Activation of toll-like receptor, RAGE and HMGB1 signalling in malformations of cortical development

Emanuele Zurolo,1,* Anand Iyer,1,* Mattia Maroso,1,2 Caterina Carbonell,1 Jasper J. Anink,1 Teresa Ravizza,2 Kees Fluiter,3 Wim G. M. Spliet,4 Peter C. van Rijen,5 Annamaria Vezzani2 and Eleonora Aronica1,6

1 Department of (Neuro) Pathology and Academic Medical Centre, University of Amsterdam, Amsterdam, 1105 AZ, The Netherlands
2 Department of Neuroscience, Mario Negri Institute for Pharmacological Research, 20156 Milano, Italy
3 Department of Neurogenetics, Academic Medical Centre, University of Amsterdam, Amsterdam, 1105 AZ, The Netherlands
4 Department of Pathology and Neurosurgery, University Medical Centre Utrecht, 3508 AB Utrecht, The Netherlands
5 Rudolf Magnus Institute for Neuroscience, University Medical Centre Utrecht, 3508 AB, Utrecht, The Netherlands
6 Epilepsy Institute in The Netherlands Foundation (Stichting Epilepsie Instellingen Nederland, SEIN), 2103 SW, Heemstede, The Netherlands

*These authors contributed equally to this work.

Correspondence to: Dr Eleonora Aronica,
Department of (Neuro) Pathology,
Academic Medical Centre,
Meibergdreef 9,
1105 AZ Amsterdam,
The Netherlands.
E-mail: e.aronica@amc.uva.nl

Recent evidence in experimental models of seizures and in temporal lobe epilepsy support an important role of high-mobility group box 1 and toll-like receptor 4 signalling in the mechanisms of hyperexcitability leading to the development and perpetuation of seizures. In this study, we investigated the expression and cellular distribution of toll-like receptors 2 and 4, and of the receptor for advanced glycation end products, and their endogenous ligand high-mobility group box 1, in epilepsy associated with focal malformations of cortical development. Immunohistochemistry showed increased expression of toll-like receptors 2 and 4 and receptor for advanced glycation end products in reactive glial cells in focal cortical dysplasia, cortical tubers from patients with the tuberous sclerosis complex and in gangliogliomas. Toll-like receptor 2 was predominantly detected in cells of the microglia/macrophage lineage and in balloon cells in focal cortical dysplasia, and giant cells in tuberous sclerosis complex. The toll-like receptor 4 and receptor for advanced glycation end products were expressed in astrocytes, as well as in dysplastic neurons. Real-time quantitative polymerase chain reaction confirmed the increased receptors messenger RNA level in all pathological series. These receptors were not detected in control cortex specimens. In control cortex, high-mobility group box 1 was ubiquitously detected in nuclei of glial and neuronal cells. In pathological specimens, protein staining was instead detected in the cytoplasm of reactive astrocytes or in tumour astrocytes, as well as in activated microglia, predictive of its release from glial cells. In vitro experiments in human astrocyte cultures showed that nuclear to cytoplasmic translocation of high-mobility group box 1 was induced by interleukin-1β. Our findings provide novel evidence of intrinsic activation of these pro-inflammatory signalling pathways in focal malformations of cortical development, which could contribute to the high epileptogenicity of these developmental lesions.
Introduction

Increasing evidence indicates that activation of inflammatory processes in the brain is a common feature of various epileptic disorders. Activation of cells of the microglia/macrophage lineage and astrocytes, associated with concomitant induction of various inflammatory pathways, and expression of inflammatory mediators in neurons, have been observed in epileptic human tissue, including focal malformations of cortical development (MCD), which represent a major cause of paediatric epilepsy (Aronica et al., 2005a, 2007; Boer et al., 2006, 2008; Ravizza and Vezzani, 2006; Ravizza et al., 2006, 2008; Iyer et al., 2010a). Similar to the human condition, a prominent and long-lasting increase in inflammatory mediators has also been described in epileptogenic areas in different experimental models of seizures and epilepsy (Vezzani and Granata, 2005; Aronica and Gorter, 2007; Vezzani et al., 2008).

Experimental studies provide evidence that induction of specific pro-inflammatory pathways in forebrain mediates proconvulsant effects, and their pharmacological modulation represents a potential strategy to reduce seizure activity (reviewed in Vezzani et al. (2008, 2010)). Particular attention has recently focused on the role of toll-like receptor (TLR) signalling pathways in epilepsy (Ravizza et al., 2006; Boer et al., 2006, 2008; Ravizza and Vezzani, 2006; Ravizza et al., 2006, 2008; Iyer et al., 2010a). Similar to the human condition, a prominent and long-lasting increase in inflammatory mediators has also been described in epileptogenic areas in different experimental models of seizures and epilepsy (Vezzani and Granata, 2005; Aronica and Gorter, 2007; Vezzani et al., 2008).

TLRs play a key role in pathogen recognition (Kawai and Akira, 2007) as they bind various molecules of microbial origin, called pathogen-associated molecular patterns, and trigger inflammation by inducing the transcription of genes encoding cytokines, including interleukin-1β (IL-1β). Increasing evidence indicates that, in the absence of pathogens, TLR signalling can be activated by molecules released by injured tissue, namely damage-associated molecular patterns, which include high-mobility group box 1 (HMGB1) (Bianchi and Manfredi, 2009). This is almost an ubiquitous chromatin component that is passively released by necrotic cells, retained by cells undergoing apoptosis and actively secreted by cells following immune challenges or various kind of biological stress (Muller et al., 2004). Nuclear HMGB1 regulates transcription of different sets of genes, including proinflammatory genes (Pedrazzi et al., 2007; Mouri et al., 2008; Bianchi and Manfredi, 2009); secreted HMGB1 can bind the receptor for advanced glycation end products (RAGE) (Scaffidi et al., 2002) and TLR2 and TLR4 (Park et al., 2004).

Increased expression of genes and proteins involved in the TLR pathways has been detected in epileptogenic focal lesions, such as focal cortical dysplasia, gangliogliomas and cortical tubers of patients with tuberous sclerosis complex (Aronica et al., 2008; Boer et al., 2009). More recent studies in animal models of acute and chronic seizures have demonstrated that HMGB1, via activation of TLR4, plays a role in generating and perpetuating seizures (Maroso et al., 2010). The same work reported evidence of activation of HMGB1–TLR4 axis in human temporal lobe epilepsy. HMGB1 proconvulsant effects are mediated by a neuronal signaling involving tyrosine phosphorylation of the NR2B regulatory subunit of the N-Methyl-D-aspartic acid receptor complex that controls Ca2+ influx (Maroso et al., 2010). The role of HMGB1 binding to RAGE in seizures has not yet been addressed.

The present study was designed to test the hypothesis that activation of HMGB1–TLR4 axis, originally described in temporal lobe epilepsy (Maroso et al., 2010), also occurs in focal epileptogenic developmental lesions, and may additionally involve TLR2 and RAGE, possibly representing a common mechanism of epileptogenesis. A detailed analysis of the cellular expression of HMGB1 and its cognate receptors allowed us to identify both the cellular source of HMGB1 and its targets in epileptogenic tissue.

Materials and methods

Subjects

A total of 18 surgical specimens were examined: six focal cortical dysplasia type IIB, six cortical tubers from patients with tuberous sclerosis complex and six gangliogliomas. The cases included in this study were obtained from the departments of Neuropathology of the Academic Medical Centre (University of Amsterdam) in Amsterdam, the University Medical Centre in Utrecht and the Netherlands. The clinical characteristics derived from the patient’s medical records are summarized in Table 1. Patients underwent therapeutic surgical resection for refractory epilepsy and had, predominantly, medically intractable complex partial seizures. All of the patients included in our series did not have apparent seizure activity in the 24 h before surgery. Patients who underwent implantation of strip and/or grid electrodes for chronic subdural invasive monitoring before resection were excluded from the study.

For the grading of the degree of focal cortical dysplasia specimens, we followed the classification system proposed by Palmini et al. (2004). All patients with cortical tubers fulfilled the diagnostic criteria for tuberous sclerosis complex (Gomez et al., 1999). For the gangliogliomas, we used the revised WHO classification of tumours of the CNS (Louis et al., 2007). In five patients (one focal cortical dysplasia, one tuberous sclerosis complex and three gangliogliomas), a significant amount of perilesional tissue (normal-appearing cortex/white matter adjacent to the lesion) was resected. Peritumoural tissue (cortex/white matter adjacent to the lesion with reactive changes, such as astrogliosis and microglia activation, but not tumour cells) of three patients with brain tumours (two astrocytomas and one lymphoma), but without refractory epilepsy, was also analysed. We also included brain tissue from an autopsy case of tuberous sclerosis complex (34th gestational week, obtained from medically induced abortion with appropriate maternal consent for brain autopsy; kindly provided by Dr M. Sinico, Service d’anatomie pathologique, CHI de Créteil, Créteil, France). In addition, normal-appearing control cortex/white matter was obtained at autopsy from six young adult control patients (male/female 3/3; mean age 30.8; range 14–35 years), without a history of seizures or other neurological diseases. All autopsies were performed within 12 h after death. Informed consent was obtained for the use of brain tissue.
Table 1 Summary of clinical details of cases studied according to pathology

<table>
<thead>
<tr>
<th>Pathology type</th>
<th>Number of cases</th>
<th>Mean age (years) at surgery (mean ± SD)</th>
<th>Mean age (years) at seizure (mean ± SD)</th>
<th>Localization (n)</th>
<th>Mean duration (years) of epilepsy (mean ± SD)</th>
<th>Seizure frequency (per months)</th>
<th>Postoperative epilepsy: Engel’s class</th>
</tr>
</thead>
<tbody>
<tr>
<td>Focal cortical dysplasia IIB</td>
<td>6</td>
<td>26.2 ± 11.7</td>
<td>7.2 ± 5</td>
<td>Temporal (3), Frontal (3)</td>
<td>18.1 ± 7.3</td>
<td>&lt;10 (33%)</td>
<td>I (67%)</td>
</tr>
<tr>
<td>Cortical tubers (tuberous sclerosis)</td>
<td>6</td>
<td>17.8 ± 10.1</td>
<td>4.5 ± 2</td>
<td>Frontal (3), Temporal (2), Parietal (1)</td>
<td>13.5 ± 10.9</td>
<td>&lt;10 (33%)</td>
<td>I (50%)</td>
</tr>
<tr>
<td>Ganglioglioma</td>
<td>6</td>
<td>32.0 ± 10.5</td>
<td>15.8 ± 6.4</td>
<td>Temporal</td>
<td>16.1 ± 2.1</td>
<td>&gt;20 (33%)</td>
<td>II (50%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Tissue was obtained and used in a manner compliant with the Declaration of Helsinki.

**Real-time quantitative polymerase chain reaction analysis**

Real-time quantitative polymerase chain reaction analysis was performed using RNA prepared from freshly frozen histologically normal human cortex (n = 5; autopsy specimens) and specimens of patients with MCD (focal cortical dysplasia, tuberous sclerosis complex and gangliogliomas; n = 5 in each series). All the specimens used for the polymerase chain reaction analysis were carefully inspected by microscopy prior to messenger RNA extraction using both histological and immunocytochemical stainings [haematoxylin and eosin, luxol-PAS, glial fibrillary acidic protein (GFAP), neuronal nuclear protein (NeuN)] to confirm that the lesion was present in the sample and attention was taken to provide for RNA isolation equal grey/white matter tissue components. The concentration and purity of RNA (isolated with the TRIzol® LS Reagent) were determined spectrophotometrically at 260/280 nm with a nanodrop spectrophotometer (Ocean Optics, Dunedin, FL, USA). Five micrograms of total RNA were reverse-transcribed into complementary DNA with oligo dT primers. The reverse transcription was performed in 50 μl reactions. Oligo dT primers (5 nmol) were annealed to 5 μg total RNA in a total volume of 25 μl by incubation at 72°C for 10 min and cooled to 4°C. Reverse transcription was performed by the addition of 25 μl RT-mix, containing: First Strand Buffer (Invitrogen—Life Technologies), 2 mM dNTPs (Pharmacia, Germany), 30 U RNase inhibitor (Roche Applied Science, Indianapolis, IN, USA), 0.4 μM of both reverse and forward primers. The final volume was adjusted to 5 μl with H2O (polymerase chain reaction grade). The LightCycler® 480 Real-Time PCR System (Roche Applied Science) was used with a 384-multwell plate format. The cycling conditions were carried out as follows: initial denaturation at 95°C for 5 min, followed by 45 cycles of denaturation at 95°C for 15 s, annealing at 55–60°C for 20 s and extension at 72°C for 10 s. Fluorescent product was measured by a single acquisition mode at 72°C after each cycle. For distinguishing specific from non-specific products and primer dimers, a melting curve was obtained after amplification by holding the temperature at 65°C for 15 s followed by a gradual increase in temperature to 95°C at a rate of 2.5°C/s, with the signal acquisition mode set to continuous. Quantification of data was performed using the computer programme LinRegPCR in which linear regression on the Log(fluorescence) per cycle number data was applied to determine the amplification efficiency per sample (Ramakers et al., 2003). The mean efficiency per primer set and the individual cycle threshold (Ct) values were then used to estimate the starting concentration per sample (Karlen et al., 2007). The concentration of each specific product was divided by the concentration of reference genes (TATA box-binding protein and hypoxanthine phosphoribosyl transferase) and this ratio was compared between patient and control groups.

**Tissue preparation for in situ hybridization and immunocytochemistry**

The tissue was fixed in 10% buffered formalin (J.T. Baker, Davenport, The Netherlands) (autopsy tissue for 2 weeks; surgical specimens for 24 h). In all cases, a representative formalin-fixed, paraffin-embedded tissue block was studied, selecting large resection specimens containing normal cortex adjacent to abnormal cortex for comparison, as an internal control. Paraffin-embedded tissue was sectioned at 6 μm, mounted on precoated glass slides (StarFrost, Waldemar Knittel Glasbearbeitungs GmbH, Braunschweig, Germany) and two slices for each paraffin block were used for in situ hybridization and immunocytochemical staining as described below.

**In situ hybridization**

In situ hybridization for human TLR2 and TLR4 was performed with a 5’ fluorescein-labelled 19-mer antisense oligonucleotide containing locked nucleic acid and 2’ OME RNA moieties (TLR2: 5’FAM-1TmAm GICm UmCI TnGmUl AmGm AITmCmUlGmAmAG; TLR4: 5’FAM-1TmUm CITm Um UlAm Cm UlAm GmCITm CmAlITmUmCIC; capitals indicate locked nucleic acids, lower case indicates 2’OMe RNA).
The oligonucleotides were synthesized by Ribotask ApS, Odense, Denmark. The hybridizations were carried out at 59°C on 6 μm sections of paraffin-embedded material as described previously (Budde et al., 2008). The hybridization signal was detected using a rabbit polyclonal anti-fluorescein/oregon green antibody (A21253, Molecular probes, Invitrogen) and a horseradish peroxidase-labelled goat anti-rabbit polyclonal antibody (PO448 Dako, Glostrup, Denmark) as secondary antibody. Signal was detected with chromogen 3-amin-9-ethyl carbazole (AEC, Sigma, St Louis, USA).

**Immunocytochemistry**

GFAP (polyclonal rabbit, DAKO, Glostrup, Denmark; 1:4000), vimentin (mouse clone V9, DAKO; 1:400), neuronal nuclear protein (NeuN; mouse clone MAS377, IgG1; Chemicon, Temecula, CA, USA; 1:1000), neurofilament (NF, SM131; Sternberger Monoclonals, Lutheford, MD; 1:1000), microtubule-associated protein (MAP2; mouse clone HM2; Sigma 1:100), (HLA)-DP, DR (mouse clone CR3/43; DAKO, Glostrup, Denmark, 1:400), CD68 (mouse clone PG-M1, DAKO; 1:200) and CD31 (mouse JC/70A; 1:100) were used in the routine immunocytochemical analysis of epilepsy specimens.

For the detection of TLR4 we used a rabbit polyclonal antibody [Santa Cruz, Santa Cruz, CA, USA; sc10741; 1:20; (Maroso et al., 2010)], for TLR2, a polyclonal goat (R&D systems, Abingdon UK; 1:100), for RAGE, goat anti-RAGE antibody (1:200, AGE 001; Biolo, Kronshagen, Germany), and for HMGB1, rabbit polyclonal antibody (Pharmigen, San Diego, CA, USA; MRC1024). We counted the total number of GFAP and RAGE immunoreactive balloon/giant cells, as previously described (Martinian et al., 2009). An identical region in the white matter, beneath the region of dysplasia or tuber, was outlined at low magnification (×2.5 objective) on adjacent sections stained with the different antibodies. One section was randomly selected and the mean area for quantitative analysis was 5.95 mm². All balloon cells within this region were counted systematically at high magnification (×40 objective) as positive immunoreactivity (including strong or intermediate intensity of labelling) or negative. The percentage of labelled balloon cells (or giant cells) was calculated based on the total number of balloon cells (or giant cells).

Quantitative analysis was also performed for HMGB1 and the number of positive cells was quantified as previously described (Maroso et al., 2010). Briefly, two representative adjacent non-overlapping fields of the pathological tissue (focal cortical dysplasia, tuberous sclerosis complex, gangliogliomas and control cortex) were captured (magnification ×40; total area of each field: 171,600 μm²) and digitized with a laser scanning confocal microscope (Bio-Rad, Hercules, CA, USA; MRC1024). We counted the total number of GFAP and HLA-DR (human leukocyte antigen system-DP, DQ, DR)-positive cells, and those showing nuclear or extra-nuclear HMGB1 staining. We counted only activated HLA-DR cells expressing HMGB1 since the morphology of resting or weakly activated microglia (small cell bodies with extensive ramifications) did not allow for accurate counting.

**Cell cultures**

For cell culture experiments (astrocyte-enriched human cultures), foetal brain tissue (22–23 weeks of gestation) was obtained from spontaneous or medically induced abortions with appropriate maternal written consent for brain autopsy. Resected tissue samples were collected in Dulbecco’s modified Eagle’s medium/HAM F10 (1:1) (Gibco, Grand Island, NJ, USA). Cell isolation was performed as previously described (Aronica et al., 2003, 2005c). Briefly, after removal of meninges and blood vessels, tissue was dissociated by incubation at 37°C for 20 min in a Hank’s balanced salt solution containing 2.5 mg/ml trypsin (Sigma, St Louis, MO, USA) and 0.1 mg/ml bovine pancreatic...
DNase I (Boehringer Mannheim, Germany). Tissue was triturated and washed with Dulbecco’s modified Eagle’s medium/HAM F10, supplemented with 50 U/ml penicillin, 50 μg/ml streptomycin and 10% foetal calf serum. Cell suspension (containing ~0.5 g wet weight tissue/10 ml culture medium) was passed through a 70 μm cell sieve (Becton Dickinson, USA), plated into poly-L-lysine (15 μg/ml, Sigma) precoated 25 cm² flasks (Falcond, Lincoln Park, NJ, USA) and maintained in a 5% CO₂ incubator at 37°C. After 48 h, the culture medium was replaced with fresh medium and cultures were subsequently fed twice a week. Cultures reached confluence after 2–3 weeks. Secondary astrocyte cultures were established by trypsinizing confluent cultures and sub-plating onto poly-L-lysine precoated 25 cm² flasks (2 × 10⁵ cells/ml; for western blot analysis or for the generation of serial passages) and simultaneously into poly-L-lysine precoated 12 mm coverslips (Sigma) in 24-well plates (Falcond; 2 × 10⁶ cells/well; for immunocytochemistry). More than 98% of the cells in primary culture, as well as in the successive 12 passages, were strongly immunoreactive for the astrocytic marker GFAP. In the current study, astrocytes were used for immunocytochemical analyses at passages 3 and 4. Human recombinant (r)IL-1β (Peprotech, NJ, USA; 10 ng/ml) was applied and maintained in the medium for 24 h before harvesting them for immunocytochemistry. As previously shown (Aronica et al., 2005c), the viability of human astrocytes in culture was not influenced by the treatments.

The astrocytoma cell line U373 was obtained from the American Type Culture Collection (Rockville, MD, USA); cells were cultured in Dulbecco’s modified Eagle’s medium/HAM F10 (1:1) supplemented with 50 U/ml penicillin, 50 μg/ml streptomycin and 10% foetal calf serum. IL-1β (10 ng/ml) was applied and maintained in the serum-free medium for 24 h before being harvested for western blot analysis.

**Preparation of cellular extracts and western blot analysis**

Twenty-four hours after IL-1β (10 ng/ml) treatment, medium was collected and glial cells were washed twice with cold phosphate buffered saline. Nuclear and cytoplasmic extracts were prepared as previously described (Hayakawa et al., 2010). Briefly, samples were homogenized in lysis buffer containing 10 mM Tris (pH 8.0), 150 mM NaCl, 10% glycerol, 1% NP-40, Na-orthovanadate (10.4 mg/ml), 5 mM EDTA (pH 8.0), 5 mM NaF and protease inhibitor cocktail (Boehringer Mannheim, Germany) by incubating on ice for 15 min. The homogenates were centrifuged at 13 000 rpm for 15 min and the supernatant was treated as the cytoplasmic/membrane fraction and the pellet was used as the nuclear fraction. Protein content was determined by the bicinchoninic acid method (Smith et al., 1985). Western blot analysis was performed, as previously described (Aronica et al., 2005b). For electrophoresis, equal amounts of proteins (50 μg/lane) were separated by sodium dodecyl sulphate–polyacrylamide gel electrophoretic analysis. Separated proteins were transferred to nitrocellulose paper for 15–30 min at 10 V, using a semi-dry electroblotting system (BioRad, Transblot SD, Hercules, CA, USA). Blots were incubated overnight in Tris buffered saline with Tween (TBST; 20 mM Tris, 150 mM NaCl, 0.1% Tween, pH 7.5)/5% non-fat dry milk, containing the primary antibody (HMGB1 rabbit polyclonal antibody, 1:1000). After several washes in TBST, the membranes were incubated in TBST/5% non-fat dry milk/1% bovine serum albumin, containing the goat anti-rabbit coupled to horseradish peroxidase (1:2500; Dako, Denmark) for 1 h. After washes in TBST, immunoreactivity was visualized with ECL PLUS western blotting detection reagent (GE Healthcare Europe, Diegen, Belgium). Expression of β-actin (monoclonal mouse, Sigma, St Louis, MO, 1:50,000) was used as loading control.

**Statistical analysis**

Statistical analyses were performed with Statistical Package for the Social Sciences for Windows (SPSS 11.5, SPSS Inc., Chicago, IL, USA) using two-tailed Student’s t-test and to assess differences between more than two groups ANOVA and a non-parametric Kruskal–Wallis test followed by Mann–Whitney U-test. P < 0.05 was considered significant.

**Results**

**Toll-like receptor 2**

**Messenger RNA expression**

TLR2 messenger RNA expression was studied by quantitative polymerase chain reaction in MCD; an average 3- to 4-fold increase in messenger RNA expression was observed in focal cortical dysplasia, tuberous sclerosis complex and gangliogliomas as compared with control cortex (P < 0.05 versus autopsy specimens; Fig. 1A). In situ hybridization was additionally performed to study the cellular distribution of TLR2 messenger RNA in focal cortical dysplasia cases, which confirmed the protein expression pattern (see below). TLR2 messenger RNA was detected in balloon cells and in glial cells of focal cortical dysplasia specimens (Fig. 1C), but was not detected in neurons or in resting glial cells in control cortex (both autopsy material and the perilesional surgical cortex; not shown).

**Immunoreactivity**

In human control cortical autopsy specimens, TLR2 immunoreactivity was not detected either in neurons or in glial cells in both cortex and white matter, throughout all cortical layers (Figs 2A, B and 3A, C). In focal cortical dysplasia, tuberous sclerosis complex and gangliogliomas, the dysplastic neuronal cells were not labelled with TLR2 [Figs 2C, H, J (inset b) and 3A]. In contrast, 54.16 ± 11% of balloon cells in focal cortical dysplasia and 51.3 ± 14% of giant cells in tuberous sclerosis complex showed TLR2 staining (Fig. 2D–I; see immunoreactive score in Fig. 3B). TLR2 immunoreactivity was also observed in cells exhibiting glial morphology (Figs 2C, D, J and 3C). Double labelling demonstrated TLR2 expression in cells of the microglial/macrophages lineage in all series (HLA-DR positive cells; Fig. 2K–N), but not in neuronal cells (NeuN positive cells; not shown). Reactive astrocytes and endothelial cells within the dysplastic cortex only occasionally displayed TLR2 immunoreactivity (data not shown).

**Toll-like receptor 4**

**Messenger RNA expression**

An average 4-fold increase in TLR4 messenger RNA expression was observed by quantitative polymerase chain reaction in focal cortical dysplasia, tuberous sclerosis complex and gangliogliomas as compared with control cortex (P < 0.05 versus autopsy specimens; Fig. 1B). In situ hybridization analysis was additionally performed to study the cellular distribution of TLR4 messenger RNA in focal cortical dysplasia cases, which confirmed the protein expression pattern (see below). In focal cortical dysplasia, TLR4
messenger RNA was detected in neurons and in glial cells (Fig. 1D) but not in neurons or in resting glial cells in control cortex (both autopsy material and the perilesional surgical cortex; data not shown).

**Immunoreactivity**

In human control cortical autopsy specimens, TLR4 immunoreactivity was not detected in neurons throughout all cortical layers (Fig. 4A); only in one case, faint immunoreactivity was observed in few pyramidal neurons. Glial staining was not observed in both cortex and white matter (Fig. 4B and D). In focal cortical dysplasia, tuberous sclerosis complex and gangliogliomas, strong TLR4 expression was observed in a large number of dysplastic neuronal cells (62.8 ± 5.5 in focal cortical dysplasia, 61.7 ± 16 in tuberous sclerosis complex and 57.8 ± 18 in gangliogliomas; Figs 4C, F, H and 3A). In contrast, only 1.6 ± 0.9% of balloon cells in focal cortical dysplasia and 1.3 ± 0.5% of giant cells in tuberous sclerosis complex showed TLR4 staining (Figs 4D–I and 3B). TLR4 immunoreactivity was also detected in cells exhibiting glial morphology (Figs 4E, H and 3D). Double labelling demonstrated TLR4 expression in neuronal cells and in GFAP-positive astrocytes (Fig. 4I–K). Activated microglial cells within the dysplastic cortex were occasionally TLR4-positive [Fig. 4K (inset a)]. As compared with the staining in normal brain, peritumoural tissue, with evidence of astrogliosis and microglia

---

**Figure 1** TLR2 and TLR4 and RAGE messenger RNA expression in control and focal malformations of cortical development. Real-time polymerase chain reaction. (A, B and E) Expression levels were determined in duplicate, corrected for the expression levels of TATA box-binding protein and hypoxanthine phosphoribosyl transferase. Expression levels in control cortex (n = 5), focal cortical dysplasia (FCD, n = 5), cortical tubers of tuberous sclerosis complex (TSC, n = 5) and ganglioglioma (GG, n = 5). TLR2 (A), TLR4 (B) and RAGE (E) messenger RNA levels were significantly increased in epilepsy specimens as compared with controls. There were no significant differences in TLR2 and TLR4 between focal cortical dysplasia, tuberous sclerosis complex and gangliogliomas. The error bars represent SEM; *P < 0.05. In situ hybridization analysis of TLR2 (C) and TLR4 (D) expression. (C) Expression is observed in cells with glial morphology (arrows) and balloon cells (inset). (D) Expression is observed in neurons (arrows) and glial cells (arrow-head and inset). Sections are counterstained with haematoxylin. Scale bar: C = 40 μm; D = 80 μm.
Figure 2 Distribution of TLR2 immunoreactivity in control cortex and focal malformations of cortical development. (A and B) Representative photomicrographs of immunohistochemical staining for TLR2 in the control cortex (A, Ctx) and white matter (B, Wm) showing undetectable expression in neurons and in glial cells. (C-G) Representative photomicrographs of immunohistochemical staining for TLR2 in focal cortical dysplasia (FCD) specimens. (C) Positive glial cells (with the morphology of microglial cells; arrows) within the dysplastic cortex; arrowhead in C indicates a TLR2-negative dysmorphic neuron; insets show high magnification of positive cells around a blood vessel (a) and in the vicinity of a dysmorphic neuron (b, arrowhead). (D) Positive glial cells within the white matter (arrows; high magnification in inset); arrowheads indicate positive balloon cells. (E–G) Strong TLR2 immunoreactivity in balloon cells (arrows in E, high

(continued)
activation, from patients without history of epilepsy, showed low or undetectable immunoreactivity for both TLR2 and TLR4 (Fig. 7A–C).

As compared with the staining in normal brain, peritumoural tissue, with evidence of astrogliosis and microglia activation, from patients without history of epilepsy, showed low or undetectable immunoreactivity for both TLR2 and TLR4 (Fig. 7A–E). HMGB1 immunoreactivity was similar to control cortex, showing only detectable nuclear staining (Fig. 7E, inset).

Receptor for advanced glycation end products

Messenger RNA expression

An average 2- to 3-fold increase in RAGE messenger RNA expression was found using quantitative polymerase chain reaction in focal cortical dysplasia, tuberous sclerosis complex and gangliogliomas as compared with control cortex ($P < 0.05$ versus autopsy specimens, Fig. 1E).

Immunoreactivity

In human control cortical autopsy specimens, variable RAGE immunoreactivity was detected in neurons throughout all cortical layers (Fig. 5A, arrows and inset a; inset b shows absence of neuronal immunoreactivity after pre-absorption); only low or undetectable staining was observed in glial cells (Fig. 5B and J). In focal cortical dysplasia, tuberous sclerosis complex and gangliogliomas RAGE immunostaining was observed in a large number of dysplastic neuronal cells ($74.6 \pm 6.3$ in focal cortical dysplasia, $73.4 \pm 13$ in tuberous sclerosis complex and $77.9 \pm 16$ in gangliogliomas) (Fig. 5C, E, G and I); $67.12 \pm 13\%$ of balloon cells in focal cortical dysplasia and $61.7 \pm 9\%$ of giant cells in tuberous sclerosis complex (TSC). The immunoreactive score represents the total score, which was taken as the product of the intensity score and the frequency score (for details refer to the 'Materials and methods' section).

**Figure 3** Semiquantitative evaluation of TLR2 and TLR4 immunoreactivity in control cortex and focal malformations of cortical development. Plots show the cellular distribution of TLR2 and TLR4 in controls, focal cortical dysplasia (FCD), cortical tubers of tuberous sclerosis complex (TSC) and ganglioglioma (GG). (A) Neurons; (B) balloon/giant cells; (C and D) glial cells. The immunoreactive score represents the total score, which was taken as the product of the intensity score and the frequency score (for details refer to the 'Materials and methods' section).
Figure 4 Distribution of TLR4 immunoreactivity in control cortex and focal malformations of cortical development. (A and B) Representative photomicrographs of immunohistochemical staining for TLR4 in the control cortex (A, Ctx) and white matter (B, Wm) showing undetectable expression in large majority of neurons (arrows) and in glial cells (arrowheads). (C–G) Representative photomicrographs of immunohistochemical staining for TLR4 in focal cortical dysplasia (FCD) specimens. (C) Positive neuronal cells within the dysplastic cortex; inset: a positive dysmorphic neuron (arrow; arrowhead, positive glial cell). (D) Balloon cells (arrows) with faint TLR4 immunoreactivity; faint immunoreactivity is occasionally detected in a few balloon cells (inset). (E) A negative balloon cell (arrow), surrounded by positive glial cells (arrowheads). (F and G) Representative photomicrographs of TLR4 immunoreactivity in cortical tubers of tuberous sclerosis complex with neuronal (arrows in F) and glial immunoreactivity (inset in F), whereas the large majority of giant cells are negative (arrow in G). (H) TLR4 immunoreactivity in ganglioglioma (GG) showing different positive glial (arrowheads and neuronal cells (inset)). Sections were counterstained with haematoxylin. (I–K) GFAP (I, green), TLR4 (J, red) and merged image (K) showing colocalization in focal cortical dysplasia. (K) Merged images showing TLR4 positive cells (red) in focal cortical dysplasia, but lack of colocalization with HLA-DR (green); occasionally expression is observed in HLA-DR positive cells (inset a); inset b shows colocalization with the neuronal marker NeuN. Scale bar: A, B and D: 80 μm; C: 160 μm; E: 20 μm; F–H, K–L: 40 μm.
Figure 5  Distribution of RAGE immunoreactivity in control cortex and focal malformations of cortical development. (A and B) Representative photomicrographs of immunohistochemical staining of RAGE in the control cortex (A, Ctx) and white matter (B, Wm) showing moderate expression in some pyramidal neurons (arrows; inset in A; inset b shows absence of neuronal immunoreactivity after pre-absorption) and low or undetectable expression in glial cells (arrows in B and inset). (C and D) Representative photomicrographs of...
sclerosis complex showed RAGE staining. RAGE immunoreactivity was also detected in cells exhibiting glial morphology (Fig. 5C–H and J) phenotypically identified by double labelling as GFAP-positive cells (Fig. 5H) and activated microglial cells (Fig. 5H, inset).

HMGB1

**Messenger RNA expression**

No statistically significant differences were detected using quantitative polymerase chain reaction in focal cortical dysplasia, tuberous sclerosis complex as compared with control cortex ($P > 0.05$ versus autopsy specimens, not shown).

**Immunoreactivity**

In human control cortical autopsy specimens, HMGB1 immunoreactivity was detected in nuclei of both neurons (Fig. 6A; arrows) and glial cells (Fig. 6A; arrowheads). In focal cortical dysplasia, tuberous sclerosis complex and gangliogliomas, neuronal cells, balloon and giant cells displayed exclusively nuclear HMGB1 staining, whereas cytoplasmic staining was substantially increased in glial cells (Fig. 6C–K; see quantification in Q and R). Double labelling confirmed the HMGB1 expression in neuronal cells, and in both astrocytes and activated microglial cells (Fig. 6L–P).

No significant correlation was found between the increased cytoplasmic HMGB1 staining in glia or TLR2, TLR4 and RAGE staining in tissue specimens, and the seizure frequency before surgery, or postoperative seizure outcome (data not shown). The pattern of immunoreactivity observed for HMGB1, TLR2 and TLR4, and RAGE in control autopsy specimens was similar to that observed in normal appearing cortex adjacent to the lesions (data not shown).

As compared with the staining in normal brain, peritumoral tissue with evidence of astrogliosis and microglia activation from patients without history of epilepsy showed low or undetectable immunoreactivity for both TLR2 and TLR4; the HMGB1 immunoreactivity was similar to control cortex, showing only detectable nuclear staining (Fig. 7A–E). A pattern of immunoreactivity similar to control was also observed for RAGE (data not shown). In contrast, increased expression of TLR2, TLR4 and HMGB1 was observed within the tuber, compared with non-tuberal cortex, in a case of tuberous sclerosis complex (34th gestational week, before the development of seizures; Fig. 7F–J).

**Cell cultures**

Astrocyte-enriched human cell cultures and glioma cells were exposed to IL-1$\beta$ to study whether this cytokine, which is prominently expressed in MCD (Ravizza et al., 2006; Aronica et al., 2008; Boer et al., 2008), affects the cellular localization of HMGB1, as suggested by previous evidence in rat astrocytes (Hayakawa et al., 2010) (Fig. 7). In unchallenged astrocytes, HMGB1 was localized only in the nuclei (Fig. 8A and C), whereas after IL-1$\beta$ exposure, HMGB1 signal was also localized in the cytoplasm (Fig. 7B and E), indicating nuclear to cytoplasmic translocation, as detected in surgical specimens, and predictive of its subsequent release (Muller et al., 2004; Hayakawa et al., 2010). Western blot analysis demonstrated that IL-1$\beta$ increased the expression of HMGB1 in the cytoplasmic fraction of glial cells and showed that the response to this cytokine involved an active release of HMGB1 into the culture media (Fig. 8E).

**Discussion**

Recent data obtained in different experimental models of acute and chronic seizures, identified the crucial role played by the activation of HMGB1–TLR4 signalling in the hippocampus in the generation and recurrence of seizures (Maroso et al., 2010). This evidence is corroborated by findings of increased levels of HMGB1 and TLR4 in surgical specimens from patients with temporal lobe epilepsy and hippocampal sclerosis, supporting the involvement of the HMGB1–TLR4 axis in human epilepsy (Maroso et al., 2010).

In the present study, we demonstrate the intralesional overexpression and cellular distribution of HMGB1 and its cognate receptors TLR2, TLR4 and RAGE in focal cortical dysplasia, tuberous sclerosis complex and ganglioglioma specimens from patients with medically intractable epilepsy. These findings provide evidence of a chronic inflammatory state involving these novel pathways in human epileptic developmental lesions.
Figure 6  High-mobility group box 1 (HMGB1) immunoreactivity in control cortex and focal malformations of cortical development. (A and B) Representative photomicrographs of immunohistochemical staining for HMGB1 in the control cortex (A, Ctx) and white matter (B, Wm) showing nuclear immunoreactivity in pyramidal neurons (arrows and inset in A) and in glial cells (arrowheads in A and B; inset in B). (C–F) Representative photomicrographs of immunohistochemical staining of HMGB1 in focal cortical dysplasia (FCD) specimens. (C and D) Nuclear expression is observed in both neuronal and glial cells. Insets (a and b) in C show high magnifications of dysmorphic neurons with nuclear immunoreactivity; arrows in C (inset a) and in D show glial cells with both nuclear and cytoplasmic staining. (D) High magnification of an immunoreactive glial cells and glial processes. (E) Nuclear immunoreactivity in a balloon cell. (F) Nuclear and cytoplasmic staining in perivascular glial cells (arrows). (G–J) Representative photomicrographs of HMGB1 immunoreactivity in cortical tubers of tuberous sclerosis complex (TSC). (G) Dysmorphic neurons with nuclear immunoreactivity (arrows) and glial cells with both nuclear and cytoplasmic staining (arrowheads). (H and I) HMGB1 immunoreactive glial cells and processes (arrowheads) within the white matter, with occasionally few negative dysmorphic neurons (arrow in H). (J) Giant cell showing only nuclear staining. (K) HMGB1 immunoreactivity in ganglioglioma (GG) showing neurons with nuclear immunoreactivity (arrows) and glial cells with both nuclear and cytoplasmic staining (arrowheads). Inset (a) shows glial cells with prominent cytoplasmic immunoreactivity; inset (b) shows high magnification of a positive neuron and a glial cell with immunoreactive processes. Sections were counterstained with haematoxylin. (L–N) Merged images showing colocalization of HMGB1 (red) with NeuN (green; L), GFAP (green; M), HLA-DR (green; N) in focal cortical dysplasia. (O and P) Merged images showing colocalization of HMGB1 (red) with GFAP (green; O), and HLA-DR (green; P) in gangliogliomas. (Q and R) Quantification bar chart of HMGB1-positive cells (astrocytes in Q and microglia/macrophages in R) in control, focal cortical dysplasia, tuberous sclerosis complex and gangliogliomas specimens. Extranuclear staining: *P < 0.05 versus control, one-way ANOVA followed by Tukey’s test. nd = not detectable. Scale bar: A, B and K: 160 μm; C, E, H, I, L, N and P: 80 μm; G and J: 20 μm; D, F, M and O: 40 μm.
Differential cellular distribution of toll-like receptors and receptor for advanced glycation end products in malformations of cortical development

Toll-like receptors 2 and 4

These receptors play a critical role in determining the pathological outcomes in several neurological disorders, including autoimmune diseases, neurodegeneration, trauma, stroke and more recently, epilepsy (Bsibsi et al., 2002; Aravalli et al., 2007; Crack and Bray, 2007; Andersson et al., 2008; Drexler and Foxwell, 2010; Maroso et al., 2010).

In the present study, we provide the first evidence of cell-specific upregulation of both messenger RNA and protein levels of TLR2 and TLR4 in different MCD. In histologically normal cortex (autopsy and surgical tissue samples), both receptors could be detected by real time-polymerase chain reaction while messenger RNAs and respective proteins were below detection levels in neuronal and resting glial cells as assessed by in situ hybridization and immunohistochemistry. In agreement, the expression of TLR2 and TLR4 messenger RNA has been shown to be generally low in brain, as compared with other tissues (Nishimura and Naito, 2005).

Immunocytochemical analysis showed a differential cellular distribution of TLR2 and TLR4 in different MCD. In histologically normal cortex (autopsy and surgical tissue samples), both receptors could be detected by real time-polymerase chain reaction while messenger RNAs and respective proteins were below detection levels in neuronal and resting glial cells as assessed by in situ hybridization and immunohistochemistry. In agreement, the expression of TLR2 and TLR4 messenger RNA has been shown to be generally low in brain, as compared with other tissues (Nishimura and Naito, 2005).

Immunocytochemical analysis showed a differential cellular distribution of TLR2 and TLR4; TLR2 protein was consistently and predominantly expressed in activated microglia within the different epileptogenic lesions, in agreement with previous studies in microglial cell cultures [for review see Kielian (2006)]. Notably, TLR2 has been suggested to play a role in mediating microglia activation (Babcock et al., 2006; Kielian, 2006; Aravalli et al., 2007; Mallard et al., 2009). Moreover this receptor is involved in the induction of inflammatory pathways (O’Neill et al., 2009) and thus it may contribute to the increase in inflammatory mediators in microglia previously described in epileptogenic human tissue (Aronica et al., 2005a, 2007; Boer et al., 2006, 2008; Ravizza et al., 2006, 2008).

In contrast to its commonly recognized microglial expression, the expression of TLR2 in astrocytes is more controversial [for review see Kielian (2006)]. Accordingly, we have been able to detect sporadic expression of TLR2 in reactive astrocytes, which were abundantly present in the epileptic lesions examined. However, we report expression of TLR2 in balloon and giant cells, but not in dysplastic neurons, in higher proportion as compared with TLR4. Since balloon and giant cells represent a significant source of proinflammatory molecules (Ravizza et al., 2006; Boer et al., 2008), the evaluation of the TLR2 function in these cell types deserves further investigation, with a recently described culture system (Yasin et al., 2010).

TLR4, differently from TLR2, showed a prominent expression in both reactive astrocytes and neuronal cells. Studies examining TLR4 expression in astrocytes in vitro have produced conflicting results [reviewed in Crack and Bray (2007)]; in some studies astroglial expression of TLR4 could not be demonstrated (Farina et al., 2005; Kielian, 2006) whereas other studies have shown a constitutive expression of TLR4 in astrocytes and its upregulation following cell activation (Bsibsi et al., 2002; Bowman et al., 2003; Carpentier et al., 2005). These discrepancies may reflect differences in cell source and culture conditions (Kielian, 2006); alternatively, lack of TLR4 induction may be ascribed to insufficient release of pro-inflammatory cytokines (such as IL-1β) or damage-associated molecular patterns (such as HMGB1) by the injured/activated cells in some of these studies. Accordingly, TLR4 expression in astrocytes has been observed in experimental models of seizures associated with release of IL-1β and HMGB1 by neurons and astrocytes (Maroso et al., 2010).

TLR4 expression in dysplastic neuronal cells may critically affect neural homeostasis [for reviews see Crack and Bray (2007);
Figure 7 TLRs and HMGB1 in brain tumour or prenatal tuberous sclerosis cases without history of chronic seizures. (A–E) Peritumoural cortex. (A and B) Representative photomicrographs of immunohistochemical staining for glial fibrillary acidic protein (A) and HLA-DR (B) showing astrogliosis (A) and activation of microglia (B). (C–E) Representative photomicrographs of immunohistochemical staining of TLR2 (C), TLR4 (D) showing the low or undetectable immunoreactivity in reactive glial cells (arrows in insets). (E) HMGB1 immunoreactivity was similar to control cortex, showing only detectable nuclear staining (inset in E). (F–J) Representative photomicrographs of TLR2 (F and G), TLR4 (H and I) and HMGB1 in a cortical tuber of tuberous sclerosis complex (TSC) at 34 weeks of gestation (before the development of seizures). (F) TLR2 positivity only in the tuber; high magnification of TLR2 positive giant cells is shown in G. (H and I) TLR4 immunoreactivity within the tuber in both giant cells (arrows in I) and reactive glia (arrow heads in I). (J) HMGB1 immunoreactive glial cells and processes (arrows) within the tuber. Scale bar: A–E: 300 μm; F–H: 250 μm; G: 160 μm; I–J: 40 μm.
Mallard et al. (2009). The activation of this receptor may indirectly enhance neuronal Ca\(^{2+}\) influx through the phosphorylation of NMDA-NR2B receptors, supporting neuronal hyperexcitability (Maroso et al., 2010), and thus playing a role in the epileptogenicity of focal MCD. Notably, dysplastic neurons in focal cortical dysplasia and gangliogliomas specimens express high levels of NR1 and NR2A/B subunit proteins (Ying et al., 1999; Aronica et al., 2001b, c), and NR2A/B distribution correlates with in situ epileptogenicity in patients with focal cortical dysplasia (Najm et al., 2000; Moddel et al., 2005). Although a rapid induction of TLR4 has been shown in different experimental models of lesional and non-lesional seizures (Maroso et al., 2010), seizures alone may not account for changes in its neuronal and glial expression in MCD since perilesional tissue, although exposed to seizures, did not show significant upregulation of TLR4. Therefore, the lesion per se, or the concomitant presence of the lesion and the epileptic activity, is likely to play a role in modulating the TLR system in these developmental disorders.

**Receptor for advanced glycation end products**

In human control brain, RAGE expression has been found in neurons and glial cells, and its expression is increased in activated astrocytes and microglial cells in tissue of patients with neurodegenerative disorders, such as Alzheimer’s disease and Huntington’s disease (Sasaki et al., 2001; Ma and Nicholson, 2004; Lue et al., 2005).

In our study, prominent RAGE expression was observed in both glial and neuronal cells in different MCD. RAGE was consistently expressed in activated astrocytes and microglial cells, as well as in dysplastic neurons and balloon/giant cells in focal cortical dysplasia and tuberous sclerosis complex. RAGE is a receptor that binds different molecules, including HMGB1 and members of the S100 protein family (Sims et al., 2010). Interestingly, overexpression of both HMGB1 and S100B has been observed in human temporal lobe epilepsy, and our study showed changes in HMGB1 in MCD (see below) (Griffin et al., 1995; Maroso et al., 2010). While HMGB1–TLR4 interaction has been studied in experimental model of seizures, the role of the HMGB1–RAGE axis, and possibly S100 ligands, in epilepsy requires further investigation (Dyck et al., 2002; Sakatani et al., 2007, 2008; Sims et al., 2010).

**Cellular distribution of HMGB1 in control cortex and malformations of cortical development**

HMGB1 is an almost ubiquitous structural non-histone chromatin protein involved in the regulation of transcription of a set of inflammatory genes (Bianchi and Manfredi, 2007; Pedrazzi et al., 2007; Mouri et al., 2008). However, HMGB1 can be released from cells upon its cytoplasmic translocation under immune/inflammatory challenges or injurious conditions (Czura et al., 2001; Bianchi and Manfredi, 2009). Extracellular HMGB1 acts as a ‘danger signal’ to orchestrate a homeostatic defensive response in challenged tissues; however, its pro-inflammatory properties, acquired upon its cellular release and consequent TLR/RAGE stimulation, appear to contribute to the pathogenesis of various...
inflammatory and CNS diseases (Bianchi and Manfredi, 2007; Bianchi, 2009; Hrengvildsdottir et al., 2009).

In histologically normal temporal cortex (autopsy and surgical tissue samples), HMGB1 is expressed in nuclei of neuronal and glial cells. Accordingly, a similar pattern of expression has been reported in human control hippocampus (Maroso et al., 2010). In MCD, HMGB1 remained predominantly nuclear in dysplastic neurons and balloon/giant cells, suggesting that it may contribute to regulate inflammatory gene transcription in these cells (Ravizza et al., 2006; Pedrazzi et al., 2007; Mouni et al., 2008; Bianchi and Manfredi, 2009). Differently, we observed a cytoplasmic translocation of HMGB1 in reactive astrocytes in focal cortical dysplasia and tuberous sclerosis complex, in tumour astrocytes in gangliogliomas and in activated microglia in all MCD cases. A cytoplasmic translocation in activated glia has recently been reported in experimental models of seizures and in hippocampal tissue from patients with temporal lobe epilepsy (Maroso et al., 2010). These findings indicate that glial cells are a major source of extracellular HMGB1 in epileptic pathologies.

We show that IL-1β induces the relocation of nuclear HMGB1 to the cytoplasm and release in human cultured astroglial cells, as previously reported in rat cultured astrocytes (Hayakawa et al., 2010). Since IL-1β is upregulated in epileptogenic tissue from MCD and temporal lobe epilepsy (Vezzani et al., 2008), this pro-inflammatory cytokine may play a pivotal role in inducing HMGB1 release from glia in human epilepsy. Since microglial and astrocytes have been shown to respond to HMGB1 stimulation with production of several inflammatory mediators (Kim et al., 2006; Pedrazzi et al., 2007; Andersson et al., 2008), these cells are likely to provide a positive feedback loop that amplifies the inflammatory response in epilepsy-associated MCD.

HMGB1 has been suggested to promote stem cell migration and differentiation (Huttunen et al., 2002; Palumbo et al., 2004; Chavakis et al., 2007), raising the possibility that HMGB1 release within the epileptogenic lesions interferes with neuronal migration and differentiation during brain development (Flores-Sarnat et al., 2003; Lamparello et al., 2007; Yasin et al., 2010). Although the activation of HMGB1–TLR/RAGE pathways has been shown in several neurological disorders associated with gliosis (Bisbisi et al., 2002; Aravalli et al., 2007; Crack and Bray, 2007; Andersson et al., 2008; Drexler and Foxwell, 2010), this phenomenon is not simply the result of glial activation, since non-epileptic perisialional tissue with reactive gliosis show immunoreactivity patterns similar to control tissue. Seizures alone may not be the only determinants of the observed effects since perisialional tissue with normal morphology, but probably exposed to similar seizure activity, did not show detectable changes in TLR/HMGB1 expression as compared with control tissue. Moreover, prenatal TLRs and HMGB1 expression was found in giant cells within the tuber in tuberous sclerosis complex. It is likely, therefore, that the induction of these signalling pathways is intrinsic to the developmental lesion per se, and the concomitant occurrence of seizures could contribute to perpetuate this activation. Interestingly, the mTOR (mamalian target of rapamycin) pathway is concomitantly activated in these lesions (Iyer et al., 2010b), and this signalling plays a role both in regulating cell growth and size, as well as in activating immune/inflammatory responses (Lim et al., 2003; Schmitz et al., 2008; Weichhart and Saemann, 2009).

This study, together with our former functional and pharmacological evidence obtained in experimental models of temporal lobe epilepsy (Maroso et al., 2010), supports the role of HMGB1–TLR/RAGE pathways in the mechanisms underlying the intrinsic high epileptogenicity of focal developmental glioneuronal lesions. In the absence of genuine models of MCD (Wong, 2009), we believe that the HMGB1-mediated proconvulsant mechanism recently described in temporal lobe epilepsy models, and concordant with data in temporal lobe epilepsy human tissue, would reasonably support that the same mechanism is likely to be operative in MCD epileptogenic areas. Thus, pharmacological modulation of the HMGB1–TLR/RAGE axis with receptor antagonists or inactivating antibodies (Yang et al., 2002; Hennessy et al., 2010) may represent a potential novel antiepileptic strategy in MCD. The evidence we provide here using human epileptogenic surgical tissue offers instrumental information to guide future studies into the possibility of interfering with inflammatory mechanisms in humans to prevent or alleviate seizures associated with developmental lesions [ClinicalTrials.gov Study of VX-765 in Subjects with Treatment-resistant Partial Epilepsy [online], http://clinicaltrials.gov/ct2/show/NCT01048255 (2010)].

**Funding**

National Epilepsy Fund, NEF 05-11 (E.A.); EU-FP7-project 202167 and Stichting Michelle M06.011, M07.016 (to E.A.); Cariplo Foundation and PACE (to A.V.).

**References**


Riaz-K, Galic MA, Pittman OJ. Contributions of peripheral inflammation to seizure susceptibility: cytokines and brain excitability. Epilepsy Res 2010; 89: 34–42.


