T cell numbers correlate with neuronal loss rather than with seizure activity in medial temporal lobe epilepsy

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Abstract

Objective: Medial temporal lobe epilepsy (MTLE) is a drug-resistant focal epilepsy that can be caused by a broad spectrum of different inciting events, including tumors, febrile seizures, and viral infections. In human epilepsy surgical resections as well as in animal models, an involvement of the adaptive immune system was observed. We here analyzed the presence of T cells in various subgroups of MTLE. We aimed to answer the question of how much inflammation was present and whether the presence of T cells was associated with seizures or associated with hippocampal neurodegeneration.

Methods: We quantified the numbers of CD3\(^+\) T cells and CD8\(^+\) cytotoxic T cells in the hippocampus of patients with gangliogliomas (GGs; intrahippocampal and extrahippocampal, with and without sclerosis), febrile seizures, and postinfectious encephalitic epilepsy and compared this with Rasmussen encephalitis, Alzheimer disease, and normal controls.

Results: We could show that T cell numbers were significantly elevated in MTLE compared to healthy controls. CD3\(^+\) as well as CD8\(^+\) T cell numbers, however, varied highly among MTLE subgroups. By comparing GG patients with and without hippocampal sclerosis (HS), we were able to show that T-cell numbers were increased in extrahippocampal GG patients with hippocampal neuronal loss and HS, whereas extrahippocampal GG cases without hippocampal neuronal loss (i.e., absence of HS) did not differ from healthy controls. Importantly, T cell numbers in MTLE correlated with the degree of neuronal loss, whereas no correlation with seizure frequency or disease duration was found. Finally, we found that in nearly all MTLE groups, T cell numbers remained elevated even years after the inciting event.

Significance: We here provide a detailed histopathological investigation of the involvement of T cells in various subgroups of MTLE, which suggests that T cell influx correlates to neuronal loss rather than seizure activity.
INTRODUCTION

Medial temporal lobe epilepsy (MTLE) is the most common form of drug-resistant focal epilepsy, and patients often undergo resection of the affected area.\(^1\) In a large international consortium, the underlying histopathological changes in these resections have been evaluated and classified. The most common ones in adults were hippocampal sclerosis (HS), tumors, and cortical malformations. Among those, HS was identified in 44.5% of adult hippocampal resections.\(^1\) Tumors, as the second most frequent histopathological diagnosis, were found in 23.6%, with the largest subgroup being gangliogliomas (GGs).\(^1\) Other reasons for the development of MTLE include postinfectious encephalitic epilepsy (PEE) and febrile seizures (FS) during childhood.\(^2\)–\(^4\) The International League Against Epilepsy (ILAE) has classified HS into three categories, depending on the hippocampal subfields affected most severely by neuronal loss. In the most common type, ILAE Type 1, CA1 and CA4 regions are most heavily and similarly affected.\(^5\) Tumors, as the second most frequent histopathological diagnosis, were found in 23.6%, with the largest subgroup being gangliogliomas (GGs).\(^1\) Other reasons for the development of MTLE include postinfectious encephalitic epilepsy (PEE) and febrile seizures (FS) during childhood.\(^2\)–\(^4\) The International League Against Epilepsy (ILAE) has classified HS into three categories, depending on the hippocampal subfields affected most severely by neuronal loss. In the most common type, ILAE Type 1, CA1 and CA4 regions are most heavily and similarly affected.\(^5\) Unfortunately, HS itself does not provide any information on the etiology of the disease that leads to the neuronal loss and gliosis in the hippocampus. Because MTLE can be classified into these different ILAE types, it is possible that these types result from different underlying etiologies.\(^6\) Inflammation, especially mediated by the innate immune system, has been shown to be involved in various forms of epilepsy, among them MTLE. Several cytokines, such as interleukin (IL)-1β, IL-6, IL-10, and tumor necrosis factor-α, and various chemokines, such as the chemokines C-C motif ligand (CCL)3 and CCL4, were elevated in resected hippocampi.\(^7\)–\(^12\) Moreover, differences in cytokine and chemokine levels between patients with MTLE of different etiologies were found in resected hippocampi as well as in the serum.\(^12\),\(^13\) In addition to the innate, also the adaptive immune system is thought to be involved in epilepsy. T cell infiltrates have been reported in various forms of epilepsy, among them MTLE, in highly varying numbers.\(^7\),\(^14\) These numbers of infiltrating T cells were seen to correlate with the amount of neuronal loss and to vary among ILAE HS classifications. Moreover, drastically higher numbers of cytotoxic CD8\(^+\) T cells were found in the resected hippocampi of MTLE patients.\(^15\),\(^16\) In addition, in animal models of epilepsy, leukocytes were shown to contribute to seizure generation, as animals treated with anti-integrin antibodies, thereby countering infiltration of leukocytes in the central nervous system (CNS), showed milder forms of epilepsy.\(^17\),\(^18\) Furthermore, it was shown that CD8\(^+\) T cells preferentially infiltrate the hippocampus after only a single seizure. T cell numbers peaked after 48 h but went back to baseline numbers (preseizure) after 7 days.\(^19\)

Here, we addressed the presence of T lymphocytes among different MTLE etiologies. Specifically, our aim was to evaluate whether the number of infiltrated T cells is increased and whether T cell numbers are linked with seizure activity, as described for epilepsy animal models,\(^17\)–\(^19\) or alternatively, whether inflammatory infiltrates are correlated with neurodegeneration, as was repeatedly shown in Alzheimer disease.\(^20\)–\(^22\)

To this end, we selected hippocampal biopsies of MTLE patients with different etiologies, such as GG patients with both intrahippocampal GG and HS, patients with extrahippocampal GG with and without HS, patients with childhood FS, and postencephalitis patients who developed PEE. Our results confirm previous findings of significantly elevated T cell numbers in MTLE compared to nonseizure controls. By analyzing T cell numbers in different underlying etiologies of MTLE, we could show that the T cell increase differed strongly among the different MTLE groups. The number of T cells strongly correlated to the degree of neuronal loss, rather than seizure activity or disease duration.

MATERIALS AND METHODS

2.1 Patients

Patient samples were collected at the Bethel Epilepsy Center, Department of Epileptology at the University Hospital Bonn as well as at the Medical University of Vienna between 1989 and 2015.

KEYWORDS

Ammon’s horn sclerosis, febrile seizure, ganglioglioma, medial temporal lobe epilepsy, neurodegeneration, neuroinflammation, postencephalitic epilepsy

Key Points

- The numbers of T cells in MTLE with HS are increased compared to normal controls
- T cell influx seems to be associated with neurodegeneration
- T cell numbers correlate neither with seizure frequency nor with disease duration
- There is no specific ILAE HS type in any of the etiological MTLE subgroups
For this study, hippocampal resections from MTLE patients from various etiologies were compared to specimens from autopsy controls (nonneurological disease controls and neurodegeneration controls) as well as to hippocampal resections from patients with Rasmussen encephalitis (RE; inflammatory controls). Patient data including gender, age, disease duration, frequency of seizures, and whether invasive electroencephalographic recordings were performed are summarized in Table S1.

Forty-one patients who presented with recurrent unprovoked seizures, compatible with a mesiotemporal origin, were further classified into etiological subgroups. Two of these patients have been included in a previous study.14

The following groups were established:

• MTLE after FS ($n = 13$). All these hippocampi showed neuronal cell loss and gliosis.
• Intrahippocampal GG with HS (iGG + HS): tumor infiltration into hippocampus. These hippocampi showed neuronal cell loss and gliosis ($n = 4$).
• Extrahippocampal GG with HS (eGG + HS): no tumor infiltration into hippocampus. These hippocampi showed neuronal cell loss and gliosis ($n = 8$).
• Extrahippocampal GG without HS (eGG − HS): no tumor infiltration into hippocampus. These hippocampi were without neuronal cell loss and without gliosis; hippocampi were resected because presurgical investigations suggested that hippocampi were part of the epileptogenic areas ($n = 8$).
• PEE (postinfectious encephalitic epilepsy): a group of patients who developed MTLE after infection with herpes simplex virus ($n = 3$), mumps ($n = 1$), smallpox ($n = 3$), and tuberculosis ($n = 1$). All these hippocampi showed neuronal cell loss and gliosis ($n = 8$).

As controls for the histopathological evaluation of surgically obtained hippocampal specimens, we used 23 control hippocampi of the following individuals:

• Autopsy cases without CNS disorders and without neuronal loss (healthy controls [HCs], $n = 8$);
• Autopsy specimens from patients with moderate to severe hippocampal neuronal loss unrelated to MTLE: five patients with Alzheimer disease and one patient with Alzheimer and Parkinson disease, all without seizures (neurodegeneration controls [NDs], $n = 6$); and
• Surgical resections from patients with RE (inflammatory controls, $n = 9$).

2.2 | Tissue collection

Tissue resected during surgeries was immersed in Ringer solution on ice in the operating theater, from where it was picked up directly after resection, and then transferred to the pathology department for overnight fixation and paraffin embedding. Autopsy tissue (Alzheimer patients) had an average postmortem delay of 42 h. In these autopsy cases, fixation was done for multiple days in buffered formalin as described earlier.23

2.3 | Ethics statement

Patients gave informed consent to use of their brain tissue for research. The study was approved by the ethical committees of the Medical University of Münster (2015-088-f-S), the University of Bonn (360/12), and the Medical University of Vienna (EK1206/2013).

2.4 | Immunohistochemistry

Available surgical specimens from MTLE patients and control hippocampi were stained for inflammatory markers and neuronal loss as previously described.24 In short, sequential 4-μm sections of paraffin-embedded hippocampal specimens were deparaffinized, and antigen retrieval was performed by steaming in a conventional household food steamer with the corresponding antigen retrieval solution. Primary antibody was applied overnight at 4°C. Sections were stained for all T cells (anti-CD3), for cytotoxic T cells (anti-CD8), and for neurons (anti-NeuN). Incubation with primary antibodies was followed by the corresponding biotinylated secondary system and 3,3′-Diaminobenzidine (DAB) development. For cytotoxic T lymphocyte (CTL) staining, an additional tyramide enhancement was used (for staining details, see Table S2). The highly variable tissue quality of biopsy and autopsy tissue is a common problem in histochemical studies of human tissue. NeuN, which was used here to stain for neurons, is highly formalin sensitive and requires citrate pre-treatment for antigen retrieval but disappears when stronger antigen retrieval with EDTA pH 9.0 is used. On the other hand, the antigens recognized by the CD3 and CD8 antibodies (unlike for instance CD4) are very robust, less sensitive to formalin or formaldehyde, and can easily be recognized in tissues with long fixation times and long postmortem delays, as shown in our previous work with archival autopsy material.25 Thus, we are confident that the T cell stainings we performed are methodically sound and the results are optimal presentations of the amount of T cells in these cases.

2.5 | Histochemistry

As the NeuN antibody showed highly variable staining intensity for autopsy cases, they were in addition stained with
Cresyl violet to identify neurons. To this end, sections were deparaffinized and rehydrated. Cresyl violet solution (.25% solution + 10 drops of 10% acetic acid) was applied for 4 min at 56°C and rinsed in deionized water containing some drops of glacial acetic acid. Sections were again dehydrated by consecutive ethanol steps before incubation in isopropyl alcohol for 1 min. Finally, sections were rinsed in xylene and mounted.

2.6 Cell quantification

T cell infiltration (for all T cell subsets with anti-CD3 and for cytotoxic T cells with anti-CD8 antibodies) was assessed in a semiautomated manner. To this end, all stained slides were scanned with a slide scanner at ×200 magnification (NanoZoomer Digital Pathology, Hamamatsu Photonics), and the area of interest (hippocampus) was selected. Within this area, 7–10 images (×100 magnification) were exported, depending on the size of the hippocampus (≥80% coverage of whole hippocampal area). Next, the T cell number was semiautomatically counted by manually setting a threshold for staining intensity and cell size with Image-Pro Premier 9.2 (Media Cybernetics). Neuronal loss was determined based on NeuN or Cresyl violet stainings in a semiquantitative manner by two independent, experienced histopathologists (J.B. and A.R.T.) and classified according to the ILAE.26

2.7 Analysis of seizure frequency

Total seizure frequency (see also Table S1) for the months prior to surgery was obtained from the discharge letters of the presurgical evaluation. For the seizure frequency, a pseudocategorical value was calculated, referred to as “seizure frequency coefficient” throughout the article. To this end, the mean value of seizures per month (focal and generalized combined) was evaluated and the square root calculated for easier graphical representation. For patients with continuous seizures, an arbitrary value was chosen (100,000), which was far higher than all other values.

2.8 Statistics

As all data in this study are nonparametric, statistical tests were performed accordingly. For the analyses of T cell infiltration and neuronal loss among various MTLE etiologies, Kruskal–Wallis tests with multiple comparisons were performed, and every group was compared to HCs. Dunn post hoc correction was applied after multiple testing. To evaluate significant enrichment of the cytotoxic T cell population among the total T cell population (percentage of CD8+ among CD3+), a one-sample t-test was performed and tested against a hypothetical value of 33%. For the correlation between the number of T cells and disease duration, seizure frequency coefficient, and neurodegeneration, a Spearman correlation analysis was performed, where all samples from all MTLE etiologies and the inflammation controls were pooled. Hierarchical clustering was performed with MEV software (MeV4.8.1, TM4). Probability values less than .05 were considered to be significant. All statistical analyses were performed with GraphPad Prism 6.

3 RESULTS

3.1 T cell infiltration in MTLE varies depending on etiology

CD3+ T cell (all T cells) and cytotoxic T cell (CD8+) infiltration in the hippocampus was determined in the different MTLE subgroups and compared to autopsy controls, NDs, and inflammatory (RE) controls (Figure 1). In autopsy controls (Figure 1A), almost no T cells were found. Some of the NDs showed a small increase in the number of T cells. Most of these cells were found in the perivascular space, with some sparse parenchymal T cell infiltrates (Figure 1B). FS subjects (Figure 1C) also showed inflammatory T cells with occasional perivascular cuffs and sparse parenchymal infiltration throughout the hippocampus. In RE, we found very high T cell numbers in the parenchyma; T cells were often attached to or in close vicinity to neurons, as previously described (Figure 1D).27–29 In eGG − HS (Figure 1E) and eGG + HS (Figure 1F), the pattern of T cell infiltration looked very similar to that in FS, with diffuse infiltration of T cells in the parenchyma and an occasional perivascular cuff. In iGG + HS (Figure 1G), there were cases with both moderate and high numbers of parenchymal T cells, occasionally forming small clusters. Also, in PEE, the number as well as the T cell infiltration pattern differed widely throughout the cohort. In the samples with low T cell numbers, the infiltration pattern looked similar to what was observed in eGG − HS and FS, with some perivascular cuffs, and sparsely distributed T cells in the parenchyma but without appositions to neurons (Figure 1H). On the other hand, in the PEE case with by far the highest number of T cells (post-herpes simplex encephalitis case), the whole tissue section was filled with T cells, and the tissue morphology was completely destroyed (not shown). In the case with the second highest number, T cells infiltrated in high numbers and formed T cell clusters, similar to what can be observed in RE (Figure 1I).

We also quantified the numbers of T cells between nonseizure controls (autopsy and neurodegenerative controls) as well as between the different MTLE subgroups. CD3+ and CD8+ T cell numbers were significantly elevated in MTLE compared to...
controls (Figure 2A,C). Furthermore, we quantified the T cell numbers in every MTLE subgroup and could show that in FS, eGG + HS, iGG + HS, and PEE, T cells were significantly elevated compared to HCs. Surprisingly, the numbers of T cells in NDs were comparable to the numbers in eGG − HS. Although seemingly slightly enhanced, these T cell numbers in NDs and eGG − HS were not significantly different from numbers in HCs (Figure 2B,D). When comparing the three different GG groups among each other, no significant difference was found for the number of CD3+ T cells. The number of CD8+ T cells, however, was significantly increased in iGG + HS compared to eGG − HS (Kruskal–Wallis test with Dunn post hoc correction for multiple testing: \( p = .008 \)). A similar pattern was observed for all T cells (CD3+, Figure 2B) as well as for the CTL (CD8+, Figure 2D) subgroup (also see Table S3).

The percentage of CTLs as part of the total T cell population varied among the MTLE etiological groups. Overall, the tendency of CD8+ T cells, rather than CD4+ T cells, to infiltrate the CNS parenchyma can also be observed here, with a significantly increased percentage of CD8+/CD3+ cells compared to the periphery (about one third of CD3+ cells are CD8+ cells in the blood\(^{30,31} \)). This was also found in the autopsy and ND groups. Interestingly, FS subjects, with a nearly 82% percentage of CD8+/CD3+ T cells, had nearly similar levels to RE subjects, although absolute numbers of infiltrating T cells were drastically higher in RE. On the other hand, in PEE, the CD8+ T cell population made up only around 45% of the T cells, which is marginally higher than in the blood. In eGG + HS, the CD8+ population was nearly doubled compared to the peripheral blood (62%). In iGG + HS and eGG − HS (with 48.36% and 38.64%, respectively), no significant enrichment of CTLs among the overall CD3+ T cell population was found (Table 1).
3.2 | T cells do not correlate with seizure frequency or disease duration

To identify parameters correlating with hippocampal T cell numbers, we performed a hierarchical clustering analysis of CD3⁺ and CD8⁺ cell numbers, seizure frequency coefficient, neurodegeneration, and disease duration (Figure 3A). As expected, numbers of CD3⁺ and CD8⁺ cells correlated strongly ($R = .89$). On the other hand, neither seizure frequency nor disease duration clustered closely with inflammatory cell numbers (Figure 3A). This was further confirmed by analyzing the correlation between CD3⁺ and CD8⁺ T cell numbers with the disease duration (Figure 3B,C). Although some MTLE subgroups showed very high numbers at the beginning of the disease (most prominently PEE), no overall correlation between T cell numbers and disease duration was found (Figure 3B: $R = -.04, p = .77$; Figure 3C: $R = -.06, p = .66$). Similarly, we analyzed the correlation between T cell numbers and the seizure frequency coefficient. Neither for CD3⁺ (Figure 3D, $R = .17, p = .22$) nor for CD8⁺ T cells (Figure 3E, $R = .12, p = .4$) could a correlation be found.
3.3 | T cell numbers are highly associated with neurodegeneration

As shown in the hierarchical clustering, the factor that correlates most strongly with T cell influx is neuronal loss (Figure 3A). We therefore further analyzed how T cell influx and neurodegeneration relate in different MTLE etiologies. As per its definition, the eGG − HS group had no neuronal loss, whereas all other MTLE subgroups showed moderate to severe neuronal loss. There was a large variability in the amount of neuronal loss in hippocampal subfields within a specific group as well as between groups. Neuronal loss ranged from mild (below 20%, in two RE, three eGG + HS, and one PEE case), to very severe (more than 50%) neuronal loss (in three RE, five PEE, five FS, one eGG + HS, and two iGG + HS cases; Figure 4A, Table S3). Furthermore, also the degree of neuronal loss between hippocampal subfields was highly variable for all MTLE etiologies (Figure 4B, Table S3). NDs, RE, and PEE showed no clear trend.

### Table 1

| Etiology          | Average CD8 (CD3) | %     | p      |
|-------------------|-------------------|-------|--------|
| Autopsy           | .77 (1.57)        | 55.40 | .01    |
| ND                | 2.43 (3.35)       | 65.73 | .03    |
| eGG − HS          | 1.84 (5.16)       | 38.64 | ns     |
| eGG + HS          | 8.22 (12.76)      | 62.00 | >.001  |
| iGG + HS          | 9.07 (18.47)      | 48.36 | ns     |
| FS                | 5.73 (6.75)       | 81.97 | >.0001 |
| RE                | 32.17 (52.12)     | 64.78 | >.0001 |
| PEE               | 277.11 (544.28)   | 45.61 | .03    |

Abbreviations: eGG − HS, extrahippocampal ganglioglioma without hippocampal sclerosis; eGG + HS, extrahippocampal ganglioglioma with hippocampal sclerosis; FS, febrile seizures; iGG + HS, intrahippocampal ganglioglioma with hippocampal sclerosis; ND, neurodegenerative controls; ns, not significant; PEE, postinfectious encephalitic epilepsy; RE, Rasmussen encephalitis.

**Figure 3** T cell infiltrates do not depend on seizure frequency or disease duration. (A) Hierarchical cluster analysis of various factors and their correlation with CD3⁺ and CD8⁺ T cell numbers. For better graphical representation, all values were log10 transformed. Spearman correlation coefficient is indicated on the right of the dendrogram. (B) Numbers of CD3⁺ T cells do not correlate with the disease duration. (C) Numbers of CD8⁺ T do not correlate with the disease duration. (D) CD3⁺ T cell numbers do not correlate with seizure frequency coefficient. (E) CD8⁺ T cell numbers do not correlate with seizure frequency coefficient. Spearman correlations were performed. DD, disease duration; eGG − HS, extrahippocampal ganglioglioma without hippocampal sclerosis; eGG + HS, extrahippocampal ganglioglioma with hippocampal sclerosis; FS, febrile seizures; iGG + HS, intrahippocampal ganglioglioma with hippocampal sclerosis; ND, neurodegeneration; PEE, postinfectious encephalitic epilepsy; RE, Rasmussen encephalitis; SF, seizure frequency.
toward one specifically affected subfield. In most of the FS and eGG + HS cases, the CA1 region was highly affected in contrast to other subfields. In the iGG + HS group, no clear pattern could be observed, as the number of cases was low and the CA2 and CA3 subfields in two cases could not be clearly discriminated in the tissue resections available. This large variability was also reflected by the finding that no clear HS type, as defined by the ILAE, could be identified for any of the groups (Table 2). Because the number of T cells as well as the neuronal loss was highly variable within and between the various groups, we further analyzed the correlation between the number of T cells infiltrating the hippocampus and the percentage of neurons lost. This analysis revealed a strong correlation between the two variables, independent of the HS subtype (Figure 4C). Finally, we also tested the correlation between the age of the patients and the overall loss but did not find a significant correlation ($R = -.2, p = .09$, Figure 4D).

4 | DISCUSSION

Drug-resistant MTLE is a histopathologically diverse disease spectrum, ranging from exclusively hippocampal gliosis to severe neuronal loss and innate immune activation. In addition to this, MTLE develops on a background of a variety of etiologies, such as tumors, FS, or encephalitis. Moreover, several studies show an enhanced number of T cells in the brains of patients with MTLE. First, a previous study that focused on mitochondrial DNA deletions in MTLE patients...
showed that T cell numbers were higher in patients with hippocampal lesions compared to patients without lesions.\textsuperscript{14} Second, also in studies that compared MTLE with different ILAE HS classifications, the amount of T cells was found to be increased.\textsuperscript{15,16} These studies as well as animal models of epilepsy\textsuperscript{17,18} show that T cells are increased. However, in human MTLE and in our study, the role of these T cells is unclear in several ways. Although in the abovementioned animal studies it was shown that counteracting the infiltration of T cells in the CNS reduced seizure frequency, in human cases it is unknown whether the infiltrating T cells are a cause or result of the seizures. Furthermore, whether the infiltration of these T cells is caused by seizures or by the neuronal damage often accompanying seizures remains to be elucidated. Here, we thus studied whether the infiltrating T cells correlate to the seizures or to the amount of neuronal degeneration and whether there were differences between MTLE patients with different etiological backgrounds.

All resected hippocampi of MTLE patients were surgically removed due to seizure activity. If T cell infiltration would be correlated to the seizure activity, one would expect more infiltration in patients with higher numbers of seizures. However, in our study, no correlation between seizure frequency and T cell numbers could be found, which is in contrast to previous results from animal models.\textsuperscript{18,19} In addition, no positive or negative correlation between disease duration and T cell numbers was found. Here, our study revealed continuously increased levels of T cells, even years after the inciting event.

In contrast to the seizure frequency and disease duration studies, our analysis of neurodegeneration in MTLE showed a strong correlation between the percentage of neurons lost and the number of T cells in the brain. Neurodegeneration has repeatedly been linked to parenchymal T cell influx, as previously shown in Alzheimer disease and other neurodegenerative diseases.\textsuperscript{20–22} Also, in MTLE a correlative link between neuronal loss and T cell numbers has been described. T cell numbers in that study varied between different HS ILAE classification subtypes.\textsuperscript{15} Here, we also analyzed ILAE HS classification subtypes and found that approximately 50% were ILAE Type 1 and 50% ILAE Type 2, whereas ILAE Type 3 was absent. These classification findings fit with the descriptions of previous studies.\textsuperscript{1,15} However, we did not find any of the ILAE HS types to be specifically prominent in any of the MTLE subgroups. In addition, we did not find a direct link of ILAE HS subtype to T cell numbers.

Our results show increased but highly variable T cell infiltration in MTLE etiologies with HS, as reported previously.\textsuperscript{14} In our study, we specifically compared MTLE cases with different etiological backgrounds. As shown, the number of CD3$^+$ T cells as well as CD8$^+$ cytotoxic T cells was significantly increased in hippocampi of FS, eGG + HS, iGG + HS, RE, and PEE. In addition, as previously reported for immune-mediated disorders, all groups, except for eGG – HS and iGG + HS, showed a parenchymal enrichment of the CD8$^+$ T cell population compared to the blood of normal controls.\textsuperscript{25,30–32} iGG + HS showed an average CD8$/CD3^+$ T cell ratio of nearly 50%, and hence an increase compared to the peripheral ratio in HCs, where about one third of T cells are CD8$^+$ T cells. However, possibly due to the low sample number, no significant enrichment of the CD8$^+$ CTL population in the CNS was observed. As shown earlier, T cells formed the repeatedly described infiltrates with neuronal appositions in RE.\textsuperscript{27–29} This was also observed in the early cases of PEE and to a lesser degree in iGG + HS, indicating a directed T cell response against a specific target.\textsuperscript{27,33} In the other groups, T cell infiltrates were sparsely distributed throughout the parenchyma without appositions. Most important, in this study we used three different GG groups, one group with GG outside the hippocampus and without HS (eGG – HS), a second extrahippocampal group with HS (eGG + HS), and a GG group with tumor cells within the hippocampus and with HS (iGG + HS). As all included patients underwent surgery due to recurrent seizures, these three different GG groups allowed us to evaluate whether seizure activity or neurodegeneration were more prominently affecting the degree of T cell influx. Interestingly, comparison of these three groups showed that in eGG – HS, T cell numbers did not differ from HCs, whereas in eGG + HS as well as in iGG + HS, T cell numbers were increased. Separate statistical analysis of T cells of solely these three GG groups only showed a difference in CD8$^+$ T cell numbers between the eGG – HS and iGG + HS groups. This difference in CD8$^+$ T cells most likely results from the presence of tumor cells in the hippocampus, although an influence by neurodegeneration cannot be ruled out.

### TABLE 2

| Disease type                                      | ILAE Type 1 | ILAE Type 2 |
|--------------------------------------------------|-------------|-------------|
| Rasmussen encephalitis                           | 4 (57%)     | 3 (43%)     |
| Febrile seizures                                 | 4 (31%)     | 9 (69%)     |
| eGG – HS                                        | ND (no HS)  | 0 (0%)      |
| eGG + HS                                        | 3 (37.5%)   | 5 (62.5%)   |
| iGG + HS                                        | 1 (25%)     | 3 (75%)     |
| PEE                                             | 5 (71%)     | 2 (29%)     |

Note: ILAE types were characterized for patient samples from the various groups. ILAE Type 3 was not found in our samples and thus is not shown in the table.

Abbreviations: eGG – HS, extrahippocampal ganglioglioma without HS; eGG + HS, extrahippocampal ganglioglioma with HS; HS, hippocampal sclerosis; iGG + HS, intrahippocampal ganglioglioma with HS; ILAE, International League Against Epilepsy; ND, neurodegeneration controls; PEE, postinfectious encephalitis epilepsy.
these findings point at a correlation between T cell influx and neuronal loss rather than epileptic seizures.

Unfortunately, identifying whether a certain T cell influx is a cause or a result of the concomitant HS remains uncertain in human MTLE. This is a limitation of this study. As previously reported, different inflammatory profiles were found in various MTLE etiologies.12,13 This difference in cytokine and chemokine profiles could also be mediated by the varying degree of neuronal loss, as innate immune activation has repeatedly been described in neurodegeneration.34 In patients with severe neuronal loss, increased innate immune activation could trigger enhanced T cell recruitment to the CNS. Therefore, the diverse innate immune activation previously reported in different MTLE etiologies13 could lead to highly diverse T cell attraction into the CNS, resulting in the broad spectrum of T cell dynamics and T cell subset composition found in this study. On the other hand, it has been proposed that the T cells directly mediate the neuronal loss in MTLE,15 which could also affect the diverse inflammatory profile observed among MTLE etiologies.12,13 However, this mechanism is most likely only applicable in specific MTLE etiologies, such as in PEE. It is not unthinkable that in PEE, shortly after loss of the virus in infected cells, T cell appositions, possibly of antigen-specific T cells, still appear in the infected areas.35 In the other etiologies, we suggest a rather secondary T cell influx caused by ongoing innate immune activation, as no T cell appositions or granzyme B-positive T cells have been found in this and in previous studies.36

In summary, we here provide a detailed analysis of the T cell response over time in diverse MTLE etiologies, adding to previous studies on the involvement of the immune system in MTLE. Our findings suggest that T cell influx, rather than being correlated to epileptic seizures, is correlated to neurodegeneration. Whether T cell influx is a cause of or results from the neurodegeneration cannot be answered with certainty. It may be dependent on the etiological background of MTLE.

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CONFLICT OF INTEREST

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AUTHOR CONTRIBUTIONS

Study concept and design: Jan Bauer, Christian G. Bien; analysis and interpretation of data: Anna R. Tröscher, Eirini Sakaraki, Katharina M. Mair, and Jan Bauer; drafting of the manuscript: Anna R. Tröscher and Jan Bauer; immunohistochemistry and data collection: Eirini Sakaraki, Anna R. Tröscher, Katharina M. Mair, and Ulrike Köck; clinical data and sample collection: Christian G. Bien, Thomas Cloppenborg, Attila Racz, Valeri Borger, and Albert J. Becker; critical revision of the manuscript for important intellectual content: Jan Bauer and Christian G. Bien; statistical analysis: Anna R. Tröscher; obtained funding: Jan Bauer; study supervision: Jan Bauer.

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SUPPORTING INFORMATION
Additional supporting information may be found in the Supporting Information section.

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