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 GAD65114–123 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.
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). 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. LE typically presents with adult-onset mesial temporal lobe seizures, progressive episodic memory disturbance, and a variety of other neuropsychiatric symptoms. 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.

Glutamic acid decarboxylase is expressed in interneurons and catalyzes the conversion of glutamate to $\gamma$-aminobutyric acid (GABA). The brain contains two isoforms, GAD65 and GAD67, which display characteristic differences in localization and activity patterns. 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. 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. Moreover, GAD65-expressing interneurons have been implicated in hippocampus- and amygdala-based synaptic plasticity, learning, and memory.

Due to the intracellular localization of GAD65, direct pathogenic effects of aabs have been questioned. 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 immunogenetic findings.

Subjects and Methods

Patients

According to the consensus criteria for autoimmune encephalitis by Graus et al. from 2016, 10 immunotherapy-naive 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 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, $3^2$, repetition time 1570 msec, echo time 3.42 msec, flip angle 15 degrees, and field of view $256 \times 256$ mm. The analysis of the structural image data was done using FreeSurfer (V.5.1.0, Martinsos 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. 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-R15 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, Amphiphysin, 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 (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-antirabbit; 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...
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).

**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 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, orofacial automatisms, fumbling | Left temporal slowing     |
| 5   | f      | 61                         | 5.80                     | 0                       | 0         | 0         | 0          | >2000 iU/mL              | IFT: 1:3.2             | Orofacial 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 temporal slowing      |
| 9   | f      | 42                         | 5.00                     | 1                       | 0         | 1         | 0          | >2000 iU/mL              | IFT: 1:3.2             | Epigastric aura, loss of consciousness, orofacial automatisms, fumbling | Left temporal slowing     |
| 10  | m      | 24                         | 7.00                     | 3                       | 0         | 3         | 0          | >2000 iU/mL              | IFT: 1:3.2             | Loss of consciousness, orofacial 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.

**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
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.
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B

CD4+ T cells

CD8+ T cells

HLADR+CD4+ T cells

HLADR+CD8+ T cells

PB

CSF

C

FLAIR

T2

D

Relative Hippocampal Volume

CTR

GAD65

E

F

G

r = -0.782
p = 0.008

r = -0.818
p = 0.004

r = 0.733
p = 0.016
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. 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. 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 pharmaco-resistant 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.
Figure 2. CD8<sup>+</sup> 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<sup>+</sup> and CD8<sup>+</sup> T cells demonstrated strong parenchymal T cell infiltrates in the CA (B) and DG (C) regions with CD8<sup>+</sup> 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 naive 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.
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 naive 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) within the population of antigen-experienced memory CD8+ T cells but not in naive 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 GAD65114–123 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*02:01 molecule is known to present the immunodominant GAD65114–123 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...
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 destruction of the affected hemisphere(s).

Parenchymal infiltrates of CD8+ T cells in part with direct apposition to MHC class I+ neurons 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. 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. 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. 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.

In addition to various neurological syndromes such as LE, autoantibodies to GAD65 are also found in patients with type 1 diabetes mellitus (T1DM). 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. 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. 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. 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). Indeed, GAD65 autoreactive T cell responses could be specifically detected in T1DM patients using assays that selectively measure memory T cell responses. 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, whereas CD4+ T cells in T1DM recognize diverse GAD65 epitopes depending also on post-translational modifications and the individual HLA class II haplotypes, the HLA class I-restricted GAD65114-123 peptide seems to represent the immunodominant peptide in T1DM patients owing to the very common HLA-A*02:01 molecule. The GAD65114-123 peptide has been demonstrated to be processed from whole GAD65 protein and presented by HLA-A*02:01 expressing pancreatic β-cells that are subsequently recognized and killed by autoreactive CD8+ T cells.

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 well 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...
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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 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.

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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.