Molecular alterations in epilepsy-associated malformations of cortical development
Boer, K.

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Inflammatory processes in cortical tubers and subependymal giant cell tumors of tuberous sclerosis complex

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ABSTRACT

Cortical tubers and subependymal giant cell tumors (SGCTs) are two major cerebral lesions associated with tuberous sclerosis complex (TSC). In the present study we investigated immunocytochemically the inflammatory cell components and the induction of two major pro-inflammatory pathways (the interleukin (IL)-1β and complement pathways) in tubers and SGCTs resected from TSC patients. All lesions were characterized by the prominent presence of microglial cells expressing class II-antigens (HLA-DR) and, to a lesser extent, the presence of CD68-positive macrophages. We also observed perivascular and parenchymal T-lymphocytes (CD3+ with a predominance of CD8+ T-cytotoxic/suppressor lymphoid cells. Activated microglia and reactive astrocytes expressed IL-1β and its signaling receptor IL-1RI, as well as components of the complement cascade, such as C1q, C3c and C3d. Albumin extravasation, with uptake in astrocytes, was observed in both tubers and SGCTs, suggesting that alterations in blood-brain barrier permeability are associated with inflammation in TSC-associated lesions. Our findings demonstrate a persistent and complex activation of inflammatory pathways in cortical tubers and SGCTs.

INTRODUCTION

Tuberous sclerosis complex (TSC) is an autosomal dominant, multisystem disorder resulting from a mutation in the TSC1 or TSC2 gene [21, 22]. Hamartin and tuberin, the TSC1 and TSC2 gene products, form a protein complex that inhibits signal transduction through the mammalian target of rapamycin (mTOR). mTOR is an important protein kinase involved in cell growth, proliferation, motility and survival. Although TSC affects different organ systems, disability in TSC patients results most often from cerebral involvement, giving rise to neurological and psychiatric manifestations including epilepsy, mental retardation and autism [301, 302]. The characteristic brain lesions of TSC are cortical tubers, subependymal nodules, and subependymal giant cell tumors (SGCTs) [23, 24]. Cortical tubers, which often represent the cause of epilepsy in TSC patients, are neuropathologically characterized by disordered cortical lamination, astrogliosis, dysplastic neurons and the presence of cytomegalic cells (giant cells; for review see [53, 303]). Abnormal giant cells with an immature, neuroglial phenotype are also a feature of SGCTs, which are low-grade, slow-growing tumors that arise from the periventricular region and can cause obstructive hydrocephalus [304]. Previous studies have focused on the roles and neurochemical features of dysplastic neurons and giant cells within TSC cerebral lesions [114, 305, 306]. Recently, activation of cells of the microglia/macrophage lineage and induction of inflammatory pathways have been described in tissue from epileptic patients, including individuals with malformations of cortical development [82, 108, 109, 189, 190]. In addition, activation of inflammatory pathways in cortical tubers has been suggested by the presence of macrophages and alterations in the expression of tumor necrosis factor-α, nuclear factor kappa B and cell adhesion molecules in these lesions [107]. In the present study, we investigated the inflammatory cell components of human cortical tubers and SGCTs in detail, with markers of both innate and adaptive immunity. We also analyzed the intralesional expression and cellular distribution of components of IL-1β signaling and the complement cascade, as well as the permeability of the blood-brain barrier (BBB).
MATERIALS AND METHODS

Subjects
The patients included in this study were obtained from the databases of the Departments of Neuropathology of the Academic Medical Center (University of Amsterdam) in Amsterdam, the University Medical Center in Utrecht (UMCU) and the Free University Medical Center (VUMC) in Amsterdam. We examined a total of 15 specimens, 9 cortical tubers and 6 SGCTs, resected from patients undergoing epilepsy surgery or surgery for obstructive hydrocephalus. One tuber specimen was obtained post-mortem (age 32 years; male). Informed consent was obtained for the use of brain tissue and for access to medical records for research purposes. Tissue was obtained and used in a manner compliant with the Declaration of Helsinki. For the SGCTs we used the revised WHO classification of tumors of the nervous system [131]. All patients fulfilled the diagnostic criteria for TSC [307]. Information concerning which of the two genes is mutated is reported in Table 1. In 4 patients, a significant amount of perituberal tissue (normal-appearing cortex/white matter adjacent to the lesion) was resected as well. In addition, normal-appearing control cortex/white matter was obtained at autopsy from one TSC patient. This material represents good control tissue, since it is exposed to the same seizure activity, drugs and fixation protocol, and age and gender are the same.

Normal-appearing control cortex/white matter from the temporal region was obtained at autopsy from 5 young adult control patients (male/female: 3/2; mean age 29; range 14-35), without a history of seizures or other neurological diseases. All autopsies were performed within 12 h after death. We also included control material from patients with age < 10 years (1, 3 and 8 years). The clinical characteristics derived from the patient’s medical records are summarized in Table 1. Seizures (present in 14 out 15 patients) were resistant to maximal tolerated doses of antiepileptic drugs (AEDs; carbamazepine, valproic acid, phenytoin, levetiracetam, oxcarbazepine, clobazam, vigabatrin, lamotrigine, gabapentin and clonazepam). Fourteen patients underwent presurgical evaluation [144]. We classified the postoperative seizure outcome according to Engel [145]. Class I consists of patients who remain completely seizure-free and class II includes patients who are almost seizure free or have rare or nocturnal seizures only. Class IV includes patients who have no worthwhile improvement postoperatively. Follow-up period ranged from 1 to 6 years.

Table 1 Summary of clinical characteristics of TSC patients

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>Tuber (n=9)</th>
<th>SGCTs (n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male/female</td>
<td>4/5</td>
<td>4/2</td>
</tr>
<tr>
<td>Mean age at surgery (yrs; n=8)</td>
<td>13.8 (1-35)</td>
<td>13.2 (1-23)</td>
</tr>
<tr>
<td>Age at autopsy (n=1)</td>
<td>32</td>
<td></td>
</tr>
<tr>
<td>Location</td>
<td>Frontal: 5; Temporal: 2; Parietal: 2</td>
<td>LV</td>
</tr>
<tr>
<td>Seizure type</td>
<td>CPS (67%); GTCS (22%); TS/MS (11%)</td>
<td>CPS (33%); GTCS (67%)</td>
</tr>
<tr>
<td>Mean age at seizure onset (yrs)</td>
<td>4.3 (0.1-12)</td>
<td>6.0 (0.3-16)</td>
</tr>
<tr>
<td>Duration of epilepsy (yrs)</td>
<td>11.5 (0.9-34)</td>
<td>7.9 (0.7-20)</td>
</tr>
<tr>
<td>Seizure frequency (months)</td>
<td>&lt; 10 (33%); &gt;30 (67%)</td>
<td>&lt; 10 (50%); &gt;30 (50%)</td>
</tr>
<tr>
<td>Mutation TSC1/TSC2</td>
<td>4/5</td>
<td>2/4</td>
</tr>
<tr>
<td>Postoperative epilepsy (n=8):</td>
<td>1 (50%); II (38%); IV (12%)</td>
<td>I (67%); II (33%)</td>
</tr>
</tbody>
</table>

CPS, complex partial seizures; GTCS, generalized tonic-clonic seizures; TS, tonic seizures; MS, multiple seizures; LV, lateral ventricle.
Tissue preparation
Tissue was fixed in 10% buffered formalin and embedded in paraffin. To avoid differences in labeling related to technical variables such as tissue fixation, we used the same fixation protocol for both autopsy and surgical material; small samples of selected regions (temporal cortex/hippocampus) were collected at autopsy and immediately fixed in formalin for 24 hours (same fixation time used for the surgical specimens). Paraffin-embedded tissue was sectioned at 6 µm, mounted on organosilane-coated slides (SIGMA, St. Louis, MO) and used for histological and immunocytochemical reactions as described below.

Antibody characterization
Antibodies specific for glial fibrillary acidic protein (GFAP; polyclonal rabbit, DAKO, Glostrup, Denmark; 1:4000), vimentin (mouse clone V9; DAKO; 1:1000), neuronal nuclear protein (NeuN; mouse clone MAB377; Chemicon, Temecula, CA, USA; 1:2000), synaptophysin (mouse clone Sy38; DAKO; 1:200), neurofilament (SMI311; Sternberger Monoclonals, Lutherville, MD; 1:1000), phospho-S6 ribosomal protein (Ser235/236, pS6; rabbit polyclonal, Cell Signaling Technology, Beverly, MA, USA; 1:50) and cleaved caspase-3 (rabbit polyclonal, Cell Signaling Technology; 1:100) were used in the routine immunocytochemical analysis of TSC specimens to document the presence of a heterogeneous population of cells, the activation of the mTOR pathway [63] and the activation of the cell death cascade. For the detection of the inflammatory cells we used the following antibodies (Abs): anti-human leukocyte antigen (HLA)-DP, -DQ, -DR (mouse clone CR3/43; DAKO; 1:400), anti-HLA-DR (mouse clone Ta1b5; SIGMA; 1:100), anti-CD68 (mouse monoclonal, clone PG-M1; DAKO; 1:200; monocytes, macrophages, microglia), anti-CD3 (mouse monoclonal, clone F7.2.38; DAKO; 1:200; T-lymphocytes), anti-CD4 (mouse monoclonal, clone 4B12; Neomarkers; 1:100; helper/inducer T-lymphocyte subset), anti-CD8 (mouse monoclonal, clone C8/144B; DAKO; 1:100; cytotoxic/suppressor T-lymphocyte subset), anti-CD20 (mouse monoclonal, clone L26; DAKO; 1:400; B-lymphocytes), anti-CD15 (mouse monoclonal, clone MMA; Neomarkers; 1:100; granulocytes). For the detection of the IL-1β signaling components, the following Abs were used: anti-human IL-1β (goat polyclonal, sc-1250, Santa Cruz Bio., CA, USA; 1:70), anti-human IL-1RI (goat polyclonal, R&D Systems, Abingdon UK; 1:50; [190]). For the detection of the complement system components the following antibodies were used: anti-C1q, anti-C3c and anti-C3d (rabbit polyclonal, DAKO; C1q, 1:100; C3c, C3d, 1:200 [189]). To determine BBB permeability we used anti-albumin (rabbit polyclonal, DAKO; 1:20.000).

Immunocytochemistry
Immunocytochemistry was carried out as previously described [113, 256]. Single-label immunocytochemistry was performed with the avidin-biotin peroxidase method for the polyclonal goat antibodies and Powervision (Immunologic, Duiven, The Netherlands) for the monoclonal mouse and polyclonal rabbit antibodies. 3,3’-Diaminobenzidine was used as chromogen. Sections were counterstained with hematoxylin. Sections incubated without the primary Ab or with the primary Ab and an excess of the antigenic peptide were essentially blank. For double-labeling studies, sections were incubated with the primary antibodies (anti-HLA-DR, -C1q, -C3c, -C3d, -IL-1β, -IL-1RI or -albumin combined with anti-pS6, -NeuN, -GFAP, -vimentin, -caspase3 or -HLA-DP, -DQ, -DR) for 2 h at room temperature with Alexa Fluor® 568-conjugated anti-rabbit, anti-goat or anti-mouse IgG and Alexa Fluor® 488 anti-mouse or anti-rabbit (1:100, Molecular Probes, The Netherlands). Sections were
then analyzed by means of a laser scanning confocal microscope (Bio-Rad, Hercules, CA, USA; MRC1024) equipped with an argon-ion laser. Labeled tissue sections were examined with respect to the presence or absence of various histopathological parameters and specific immunoreactivity (IR) for the different markers. Neuronal cell bodies were differentiated from glia and glioneuronal giant cells on the basis of morphology. Only neurons in which the nucleolus could be clearly identified were included. Giant cells have eccentric nuclei and ballooned opalescent eosinophilic cytoplasm. Reactive astrocytes were detected according to their morphology and the upregulation of GFAP and vimentin. The frequency of HLA-DR-, CD68-, CD3-, CD8-, CD4-, CD15-, and CD20-positive cells was evaluated as (1) rare, (2) sparse, (3) high and (4) very high, to give information about the relative number of inflammatory cells within the dysplastic area (Table 2). As previously reported [189, 190], the intensity of immunoreactive staining was evaluated using a semi-quantitative three-point scale where immunoreactivity was defined as: -, absent; +, moderate; ++, strong staining (Table 3). Quantitative analysis was also performed. The numbers of HLA-DR-positive microglia/macrophages and CD3-positive T-lymphocytes were quantified as previously described [107, 108]. Statistical analysis was performed with SPSS for Windows. Data were analyzed with the Kruskal-Wallis test, followed by a Mann-Whitney test to assess the difference between groups. Correlations between immunostaining (number of HLA-DR-positive microglial cells) and different clinical variables (duration of epilepsy, seizure frequency, age at surgery, age at seizure onset, seizure outcome) were assessed with the Spearman’s rank correlation test. The value of p < 0.05 was defined statistically significant.

**RESULTS**

**Case material and histological features**

The clinical characteristics of the patients are summarized in Table 1. All the 9 tubers displayed similar histopathological features with astrogliosis, loss of lamination, giant cells with pale eosinophilic cytoplasm and dysplastic neurons (Fig. 1A-C). The SGCTs were composed of large, plump glial cells with a broad spectrum of histopathological features, including cells resembling gemistocytic astrocytes, multi-nucleated cells, elongated cells and in 4 out of 6 SGCTs, giant cells (Fig. 6A). Calcifications and signs of previous intratumoral hemorrhage were also observed. Cells showing strong staining for phosphorylated S6 (pS6) ribosomal protein and therefore activation of the mTOR signal transduction pathway were identified within all tubers, in both cortex and subcortical white matter. Dysplastic neurons, giant cells and some dysplastic astrocytes (expressing immature glial markers); but nor reactive astrocytes or normal appearing neurons showed expression of pS6. Similar pS6 immunoreactivity (IR) patterns were observed in the different tuber specimens included in this study. We did not observed correlations between the expression intensity or number of pS6 positive cells and mutations in either TSC1 or TSC2. Tumor cells within all the SGCT samples showed robust pS6 staining.
**Inflammation in tuberous sclerosis complex**

Cortical tubers showed prominent expression of the astroglial marker GFAP in cells with the morphology of reactive astrocytes, as well as in a subpopulation of giant cells (Fig. 1D-F). Cells of the microglia/macrophage cell system, showing strong IR for the major histocompatibility complex (MHC) class II-antigens (HLA-DR), were identified in all tubers examined (Table 2; Fig. 1G-K). In the majority of tubers we observed a diffuse distribution of HLA-DR IR throughout the dysplastic region, with a relatively high number of positive cells (Fig. 1G). Cells strongly positive for HLA-DR and with the morphology of activated microglial cells (showing short stout processes) were often clustered around dysplastic neurons (Fig. 1H), around blood vessels (Fig. 1I) and around giant cells with a balloon-like appearance (Fig. 1J-K).

**Table 2. Immunocytochemical profile of inflammatory cells in cortical tubers and SGCTs**

<table>
<thead>
<tr>
<th></th>
<th>Tuber (n=9)</th>
<th>SGCT (n=6)</th>
<th>Perituberal (n=4)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 1+ 2+ 3+ 4+</td>
<td>0 1+ 2+ 3+ 4+</td>
<td>0 1+ 2+ 3+ 4+</td>
</tr>
<tr>
<td>HLA-DR</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD68</td>
<td>- - 44 56  -</td>
<td>- - 100  -</td>
<td>50 50 -</td>
</tr>
<tr>
<td>CD3</td>
<td>33 56 11</td>
<td>- - 67 33</td>
<td>75 25 -</td>
</tr>
<tr>
<td>CD8</td>
<td>33 56 11</td>
<td>- - 67 33</td>
<td>75 25 -</td>
</tr>
<tr>
<td>CD14</td>
<td>89 11</td>
<td>83 17</td>
<td>-</td>
</tr>
<tr>
<td>CD15</td>
<td>89 11</td>
<td>67 33</td>
<td>-</td>
</tr>
<tr>
<td>CD20</td>
<td>- - -</td>
<td>- - -</td>
<td>-</td>
</tr>
</tbody>
</table>

Double-labeling experiments confirmed HLA-DR positive cells within the tubers around cells expressing pS6 (used as a marker of mTOR activation; Fig. 2). Some cells surrounded by microglia showed expression of the apoptotic marker caspase-3 (Fig. 2G-I). Caspase-3 expression was also observed in some microglia (colocalization with HLA-DR; Fig. 2G-I). CD68 IR was increased in tuber specimens compared to control cortex (Table 2; Fig. 3A). CD68-positive cells (with macrophage-like morphology) were less frequently encountered than HLA-DR-positive cells and were located around dysplastic cells and blood vessels (Fig. 3B-C). Whether the macrophages represent transformed microglia or infiltrated monocyte-derived cells cannot be established by immunocytochemistry [308]. Neither HLA-DR, nor CD68-positive reaction products were detected in astroglial cells, dysplastic neurons or giant cells (Fig. 1, 2 and 3). CD15-positive cells were observed in only 1 tuber (Table 2). As previously reported [107-109], we observed only scattered HLA-DR- and CD68-positive cells in control autopsy tissue (data not shown). Similarly, histologically normal perituberal cortex displayed only a few HLA-DR- and CD68-positive cells and the IR was often restricted to the perivascular space (Fig. 1J). The number of HLA-DR immunoreactive cells in cortical tubers and control specimens was quantified and the mean number of positive cells across three distinct 1 mm² areas was found to be significantly higher in tuber specimens than in control tissue (p < 0.05; Fig. 4A). Differences between control (autopsy) and perituberal specimens were not observed. The number of HLA-DR-positive cells in cortical tubers was positively correlated with the frequency of seizures prior to surgical resection (Spearman rank
correlation coefficient: \( r = 0.809, \ p < 0.01 \). No significant correlation was found between the number of HLA-DR-positive cells and other clinical variables such as age at surgery, age at seizure onset, duration of epilepsy, the AED regimens, seizure outcome after surgery and mutations in either \textit{TSC1} or \textit{TSC2}.

**Figure 1. Histopathological features of cortical tubers: distribution of cell-specific glial markers**

A-C: Hematoxylin/Eosin (HE) staining showing a low magnification view of the dysplastic cortex (A), dysmorphic neurons with Nissl-like cytoplasm (B), and giant cells with pale eosinophilic cytoplasm and balloon-like appearance (C). D-F: GFAP staining showing a low magnification view of the dysplastic cortex with strong GFAP IR (D), the presence of large reactive GFAP-positive astrocytes (arrows) among dysplastic neurons and around blood vessels (E), and GFAP-positive (arrow) and negative (arrow-head) giant cells within the white matter (F).

G-L: HLA-DR staining in cortical tuber (G-K) and perituberal cortex (L). G: low magnification view of the dysplastic cortex with strong HLA-DR IR. H-K: HLA-DR-positive cells (arrows) localized around dysplastic neurons (asterisk in H), blood vessels (I), giant cells (asterisk in J and K) within the white matter. L: low magnification view of the perituberal cortex with low HLA-DR IR; the IR was restricted to the perivascular space, showing few positive perivascular macrophages and microglial processes (arrows and arrowhead, insert in L). Sections (D-L) are counterstained with hematoxylin. Scale bar in A: A, D, G, L: 400 µm; B, C, H, J, K: 40 µm. E, F, I: 45 µm.

**Distribution of cellular components of adaptive immunity in cortical tubers**

Lymphocytes were detected in cortical tubers, with parenchymal and perivascular distribution involving both grey and white matter (Fig. 3D-F; Table 2). The lymphocyte population was represented by a T-lymphocyte infiltrate (as shown by immunocytochemical analysis performed with anti-CD3 antibody) and was predominantly composed of CD8-positive cells (Fig. 3D-F; Table 2). Infrequent CD4-positive cells were observed, B-lymphocytes were not
detected (CD20; Table 2). The number of CD3 immunoreactive cells in cortical tubers and control specimens was quantified and the mean number of positive cells across three distinct 1 mm² areas was found to be significantly higher in tuber specimens than in control tissue (p < 0.05; Fig. 4B). Differences between control (autopsy) and perituberal specimens were not observed. No significant correlation was found between the number of CD3-positive cells and different clinical variables.

Figure 2. Distribution of microglial cells and phospho-S6 (pS6) positive cells in human cortical tubers
Confocal images. A-C: low magnification view of the dysplastic cortex with prominent HLA-DR IR (A) and several pS6-positive cells (B; C, merged image). D-F: High magnification showing HLA-DR-positive microglial cells (D) surrounding cytomegalic pS6-positive cells (E; F, merged image). G-I: HLA-DR-positive microglial cells surrounding a caspase-3 (casp-3)-positive cell (arrow in I, merged image). Caspase-3 expression was occasionally also co-localized with HLA-DR in microglial cells (arrow-head in I). Scale bar in A: A-C: 133 µm; D-F: 45 µm; G-I: 80 µm.

Figure 3. Distribution of CD68-positive cells and T-lymphocytes in human cortical tubers
A-C: CD68 staining. A: low magnification view of the dysplastic cortex with strong CD68 immunoreactivity. B-C: CD68 immunoreactivity (arrows) surrounding a dysplastic neuron (B; asterisk) and around blood vessels (C). D-E: CD3 staining. D: low magnification view of the dysplastic cortex with focal CD3 immunoreactivity (arrow and insert). E: CD3-positive T-lymphocytes (arrows) around blood vessels and surrounding a giant cell (asterisk). F: CD8-positive T-lymphocytes (arrows) within the tuber tissue, surrounding dysplastic neurons (arrow-heads). Scale bar in A: A and D: 400 µm; B, C, E and F: 40 µm.
Figure 4. Evaluation of cells of the microglia/macrophage lineage and T-lymphocytes in TSC cerebral lesions
(A): graph showing HLA-DR cell counting in control cortex, cortical tubers and SGCTs. (B): graph showing CD3 cell counting in control cortex, cortical tubers and SGCTs (* p < 0.05).

Complement expression in cortical tubers: C1q, C3c, C3d immunoreactivity
In agreement with our previous report [189], both neurons and resting glial cells in control autopsy (as well as in surgical perituberal specimens) did not express detectable levels of any of the three complement components examined. In contrast, moderate to strong C1q, C3c and C3d IR was detected within all the tubers examined (Table 3). Expression of all three complement components was observed in astrocytes, cells of the microglia/macrophage lineage, neurons and giant cells (Table 3; Fig. 5A-E).

Table 3. Cell-type distribution of IL-1β, IL-1RI and complement components in cortical tubers and SGCTs
(% of cases with immunoreactive cells)

<table>
<thead>
<tr>
<th>Components</th>
<th>Tubers (n = 9)</th>
<th>SGCT (n = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Astrocytes</td>
<td>Microglia/macrophages</td>
</tr>
<tr>
<td>C1q</td>
<td>-  + ++</td>
<td>-  + ++</td>
</tr>
<tr>
<td>C3c</td>
<td>0  44  56</td>
<td>0  67  33</td>
</tr>
<tr>
<td>C3d</td>
<td>0  44  56</td>
<td>0  67  33</td>
</tr>
<tr>
<td>IL-1β</td>
<td>0  22  78</td>
<td>0  22  78</td>
</tr>
<tr>
<td>IL-1RI</td>
<td>0  11  89</td>
<td>0  11  89</td>
</tr>
</tbody>
</table>

Staining: -, absent; +, moderate; ++, strong staining

Components of IL-1β signaling in cortical tubers: IL-1β and IL-1RI immunoreactivity
In agreement with previous reports [190, 261, 262], both neurons and resting glial cells in control autopsy (as well as in surgical perituberal specimens) did not express detectable levels of IL-1β or its functional receptor IL-1RI. Moderate to strong IL-1β and IL-1RI immunoreactivity was detected in all tubers (Table 3). Expression of both IL-1β and its receptor was observed in astrocytes, cells of the microglia/macrophage lineage, neurons and giant cells, which showed particularly prominent staining (Table 3; Fig. 5F-K).

Distribution of cellular components of the innate and adaptive immune system in SGCT
Innate immunity
Cells of the microglia/macrophage cell system, showing strong IR for HLA-DR and CD68 were immunocytochemically identified in all SGCT specimens (Table 2). Cells strongly positive
for HLA-DR and with the morphology of activated microglial cells or macrophages were clustered around the tumor cells and around blood vessels (Fig. 6B-C). CD15-positive cells were observed in 2 cases, associated with focal intratumoral hemorrhage (Table 2). The number of HLA-DR immunoreactive cells in SGCTs and in control specimens was quantified. The mean number of positive cells across three distinct 1 mm² areas was significantly higher in SGCT specimens than in control tissue (p < 0.05; Fig. 4A). No significant correlation was found between the number of HLA-DR-positive cells and different clinical variables.

**Adaptive immunity**

Lymphocytes were detected within the SGCT specimens and were represented by a T-lymphocyte infiltrate (as shown by immunocytochemical analysis performed with the anti-CD3 antibody), predominantly composed of CD8-positive cells (Table 2; Fig. 6D-E). Infrequent CD4-positive cells were observed, but B-lymphocytes were not detected (CD20; Table 2). The number of CD3 immunoreactive cells in SGCTs and control specimens was quantified and the mean number of positive cells across three distinct 1 mm² areas was found to be significantly higher in SGCT specimens than in control tissue (p < 0.05; Fig. 4B). No significant correlation was found between the number of CD3-positive cells and clinical variables.

**Complement expression in SGCT**

Prominent expression of C1q, C3c and C3d was detected within all SGCTs (Table 3). Expression of all three complement components was observed in cells of the microglia/macrophage lineage and in tumor cells (Fig. 6F-H).

**Components of IL-1β signaling in SGCT**

Prominent IL-1β and IL-1RI IR was detected within the tumor in all the specimens examined (Table 3). Expression of both IL-1β and its receptor was observed in cells of the microglia/macrophage lineage and tumor cells (Table 3; Fig. 6l-J).

**Albumin immunoreactivity in cortical tubers and SGCT**

Alterations in BBB permeability were detected using albumin immunocytochemistry. As previously reported [217], albumin extravasation was not observed in control autopsy cortical specimens, as well as in control hippocampal surgical specimens (data not shown). In contrast, in cortical tubers and SGCT specimens, albumin IR was often observed next to blood vessels (Fig. 7). Glial cells located around these vessels (and tumor cells in SGCT) were also albumin positive (Fig. 7B). Double-labeling confirmed albumin localization in astrocytes (vimentin and GFAP-positive cells). Colocalization with neuronal markers was not observed (data not shown).

**DISCUSSION**

Recent observations indicate that activation of inflammatory processes is a common feature of various epileptic disorders with different etiologies, but without a primary inflammatory pathogenesis [82]. Inflammatory components, suggesting a prominent and persistent...
activation of the innate immune response, have been described in both experimental and human temporal lobe epilepsy associated with hippocampal sclerosis [93, 189, 218, 279, 309-311]. Activation of inflammatory pathways, such as the IL-1β system, has been shown to have important functional consequences on neuronal excitability and cell survival. IL-1β decreases the threshold for induction of febrile seizures in immature rodents [91, 92], prolongs seizure activity in experimental models [93, 95, 97] and contributes to excitotoxic neuronal cell death [83, 267]. In addition, pro-inflammatory pathways, involving cells of the microglia/macrophage lineage, and, in particular, the activation of IL-1β signaling, have been observed in malformations of cortical development such as focal cortical dysplasia (FCD) and glioneuronal tumors (GNT) that are associated with chronic intractable epilepsy [108, 109, 190].

Figure 5. Distribution of components of the complement cascade and IL-1β signaling in human cortical tubers
A-C: C1q immunoreactivity (IR). A: low magnification view of the dysplastic cortex with C1q IR. B and C: C1q IR in neurons (arrows in B), giant and glial cells (arrow and arrowheads in C) within the cortical tuber. D: C3c IR in neurons (arrow), giant cells (insert) and glial cells (arrowheads). E: C3d IR in giant cells (arrows) within the subcortical white matter. Insert a in E: merged image, showing expression of C3d in HLA-DR (HLA)-positive cells; insert b in E: merged image, showing expression of C3d in GFAP-positive cells. F-H: IL-1β IR. F: low magnification view of the dysplastic cortex with prominent IL-1β IR. Insert a in G: merged image, showing expression of IL-1β in GFAP-positive cells. Insert b in G: merged image, showing expression of IL-1β in HLA-DR (HLA)-positive cells. H: strong IL-1β IR in giant and glial cells (arrow and arrowhead) within the subcortical white matter. I-K: IL-1RI IR. I: low magnification view of the dysplastic cortex with IL-1RI IR. J: high magnification of cortex with prominent IL-1RI IR. Insert a in J: merged image, showing expression of IL-1RI IR in HLA-DR (HLA)-positive cells. Insert b in J: merged image, showing expression of IL-1RI IR in a NeuN-positive neuron. Insert c in J: merged image, showing expression of IL-1RI in a GFAP-positive cell. K: strong IL-1RI IR in a giant cell (arrow) within the subcortical white matter. Scale bar in K: A: 400 µm; B, E, G, J: 80 µm; C: 90 µm; F and I: 350 µm; D, H, K: 40 µm.
Inflammation in tuberous sclerosis complex

Figure 6. Distribution of components of inflammatory pathways in SGCT
A: Hematoxylin/Eosin (HE) staining of a SGCT. B: HLA-DR staining showing prominent IR within the tumor; insert in B shows HLA-DR-positive cells around a negative tumor cell. C: CD68 staining showing prominent IR within the tumor; insert in C shows CD68-positive cells around negative tumor cells. D and E: CD3- and CD8-positive T-lymphocytes within the tumor. F-H: strong C1q, C3c and C3d IR in SGCT. I and J: IL-1β and IL-1RI IR in tumor cells; insert in J shows a tumor cell with strong IL-1RI membrane staining. Scale bar in A: A - C: 160 µm; D-J: 80 µm.

Figure 7. Albumin immunoreactivity in cortical tubers and SGCT
A and B: Albumin IR around blood vessels and in perivascular glial cells within cortical tubers. Insert in A shows high magnification of a blood vessel surrounded by albumin IR. C: strong and diffuse albumin IR in SGCT. D: confocal images showing colocalization of albumin (Alb; red) with vimentin (Vim; green; arrows) and GFAP (insert in D) in perivascular and parenchymal astrocytes. Scale bar in A: A-D: 50 µm. Insert in D: 30 µm.
Innate immunity in cortical tubers and SGCT

Microglial cells are the key components of the innate immune response (for review see [80]). In the present study, we demonstrate that both cortical tubers and SGCT specimens show a large number of activated microglial cells, expressing MHC class II-antigens (HLA-DP, -DQ, -DR). The immunophenotype of microglial cells, with prominent HLA-DR-positive cells, including a subpopulation of CD68-positive macrophages, is in line with previous studies in FCD and low-grade glial tumors, including GNT [108, 109, 308, 312]. Similarly to FCD and GNT, the HLA-DR positive activated microglial cells in cortical tubers and SGCTs were distributed throughout the dysplastic and tumor area, and were not exclusively restricted to the perivascular compartments. In particular, as previously shown for CD68-immunoreactive macrophages in tubers [107], the HLA-DR positive microglial cells were localized around cells (giant cells, dysplastic neurons and tumor cells) displaying mTOR activation, as indicated by the high levels of pS6 expression. Interestingly, the mTOR pathway not only plays a role in regulating cell growth and size (reviewed in [313, 314]), but also regulates the induction of inflammatory mediators [315-317]. Thus, the cellular components of the TSC-associated lesions could also potentially contribute to the inflammatory response, as a result of TSC1 or TSC2 inactivation followed by deregulation of mTOR signaling. Accordingly, activated microglial cells and macrophages were predominantly observed within the dysplastic cortex and within the tumor. The possibility to examine the perituberal cortex confirmed the localization of the inflammatory process within the pathological brain regions. It is clear that various potential other mechanisms may underlie the presence of inflammatory cells in the TSC-associated lesions and further investigation is required to ascertain the involvement of the mTOR pathway in the regulation of the immune responses occurring in TSC lesions.

Since cortical tubers are highly epileptogenic, it is possible that chronic seizure activity triggers the microglial cell activation observed within the dysplastic cortex. Several studies in experimental models of seizures indicate the occurrence of an inflammatory response in brain areas involved in the epileptic activity (reviewed in [82]). In addition, the epileptogenicity of cortical tubers, as measured by seizure frequency, correlates with the prominent presence of microglial cells within the tuber, as previously shown for FCD and GNT [108, 109]. Comparison with nonepileptogenic tubers could not be performed. In vivo measurements of activated microglia, using brain imaging techniques, may represent an interesting approach to study the distribution of inflammatory processes and their clinical significance in a larger number of TSC patients [318]. Furthermore, since recently influence of AEDs (such as carbamazepine and valproic acid) on the production of cytokines has been reported [319], a careful evaluation, in a larger group of epileptic patients, of a possible correlation between deregulation of the inflammatory response and the type, the dose or the level of AEDs is required.

The expression of apoptotic markers within the cortical tubers (present results and [107]), together with the prominent activation of cells of the microglia/macrophage lineage suggests a role for these cells and their associated inflammatory pathways in tuber pathogenesis. Whether the presence of a prominent population of inflammatory cells may contribute to the pathological process in TSC, including the development of cerebral calcifications, deserves further investigation. In addition, immunological dysfunctions and microglial activation occurring early during development have been implicated in several developmental brain disorders, including the diseases process in autism which is common in TSC patients (for reviews see [320, 321]).
Adaptive immune system in cortical tubers and SGCT
The adaptive immune response represents a specialized component of the immune system (for review see [80]). In the present study, we demonstrate the existence, in both cortical tubers and SGCTs, of mechanisms of adaptive immunity, mediated by T-lymphocytes. Lymphocytes, stained with markers of T-cell differentiation were observed in the perivascular zone, but also in the adjacent parenchyma, with involvement of both grey and white matter in cortical tubers. The presence of T-lymphocytes has been previously reported in a large series of SGCTs [322]. We investigated the nature of the lymphoid population and showed that the large majority of lymphocytes displayed a T-cytotoxic/suppressor immunophenotype, as indicated by CD8 immunoreactivity. A predominant cellular response mediated by cytotoxic T cells has also been observed in Rasmussen encephalitis [323] and in low grade astrocytomas [312], including GNT (unpublished data). Interestingly, both these conditions are associated with intractable epilepsy. The factors that trigger these T-lymphocyte infiltrations are not known. However, alterations in BBB permeability, resulting from seizure activity and/or induction of inflammatory mediators by activated glial cells, may facilitate the entry of these components of the adaptive immune system.

Activation of the complement cascade in cortical tubers and SGCT
The present study demonstrates prominent activation of the complement cascade in cortical tubers and SGCTs. Complement components C1q, C3c and C3d were observed within both lesions, with expression in tumor cells in SGCTs and in both glial (astrocytes and microglia) and neuronal cells in tubers. Components of the complement cascade are known to critically regulate the inflammatory response in different pathological conditions [79, 324, 325]. The presence of biologically active fragments of C3 (C3c and C3d) within the TSC-associated lesions indicates that the activation of the complement cascade has reached a point which may support a sustained inflammatory process. Products of C3 have been shown to regulate cytokine synthesis by monocytes (for review see [79]), indicating the existence of a reinforcing feedback mechanism between the complement cascade and the proinflammatory cytokine system, which is critical for the propagation of the inflammatory response. Interestingly, a persistent activation of the complement cascade has been reported recently in both experimental and human temporal lobe epilepsy [189] and in GNT (unpublished results). In addition, sequential infusion of components of the complement cascade into the hippocampus of rats has been shown to induce both behavioral and electrographic seizures [325]. Activation of the complement system may represent a common mechanism for initiating a sustained inflammatory process in chronic epileptic pathologies and therefore the complement system may provide an interesting target to control inflammation and eventually modify seizure progression in TSC and other diseases. Thus, evaluation of the role of complement activation in epilepsy might be worthwhile and requires careful preclinical functional and pharmacological studies.

Activation of IL-1β signaling in cortical tubers and SGCT
The complexity of the inflammatory pathways activated in TSC-associated brain lesions is indicated by the observed induction of IL-1β signaling. We showed that IL-1β and its functional receptor, IL-1RI, are expressed in cortical tubers and SGCTs. IL-1β and IL-1RI were detected in tumor cells in SGCTs and in both glial (astrocytes and microglia) and neuronal
cells in tubers. Induction of IL-1β signaling, with a similar cellular distribution, has been reported recently in other malformations of cortical development [27, 190]. The presence of both IL-1β and IL-1R1 in glial and neuronal cells, suggests that IL-1β may exert both autocrine and paracrine-like actions. Although the components of IL-1β signaling are known to be rapidly up-regulated following seizures [82], seizures alone cannot account for these changes in glial and neuronal expression of IL-1β signaling components, since perilesional tissue, also exposed to seizure activity, was devoid of detectable immunoreactivity. Therefore, as previously discussed for the activation of microglia, the cellular components of the TSC-associated lesions and the associated epileptic activity together could play a role in establishing a chronic inflammatory state.

**Alterations in BBB permeability**

The prominent activation of inflammatory pathways, such as the complement cascade and IL-1β signaling, may have important consequences on the permeability function of the BBB [82, 326]. Alterations in BBB permeability have been demonstrated recently in both human and experimental temporal lobe epilepsy, with the accumulation of serum proteins within the parenchyma and a positive correlation between BBB permeability and the occurrence of spontaneous seizures in chronic epileptic rats [182, 217, 218]. Serum proteins, such as albumin, may critically contribute to neuronal hyperexcitability, playing a role in the pathogenesis of focal epilepsy [327]. We report focal changes in BBB permeability in both cortical tubers and SGCTs, as demonstrated by perivascular parenchymal leakage of serum albumin, with uptake into astrocytes. Interestingly, albumin uptake into astrocytes has been shown to affect K+-homeostasis, facilitating neuronal hyperexcitability and epileptiform activity [328].

**CONCLUSIONS**

Our results confirm and expand previous findings that indicate the occurrence of a complex and sustained inflammatory reaction in tubers and SGCTs of patients with TSC. The prominent inflammatory changes observed in these lesions include both the innate and the adaptive immune response and involve the activation of both the complement cascade and IL-1β signaling. Understanding the components and mediators of the inflammatory response in TSC-associated cerebral lesions may have great importance for the development of new therapeutic strategies.

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