs) that arose throughout affinity maturation, we aligned the sequences of the heavy chains and light chains of our GAD65-reactive mAbs against the 2021-10-11 germline reference set with the international ImMunoGeneTics information system/HighV-Quest v.3.5.27. We selected 2 mAbs (5D1 and 7A2) and reverted the sequences in FR1, FR2, FR3, CDR1, CDR2, and flanking regions of CDR3 to the germ-line sequence by nucleotide synthesis (GeneArt, Thermo Fisher Scientific) and performed cloning into the pTT5 expression system as described above.

Statistics

Statistical significance was assessed with Prism Software (GraphPad) by unpaired or paired, nonparametric or parametric *t*

test analysis, as appropriate. *p* Values of **p* < 0.05, ***p* < 0.01, ****p* < 0.001, and *****p* < 0.0001 were considered significant and designated accordingly. Kd values were calculated by nonlinear regression using Prism Software (GraphPad).

Data Availability

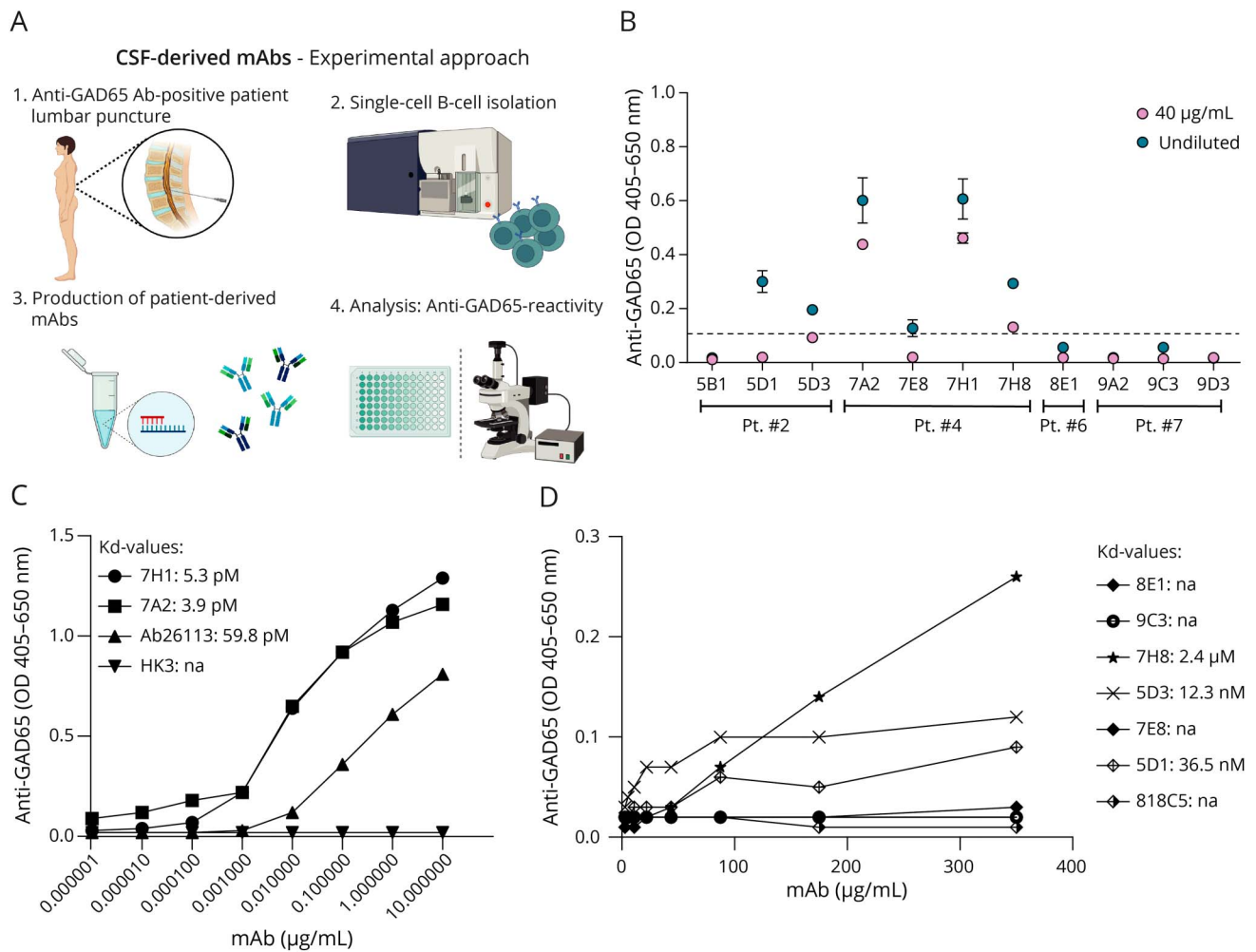
The data that support the findings of this study are available from the corresponding author on reasonable request. No deidentified patient data will be shared.

Results

Patient Cohort Characteristics and GAD65 Reactivity

We aimed to generate mAbs derived from CSF from patients with GAD65-Ab SD. We included 7 patients with GAD65-Abs and an associated neurologic disorder, 4 with LE, 2 patients with CA, and 1 patient with recurrent neuritis nervi optici (NNO). The patient with NNO (no. 5) is the daughter of a patient with high-titer GAD-Abs and an associated CA. The patient was tested negative for Abs against myelin oligodendrocyte glycoprotein and aquaporin-4. One patient received azathioprine at the time of sampling; the other patients were untreated. As described,¹⁸ autoimmune comorbidities were common. Specific oligoclonal bands (OCBs) in CSF were present in 3/7 patients (Table 1). All patients exhibited high GAD65-Ab levels both in serum and CSF detected by GAD65 ELISA (eFigure 1A, links. lww.com/NXI/A808) and tissue-based assay using primate

Figure 1 Evaluation of GAD65 Reactivity of Generated mAbs by ELISA



(A) Overview of the technical workflow to generate CSF-derived mAbs (created with BioRender). (B–D) Patient-derived mAbs were tested for GAD65 reactivity in ELISA. The horizontal dashed line indicates 4× OD of the negative control. Data are shown as the mean of at least 3 independent experiments; error bars indicate the SEM. Kd values were calculated using nonlinear regression analysis. (B) MABs were tested at a concentration of 40 µg/mL and undiluted (concentrations ranging from 0.35 to 5.29 mg/mL). (C and D) MABs positive in the screening-ELISA and the mAb HK3⁴¹ and the myelin oligodendrocyte glycoprotein-specific mAb 8.18C5 as controls were tested in different dilutions with high-affinity GAD65-reactive Abs presented in (C) and low-affinity GAD65-reactive Abs in (D). GAD = glutamic acid decarboxylase; Kd = dissociation constant; mAb = monoclonal antibody; na = nonapplicable; OD = optical density.

cerebellar slices (eFigure 1, B and C, links.lww.com/NXI/A808) and showed evidence of an intrathecal GAD65-Ab production (Table 1).

GAD65-Ab-Producing B Cells Are Present in the CSF at Early Stages of the Disease

Next, we aimed to analyze GAD65 reactivity on a single-cell level. We performed single-cell sorting of CSF cells from all 7 patients and produced patient-derived mAbs (Figure 1A). The gating strategy to define B-cell subpopulations is illustrated in eFigure 2, links.lww.com/NXI/A808. We were able to generate 12 mAbs derived from CSF B cells from 3 patients (3 mAbs from patient 2, 5 mAbs from patient 4, 1 mAb from patient 6, and 3 mAbs from patient 7) (Table 2). The generated mAbs were screened for their binding capacity to GAD65 using ELISA. Hereby, we identified 4 mAbs with low affinity (5D1, 5D3, 7E8, and 7H8) and 2 mAbs with high

affinity (7A2 and 7H1) to GAD65 in ELISA (Figure 1B, Table 2). We tested our mAbs in dilution series to determine their affinity and detected dissociation constant (Kd) values ranging from 3.9 pM to 2.4 µM (Figure 1, C and D). Five mAbs tested positive in a cell-based assay using GAD65-transfected HEK293 cells (Figure 2, A and B, Table 2). These 5 mAbs also showed a GAD65-specific staining pattern in tissue-based assay using primate cerebellar slices (Figure 2C, Table 2). Of interest, 2 mAbs (7E8 and 7H8) showed GAD65 reactivity in ELISA but were negative both in cell-based and tissue-based assays. On the contrary, another mAb (9C3) tested negative in ELISA but was positive both in cell-based and tissue-based assays. Together, 58% (7/12) of CSF-derived mAbs showed GAD65 reactivity when all detection assays were considered. Thirty-three percent (4/12) of CSF-derived mAbs showed GAD reactivity in all 3 applied detection assays (Table 2). GAD-reactive mAbs were derived from 3 patients (patients 2, 4, and 7) with short disease duration (2, 3,

Table 2 Characterization of Monoclonal Antibodies

mAb	Pt.	Cell type	HC AA junction	LC AA junction	LC type	CDR3 length HC	CDR3 length LC	VH family	SHM count IGHV	SHM count IGL/LV	IgG subclass	GAD65 ELISA	TBA/ IIFT	CBA/ IIFT
5B1	2	PC	CARRDDFSRSFKYW	CMQALQTPXTF	κ	12	9	4	34 (18)	7 (6)	IgG1	Neg	Neg	Neg
5D1	2	PB	CARPRSDLWSGYNQNWFAFW	CQQASSFPGLTF	κ	18	10	7	10 (6)	5 (5)	— ^a	++	Pos	Pos
5D3	2	PC	CASCSTTCLTWFDPW	CQVWDSTSDHQVF	λ	14	11	1	15 (14)	7 (6)	IgG1	++	Pos	Pos
7A2	4	MBC	CARRRGYSGYDPYFDYW	CGTWDSLSLSSWVF	λ	15	11	4	10 (10)	3 (2)	IgG1	+++	Pos	Pos
7E8	4	PB	CARDNSKWSWDSW	CQHLHLPPAF	κ	11	9	3	28 (24)	16 (10)	IgG2	+	Neg	Neg
7G2	4	PC	CTRGRVPLGGFYNQFDYW	CLLSYGGARVAF	λ	17	10	3	26 (17)	15 (8)	IgG1	Neg	Neg	Neg
7H1	4	MBC	CARHQNTIYSYGMVDVW	CMQALETSTF	κ	15	9	5	24 (17)	9 (7)	IgG1	+++	Pos	Pos
7H8	4	MBC	CARASWFGDLTVDNW	CLQHSNWGFTF	κ	13	9	3	24 (18)	12 (9)	IgG1	++	Neg	Neg
8E1	6	PB	CARDFFSGGYFSGTPVHGFDWSW	— ^b	κ	21	6	4	34 (15)	24 (13)	IgG1	Neg	Neg	Neg
9A2	7	PC	CARDAGHCTSINCPYNMDVW	CQXQDTF	κ	19	5	4	22 (13)	23 (13)	IgG1	Neg	Neg	Neg
9C3	7	MBC	CASERMVGRVRLRYFDNW	CQXQDNVPMYTF	κ	15	10	1	34 (19)	22 (10)	IgG1	Neg	Pos	Pos
9D3	7	PC	CARRVVIATSPYYYYLVDVW	CSSIPRGSALVF	λ	20	10	5	36 (22)	33 (22)	IgG1	Neg	Neg	Neg

Abbreviations: CBA = cell-based assay; CDR3 = complementarity determining region 3; HC = heavy chain; IGHV = immunoglobulin heavy chain V gene segment; IGL/LV = immunoglobulin kappa or lambda light chain V gene segment; IIFT = indirect immunofluorescence test; κ = kappa; λ = lambda; LC = light chain; mAb = monoclonal antibody; MBC = memory B cell (CD3⁺CD14⁺CD56⁺CD27⁺CD38⁺); PB = plasmablast (CD3⁺CD14⁺CD56⁺CD27⁺CD38⁺); PC = plasma cell (CD3⁺CD14⁺CD56⁺CD138⁺); Pt. = patient; SHM = somatic hypermutation; TBA = tissue-based assay.

^a The IgG subclass of mAb 5D1 could not be determined. Replacing mutations are specified in parentheses.

^b The LC AA junction of mAb 8E1 could not be determined.

and 10 months). In patient 6 with a disease duration of 5 months, 1 non-GAD65-reactive mAb could be generated. In patients 1, 3, and 5 with disease durations of 81, 122, and 172 months, respectively, we could not generate mAbs derived from CSF B cells.

Detailed Characterization of mAbs Reveals a High Rate of SHMs in GAD65-Reactive mAbs

We analyzed the sequence characteristics of all generated mAbs. IgG subclass analysis revealed that all mAbs, except 7E8, belonged to the IgG1 subclass (Table 2). No clonal relationships were identified among the mAb sequences, indicating a polyclonal anti-GAD65 response (Table 2). The number of SHMs was determined in the V region of all Ab chains. Hereby, GAD65-specific mAbs revealed a high number of SHMs (*immunoglobulin heavy chain V gene segment*: mean: 19.0, standard error of the mean: 2.8; *immunoglobulin kappa or lambda light chain V gene segment*: mean: 10.7, SEM: 2.6), indicating that these mAbs had undergone affinity maturation (Table 2).

Affinity Maturation Is Required for GAD65 Recognition

To determine the role of affinity maturation for GAD65 reactivity, we reverted 2 monoclonal GAD65-reactive Abs (5D1 and 7A2) to their corresponding unmutated common ancestors (UCAs) (Figure 3A). Next, affinities to GAD65 of the patient-derived sequences and their UCA counterparts were compared by dilution series in GAD65 ELISA. Here, we found that reactivity to GAD65 was completely abolished for

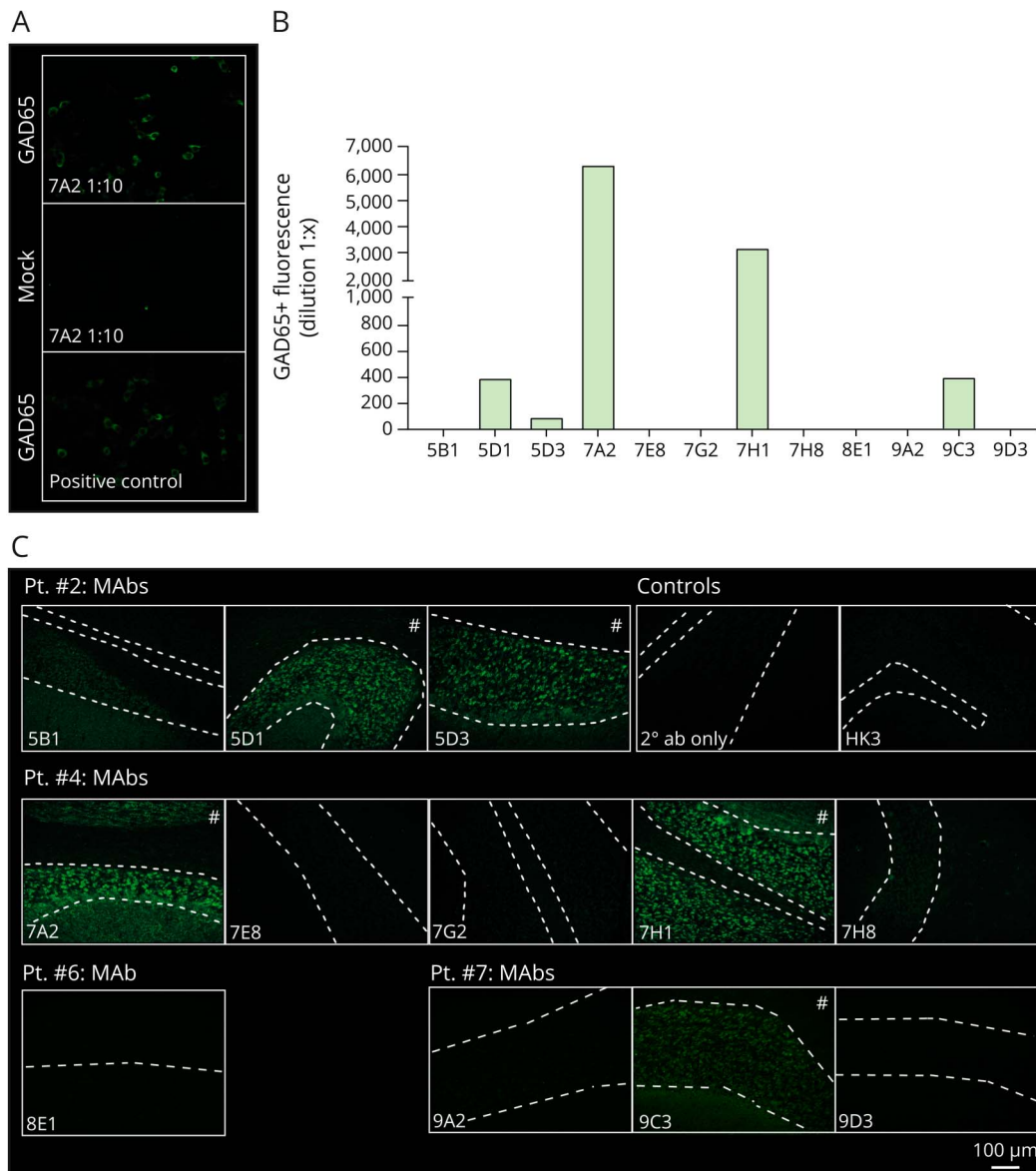
the UCA of mAb 7A2 (binding of mAb 5D1 UCA in ELISA could not be determined) (Figure 3B). In a cell-based assay using GAD65-transfected HEK293 cells, GAD65 reactivity was abolished for both UCAs (Figure 3C). Moreover, binding to primate brain tissue and pancreas islet cells was also abolished for both UCAs (Figure 3D).

Discussion

Our study provides insights into the CSF GAD65-specific B-cell receptor repertoire in patients with GAD65-Ab SD. An intrathecal production of GAD65-Abs is currently considered an essential diagnostic criterion in GAD65-Ab SD at least in LE/TLE and CA.¹¹ Furthermore, it was shown that GAD-specific OCBs are exclusively or predominantly present in the CSF compared with serum in patients with SPS, indicating that GAD-Abs are produced by B-cell clones within the CNS.¹⁹ However, direct proof of an intrathecal production of GAD65-specific Abs by CSF B cells was lacking so far. We were able to directly demonstrate that GAD65-Ab-producing cells are present in the CSF of patients with GAD65-Ab SD. We hereby provide evidence that GAD65-Abs can be produced by B cells within the CSF compartment resulting in a polyclonal anti-GAD65 response.

Our CSF-derived GAD65-specific mAbs showed comparable features to mAbs derived from patients with leucine-rich glioma-inactivated 1 (LG11) encephalitis²⁰ and GABA_A receptor encephalitis.²¹ Here, high proportions of Ab-secreting cells

Figure 2 Evaluation of GAD65 Reactivity of Generated mAbs by Tissue- and Cell-Based Assay

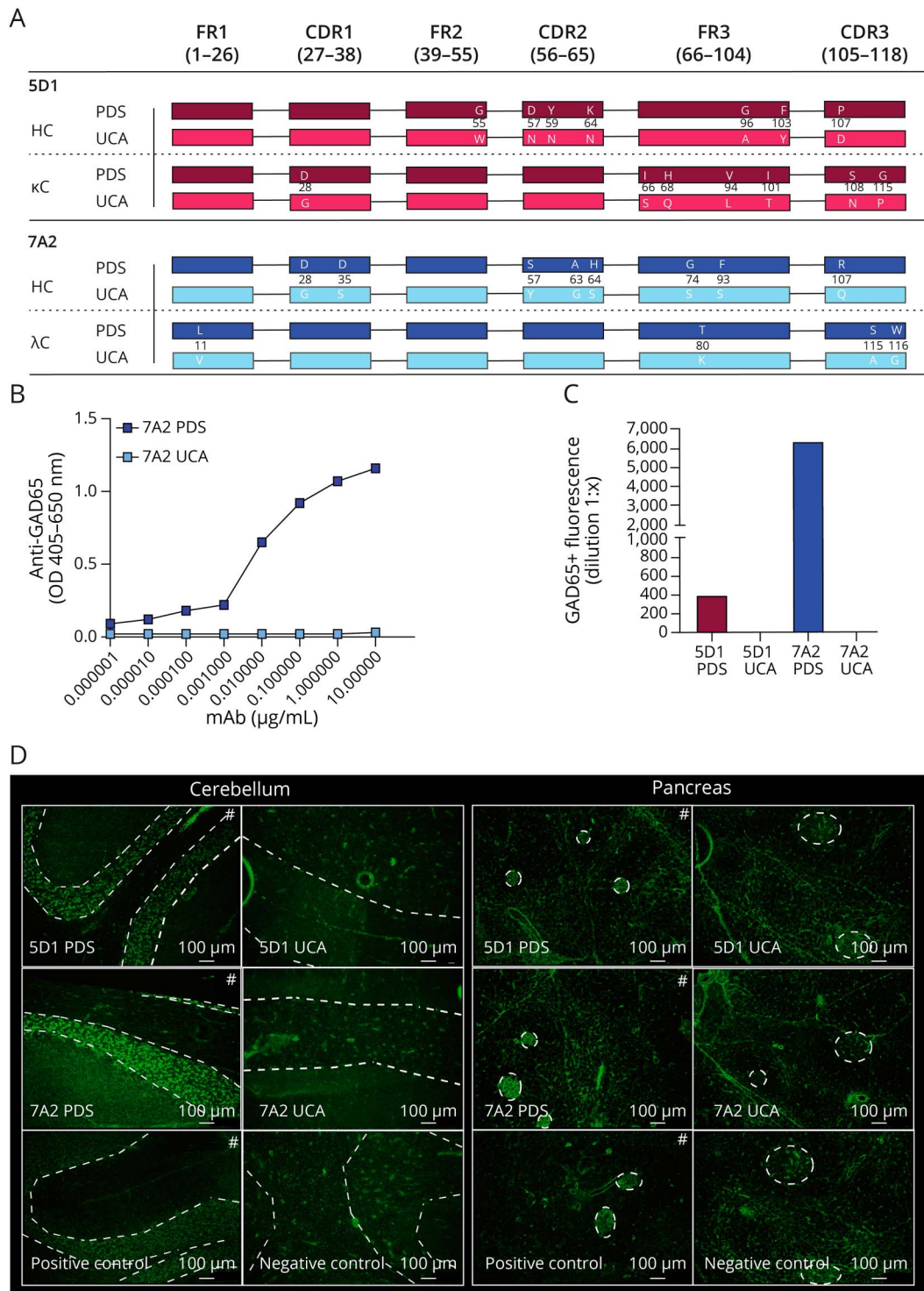


(A and B) Cell-based assay using HEK293 cells transfected with GAD65 or mock transfected was performed. MABs were used at a concentration of 50 µg/mL and in a first step diluted 1:10 followed by dilutions of 1:100/1:400/1:1,600/1:6,400/1:25,600 if positive and applied undiluted if negative. (A) Representative pictures for mAb 7A2 are shown. (B) The highest dilutions for all mAbs that revealed a positive staining are depicted. (C) Tissue-based assay with indirect immunofluorescence on primate cerebellar slices was performed to detect anti-GAD65-specific staining patterns using the patient-derived mAbs (50 µg/mL). The mAb HK3⁴¹ was used as a negative control. A condition with only secondary antibody was used as an additional negative control. #Marks mAbs that show a GAD65-specific staining pattern characterized by a leopard-like granular layer staining. GAD = glutamic acid decarboxylase; mAb = monoclonal antibody.

expressing LGI1-Abs or GABA_AR-Abs and high numbers of SHMs were observed. In contrast, in patients with NMDAR encephalitis, conflicting results exist. Whereas 1 study reported the presence of clonally expanded plasma cells with hypermutated antigen receptors in the CSF of patients with NMDAR encephalitis,²² another group showed that CSF-derived NMDAR-reactive mAbs often lacked any mutation compared with the germline-sequence^{23,24} and only a small fraction of Ab-secreting cells within the CSF exhibited Abs against NMDAR.^{23,24} The high numbers of SHMs of our mAbs indicate that GAD65-Ab-producing B cells have

undergone affinity maturation during germinal center reactions. Germinal centers constitute microenvironments that allow diversification of antigen-reactive B cells and Ab maturation via SHMs with the help of T follicular helper cells.²⁵ Recently, the relevance of germinal center reactions in autoantibody-mediated diseases was studied in neuromyelitis optica spectrum disorders, where a germinal center-based generation of aquaporin-4 Abs was shown by analyzing paired blood and deep cervical lymph node samples. Hereby, rituximab effectively eliminated the intranodal synthesis of aquaporin-4 Abs and aquaporin-4-reactive B cells, which might

Figure 3 Unmutated Common Ancestors (UCAs) of 2 GAD65-Reactive mAbs



(A) The differences of the amino acid (AA) sequences of heavy and light chains between the patient-derived sequences (PDS) and their corresponding UCAs are illustrated. The differing AAs are depicted in white letters and the AA positions in black numbers. (B) GAD65 reactivity of PDS of mAb 7A2 and the corresponding UCA were compared by GAD65 ELISA. MAb 5D1 is not included due to weak reactivity in ELISA with positivity only at high concentrations (>350 $\mu\text{g/mL}$); however, the corresponding UCA was not producible at such a high concentration. (C) Cell-based assay using HEK293 cells transfected with GAD65 was performed. MAbs representing PDS or UCAs were used at a concentration of 50 $\mu\text{g/mL}$ and in a first step diluted 1:10 followed by dilutions of 1:100/1:400/1:1,600/1:6,400/1:25,600 if positive and applied undiluted if negative. The highest dilutions that revealed a positive staining are depicted. (D) Comparison of binding to primate cerebellar and pancreas tissue of patient-derived sequences (PDS) and unmutated common ancestors (UCAs). For the mAbs 5D1 and 7A2, binding of the PDS and UCAs to primate cerebellar tissue and pancreas tissue was compared by indirect immunofluorescence with concentrations of 50 $\mu\text{g/mL}$ of the mAbs. Left panel: Binding to primate cerebellar tissue is illustrated. The dashed white lines confine the leopard-like staining pattern of the granular layer that is specific for GAD65-Abs. Right panel: Binding to primate pancreas islets is illustrated. Pancreas islet cells are marked with a dashed white circle. GAD65-specific staining pattern is marked with #. CDR = complementarity determining region; ctrl = control; FR = framework region; GAD = glutamic acid decarboxylase; HC = heavy chain; mAb = monoclonal antibody; neg = negative; pos = positive; κC = kappa light chain; λC = lambda light chain.

explain how rituximab can be clinically effective without concomitant reduction of serum autoantibody levels.²⁶ In NMDAR encephalitis, tertiary lymphoid architectures in ovarian teratomas were suggested, and a production of NMDAR autoantibodies was detected from cultured teratoma explants and dissociated intratumoral B cells and from 3/7 cultures of cervical lymph nodes.²⁷ Our data point to a relevance of germinal center reactions also in GAD65-Ab SD.

During B-cell development, 2 tolerance checkpoints with elimination of autoreactive B cells from the maturing B-cell repertoire exist. The central tolerance checkpoint affects immature B cells in the bone marrow, whereas the peripheral tolerance checkpoint relates to B cells at the transition from new immigrant to mature naive cells in the periphery.²⁸ Defects in B-cell tolerance checkpoints have been identified as essential characteristics in the development of autoimmunity.²⁹⁻³⁴ We sought to determine the relevance of early defects in B-cell tolerance in the development of GAD65-Abs. To this aim, we reverted the sequence of 2 of our CSF-derived GAD65-reactive mAbs to their UCAs, thereby generating mAbs corresponding to the B-cell receptors expressed by naive B-cell precursors of these GAD65-Ab-producing cells. Hereby, GAD65 reactivity was abolished. SHMs, therefore, play an important role in the generation of GAD65 reactivity. Similarly, an essential role of SHMs in introducing autoantigen-specific reactivity has been described in other autoimmune disorders like for Abs against aquaporin-4,³⁵ for Abs against desmoglein-3 in pemphigus vulgaris,³⁶ and for Abs against double-stranded DNA or extractable nuclear antigen in systemic lupus erythematosus.^{37,38}

In our patient cohort, we were only able to generate mAbs from patients with a short disease duration (2, 3, 5, and 10 months vs 81, 122, and 172 months). Most mAbs were isolated from plasmablasts and plasma cells. In 3 of the patients, we were able to isolate GAD-reactive mAbs derived from CSF B cells. Our patient cohort is small, and we cannot exclude that technical limitations account for our inability to generate mAbs in some patients. However, the fact that all patients who exhibited GAD-specific B cells in the CSF had a rather short disease duration supports the idea that B cell-mediated or Ab-mediated mechanisms—if relevant in disease pathogenesis—are more pronounced in early disease stages. Of note, the number of mAbs that we could retrieve per patient was lower compared with published data for LGI1 encephalitis²⁰ and NMDAR encephalitis.²³ This might be related to differences in the technical approach; however, it might also be related to a relatively small fraction of B cells within the CSF cells in GAD65-Ab SD even at early stages of disease. Previously, it was shown that in patients with long-standing GAD65-LE, numbers of activated CD8⁺ T cells and CD4⁺ T cells are increased in CSF and peripheral blood.³⁹ On the contrary, numbers of B cells and plasma cells were not altered in the CSF and peripheral blood of patients with long-standing GAD65-Ab-associated LE.³⁹ Recently, it was shown that patients with GAD65-associated TLE exhibit high numbers of plasma cells within the brain parenchyma in the first 6

years after symptom onset, albeit no signs of Ab-mediated tissue damage were found. In addition, dense infiltration of CD8⁺ cytotoxic T cells was observed. In contrast, with longer disease duration, the density of all lymphocytes decreased. As a conclusion, an early active inflammatory stage (≤ 6 years) with neuronal loss followed by an immunologically inactive or low-active stage was proposed.⁴⁰ Fitting to these observations, it was shown that an early initiation of immune therapy and a subacute onset in GAD65-Ab-associated CA were associated with therapy response.¹³ Also, in line with these findings, we previously found no clinical response to rituximab treatment in long-lasting GAD65-associated neurologic disorders when the modified Rankin Scale is used as the outcome measure.¹²

Limitations of our study are the small patient number with heterogeneous clinical phenotypes, which is attributed to the rareness of GAD65-associated neurologic disorders, the requirement of direct processing of the CSF, and the elaborate procedure to generate the mAbs. Furthermore, no patients with SPS were included in our study. Therefore, our results cannot readily be translated to this patient subgroup.

Together, this study indicates that GAD65-specific B cells are generated during a normal immune reaction from B cells that are not specific for GAD65. Our data underline the importance of SHMs in introducing autoantigen-specific reactivity during an ongoing immune response. In part of the patients with a short disease duration, GAD65-specific B cells represent a remarkably high proportion of CSF B cells. The pathogenic relevance of these GAD65-specific B cells remains to be established.

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Disclosure

The authors report no disclosures relevant to the manuscript. Go to [Neurology.org/NN](https://www.neurology.org/NN) for full disclosures.

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Appendix (continued)

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