Inflammation and epilepsy: the contribution of astrocytes
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2.2 Evaluation of the Innate and Adaptive Immunity in Type I and Type II Focal Cortical Dysplasias

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Summary
Purpose: Induction of inflammatory pathways has been reported in epileptic patients with focal malformations of cortical development. In the present study we examined the innate and adaptive immune responses in focal cortical dysplasia (FCD) with different histopathologic and pathogenetic features.

Methods: The inflammatory cell components and the induction of major proinflammatory pathways and molecules [complement pathway, interleukin (IL)-1β, and chemokine monocyte chemotactic protein-1 (MCP1)] was investigated in surgical specimens of sporadic type IA and type IIB FCD using immunocytochemical methods.

Results: FCD II but not FCD I cases exhibit activation of the mammalian target of rapamycin (mTOR) cascade with strong neuronal expression of the phosphorylated isoform of S6 protein. Microglia reactivity was increased in all lesions (FCD I and II) compared to control tissue; however, the number of HLA-DR–positive cells was significantly higher in FCD II than in FCD I. In FCD II specimens we also observed perivascular and parenchymal T lymphocytes (CD3+), with a predominance of CD8+ T-cytotoxic/suppressor lymphocytes, as well as a few dendritic cells. Expression of components of the complement cascade, IL-1β, and MCP1 was prominent in FCD II cases.

Discussion: Our findings indicate a prominent activation of both innate and adaptive immunity, with involvement of different inflammatory pathways in FCD II cases, supporting the possible involvement of inflammation in the epileptogenesis of these lesions, as well as the notion that FCD II is pathologically distinct from FCD I.
**INTRODUCTION**

Focal cortical dysplasias (FCD) represent sporadic architectural and cytoarchitectural malformations of the cerebral cortex, usually confined to a single hemispheric lobe, which are recognized causes of chronic medically intractable epilepsy in children and young adults (Thom, 2004, Fauser et al., 2006, Najm et al., 2007, Blümcke et al., 2009). According to the current histopathological classification system, FCD have been classified into Type I, characterized by cortical dyslamination, and Type II, characterized by additional cytoarchitectural abnormalities, i.e. the presence of dysmorphic neurons and balloon cells (Palmini et al., 2004). The molecular pathogenesis of FCD type I and type II remains to be clearly elucidated. However, recent molecular-genetic and histopathological studies indicate the involvement of the phosphatidyl-inositol 3-kinase (PI3K)-mTOR (mammalian target of rapamycin) pathway in FCD type II (Becker et al., 2006, Schick et al., 2007). In contrast, activation of the mTOR pathway has not been reported in FCD type I. The cellular mechanism(s) underlying the epileptogenicity of FCD has also not been fully elucidated. Previous studies support the role of developmental alterations of the balance between excitation and inhibition (Wong, 2008). In particular, impairment of the GABAergic system has been suggested as a possible mechanism for the epileptogenicity of both type I and II FCD (Spreafico et al., 2000, Garbelli et al., 2006). Recent evidence strongly suggest the involvement of inflammatory processes in the etiopathogenesis of seizures (Vezzani and Granata, 2005, Vezzani et al., 2008). Activation of cells of the microglia/macrophage lineage and induction of different inflammatory pathways have been described in epileptogenic tissue from temporal lobe epilepsy with hippocampal sclerosis (Ravizza et al., 2008b), as well as in malformations of cortical development (MCD), including FCD type II (Aronica et al., 2005, Boer et al., 2006, Ravizza et al., 2006, Aronica et al., 2007, Boer et al., 2008). Whether these inflammatory changes represent an intrinsic feature of FCD or they are triggered by chronic seizure activity is still unclear. In addition the involvement of the mTOR pathway in the regulation of the inflammatory response in malformations associated with activation of this pathway cannot be excluded (Weinstein et al., 2000, Potter et al., 2001, Lim et al., 2003).

The aim of the present study was to determine whether components of innate and adaptive immunity were present and similarly distributed in brain tissue from patients with FCD type I and FCD type II and chronic medically intractable epilepsy. In addition, the expression and cellular distribution of major pro-inflammatory pathways and molecules known to be up-regulated in MCD associated with activation of the mTOR pathway were also analyzed.
**Materials and Methods**

**Subjects**

The cases included in this study were obtained from the archives of the departments of neuropathology of the Academic Medical Center (University of Amsterdam), the University Medical Center in Utrecht, and the VU University Medical Center (VUMC) in Amsterdam. Twenty-seven brain tissue specimens, removed from patients undergoing surgery for intractable epilepsy, were examined. Informed consent was obtained for the use of brain tissue and for access to medical records for research purposes. All cases were reviewed independently by two neuropathologists, and the diagnosis was confirmed according to the system recently proposed by Palmini et al. for grading the degree of FCD (Palmini et al., 2004). Table S1 summarizes the clinical findings of epilepsy patients and controls. None of the patients with FCD fulfilled the diagnostic criteria for tuberous sclerosis complex (TSC). Table S2 summarizes the neuropathologic findings of FCD specimens and the standard stains used.

All patients underwent presurgical evaluation with phase I investigations consisting of non-invasive tests, history, medical, neurological and neuropsychological assessment, structural neuroimaging, and extensive interictal and ictal electroencephalography (EEG) studies with video monitoring. In phase II, an intracarotid sodium amyntal test (Wada test), interictal positron emission tomography (PET), (inter)ictal single photon emission computed tomography (SPECT), and functional magnetic resonance imaging (fMRI) were performed on indication (van Veelen et al., 1990). Patients who underwent implantation of strip and/or grid electrodes for chronic subdural invasive monitoring before resection were excluded from this study. Patients had complex partial seizures (CPS), and all patients had daily seizures, which were resistant to maximum doses of antiepileptic drugs. Seizure duration represents the interval in years from age at seizure onset and age at surgery. The postoperative seizure outcome was classified according to Engel (1993).

Normal-appearing control cortex was obtained at autopsy from six age-matched patients without history of seizures or other neurologic diseases. Autopsied brain tissues from patients with neuroinflammatory pathologies [viral encephalitis and multiple sclerosis (MS)] were also examined (as positive controls). All autopsies were performed within 12 h after death. Histologically normal temporal neocortex (without evidence of significant neuronal loss, gliosis or malformation; epilepsy controls) from four patients undergoing extensive surgical resection of the mesial structures for the treatment of medically intractable complex partial epilepsy was also used for immunocytochemical analysis. This material represents good control tissue, since it is exposed to similar seizure activity, duration of epilepsy, and fixation protocol, and is also useful for investigating whether seizure activity itself triggers the inflammatory response.
**Tissue preparation**

One representative paraffin block per case (containing the complete lesion or the largest part of the lesion resected at surgery) was sectioned, stained, and assessed. Formalin fixed, paraffin-embedded tissue was sectioned at 6 μm and mounted on precoated glass slides (Star Frost, Waldemar Knittl GmbH, Barunschweig, Germany). Sections of all specimens were processed for hematoxylin and eosin (H&E), Luxol fast blue (LFB), and Nissl stains as well as for immunocytochemical stainings for a number of neuronal and glial markers as described in subsequent text.

**Antibody characterization and immunocytochemistry**

Antibodies (Abs) specific for glial fibrillary acidic protein (GFAP; polyclonal rabbit, DAKO, Glostrup, Denmark; 1:4,000), vimentin (mouse clone V9; DAKO; 1:1,000), neuronal nuclear protein (NeuN; mouse clone MAB377; Chemi-Con, Temecula, CA, U.S.A.; 1:2,000), neurofilament (SMI311; Sternberger Monoclonals, Lutherville, MD, U.S.A.; 1:1,000), cleaved caspase-3 (rabbit polyclonal, Cell Signaling Technology, Beverly, MA, U.S.A.; 1:100), and phospho-S6 ribosomal protein (Ser235/236; pS6, rabbit polyclonal, Cell Signaling Technology; 1:50) were used in the routine immunocytochemical analysis of FCD cortical specimens to document the presence of a heterogeneous population of cells, apoptosis, and activation of the mTOR pathway (Baybis et al., 2004).

For the detection of the inflammatory cells and proinflammatory pathways the following Abs were used: anti-human leukocyte antigen (HLA)-DP, DQ, DR (HLA-DR; mouse clone CR3/43; DAKO, Glostrup, Denmark; 1:400), 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), DC-SIGN (CD209; monoclonal mouse, BD Pharmingen, San Diego, CA, U.S.A.), MCP1 (MCP1/CCL2; monoclonal mouse, R&D Systems, Minneapolis, MN, U.S.A.; 1:10), interleukin (IL)-1β [goat polyclonal, sc-1250, Santa Cruz Bio., CA, U.S.A.; 1:70, (Ravizza et al., 2006)], anti-C1q and anti-C3d [rabbit polyclonal, DAKO, Glostrup, Denmark; C1q, 1:100; C3c, C3d, 1:200, (Aronica et al., 2007)].

Immunocytochemistry was carried out as described previously (Aronica et al., 2003). Single-label immunocytochemistry was performed using the Powervision kit (Immunologic, Duiven, The Netherlands) and 3,3-diaminobenzidine as chromogen. Sections were counterstained with hematoxylin. Sections incubated without the primary Ab, with preimmune serum, or with the primary Ab (for IL-1β and MCP1) and an excess of the antigenic peptide
were essentially blank. A similar pattern of immunoreactivity was observed in surgical and autopsy control specimens included in this study.

For double-label immunocytochemistry with DC-SIGN (IgG2b) and CD3 (IgG1), we used secondary Ig subtype specific Abs and as chromogens 3-amino-9-ethyl carbazole (AEC, Sigma, St. Louis, MO, U.S.A.) and Fast Blue B salt (Sigma). For double-labeling with HLA-DR and pS6 (as well as for caspase-3 with GFAP, HLA-DR, or NeuN, not shown), we used, as secondary antibodies, Alexa Fluor 568-conjugated anti-rabbit IgG and Alexa Fluor 488 anti-mouse IgG (1:100; Molecular Probes, Leiden, The Netherlands). Sections were mounted with Vectashield containing DAPI (targeting DNA in the cell nucleus; blue emission) and analyzed by means of a laser scanning confocal microscope (Leica TCS SP2; Wetzlar, Germany).

RESULTS

Case material and histological features

The clinical features of the cases included in this study are summarized in Table 1. All patients had a history of chronic pharmaco-resistant epilepsy. Post-operatively, 13 patients (76%) in this cohort were completely seizure free. Age at surgery, seizure duration and seizure frequency were not statistically different between patients with FCD I and FCD II in this cohort. In this study we excluded patients with mild degree of cortical dysplasia (mild malformation of cortical development; (Palmini et al., 2004). The FCD cases included had all the previously described histopathological features of Type IA or Type II B FCD (Palmini et al., 2004). FCD IA is characterized by cortical dyslamination and often presence of ectopic white matter neurons; the Type II B is characterized by additional cytoarchitectural abnormalities, including the presence of dysmorphic neurons and balloon cells (Fig. 1). We only included

Table 1. Summary of clinical findings of epilepsy patients and controls

<table>
<thead>
<tr>
<th>Pathology type</th>
<th>Number of cases</th>
<th>Gender (M/F)</th>
<th>Mean age at surgery (range/years)</th>
<th>Localization</th>
<th>Mean duration of epilepsy (range/years)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FCD I</td>
<td>8</td>
<td>4/4</td>
<td>27.8 (19-39)</td>
<td>5 Temporal</td>
<td>7.7 (6-13)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>3 Frontal</td>
<td></td>
</tr>
<tr>
<td>FCD II</td>
<td>9</td>
<td>5/4</td>
<td>26.2 (18-36)</td>
<td>Temporal</td>
<td>8 (6-14)</td>
</tr>
<tr>
<td>Epilepsy control (no MCD)</td>
<td>4</td>
<td>2/2</td>
<td>29.5 (25-31)</td>
<td>Temporal</td>
<td>8.1 (7-13)</td>
</tr>
<tr>
<td>Controls (no epilepsy)</td>
<td>6</td>
<td>3/4</td>
<td>30.1 (20-41)</td>
<td>Temporal</td>
<td>-</td>
</tr>
</tbody>
</table>

FCD = Focal Cortical Dysplasia.
cases of FCD which were isolated and not associated with a primary lesion (i.e. tumor or hippocampal sclerosis). As described previously (Baybis et al., 2004, Miyata et al., 2004, Ljungberg et al., 2006, Schick et al., 2007a, Schick et al., 2007b), both dysplastic neurons and balloon cells in FCD type II displayed enhanced S6 protein phosphorylation (Fig. 1 D). In contrast, phospho-S6 protein expression was not detected in FCD type I cases, similarly to epilepsy (no MCD) or normal control specimens (Fig. 1 B).

Figure 1. Histopathological features of focal cortical dysplasia type I (FCD I) and FCD II: distribution of cells of the microglia/macrophage lineage. (A, C) NeuN staining showing the disorganization of the neuronal component within the dysplastic cortex in FCD I (A) and FCD II (C). (B, D) Phospho-S6 (pS6) staining showing absence of immunoreactivity (IR) in FCD I (B), but several pS6-positive cells in FCD II (D), including dysplastic neurons and balloon cells (insert in D). (E–H) HLA-DR staining. (E, F) FCD I showing moderate presence of HLA-DR–positive cells in both cortex (CTX; E) and white matter (Wm; F) regions; insert in E shows positive perivascular cells. (G, H) FCD II showing strong presence of HLA-DR–positive cells in both cortex (G) and white matter (H); inserts in G and H show high magnification photographs of HLA-DR–positive cells surrounding dysplastic neurons (insert a in G) and balloons cells (insert a in H). Insert b in H: merged image showing HLA-DR–positive microglial cells (green) surrounding a pS6-positive balloon cell (red). Scale bar in F: A–C, E, G–H: 200 μm; B–D: 120 μm; F: 80 μm.
Microglial reactivity in FCD type I and type II
As previously reported (Boer et al., 2006) areas of reactive microglia were often encountered in specimens of patients with FCD type II, throughout the dysplastic region (Fig. 1G-H). Cells strongly positive for HLA-DR and with the morphology of activated microglial cells (showing bushy morphology with dense and short stout processes) were often clustered around dysplastic neurons (Fig. 1G) and around balloon cells (Fig. 1H). In contrast, little microglial activation was observed in specimens of patients with FCD type I. HLA-DR IR was often restricted to the perivascular space, sparse reactive microglial cells were mainly observed within the subcortical white matter (Fig. 1E-F).

The number of HLA-DR immunoreactive cells was quantified in four categories of neocortex examined in this study, including FCD type I, FCD type II and control specimens (histologically normal temporal neocortex of patients with and without history of chronic epilepsy; Fig. 4A). Statistical analysis applied to the mean number of positive cells across three distinct 1 mm² areas indicated increased microglial reactivity in epilepsy specimens compared to control non-epileptic tissue. The number of HLA-DR positive cells was found to be significantly higher in FCD type II compared to FCD type I and epilepsy surgical specimens (Fig. 4A). In contrast, no significant differences were observed between FCD type I and epilepsy surgical specimens. In this cohort (including cases matched in terms of age, duration of epilepsy and preoperative seizure frequency) no significant correlation was found between the number of HLA-DR-positive cells clinical variables such as age at surgery, age at seizure onset, duration of epilepsy and seizure outcome after surgery.

T-lymphocyte Infiltration in FCD type I and type II
Lymphocytes were detected within the dysplastic regions, with more prominent presence in FCD type II specimens (Fig. 2; Fig. 4B). The lymphocyte population was represented by T lymphocytes (as shown by immunocytochemical analysis performed with anti-CD3 antibody; Fig. 2A-F) and was predominantly composed of CD8-positive cells (Fig. 2G-H). T lymphocytes were detected around blood vessels, but also in the tissue parenchyma in FCD type II specimens, and were often clustered around balloon cells (Fig. 2D-H). CD4-positive cells and B lymphocytes were not detected (data not shown). The number of CD3 immunoreactive cells in FCD 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 FCD specimens than in control non-epileptic tissue (Fig. 4B). The number of CD3 positive cells was found to be significantly higher in FCD type II compared to FCD type I and epilepsy surgical specimens (Fig. 4B). In contrast, no significant differences were observed between FCD type I and epilepsy surgical specimens. Differences between control non-epileptic (autopsy) and epilepsy
surgical specimens were not observed. No significant correlation was found between the number of CD3-positive cells and the amount of microglial reactivity in FCD type I and II specimens (analysis was performed in serial sections stained with CD3 and HLA-DR). No significant correlation was found between the number of CD3-positive cells and the different clinical variables in the different patient groups.

Figure 2. Distribution T lymphocytes in FCD I and FCD II. (A–F) CD3 staining. (A–C) Focal cortical dysplasia type I (FCD I) with few CD3 positive cells in cortex (CTX; arrow) and white matter (Wm; arrows). (D–F) FCD II with several CD3 positive cells in cortex (CTX; D–E, arrows) and white matter (Wm; F); arrows in E show lymphocytes surrounding a balloon cell (asterisk) and a dysmorphic neuron (arrowhead); insert in F shows perivascular CD3 positive infiltrates in Wm. (G, H) CD8-positive T lymphocytes in FCD II. (G) Perivascular CD8 positive cells. (H) CD8 T lymphocytes around balloon cells (asterisks). Scale bar in H: A, B: 40 μm; C: 50 μm. E D–F: 80 μm. G, H: 20 μm.
Figure 3. Distribution of dendritic cells in focal cortical dysplasia type I (FCD I) and FCD II. (A–C) DC-SIGN staining in control tissue. (A) Tonsil; (B) choroid, plexus; (C) cortex (CTX) with DC-SIGN–positive cells in meninges. (D–F) FCD I with DC-SIGN–positive cells in the leptomeninges, but absence of positive cells in cortex (E) and white matter (F). (G, H) FCD II showing perivascular DC-SIGN–positive cells and occasionally few cells with parenchymal location (insert in G). I and insert in H: CD83 staining showing perivascular positive cells. (J) Perivascular distribution of DC-SIGN (red) and CD3 (blue) positive cells in FCD II. Scale bar in J: A: 25 μm B: 30 μm; C: 40 μm; D–F: 80 μm; G–I: 20 μm I, J: 40 μm.
Dendritic cells in FCD type I and type II
Dendritic cells (DCs) were identified using DC-SIGN (CD209, a marker for both immature and mature DCs). DC-SIGN stains dendritiform cells in T-cell zones of lymph node (Fig. 3A), in the choroid plexus (Fig. 3B), and a few cells in the leptomeninges of control cortical specimens (Fig. 3C). No DC-SIGN IR was detected in the gray and white matter of the large majority of control and FCD I specimens (Fig. 3D–F). Differences between FCD I, control nonepileptic (autopsy), and epilepsy surgical specimens were not observed (Fig. 4C). However, in FCD II specimens, few DC-SIGN–positive cells, with an irregular and ramified morphology were detected around blood vessels (Figs. 3G–H and 4C). No DC-SIGN IR was detected on vascular endothelia. We did not observe parenchymal deposits of DC-SIGN IR. The intrameningeal DC-SIGN cell distribution (with scattered positive cells) did not differ between the different patient groups. The presence of mature DCs in the FCD specimens was also investigated using an anti-CD83 Ab (Serafini et al., 2006; Cudrici et al., 2007). Only in FCD II, a few CD83-positive cells were present around blood vessels. No CD83-positive cells were detected in the tissue parenchyma. DC-SIGN–positive cells were consistently and abundantly detected in the meninges, as well as around intraparenchymal blood vessels, and in tissue specimens from patients with MS or viral encephalitis. To determine a possible morphologic correlation between the presence of DCs and T-cell infiltrates, we used double staining for DC-SIGN and CD3. Indeed many cells situated close to DC-SIGN–positive cells were positive for CD3 (Fig. 3J).

Figure 4. Evaluation of cells of the microglia/macrophage lineage and T lymphocytes and dendritic cells in focal cortical dysplasia type I (FCD I) and FCD II. (A) Graph showing HLA-DR cell counting in control cortex, epilepsy control (no malformation of cortical development, MCD), FCD I, and FCD II. (B) Graph showing CD3 cell counting in control cortex, epilepsy control, FCD I, and FCD II. (C) Graph showing DC-SIGN cell counting in control cortex, epilepsy control, FCD I, and FCD II (*p < 0.05).
Figure 5. Distribution of interleukin (IL)-1β and monocyte chemotactic protein-1 (MCP1) in focal cortical dysplasia type I (FCD I) and FCD II. (A, B and E, F) FCD I. A and B (IL-1β staining) showing absence of glial or neuronal immunoreactivity (IR) in both cortex (CTX, A) and white matter (Wm, B). E and F (MCP1 staining) showing very light neuronal IR in cortex (E), without detectable glial IR in both cortex and white matter (F). (C, D and G, H) FCD II. C and D (IL-1β staining) and G, H (MCP1 staining) showing strong IR within cortex (C and G) and white matter (D and H). Arrows in C and G indicate positive dysplastic neurons; arrowheads in C and G indicate positive glial cells; arrows in D and H indicate positive balloon cells. Insert in D shows positive astrocytes. Scale bar in H: B, C, E, F, G, H: 80 μm; A and D: 150 μm.
Complement activation in FCD type I and type II
In agreement with our previous report (Aronica et al., 2007; Boer et al., 2008), both neurons and resting glial cells in control nonepileptic (autopsy) specimens did not express detectable levels of any of the two (C1q and C3d) complement components examined. Occasionally, C1q and C3d IR was detected in a few glial cells and blood vessels in epileptic surgical control and FCD I specimens, but not in neurons (Fig. S1A–D). In contrast, moderate to strong C1q and C3d IR was detected within all FCD II specimens examined (Figs. S1E–J and 2A,B). Expression of the two complement components was observed in glial cells, neurons, and in balloon cells (Fig. S1G,J).

IL-1β immunoreactivity in FCD type I and type II
In agreement with previous reports (Kadhim et al., 2003; Ravizza et al., 2006; Boer et al., 2008), both neurons and resting glial cells in control nonepileptic (autopsy) specimens did not express detectable levels of IL-1β (Fig. S2C). Occasionally, IL-1β IR was detected in a few glial cells in epileptic surgical control and FCD I specimens, but not in neurons (Figs. 5A,B and S2C). In contrast, moderate to strong IL-1β IR was detected in all the FCD II specimens (Figs. 5C,D and S2C). Expression of IL-1β was observed in glial cells, neurons, and giant cells (Fig. 5D).

MCP-1 immunoreactivity in FCD type I and type II
Light neuronal MCP1 IR was observed in both control and FCD I specimens; resting glial cells in controls and in the large majority of FCD I cases did not express detectable levels of MCP1 IR (Figs. 5E–F and S2D). In contrast, moderate to strong MCP1 IR was detected in all FCD II specimens (Figs. 5G,H and S2D). Expression of MCP1 was observed in glial cells, neurons, and giant cells (Fig. 5H).

DISCUSSION
We examined the features and the severity of the inflammatory response occurring in tissue from patients with FCD and chronic medically intractable epilepsy. Several observations indicate that activation of inflammatory processes is a common feature of various experimental models of seizures and in human epileptic disorders with different etiologies, but without a primary inflammatory pathogenesis (Vezzani & Granata, 2005; Ravizza et al., 2008). Activation of both innate and adaptive immune response has been recently reported in focal MCDs (Aronica et al., 2005, 2007; Boer et al., 2006, 2008; Ravizza et al., 2006).
In this study, we confirmed the occurrence of complex inflammatory changes, involving both the innate and the adaptive immune response in FCD specimens, and we demonstrate that the severity of these changes is greater in FCD II than in specimens from patients with FCD I (despite the absence of significant differences in seizure frequency and duration). These results indicate that activation of inflammatory processes is not simply an effect of seizure activity, although the presence of activated microglia in temporal cortex from patients with epilepsy (without malformations) does not exclude that this could be triggered by chronic seizure activity. However, the prominent activation of microglia observed within the FCD II specimens and the close association of activated immune cells with abnormal cell types, such as dysplastic neurons and balloon cells (displaying mTOR activation) suggests a diverse nature of activation in this type of pathology. In the present study we observed aberrant phosphorylation of S6 ribosomal protein (a known marker of mTOR activation) only in the FCD II specimens, suggesting a pathogenetic distinction between the two types. Interestingly, the mTOR pathway does not only play a role in regulating cell growth and size, but also in the innate and adaptive immune response (Lim et al., 2003; Schmitz et al., 2008; Weichhart & Saemann, 2009). One could speculate that mTOR activation within the cellular components of FCD II also contributes to the inflammatory response. Activated microglial cells are often observed around pS6 positive cells (dysplastic neurons and balloon cells; present results and Boer et al., 2008). However, further investigation is required to ascertain this possibility. Interestingly, the mTOR inhibitor rapamycin is an effective immunosuppressive agent (Thomson et al., 2009) and has been recently shown to suppress seizures or even prevent the development of epilepsy in a mouse model of TSC (Zeng et al., 2008), and reduction in seizure frequency has been reported in a young patient with TSC (Muncy & Butler, 2009). Because our study includes only FCD IIB specimens, the issue of whether the induction of the inflammatory response (associated with mTOR activation) occurs also in FCD IIA still needs to be clarified. In addition, because we only analyzed the lesion, we cannot exclude that activation of proinflammatory pathways may also occur in the perilesional cortex. Accordingly, recent observations indicate increased expression of adhesion and inflammatory factors in the perilesional cortex in TSC compared to autopsy control specimens (Boer et al., 2009).

Although activated microglial cells are the major components of the inflammatory cell population in FCD, some degree of activation of components of the adaptive immunity is also observed. The presence of T lymphocytes (CD8+, T-cytotoxic/suppressor immunophenotype) was greater in FCD II specimens than in FCD I (despite the absence of differences in seizure duration). In addition, only in FCD II cases both perivascular and intraparenchymal
lymphocytes were detected and lymphocytes were often clustered around balloon cells. Inflammatory perivascular lymphocytic infiltrates are not uncommon in the leptomeninges and are occasionally present in the upper cortical layers of patients undergoing implantation of subdural electrodes for chronic monitoring before resection (Stephan et al., 2001; Rhodes et al., 2007). However, the patients included in this study did not undergo invasive monitoring before surgery and did not show signs of leptomeningitis. Moreover, the lymphocytic infiltrates were often located in deeper cortical layers or in the white matter. As expected, the T-cell response was less severe in FCD cases than in cases of MS or viral encephalitis (present results; Serafini et al., 2006). A recent study (Wirenfeldt et al., 2009) reports that microglial activation is increased in patients with active Rasmussen’s encephalitis (RE), compared with cases of FCD and TSC; however, the entity of the lymphocytic infiltrates in RE compared to FCD was not evaluated. The factors that trigger these T-lymphocyte infiltrations in FCD are not known. Alterations in blood–brain barrier permeability, resulting from seizure activity and/or induction of inflammatory mediators by activated microglia, may facilitate their entry. Few T-lymphocytes were found in tissue of patients with temporal lobe epilepsy (TLE) and hippocampal sclerosis, or in experimental models of TLE, indicating that not all types of epileptogenic causes promote accumulation of lymphocytes.

Recently attention has been focused on the role of the antigen-presenting DCs in the initiation of the adaptive immunity (Reis e Sousa, 2006). These cells normally reside in the leptomeninges and choroid plexus. However, under different inflammatory conditions, perivascular and intraparenchymal DCs can be detected in the brain (Serafini et al., 2006; Cudrici et al., 2007). A subarachnoid mixed infiltrate of DCs has been reported in a patient with MCD and chronic epileptic encephalopathy (Rhodes et al., 2007). In the present study we found DCs around intraparenchymal blood vessels only in FCD II specimens. These DCs express the maturation marker CD83 and are associated with perivascular T-lymphocytes. Although they represent a numerically minor population of immune cells compared to the number of DCs in cases of viral encephalitis or MS (Serafini et al., 2006; Cudrici et al., 2007), they deserve attention in view of their ability to drive chronic inflammation interacting with T cells and regulating chemokine production (Tang & Cyster, 1999; Penna et al., 2002). Interestingly, function of DCs is also under control of the mTOR signaling pathway (Thomson et al., 2009), which is deregulated in FCD II.

The complexity of the inflammatory response induced in FCD II is supported by the observed activation of both complement and IL-1β signaling pathways. Prominent glial and neuronal expression of key complement components, C1q and C3d, was detected within the dysplas-
tic region of FCD II specimens. In particular the presence of the biologically active fragments of C3d indicates that the activation of the complement cascade has reached a point that may support a sustained inflammatory process [for review see (Lucas et al., 2006)]. Persistent activation of the complement cascade has been reported recently in both experimental and human temporal lobe epilepsy (Aronica et al., 2007) as well as in TSC-associated cerebral lesions (Boer et al., 2008). As recently reported (Boer et al., 2007), FCD type II lesions displayed also prominent glial and neuronal expression of the proinflammatory and proepileptogenic cytokine IL-1β (Vezzani & Granata, 2005; Vezzani et al., 2008). In contrast, significant activation of complement and IL-1β pathway was not observed in FCD I, despite similar duration of epilepsy. Notwithstanding the fact that both pathways are known to be rapidly upregulated following seizures (Vezzani & Granata, 2005; Aronica & Gorter, 2007), this indicates that seizures alone cannot account for the increased expression in FCD II lesions.

In agreement with the prominent microglial reactivity observed in FCD II specimens, we also detected increased expression of MCP1 (or CCL2), which was prominently expressed in reactive glial cells, dysplastic neurons, and balloon cells. MCP1 is a chemokine that regulates migration and infiltration of monocytes/macrophages and microglial activity, and is involved in the regulation of blood–brain barrier permeability (Stamatovic et al., 2005). Prominent induction of MCP1 has been reported in both experimental and human TLE (Gorter et al., 2006; Wu et al., 2008), as well as in TSC-lesions (data not shown). MCP1 may play a role in both neurodegenerative and regenerative processes (Kalehua et al., 2004).

Our data clearly distinguish FCD I from FCD II, supporting the critical role of sustained inflammatory reaction in FCD II, with activation of both the innate and the adaptive immune response and involvement of complement, as well as, IL-1β and MCP-1 signaling pathways.

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REFERENCES


responses and increased peripheral inflammatory responses to peripheral endotoxin insult. Journal of Neuroinflammation 5:35.2008).


