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RESEARCH ARTICLE

Impact of T cells on neurodegeneration in anti-GAD65 limbic encephalitis

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Abstract

Objective: Direct pathogenic effects of autoantibodies to the 65 kDa isoform of glutamic acid decarboxylase (GAD65) in autoimmune limbic encephalitis (LE) have been questioned due to its intracellular localization. We therefore hypothesized a pathogenic role for T cells. **Methods**: We assessed magnet resonance imaging, neuropsychological and peripheral blood, and CSF flow cytometry data of 10 patients with long-standing GAD65-LE compared to controls in a cross-sectional manner. These data were related to each other within the GAD65-LE group and linked to neuropathological findings in selective hippocampectomy specimen from another two patients. In addition, full-resolution human leukocyte antigen (HLA) genotyping of all patients was performed. **Results**: Compared to controls, no alteration in hippocampal volume but impaired memory function and elevated fractions of activated $HLADR^+$ $CD4^+$ and $CD8^+$ T cells in peripheral blood and cerebrospinal fluid were found. Intrathecal fractions of $CD8^+$ T cells negatively correlated with hippocampal volume and memory function, whereas the opposite was true for $CD4^+$ T cells. Consistently, antigen-experienced $CD8^+$ T cells expressed increased levels of the cytotoxic effector molecule perforin in peripheral blood, and perforin-expressing $CD8^+$ T cells were found attached mainly to small interneurons but also to large principal neurons together with wide-spread hippocampal neurodegeneration. 6/10 LE patients harbored the HLA-A*02:01 allele known to present the immunodominant $GAD65_{114–123}$ peptide in humans. **Interpretation**: Our data suggest a pathogenic effect of $CD8^+$ T cells and a regulatory effect of $CD4^+$ T cells in patients with long-standing GAD65-LE.

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Introduction

Autoantibodies (aabs) to the 65 kDa isoform of glutamic acid decarboxylase (GAD65) have recently been described in a subset of patients with a chronic slowly progressive form of non-paraneoplastic autoimmune limbic encephalitis (LE).[1,2] Stiff-person syndrome, cerebellar ataxia, or an overlap of two or more of the former clinical phenotypes illustrate the spectrum of anti-GAD65 aab-associated autoimmune neurological syndromes.[3] LE typically presents with adult-onset mesial temporal lobe seizures, progressive episodic memory disturbance, and a variety of other neuropsychiatric symptoms.[2] MR-imaging typically reveals uni- or bilateral hyperintense signals and swelling on T2- and fluid attenuated inversion recovery (FLAIR) sequences together sometimes followed by atrophy of mesial temporal structures predominantly involving the amygdala and anterior parts of the hippocampus.[4,5]

Glutamic acid decarboxylase is expressed in interneurons and catalyzes the conversion of glutamate to γ-aminobutyric acid (GABA). The brain contains two isoforms, GAD65 and GAD67, which display characteristic differences in localization and activity patterns.[6,7] GAD67 is typically distributed throughout the neuron and almost all of it exists in its active cofactor-bound form, whereas GAD65 is predominantly found in synaptic terminals, predominantly in the form of an inactive apoenzyme.[8] In accordance with a net inhibitory effect of GAD65 expressing interneurons on neuronal network excitability, genetic deficiency of GAD65 leads to spontaneous neuronal hypersynchrony and epileptic seizures.[9] Moreover, GAD65-expressing interneurons have been implicated in hippocampus- and amygdala-based synaptic plasticity, learning, and memory.[10,11]

Due to the intracellular localization of GAD65, direct pathogenic effects of aabs have been questioned.[12] Hence, we hypothesized a pathogenic role for T cells in LE with aabs to GAD65. To this end, we investigated the immune cell profile in 10 patients with typical long-standing GAD65-autoantibody-associated limbic encephalitis (GAD65-LE) compared to controls in a cross-sectional manner in both peripheral blood (PB) and cerebrospinal fluid (CSF) by using multi-parameter flow cytometry and related these findings to structural cerebral changes, neuropsychological functioning, and neuropathological and immunogenetic findings.

Subjects and Methods

Patients

According to the consensus criteria for autoimmune encephalitis by Graus et al.[13] from 2016, 10 immunotherapy-naïve LE patients, positive for GAD65 aabs in serum and CSF, were included (Table 1).

Recruitment, clinical, neuropsychological, and imaging studies of all patients were performed at the Department of Epileptology, University Hospital Bonn, Germany (approved by the Ethical Commission of University Hospital Bonn [222/16]). PB and CSF analysis was performed at the Department of Neurology, University of Münster, Germany. The study was approved by the local ethics committee of the Medical Faculty of the University of Münster, Germany (AZ 2013 350-f-S). All participants gave written informed consent to the study including scientific evaluation and publication of all clinical and paraclinical data obtained. All procedures were conducted in accordance with the Declaration of Helsinki.

MRI volumetry

MRI was performed within 1 week before or after PB and CSF sampling in GAD65-LE as described[4] and compared to 10 age- and sex-matched controls. In brief, T1-weighted volume datasets were acquired using a 3 Tesla scanner (Magnetom Trio, Siemens, Erlangen, Germany). Sequence parameters were as follows: magnetization-prepared rapid acquisition gradient echo (MPRAGE), voxel size $1 \times 1 \times 1$ mm,^{3} repetition time 1570 msec, echo time 3.42 msec, flip angle 15 degrees, and field of view 256×256 mm^2. The analysis of the structural image data was done using FreeSurfer (V.5.1.0, Martinos Center, Harvard University, Boston, MA), and this is freely available for download online (http://surfer.nmr.mgh.harvard.edu/). To adjust for brain size and age, mean volumes of both amygdalae and hippocampi were normalized to the whole brain volume as described.[4] The mean of these two normalized bilateral values of amygdala and hippocampus was calculated for each patient (Relative Hippocampal Volume, Relative Amygdala Volume).

Neuropsychological assessment

Neuropsychological testing was performed within 1 week before or after PB and CSF sampling. For verbal memory

assessment, the "Verbal Learning and Memory Test" (VLMT), a German adaptation of the Rey Auditory Verbal Learning Test,[14] and for figural memory assessment, the revised version of the "Diagnosticum für Cerebralschädigung" (DCS-R)[15] were used. Attention and executive functions were assessed by the EpiTrack®.[16] Lower performance was rated undercutting the third standard deviation of the mean value of 35 points (<28 points).

Two verbal memory parameter (verbal learning [immediate recall over 5 learning trials], and verbal memory [loss of learned words in ½ h delayed free recall]) and one figural memory parameter (figural learning [immediate recall over 5 learning trials]) were transformed into one individual summary score being representative for memory (Memory Score).[2] To control the impact of CNS-operant medication, the individual memory score was finally related to individual medication-sensitive "executive functions" as measured by the EpiTrack® giving the individual MemScore. Since normative values of this transformed score are not yet established, normative values of VLMT,[14] DCS-R[15] and EpiTrack®[16] were used to evaluate the cognitive performance of the GAD65-LE patients in relation to a control cohort.

Multi-parameter flow cytometry of PB and CSF

PB and CSF sampling in patients was performed within 1 week before or after MRI and neuropsychological testing as described.[17] As a control, PB and CSF samples of 24 patients with somatization disorders were used. In addition to fulfilling clinical diagnostic criteria for somatization disorders, control samples also fulfilled the following laboratory criteria defining a non-inflammatory CSF: <5 cells/μL, <500 mg protein/mL, <2 mmol/L lactate, no disruption of the PB-CSF-barrier (defined by the CSF/serum albumin ratio), no intrathecal immunoglobulin IgG, IgA, or IgM synthesis (Reiber criteria)[18] and no CSF-specific oligoclonal bands on isoelectric focusing.

Potential disease-related changes in the cellular composition of both PB and CSF compartment were analyzed using multi-parameter flow cytometry. CSF samples were obtained by lumbar puncture, collected in polypropylene tubes, and were processed within 30 min. Cells were obtained from ethylene-diamine-tetra-acetic acid (EDTA) blood by erythrocyte lysis using VersaLyse buffer (Beckman Coulter, Germany) following the manufacturer's instructions. Cells were obtained from CSF by centrifugation (15 min, 290 g, 4°C) and incubation in VersaLyse buffer. Cells were stained using the following fluorochrome-conjugated antibodies: CD14-FITC, CD138-PE, HLA-DR-ECD, CD3-PC5.5, CD56-PC7, CD4-APC, CD19-APCAlexafluor700, CD16-APCAlexafluor750, CD8-PacificBlue, and CD45-KromeOrange (all Beckman Coulter) and analyzed using the Navios (Beckman Coulter).

For analysis of effector molecule expression, frozen peripheral blood mononuclear cells (PBMCs) from all 10 patients and 10 age and sex-matched controls were thawed and directly stained using the following fluorochrome-conjugated antibodies: CD56-ECD, CD8-A700, CD3-A750 (all BeckmanCoulter), CD45RO-PerCP/Cy5.5, and CD4-BV510 (both Biolegend). Following permeabilization with fixation/permeabilization buffer (eBioscience), granzymes and perforin were stained in permeabilization buffer (eBioscience) using GrB-PE, Perforin-VioBlue (Miltenyi), acquired using a Navios flow cytometer (Beckman Coulter) and analyzed with Kaluza 1.5 (Beckman Coulter).

Anti-neuronal antibody testing

Serum and CSF were tested for the presence of IgG antibodies against intracellular neuronal antigens (ANNA1 (Hu), ANNA2 (Ri), ANNA3, PCA1 (Yo), PCA2, Tr/DNER, Ma1/2, CV2/CRMP5, Amphyphysin, SOX1, GAD65) and surface membrane neuronal antigens (NMDA receptor, AMPA receptor, GABAA receptor, GABAB receptor, glycine receptor, LGI1, and CASPR2) using established tissue-based and cell-based assays together with immunoblot and enzyme-linked immunosorbent assays (Euroimmun) as described.[17,19]

Histopathology

For histopathological analysis, sections from selective hippocampectomy specimen of two patients with GAD65-LE were incubated with antibodies against CD20, CD138, CD3 and CD8 as well as against NeuN, amyloid precursor protein, and activated caspase-3 using standard methods, and counterstained with hematoxylin. The images were acquired with Zeiss Examiner microscope.

For immunohistochemical analysis, slides were incubated 2 × 10 min in xylene, ethanol (100%–95%–70%–50%), citrate buffer (10 mmol/L, pH 6.0), and blocked for 2 h at 37°C in blocking buffer consisting of 10% normal goat serum (Invitrogen) and 1% fetal calf serum (Invitrogen) in PBS. Slides were incubated with primary antibodies against CD8 (1:50; #MA5-14548; Thermo Fisher) and Granzyme B Ab-1 (1:50; #MS-1157–S1; Thermo Fisher) or Perforin Ab-2 (1:50; #MS-1834-S1; Thermo Fisher) overnight at room temperature in blocking buffer. After washing with PBS, slides were incubated with secondary antibodies (1:200 Alexa Fluor 488 donkey-anti-mouse and 1:200 Alexa Fluor 568 goat-anti-rabbit; both from Thermo Fisher) and with 0.1 μg/mL 4′,6-diamidino-2-phenylindole (DAPI, Life technologies) for 2 h at room temperature in blocking buffer. After washing with PBS, sections were coverslipped in fluorescein mounting

Table 1. Clinical features of patients with GAD65-LE.

PID	Gender	Age at sample date (years)	Disease duration (years)	Seizure-frequency/ month	SPS/ month	CPS/ month	GTCS/ month	GAD65-aabs in serum (ELISA)	GAD65-aabs in CSF (TBA)	Semiology of partial seizures	EEG interictal
1	f	24	3.00	15	15	0	0	>2000 iU/mL	IFT: 1:10	Epigastric aura	Right >left temporal sharp waves
2	m	33	12.00	0.3	0.3	0	0	2000 iU/mL	IFT: 1:3.2	Deja-vu	Left temporal theta
3	f	30	10.00	2	0	2	0	>2000 iU/mL	IFT: 1:10	Staring, pausing	Left temporal theta
4	m	38	14.00	0	0	0	0	>2000 iU/mL	IFT 1:10	Deja-vu, loss of consciousness, oroalimentary automatisms	Left temporal slowing
5	f	61	5.80	0	0	0	0	>2000 iU/mL	IFT: 1:3.2	Oroalimentary automatisms, fumbling	Rarely bitemporal sharp waves
6	f	52	8.00	8	0	8	0	>2000 iU/mL	IFT: 1:10	Epigastric aura, loss of consciousness	Right temporal sharp waves
7	f	52	3.80	1	0	1	0	>2000 iU/mL	IFT: 1:1	Loss of consciousness, fumbling	Left temporal focal dysrhythmia
8	f	27	7.00	10	10	0	0	>2000 iU/mL	IFT: 1:10	Deja-vu	Right >left temporal slowing
9	f	42	5.00	1	0	1	0	>2000 iU/mL	IFT: 1:3,2	Epigastric aura, loss of consciousness, oroalimentary automatisms	Left temporal slowing
10	m	24	7.00	3	0	3	0	>2000 iU/mL	IFT: 1:3,2	Loss of consciousness, oroalimentary automatisms, fumbling	Right temporal sharp waves

There were no other detected epileptogenic MRI lesions in GAD65-LE patients. AED, anti-epileptic drugs; CPS, complex partial seizures; EEG, electroencephalography; FDG-PET, fluorodeoxyglucose positron emission tomography; IFT, immunofluorescence test; MRI, magnetic resonance imaging; OCB, oligoclonal bands; OKB Type 1, normal CSF; OKB Type 2, oligoclonal IgG restricted to CSF; OKB Type 4, identical oligoclonal bands in CSF and serum; PID, patient identifiable data.

medium (Vectashield, Vector laboratories) and imaged with a confocal laser scanning microscope (Eclipse Ti, Nikon).

HLA-genotyping

Genomic DNA was extracted from peripheral blood mononuclear cells of patients according to standard procedures. Next-generation sequencing-based genotyping of the HLA molecules HLA-A, -B, -C, DRB1, DQB1, and DPB1 at full (8 digit) resolution was performed by a commercial provider (Creative Biolabs).

Statistics

Data on flow cytometry (MH, CCG), MRI (JW) and neuropsychological performance (JAW, CH) were obtained and analyzed each in a completely independent fashion. Statistics were performed with SPSS Statistics Version 22 (IBM) using Spearman-Rho for correlation analysis. For group comparison, GraphPad Prism Version 8.0.2 (GraphPad Software, Inc.) was used applying Mann–Whitney U test. Corresponding significant tests were all two-sided. If not explicitly stated otherwise, the pre-chosen significance level for all

EEG ictal	AED	FDG-PET	MRI at onset	CSF cells/ µL (<5)	CSF protein in mg/L (<500 mg/L)	Blood-Brain-Barrier Disturbance	Specific OCB in CSF (Type)
Right temporal seizure	LEV 3000 mg, ESL 1200 mg	–	Hyperintense amygdala and hippocampus	1	310	–	2
Right temporal seizure	LEV 3000 mg, VPA 300 mg	–	Scarred atrophy of right hippocampus	0	393	–	1
–	LEV 4000 mg, LTG 150 mg, PER 10 mg	–	hyperintense amygdala and hippocampus	0	321	–	1
Left temporal seizure	LEV 1000 mg	Left temporal hypometabolism	selective left amygdalohip-pocampectomy	0	467	–	1
–	LTG 300 mg	–	Hyperintense amygdala and hippocampus	1	431	–	1
–	PER 8 mg, ZON 200 mg	–	Hyperintense hippocampus	1	422	–	1
–	LTG 400 mg, ZON 200 mg	–	Hyperintense amygdala and hippocampus	1	469	(+)	4
–	LTG 200 mg	Slight left temporal hypometabolism	Hyperintense amygdala and hippocampus	2	313	–	2
Left temporal seizure	LTG 300 mg	–	Normal	1	292	–	2
Left and right temporal seizures	LTG 300 mg, OXC 1800 mg, ZON 400 mg	Slight right >left temporal hypometabolism	Bilateral hyperintense amygdala and hippocampus	1	492	–	1

confirmatory tests was set to $p < 0.05$. Correlation of data was performed afterwards (GW) avoiding confirmation bias.

Results

Clinical presentation of patients with long-standing GAD65-LE

Ten German patients (7 females, 3 males, 24–62 years; Table 1) of Caucasian descent presented with a 3 to 14-year history of pharmacoresistant mesial temporal lobe seizures, declarative memory disturbance and depression to variable extent. MRI revealed uni- or bilateral hyperintense T2-/FLAIR-signals and variable volume changes of mesial temporal lobe structures. In 3 of 10 patients, cerebral FDG-PET studies were performed which showed hypometabolism of the mesial temporal lobe structures. Interictal EEG demonstrated uni- or bilateral slowing and epileptic discharges, and ictal EEG showed seizures originating from the affected temporal lobe(s). Routine CSF analysis showed normal cell counts, protein levels and blood-CSF-barrier function. Isoelectric focussing of serum and CSF showed CSF-specific oligoclonal IgG bands (type 2 or type 3 pattern) in 3/10 patients.

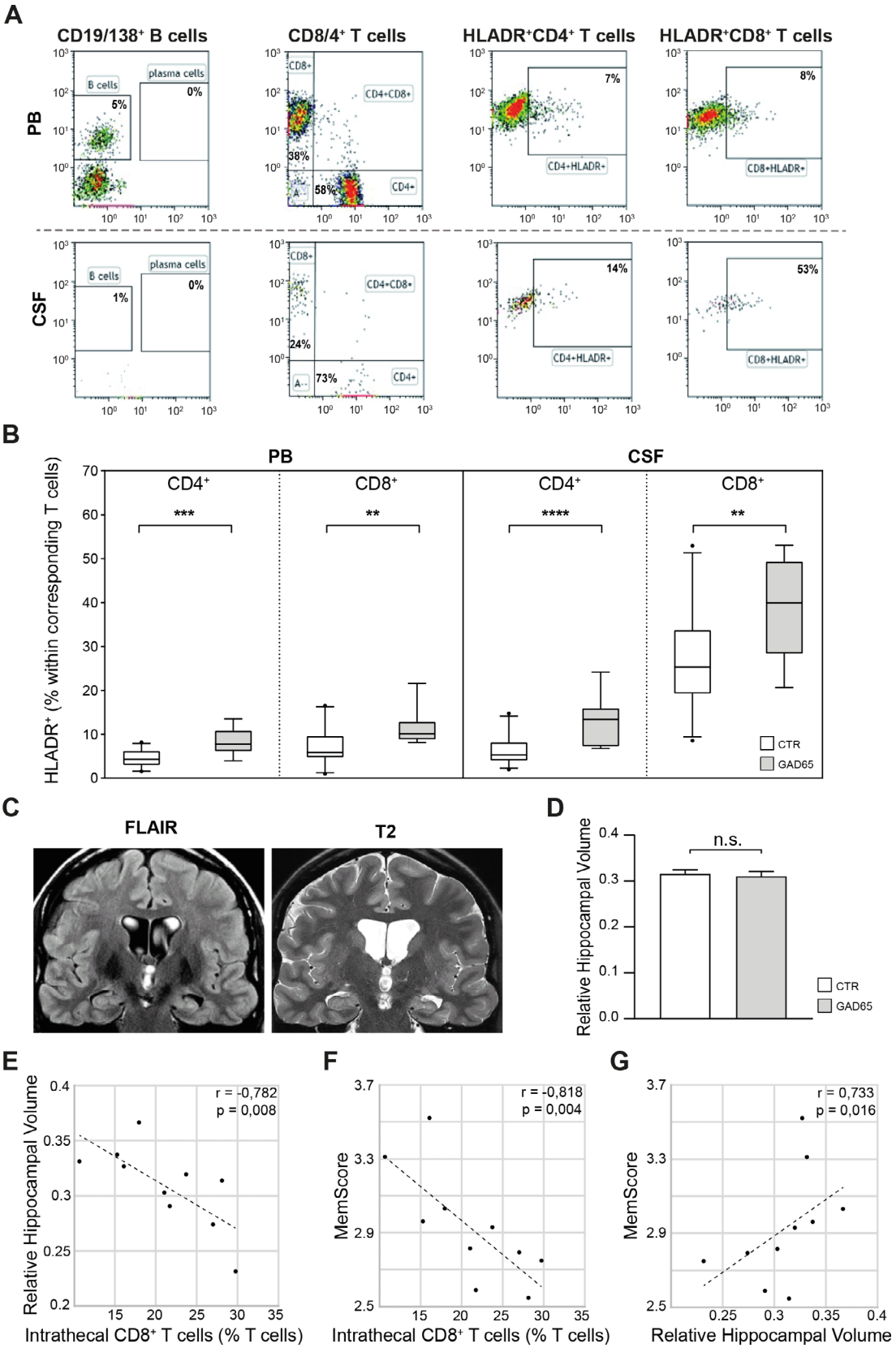

Figure 1. Elevated T cell activation correlates with hippocampal volume and memory function in patients with long-standing GAD65-LE. (A) Representative multiparameter flow cytometry analysis of peripheral blood (PB; upper panel) and cerebrospinal fluid (CSF lower panel) of one patient with long-standing GAD65-LE. (B) Group analysis of fractions of activated $HLADR^+$ $CD4^+$ T cells and activated $HLADR^+$ $CD8^+$ T cells in PB and CSF in patients with long-standing GAD65-LE and controls (CTR). (C) Representative FLAIR- and T2-MRI-sequences of the smallest hippocampus are shown. (D) Group analysis of relative hippocampal volumes in patients with long-standing GAD65-LE and controls. (E–G) Correlation analysis of the fraction of intrathecal $CD8^+$ T cells (% T cells), relative hippocampal volume and MemScore in patients with long-standing GAD65-LE as indicated.

All patients had high GAD65-autoantibody levels in serum (≥2000 IU/mL in an enzyme-linked immunosorbent assay [ELISA]), and aabs were also detected in CSF (titers ranging from 1:1 to 1:10 with a typical staining pattern in a tissue-based assay [TBA]) as typical of patients with GAD65-autoantibody-associated neurological syndromes.[20] Importantly, only 2/10 patients suffered from concomitant type 1 diabetes mellitus (T1DM).

Elevated T cell activation in PB and CSF of patients with GAD65-LE

Multi-parameter flow cytometry of PB and CSF (Fig. 1A and B) demonstrated increased fractions of activated $HLADR^+$ $CD4^+$ T cells (PB: $p = 0.0003$, CSF: $p < 0.0001$; Mann–Whitney U test) and activated $HLADR^+$ $CD8^+$ T cells (PB: $p = 0.0046$, CSF: $p = 0.0027$; Mann–Whitney U test) in both compartments as compared to controls. In contrast, no increased fractions of $CD19^+$ B cells (PB: $p = 0.569$, CSF: $p = 0.385$; Mann–Whitney U test) or $CD138^+$ plasma cells (PB: $p = 0.896$, CSF: $p = 0.913$; Mann–Whitney U test) could be detected (Table S1).

Intrathecal $CD8^+$ and $CD4^+$ T cells strongly correlate with hippocampal volume and memory function in patients with long-standing GAD65-LE in an opposite manner

In patients with long-standing GAD65-LE, considerable variation of hippocampal volumes (Fig. 1C) occurred similar to what was described.[4] Although there was no significant difference of Relative Hippocampal Volumes between GAD65-LE patients and controls (GAD65-LE: 0.315 ± 0.01; CTR: 0.31 ± 0.01; Mann–Whitney U test: $p = 0.41$; Fig. 1D), a highly significant negative correlation between the intrathecal fraction of $CD8^+$ T cells and the Relative Hippocampal Volume was detected in our GAD65-LE patient cohort ($r = -0.782$, $p = 0.008$, Spearman-Rho; Fig. 1E). Moreover, only 3/10 GAD65-LE patients showed lower performance in EpiTrack® test, and none of the GAD-LE patients displayed lower performance in VLMT and DCS-R. Nevertheless, after correction of the global (verbal and figural) memory performance by the medication-sensitive executive performance as measured by the EpiTrack® test (MemScore), there was also a highly significant negative correlation between the intrathecal fraction of $CD8^+$ T cells and the MemScore ($r = -0.818$, $p = 0.004$, Spearman-Rho; Fig. 1F). Consistently, a strong positive correlation between the Relative Hippocampal Volume and the MemScore could be verified ($r = 0.733$, $p = 0.016$, Spearman-Rho; Fig. 1G). The opposite situation was found when correlating intrathecal fractions of $CD4^+$ T cells with the Relative Hippocampal Volume ($r = 0.782$, $p = 0.008$, Spearman-Rho; data not shown) and MemScore ($r = 0.782$, $p = 0.008$, Spearman-Rho; data not shown) in GAD65-LE. These data suggest pathogenic effect of $CD8^+$ T cells and a regulatory effect of $CD4^+$ T cells in long-standing GAD65-LE. No significant correlation was found regarding the Relative Amygdala Volume ($CD8^+$: $r = 0.127$, $p = 0.726$; $CD4^+$: $r = 0.18$, $p = 0.96$; MemScore: $r = -0.42$, $p = 0.907$; Spearman-Rho).

$CD8^+$ T cell-associated neuronal degeneration in hippocampectomy specimen from patients with GAD65-LE

Selective hippocampectomy specimen (Fig. 2A–C) from two patients with pharmacoresistant mesial temporal lobe epilepsy (age 24 and 52 years, disease duration 7 months and 9 years, >2000 iU/mL GAD65 aabs in serum [ELISA], 1:32 and 1:10 in CSF [TBA], no preceding immunotherapy) that afterwards were diagnosed with GAD65-LE, revealed loss of $NeuN^+$ neurons in the cornu ammonis (CA) and dentate gyrus (DG) of the hippocampus. Consistently, staining for activated caspase-3 showed few apoptotic hippocampal neurons, whereas staining for amyloid precursor protein showed no overt axonal damage (data not shown). In both regions, strong parenchymal T cell infiltrates were detected with $CD8^+$ T cells being in close spatial proximity mainly to small interneurons (but also to large principal neurons), suggesting an antigen-specific attack towards inhibitory neuronal networks in the hippocampus (Fig. 2B and C). In contrast, only few parenchymal $CD20^+$ B cells and $CD138^+$ plasma cells were detected (data not shown). The density of these immune cell infiltrates clearly exceeded that detected in neurodegenerative and healthy controls.[21]

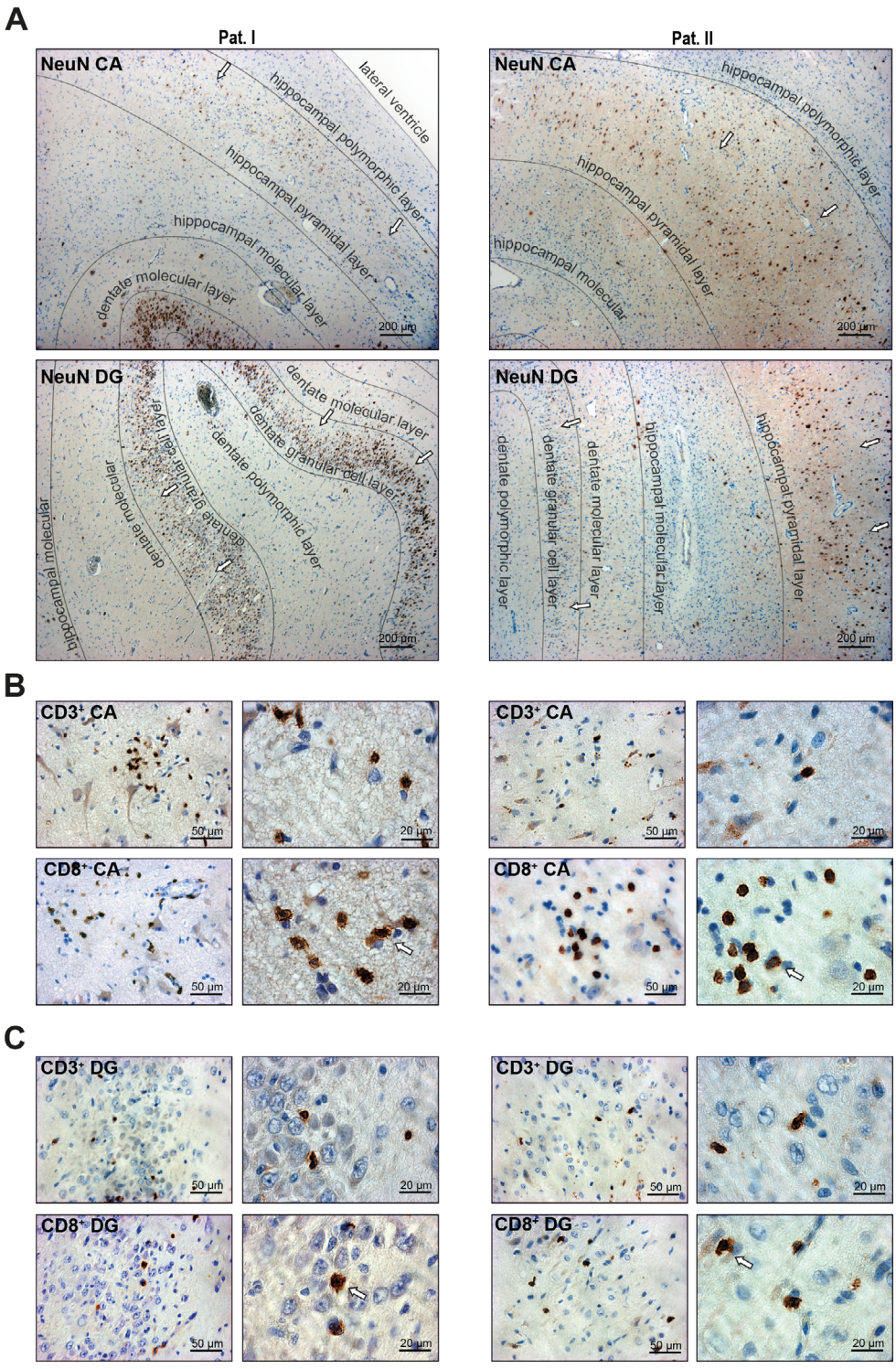

Figure 2. CD8$^+$ T cells interact with neurons in GAD65-LE. Selective hippocampectomy specimen from two patients (Pat. I left; Pat. II, right) with GAD65-LE were analyzed for inflammatory neuronal damage. (A) Staining for NeuN revealed neuronal loss (white arrows) in the hippocampal pyramidal cell layer and dentate granule cell layer (CA, cornu ammonis; DG, dentate gyrus). (B and C) Staining for CD3$^+$ and CD8$^+$ T cells demonstrated strong parenchymal T cell infiltrates in the CA (B) and DG (C) regions with CD8$^+$ T cells being in close spatial proximity mainly to small interneurons (white arrows).

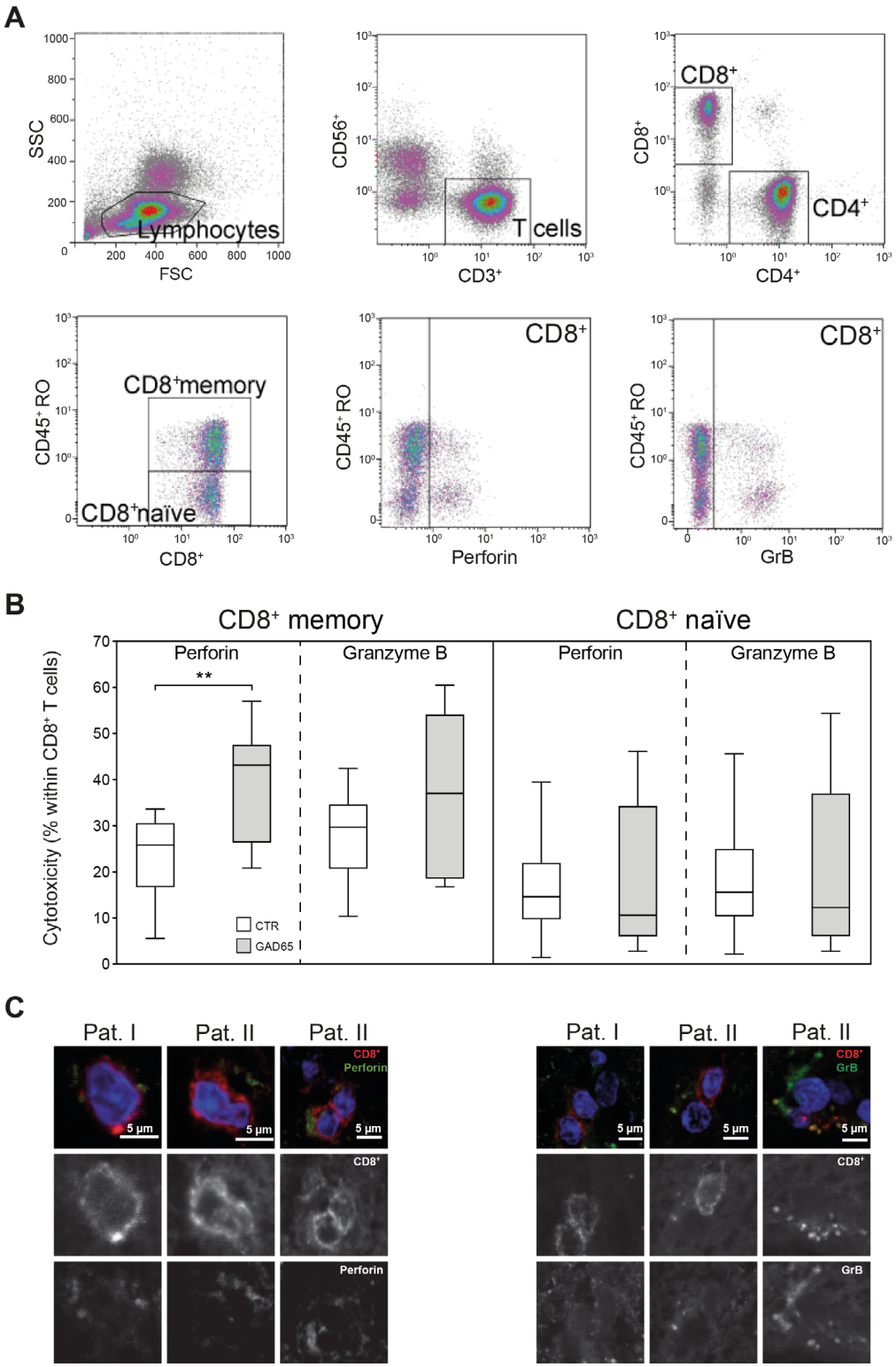

Figure 3. Peripheral CD8$^+$ T cells express increased levels of cytotoxic effector molecules in long-standing GAD65-LE. Gating strategy (A) and group analysis (B) of expression levels of cytotoxic effector molecules perforin and granzyme B (GrB) in the naïve and memory CD4$^+$ and CD8$^+$ T cell populations in PBMCs from 10 patients with long-standing GAD65-LE and controls (CTR). (C) Parenchymal CD8$^+$ T cells express cytotoxic effector molecules perforin and granzyme B in selective hippocampectomy specimen from two patients (Pat. I and Pat. II) with GAD65-LE.

CD8$^+$ T cells express cytotoxic effector molecule perforin in peripheral blood and hippocampal parenchyma of patients with long-standing GAD65-LE

To further corroborate the notion of a CD8$^+$ T cell-mediated attack towards inhibitory hippocampal interneurons, we analyzed the intracellular expression levels of the cytotoxic effector molecules perforin and granzyme B in the naïve and memory CD4$^+$ and CD8$^+$ T cell populations in PBMCs from 10 patients with long-standing GAD65-LE and controls (Fig. 3A and B). We found significantly elevated fractions of intracellularly perforin expressing cells (perforin: $p = 0.0089$; Mann–Whitney U test) and a tendency towards elevated fractions of intracellularly granzyme B expressing cells (granzyme B: $p = 0.2799$; Mann–Whitney U test) within the population of antigen-experienced memory CD8$^+$ T cells but not in naïve CD8$^+$ T cell populations (perforin: $p = 0.6842$; granzyme B: $p = 0.6842$; Mann–Whitney U test) of patients with long-standing GAD65-LE compared to controls (Fig. 3A and B). No augmented intracellular cytotoxic effector molecule expression was observed in naïve (perforin: $p = 0.3633$; granzyme B: $p = 0.7394$; Mann–Whitney U test) and memory (perforin: $p = 0.0892$; granzyme B: $p = 0.3642$; Mann–Whitney U test) CD4$^+$ T cells compared to controls (data not shown).

Consistently, parenchymal CD8$^+$ T cells approaching hippocampal neurons also expressed cytotoxic effector molecules perforin and granzyme B (Fig. 3C). These data suggest initiation and perpetuation of the parenchymal CD8$^+$ T cell response within the peripheral immune system.

Full-resolution HLA-genotyping reveals presence of the HLA-A*02:01 allele presenting the immunodominant GAD65$_{114–123}$ peptide in the majority of patients with GAD65-LE

Next-generation sequencing-based genotyping of the HLA molecules HLA-A, -B, -C, DRB1, DQB1, and DPB1 at full (8 digit) resolution revealed that 6/10 patients harbored the HLA-A*02:01 haplotype (Table 2) present in about 30% of the general population in Germany (http://www.allelefrequencies.net/). The HLA-A*02:01molecule is known to present the immunodominant GAD65$_{114–123}$ peptide in humans rendering target cells susceptible to cell death induced by GAD65-specific CD8$^+$ T cells.[22,23]

Discussion

In a cohort of patients with typical long-standing GAD65-LE, we found no significant alterations of hippocampal volumes and almost normal cognitive performance compared

Table 2. Full-resolution (8 digit) HLA-genotyping of patients with GAD65-LE.

	HLA-A		HLA-B		HLA-C		HLA-C		HLA-DRB1		HLA-DRB1		HLA-DQB1		HLA-DPB1		HLA-DPB1	
PID	D1		D1		D2		D1		D1		D2		D1		D2		D1		D2	
1	*03:01:01:01	*03:01:01:05	*18:01:01:01	*40:01:02:01	*03:04:01:01		*05:01:01:01		*03:01:01:01		*13:01:01:01		*02:01:01		*06:03:01:01		*01:01:01:01		*04:01:01:01	
2	*01:01:01:01	*30:02:01:01	*08:01:01:01	*53:01:01	*04:01:01:01		*07:01:01:01		*03:01:01:01		*13:02:01:01		*02:01:01		*03:02:01:01		*01:01:01:01		*02:01:02:01	
3	***02:01:01:01***	*24:02:01:01	*15:01:01:01	*44:02:01:01	*03:03:01:01		*05:01:01:02		*12:01:01:01		*13:01:01:01		*03:01:01:05		*06:03:01:01		*03:01:01:01		*04:01:01:01	
4	*01:01:01:01	*03:01:01:01	*08:01:01:01	*35:01:01:02	*04:01:01:01		*07:01:01:01		*01:01:01		*03:01:01:01		*02:01:01		*05:01:01:02		*03:01:01:01		*04:01:01:01	
5	***02:01:01:01***	*24:02:01:01	*07:02:01:01	*40:01:02:01	*03:04:01:01		*07:02:01:03		*01:01:01		*15:01:01:01		*05:01:01:02		*06:02:01:01		*04:01:01:01		*04:02:01:02	
6	***02:01:01:01***	*30:02:01:01	*13:02:01:01	*18:01:01:01	*05:01:01:01		*06:02:01:01		*03:01:01:01		*07:01:01:01		*02:01:01		*02:02:01:01		*02:02:01:01		*04:01:01:01	
7	***02:01:01:01***	---	*15:01:01:01	*04:01:02:01	*03:03:01:01		*03:04:01:02		*03:01:01:01		*15:01:01:01		*02:01:01		*06:02:01:01		*02:02:01:01		*04:01:01:01	
8	*11:01:01:01	*24:02:01:01	*08:01:01:01	*13:02:01:01	*06:02:01:01		*07:01:01:01		*03:01:01:01		*07:01:01:01		*02:01:01		*02:02:01:01		*02:01:02:01		*13:01:01:01	
9	***02:01:01:01***	*03:01:01:01	*07:02:01:01	*56:01:01:03	*01:02:01:01		*07:02:01:03		*04:03:01:01		*15:01:01:01		*03:05:01		*06:02:01:01		*02:01:02:01		*04:01:01:01	
10	***02:01:01:08***	*32:01:01:01	*35:03:01:01	*51:01:01:03	*04:01:01:01		*14:02:01:01		*04:03:01:01		*15:01:01:01		*03:05:01		*06:02:01:01		*02:01:02:06		*13:01:01:02	

6/10 patients harbored the HLA-A*02:01 allele (bold; present in about 30% of the general population in Germany) known to present the immunodominant GAD65$_{114–123}$ peptide in humans.
PID, patient identifiable data.

to controls. Moreover, routine CSF parameters were also largely unremarkable. However, multi-parameter flow cytometry of PB and CSF revealed significantly elevated fractions of activated $CD4^+$ and $CD8^+$ T cells.

Our findings suggest $CD8^+$ T cells as potential promoters of autoimmune epileptogenic neurodegeneration in GAD65-LE consistent with the intracellular localization of the putative target autoantigen: (i) elevated fractions of activated $HLADR^+$ $CD8^+$ T cells in PB and CSF were detected in 10 patients with GAD65-LE compared to controls; (ii) intrathecal $CD8^+$ T cells negatively correlated with hippocampal volumes and memory performance in a cross-sectional manner; (iii) peripheral and parenchymal (antigen-experienced) $CD8^+$ T cells expressed the cytotoxic effector molecule perforin and seem to cause extensive neuronal degeneration in hippocampal specimen of patients with GAD65-LE; and (iv) a majority of patients harbored the HLA-repertoire known to mediate presentation of GAD65-peptides.

(Auto-)immune epileptogenic neurodegeneration mediated by $CD8^+$ T cells has first been described in patients with Rasmussen Encephalitis (RE), a progressive epileptic disorder characterized by mostly unihemispheric cortical gray matter inflammation leading to neuronal loss and destruction of the affected hemisphere(s).[24–27] Parenchymal infiltrates of $CD8^+$ T cells in part with direct apposition to MHC class I^+ neurons[25] (and astrocytes)[28] and polar orientation of their cytotoxic granules towards neuronal perikarya have been described.

Consistently, RE patients show strong $CD8^+$ T cell expansions within the peripheral blood correlating with disease severity.[24,27] Moreover, RE patients demonstrate prominent $CD8^+$ T cell expansions also in the CNS parenchyma, and common $CD8^+$ T cell clones are shared specifically between RE patients who also share MHC class I alleles.[24,27] These data strongly support the hypothesis of an antigen-driven MHC class-I restricted $CD8^+$ T cell-mediated attack against neurons (and astrocytes) in RE. Nevertheless, thus far the specific nature of the RE (auto)antigen(s) remains elusive.

Using a murine viral encephalitis model, $CD8^+$ T cells were recently shown to cause interferon-γ (IFN-γ)-dependent, chemokine (C-C motif) ligand 2 (CCL2)-mediated neuronal deafferentation by phagocyte-mediated synaptic stripping analogous to RE.[29–31] Deafferentation has been further suggested to lower synaptic input and neuronal electrical activity below a critical threshold, thereby promoting MHC class I expression and rendering neurons susceptible to perforin/granzyme- or FasL-mediated cell death.[29–31]

In addition to various neurological syndromes such as LE, autoantibodies to GAD65 are also found in patients with type 1 diabetes mellitus (T1DM).[32–34] However, due to intracellular localization of the GAD65 autoantigen, loss of pancreatic β-cells in T1DM is considered to be mediated by GAD65 autoreactive T cells.[32–34] Early studies reported that GAD65-directed T cell responses were preferentially detectable in patients with T1DM and at-risk subjects, but rarely in healthy individuals.[35–38] Other studies, however, showed that T cell responses against GAD65 can be readily measured both in patients with T1DM and subjects without any sign of autoimmunity indicating that most individuals carry GAD65 autoreactive T cells in their naïve repertoire.[39] Later studies indicated that autoreactive T cells in patients with type 1 diabetes exhibit specific characteristics that are typical of cells that have already encountered the GAD65 antigen. These include proliferation to GAD65 in the absence of costimulatory signals and the presence of specific late activation markers (CD40, CD134).[40,41] Indeed, GAD65 autoreactive T cell responses could be specifically detected in T1DM patients using assays that selectively measure memory T cell responses.[42] To initiate a $CD8^+$ T cell response to an exogenous antigen such as GAD65, antigen-presenting dendritic cells usually need to be licensed by $CD4^+$ T cells for HLA class I-restricted cross-presentation of this antigen,[26] whereas $CD4^+$ T cells in T1DM recognize diverse GAD65 epitopes depending also on post-translational modifications and the individual HLA class II haplotypes,[43,44] the HLA class I-restricted $GAD65_{114–123}$ peptide seems to represent the immunodominant peptide in T1DM patients owing to the very common HLA-A*02:01 molecule. The $GAD65_{114–123}$ peptide has been demonstrated to be processed from whole GAD65 protein and presented by HLA-A*02:01 expressing pancreatic ß-cells that are subsequently recognized and killed by autoreactive $CD8^+$ T cells.[22,23]

The HLA-A*02:01 molecule was twice as frequent in our small cohort of patients with GAD65-LE as in the German general population (http://www.allelefrequencies.net/). Of the 10 patients with GAD65-LE included in our study, 2 also suffered from T1DM suggesting GAD65 expressing interneurons as targets of the adaptive autoimmune response. Given the activation status and effector molecules expression of peripheral $CD8^+$ T cells, the strong correlation of hippocampal structure and function with intrathecal $CD8^+$ T cells fractions as wells as the effector molecule expression of parenchymal hippocampal $CD8^+$ T cells and their peculiar association with small interneurons, it is tempting to speculate that $CD8^+$ T cells also recognize GAD65 as their cognate antigen in GAD65-LE. Subsequent predominant dysfunction and degeneration of hippocampal inhibitory interneurons might then explain the generation of epileptic seizures in these patients. Given the homogenous T cell signature in PB and CSF in anti-GAD65 LE patients compared to controls, it would be interesting to study the peripheral and intrathecal immune cell signature of T1DM patients with anti-GAD65 aabs that receive

PB and CSF examination for clinical purposes and turn out to suffer from somatization disorder much like the control group used in this study.

In addition to the pathogenic role of $CD8^+$ T cells, our data suggest the opposite effect of $CD4^+$ T cells in GAD65-LE: (i) elevated fractions of activated $HLADR^+$ $CD4^+$ T cells in PB and CSF were detected in 10 patients with GAD65-LE compared to controls; (ii) intrathecal $CD4^+$ T cells positively correlated with hippocampal volume and memory performance in a cross-sectional manner. The mechanisms underlying this effect need to be investigated in future studies.

Taken together, our data strongly argue in favor of a pathogenic role of $CD8^+$ T cells and an opposite effect of $CD4^+$ T cells in this particular form of autoimmune encephalitis.

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Author Contributions

A.D., J.P., and A.J.B.: histopathological analysis. G.W., N.H., and S.R.: patient recruitment and data analysis. J.W.: MRI data analysis. J.A.W. and C.H.: neuropsychological analysis. A.S.-M., K.S.G., M.G., C.S., C.M., and C.C.G.: sampling and analysis of biomaterial. S.G.M., H.W., C.E.E., and and N.M.: study design and supervision. All authors contributed to and approved the final version of the manuscript.

Conflicts of Interest

All authors declare no conflicts of interest.

References

1. Giometto B, Nicolao P, Macucci M, Tavolato B, Foxon R, Bottazzo GF. Temporal-lobe epilepsy associated with glutamic-acid-decarboxylase autoantibodies. Lancet. 1998;352(9126):457.
2. Malter MP, Helmstaedter C, Urbach H, Vincent A, Bien CG. Antibodies to glutamic acid decarboxylase define a form of limbic encephalitis. Ann Neurol. 2010;67(4):470-478.
3. Muñoz-Lopetegi A, de Bruijn MAAM, Boukhrissi S, et al. Neurologic syndromes related to anti-GAD65: clinical and serologic response to treatment. Neurol Neuroimmunol Neuroinflamm. 2020;7(3):e696.
4. Wagner J, Witt J-A, Helmstaedter C, Malter MP, Weber B, Elger CE. Automated volumetry of the mesiotemporal structures in antibody-associated limbic encephalitis. J Neurol Neurosurg Psychiatry. 2015;86:735–742.
5. Wagner J, Schoene-Bake J-C, Malter MP, et al. Quantitative FLAIR analysis indicates predominant affection of the amygdala in antibody-associated limbic encephalitis. Epilepsia. 2013;54(9):1679-1687.
6. Erlander MG, Tillakaratne NJK, Feldblum S, Patel N, Tobin AJ. Two genes encode distinct glutamate decarboxylases. Neuron. 1991;7(1):91-100.
7. Bowers G, Cullinan WE, Herman JP. Region-specific regulation of glutamic acid decarboxylase (GAD) mRNA expression in central stress circuits. J Neurosci. 1998;18 (15):5938-5947.
8. Kaufman DL, Houser CR, Tobin AJ. Two forms of the gamma-aminobutyric acid synthetic enzyme glutamate decarboxylase have distinct intraneuronal distributions and cofactor interactions. J Neurochem. 1991;56(2):720-723.
9. Kash SF, Johnson RS, Tecott LH, et al. Epilepsy in mice deficient in the 65-kDa isoform of glutamic acid decarboxylase. Proc Natl Acad Sci USA. 1997;94 (25):14060-14065.
10. Lange MD, Jüngling K, Paulukat L, et al. Glutamic acid decarboxylase 65: a link between GABAergic synaptic plasticity in the lateral amygdala and conditioned fear generalization. Neuropsychopharmacology. 2014;39 (9):2211-2220.
11. Sangha S, Narayanan RT, Bergado-Acosta JR, Stork O, Seidenbecher T, Pape H-C. Deficiency of the 65 kDa isoform of glutamic acid decarboxylase impairs extinction of cued but not contextual fear memory. J Neurosci. 2009;29(50):15713-15720.
12. Gresa-Arribas N, Ariño H, Martínez-Hernández E, et al. Antibodies to inhibitory synaptic proteins in neurological syndromes associated with glutamic acid decarboxylase autoimmunity. PLoS One. 2015;10(3):e0121364.
13. Graus F, Titulaer MJ, Balu R, et al. A clinical approach to diagnosis of autoimmune encephalitis. Lancet Neurol. 2016;15(4):391-404.
14. Helmstaedter C, Lendt M, Lux S. VLMT: Verbaler Lern- und Merkfähigkeitstest. Testhandbuch Hogrefe Göttingen; 2000.
15. Helmstaedter C, Pohl C, Elger CE. Eine modifizierte Version des Diagnostikums für Cerebralschäden (DCS) zur Diagnostik räumlich-visueller Gedächtnisdefizite bei Patienten mit Temporallappenepilepsie. In: Scheffner D, ed. Epilepsie 90. Einhorn-Presse Reinbek; 1991:272-279.
16. Lutz MT, Helmstaedter C. EpiTrack: tracking cognitive side effects of medication on attention and executive functions in patients with epilepsy. Epilepsy Behav. 2005;7:708-714.
17. Golombeck KS, Bonte K, Monig C, et al. Evidence of a pathogenic role for CD8(+) T cells in anti-GABAB

receptor limbic encephalitis. Neurol Neuroimmunol Neuroinflamm. 2016;3(3):e232.
18. Reiber H, Peter JB. Cerebrospinal fluid analysis: disease-related data patterns and evaluation programs. J Neurol Sci. 2001;184(2):101-122.
19. Wandinger KP, Klingbeil C, Gneiss C, et al. Neue serologische Marker zur Differentialdiagnose der Autoimmun-Enzephalitis. J Lab Med. 2011;35(6):329-342.
20. Saiz A, Blanco Y, Sabater L, et al. Spectrum of neurological syndromes associated with glutamic acid decarboxylase antibodies: diagnostic clues for this association. Brain. 2008;131(Pt 10):2553-2563.
21. Bien CG, Vincent A, Barnett MH, et al. Immunopathology of autoantibody-associated encephalitides: clues for pathogenesis. Brain. 2012;135(Pt 5):1622-1638.
22. Knight RR, Dolton G, Kronenberg-Versteeg D, et al. A distinct immunogenic region of glutamic acid decarboxylase 65 is naturally processed and presented by human islet cells to cytotoxic CD8 T cells. Clin Exp Immunol. 2015;179(1):100-107.
23. Panina-Bordignon P, Lang R, van Endert PM, et al. Cytotoxic T cells specific for glutamic acid decarboxylase in autoimmune diabetes. J Exp Med. 1995;181(5):1923-1927.
24. Schwab N, Bien CG, Waschbisch A, et al. CD8+ T-cell clones dominate brain infiltrates in Rasmussen encephalitis and persist in the periphery. Brain. 2009;132(5):1236-1246.
25. Bien CG, Bauer J, Deckwerth TL, et al. Destruction of neurons by cytotoxic T cells: a new pathogenic mechanism in Rasmussen's encephalitis. Ann Neurol. 2002;51(3):311-318.
26. Melzer N, Meuth SG, Wiendl H. CD8+ T cells and neuronal damage: direct and collateral mechanisms of cytotoxicity and impaired electrical excitability. FASEB J. 2009;23(11):3659-3673.
27. Schneider-Hohendorf T, Mohan H, Bien CG, et al. CD8(+) T-cell pathogenicity in Rasmussen encephalitis elucidated by large-scale T-cell receptor sequencing. Nat Commun. 2016;7:11153.
28. Bauer J, Elger CE, Hans VH, et al. Astrocytes are a specific immunological target in Rasmussen's encephalitis. Ann Neurol. 2007;62(1):67-80.
29. Kreutzfeldt M, Bergthaler A, Fernandez M, et al. Neuroprotective intervention by interferon-gamma blockade prevents CD8+ T cell-mediated dendrite and synapse loss. J Exp Med. 2013;210(10):2087-2103.
30. Merkler D, Horvath E, Bruck W, Zinkernagel RM, Del la Torre JC, Pinschewer DD. "Viral deja vu" elicits organ-specific immune disease independent of reactivity to self. J Clin Invest. 2006;116(5):1254-1263.
31. Di Liberto G, Pantelyushin S, Kreutzfeldt M, et al. Neurons under T cell attack coordinate phagocyte-mediated synaptic stripping. Cell. 2018;175(2):458-471.e19.
32. Lehuen A, Diana J, Zaccone P, Cooke A. Immune cell crosstalk in type 1 diabetes. Nat Rev Immunol. 2010;10(7):501-513.
33. Bluestone JA, Herold K, Eisenbarth G. Genetics, pathogenesis and clinical interventions in type 1 diabetes. Nature. 2010;464(7293):1293-1300.
34. Atkinson MA, Eisenbarth GS, Michels AW. Type 1 diabetes. Lancet. 2014;383(9911):69-82.
35. Atkinson MA, Campbell L, Gibbs KA, et al. Response of peripheral-blood mononuclear cells to glutamate decarboxylase in insulin-dependent diabetes. Lancet. 1992;339(8791):458-459.
36. Öling V, Marttila J, Ilonen J, et al. GAD65- and proinsulin-specific CD4+ T-cells detected by MHC class II tetramers in peripheral blood of type 1 diabetes patients and at-risk subjects. J Autoimmun. 2005;25(3):235-243.
37. Reijonen H, Novak EJ, Kochik S, et al. Detection of GAD65-specific T-cells by major histocompatibility complex class II tetramers in type 1 diabetic patients and at-risk subjects. Diabetes. 2002;51(5):1375-1382.
38. Seyfert-Margolis V, Gisler TD, Asare AL, et al. Analysis of T-cell assays to measure autoimmune responses in subjects with type 1 diabetes: results of a blinded controlled study. Diabetes. 2006;55(9):2588-2594.
39. Roep BO. Standardization of T-cell assays in type I diabetes. Immunology of Diabetes Society T-cell Committee. Diabetologia. 1999;42(5):636-637.
40. Endl J, Rosinger S, Schwarz B, et al. Coexpression of CD25 and OX40 (CD134) receptors delineates autoreactive T-cells in type 1 diabetes. Diabetes. 2006;55(1):50-60.
41. Viglietta V, Kent SC, Orban T, Hafler DA. GAD65-reactive T cells are activated in patients with autoimmune type 1a diabetes. J Clin Invest. 2002;109(7):895-903.
42. Monti P, Scirpoli M, Rigamonti A, et al. Evidence for in vivo primed and expanded autoreactive T cells as a specific feature of patients with type 1 diabetes. J Immunol. 2007;179(9):5785-5792.
43. Yang J, James EA, Sanda S, Greenbaum C, Kwok WW. $CD4^+$ T cells recognize diverse epitopes within GAD65: implications for repertoire development and diabetes monitoring. Immunology. 2013;138(3):269-279.
44. McGinty JW, Chow I-T, Greenbaum C, Odegard J, Kwok WW, James EA. Recognition of posttranslationally modified GAD65 epitopes in subjects with type 1 diabetes. Diabetes. 2014;63(9):3033-3040.

Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1. Multi-parameter flow cytometry data analysis of CSF and PB. No pleocytosis was detected in all samples. PID, patient identifiable data.

