Activation of the innate immune system is evident throughout epileptogenesis and is associated with blood-brain barrier dysfunction and seizure progression

Broekaart, D.W.M.; Anink, J.J.; Baayen, J.C.; Idema, S.; de Vries, H.E.; Aronica, E.; Gorter, J.A.; van Vliet, E.A.

DOI
10.1111/epi.14550

Publication date
2018

Document Version
Final published version

Published in
Epilepsia

Received: 3 April 2018 | Revised: 31 July 2018 | Accepted: 31 July 2018

DOI: 10.1111/epi.14550

FULL-LENGTH ORIGINAL RESEARCH

Epilepsia®

Activation of the innate immune system is evident throughout epileptogenesis and is associated with blood-brain barrier dysfunction and seizure progression

Diede W. M. Broekaart[1] | Jasper J. Anink[1] | Johannes C. Baayen[2] | Sander Idema[2] | Helga E. de Vries[3] | Eleonora Aronica[1,4] | Jan A. Gorter[5] | Erwin A. van Vliet[1,5]

[1]Department of (Neuro) Pathology, Amsterdam UMC, University of Amsterdam, Amsterdam Neuroscience, Amsterdam, The Netherlands

[2]Department of Neurosurgery, Amsterdam UMC, Vrije Universiteit Amsterdam, Amsterdam Neuroscience, Amsterdam, The Netherlands

[3]Department of Molecular Cell Biology and Immunology, Amsterdam UMC, Vrije Universiteit Amsterdam, Amsterdam Neuroscience, Amsterdam, The Netherlands

[4]Stichting Epilepsie Instellingen Nederland (SEIN), The Netherlands

[5]Swammerdam Institute for Life Sciences, Center for Neuroscience, University of Amsterdam, Amsterdam, The Netherlands

Correspondence
Erwin A. van Vliet, Department of (Neuro) Pathology, Amsterdam UMC, Academic Medical Center, Meibergdreef 9, 1105 AZ, Amsterdam, The Netherlands.
Email: e.a.vanvliet@uva.nl

Funding information
European Union's Seventh Framework Program/Dutch Epilepsy Foundation, Grant/Award Number: 602102 (EPITARGET) /16-05

Summary

Objective: Because brain inflammation may contribute to the pathophysiology of temporal lobe epilepsy (TLE), we investigated the expression of various inflammatory markers of the innate and adaptive immune system in the epileptogenic human and rat hippocampus in relation to seizure activity and blood-brain barrier (BBB) dysfunction.

Methods: Immunohistochemistry was performed using various immune cell markers (for microglia, monocytes, macrophages, T lymphocytes, and dendritic cells) on hippocampal sections of drug-resistant TLE patients and patients who died after status epilepticus. The expression of these markers was also studied in the electrical post–status epilepticus rat model for TLE, during the acute, latent, and chronic epileptic phase. BBB dysfunction was assessed using albumin immunohistochemistry and the BBB tracer fluorescein.

Results: Monocyte infiltration, microglia, and perivascular macrophage activation were persistently increased in both epileptogenic human and rat hippocampus, whereas T lymphocytes and dendritic cells were not or were scarcely detected. In addition to this, increased expression of C-C motif ligand 2 (CCL2) and osteopontin was observed. In humans, the expression of CD68 and CCL2 was related to the duration of epilepsy and type of pathology. In rats, the expression of CD68, CCL2, and the perivascular macrophage marker CD163 was related to the duration of the initial insult and to the number of spontaneous seizures. Interestingly, the number of CD163-positive perivascular macrophages was also positively correlated to BBB dysfunction in chronic epileptic rats.

Significance: These data suggest a proepileptogenic role for monocytes/macrophages and other cells of the innate immune response, possibly via increased BBB leakage, and indicate that T cells and dendritic cells, which are closely associated with the adaptive immune response, are only sparsely infiltrated during epileptogenesis in the electrical post–status epilepticus rat model. Future studies should reveal the relative importance of these immune cells and whether specific manipulation can modify or prevent epileptogenesis.

KEYWORDS
brain inflammation, perivascular macrophages, status epilepticus, temporal lobe epilepsy

1 | INTRODUCTION

Increasing evidence suggests an important role for brain inflammation in epilepsy.[1,2] Several studies show activation of the innate immune response, including microglia and astrocytes that produce specific inflammatory cytokines, in resected brain tissue from patients with temporal lobe epilepsy (TLE) and in experimental epilepsy models, which coincides with blood-brain barrier (BBB) disruption.[3-11] The activation of inflammatory processes has been confirmed using large-scale gene expression profiling (microarray analysis) on resected hippocampi of patients with TLE.[12] Microarray studies on epileptogenic brain regions of rats with TLE showed that brain inflammation was the most prominent process changed during different phases of epileptogenesis.[13] Furthermore, studies in rodent epilepsy models show that brain inflammation can enhance neuronal excitability,[1,14-18] possibly via disruption of the BBB.

The identification of inflammatory changes at critical phases during epileptogenesis could help to determine the role of inflammation in the progression of epilepsy and is of crucial importance when we implement anti-inflammatory strategies to prevent its development. Until now, there has been ample evidence that the innate response plays a role during the development of TLE.[1] More recently, the role of peripheral inflammation in TLE has come to attention, with a central role for the infiltration of leukocytes and/or activated circulating cytokines.[19] However, their precise contribution to epileptogenesis and BBB dysfunction is still not understood. We therefore studied the expression of various inflammatory markers of the innate and adaptive immune system in the epileptogenic human and rat hippocampus in relation to seizure activity and BBB dysfunction.

Key Points

- Monocyte infiltration, microglia, and perivascular macrophages were persistently increased in both epileptogenic human and rat hippocampus
- T lymphocytes and dendritic cells were not or were scarcely detected in both epileptogenic human and rat hippocampus
- The number of CD68+ monocytes and CD163+ perivascular macrophages, as well as CCL2 expression were related to the number of spontaneous seizures of rats
- The number of CD163+ perivascular macrophage cells was positively correlated to BBB permeability in the rat hippocampus

2 | MATERIALS AND METHODS

2.1 | Immunohistochemistry on human brain tissue

Human brain tissue was obtained from the archive of the Department of Neuropathology of the Amsterdam UMC, The Netherlands. Patients underwent resection of the hippocampus for drug-resistant TLE (TLE with hippocampal sclerosis [HS], International League Against Epilepsy type 1, n = 21; TLE no-HS, n = 6). In addition, autopsy material was used of five patients who died during acute status epilepticus (SE). This material was compared to normal-appearing hippocampi of six autopsy specimens from patients without history of seizures or other neurological diseases. Tissue was obtained and used in a manner compliant with the Declaration of Helsinki. Brain tissue was stained for immunohistochemistry using various antibodies. For details, see Appendix S1 (Materials and Methods) and Tables S1 and S2.

2.2 | Experimental animals

Adult male Sprague Dawley rats (Harlan Netherlands, Horst, The Netherlands) weighing 200-300 g at the start of the study were used. To exclude variations in seizure frequency due to differences in the estrous cycle, we did not use female rats. The study was approved by the University Animal Welfare Committee. The rats were housed individually in a controlled environment (21 ± 1°C, humidity 60%, lights on 08:00 AM-8:00 PM, food and water available ad libitum). See Figure S1 for a schematic overview of the study and Appendix S1 (Materials and Methods) for electrode implantation, SE induction, electroencephalographic monitoring, and perfusion.

2.3 | Fluorescein injection and perfusion

To determine BBB permeability, fluorescein (FSC; 100 mg/kg intravenous; Merck, Darmstadt, Germany) was injected via the tail vein under isoflurane anesthesia (4 vol %), as described previously.[5] Rats were disconnected from the electroencephalographic recording setup 3.5 hours after tracer injection and deeply anesthetized with pentobarbital (intraperitoneally, 60 mg/kg). Rats were perfused through the ascending aorta with 300 mL of 0.37% Na_2S solution and 300 mL 4% paraformaldehyde in 0.1 mol/L phosphate buffer, pH 7.4. Brains were postfixed in situ overnight at 4°C, dissected, and cryoprotected in 30% phosphate-buffered sucrose solution, pH 7.4. After overnight incubation at 4°C, brains were frozen in isopentane (−30°C) and stored at −80°C until sectioning. Brain tissue was stained

using various antibodies. For details, see Appendix S1 (Materials and Methods) and Table S2.

2.4 | Quantification of immunohistochemistry

The number of CD68-immunoreactive (IR) cells was estimated semiquantitatively in the hippocampus and classified as follows: 0 = no cells, 1 = sparse, 2 = moderate, 3 = high, 4 = very high.

The intensity of CD11b/c-IR cells was estimated semiquantitatively in the hippocampus and classified as follows: 1 = moderate, 2 = strong, 3 = very strong.

The number of C-C motif ligand 2 (CCL2)-IR glial cells in the hippocampus was estimated semiquantitatively and classified as follows: 1 = no cells, 2 = sparse, 3 = moderate, 4 = high.

The intensity of CCL2-IR neurons was classified as follows: 1 = weak, 2 = moderate, 3 = strong.

The total number of CD163-IR cells was counted throughout the hippocampus.

The immunoreactivity score for albumin staining was calculated by multiplying the intensity score by the frequency score. The intensity of albumin immunoreactivity was estimated semiquantitatively and classified as follows: 1 = no, 2 = weak, 3 = moderate, 4 = strong staining. The frequency score for albumin immunoreactivity was classified as follows: 0 = no albumin present, 1 = some spots of albumin around vessels, 2 = many albumin spots, 3 = entire hippocampus is positive for albumin.

2.5 | Quantification of BBB leakage

To detect extravasation of FSC, horizontal sections were mounted on slides (Superfrost Plus; Menzel, Braunschweig, Germany) and coverslipped with mounting medium for fluorescence (Vectashield; Vector Laboratories, Burlingame, California). Sections were counterstained with 4,6-diamidino-2-phenylindole. FSC was detected using a confocal-laser scanning microscope (LSM510; Carl Zeiss, Oberkochen, Germany) with appropriate filter settings (excitation = 488 nm, emission = 520 nm). Images were made using Zeiss software (Zeiss LSM Image Browser). A quantification of FSC was made, similar as in our previous study.[5] Briefly, a grid (230 × 230 μm^2, 15 × 15 squares) was placed on the selected brain region. Next, the intensity of the FSC signal was measured, and the number of squares that contained an FSC signal was counted. Finally, the "permeability index" was calculated (number of squares that contained an FSC signal x FSC intensity).

2.6 | Statistical comparisons

For immunohistochemistry, in which the absolute number of cells was counted or a semiquantitative analysis was performed, statistical comparisons between groups were performed using a Mann-Whitney *U* test. Correlations were tested using the Spearman rank order correlation test. $P < 0.05$ was assumed to indicate a significant difference.

3 | RESULTS

3.1 | Seizure activity in patients

Patients with TLE and HS (type 1) had 12 ± 2 seizures (mean ± SEM) per month. Patients with TLE without HS experienced 16 ± 4 seizures per month. The seizure frequency was not different between groups.

3.2 | Seizure activity in rats

Two groups of rats could be distinguished during the chronic epileptic phase (7-9 months after SE): rats with a progressive seizure development, which had on average 13 ± 3 seizures per day during the week before they were killed; and rats with a nonprogressive seizure development, which had on average 0.4 ± 0.1 seizures per day during the last week. The seizure frequency was different between these groups at this time point ($P < 0.05$). The individual seizure frequency during the last 2 weeks of electroencephalographic recording is shown in Table S5.

3.3 | CD3, CD83, and CD209 immunoreactivity in human and rat brain

CD3-(T lymphocytes), CD83-(immature and mature dendritic cells), and CD209-positive cells (mature dendritic cells) were scarce in human control hippocampus and were occasionally detected in hippocampi of patients who died after SE and in TLE patients with and without hippocampal sclerosis (data not shown). In rats, CD3-, CD83-, and CD209-positive cells were not detected in the hippocampus of controls or at any time point during epileptogenesis (data not shown). In positive control tissue (thymus), all these antibodies displayed a staining pattern as expected.

3.4 | CD68 immunoreactivity in human brain

3.4.1 | Control

CD68 is a marker for activated microglia, monocytes, or macrophages. CD68-IR cells were not present in control hippocampus. In one case, sparse CD68 immunoreactivity was observed in close proximity of blood vessels (Figure 1A).

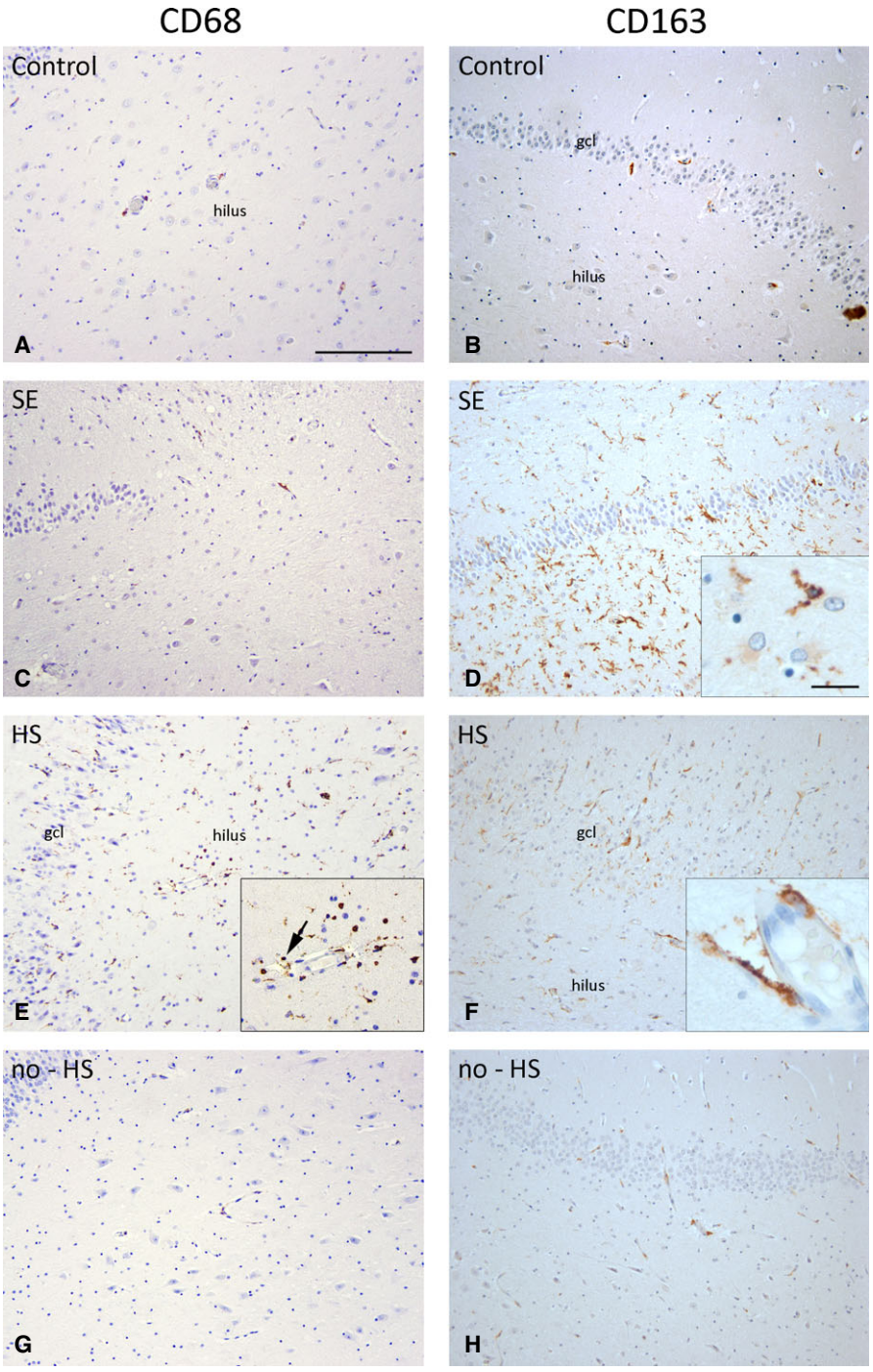

FIGURE 1 CD68 and CD163 immunoreactivity in human brain. CD68 and CD163 staining is shown in autopsy control (A, B), in the brain of a patient who died after a status epilepticus (SE; C, D), and in brains of patients with temporal lobe epilepsy and hippocampal sclerosis (HS; E, F) and without hippocampal sclerosis (no-HS; G, H). CD68 was scarcely present in controls and in the hippocampus of patients who died after SE. Higher CD68 expression was observed in both no-HS and HS cases, especially around blood vessels (inset in E, arrow indicates a CD68-immunoreactive cell next to a vessel), compared to controls. CD68 cells were more frequently observed in patients with HS compared to patients without HS (Table S3). CD163-positive cells were scarcely present around vessels in controls. CD163 immunoreactivity was higher in patients who died after SE and in patients with temporal lobe epilepsy, compared to controls. After SE, CD163-positive cells had an activated morphology with large cell bodies and coarse processes (inset in D) and were also located in the parenchyma. gcl = granular cell layer. Scale bar: A-H, 200 μm; insets, 20 μm

3.4.2 | Status epilepticus

In patients who died as a result of SE, CD68 immunoreactivity was not or was sparsely observed in the hippocampus (Figure 1C).

3.4.3 | Temporal lobe epilepsy

The number of CD68-positive cells increased in the hippocampus of TLE patients, compared to controls (Table S3). CD68 was sparsely present in the hippocampus in 50% of the patients without hippocampal sclerosis (no-HS, Figure 1G). CD68 could not be detected in the remaining no-HS cases. In HS cases, a moderate to high number of CD68-positive cells was present throughout the hippocampus (Figure 1E). More CD68-positive cells were present in HS than in no-HS (Table S3 and Figure S7B).

3.5 | CD68 immunoreactivity in rat brain

3.5.1 | Control

CD68 (ED-1) staining was not observed in controls (Figure 2A).

3.5.2 | Acute phase

CD68-positive cells were present in all hippocampal subfields, and the number of cells was increased compared to controls (Table S4 and Figure S7E). Most CD68-IR cells had a round appearance, some with IR processes. In two of five rats, CD68 was sparsely distributed. In the other rats, a moderate to high number of CD68-positive cells was observed; they were scattered throughout the parenchyma, but were also present around blood vessels.

3.5.3 | Latent phase

CD68-IR cells were abundantly present in all hippocampal layers (Figure 2C). CD68-positive cells were observed in high numbers in all rats, and these cells were distributed throughout the parenchyma, but also in close proximity of blood vessels (inset in Figure 2C). Significantly more cells were observed compared to control rats and to rats that were killed in the acute phase (Table S4 and Figure S7E). CD68 colocalized with ferritin (Figure S4A-C), indicating that these CD68-positive cells were microglia. CD68 did not colocalize with the astrocytic marker glial fibrillary acidic protein (Figure S4D-F).

3.5.4 | Early chronic phase

CD68-positive cells were present throughout the hippocampus, both in the parenchyma and near blood vessels (Figure 2E). CD68 cells had a round appearance. The number of CD68-IR cells was higher compared to controls (Table S4 and Figure S7E); however, fewer cells were IR compared to the latent phase.

3.5.5 | Late chronic phase

Sparse to moderate CD68 immunoreactivity was found in the hippocampus of chronic epileptic rats that had a nonprogressive form of epilepsy, which was more than in control rats (Table S4 and Figure S7E).

In rats that had a progressive form of epilepsy, more CD68 cells were observed (Figure 2G, Table S4, and Figure S7E) compared to rats with a nonprogressive form of epilepsy. Some CD68-IR cells were located in very near proximity of blood vessels (inset in Figure 2G).

3.6 | CD11b/c immunoreactivity in rat brain

3.6.1 | Control

CD11b/c (OX-42) specifically labels microglia cells. CD11b/c-IR cells were abundantly present throughout the hippocampus with moderate immunoreactivity, with a typical morphology of resting microglia (Figure 2B).

3.6.2 | Acute and latent phase

A dramatic increase of CD11b/c cells was observed, as a very high number of CD11b/c cells with very strong immunoreactivity were present in all hippocampal subfields, with a typical morphology of activated microglia (Figure 2D, Table S4, and Figure S7F). Especially near the hippocampal cell body layers, clusters of CD11b/c cells were found. During the acute phase, these cells had an amoeboid morphology, whereas during the latent phase CD11b/c-IR cells had a characteristic reactive bushy appearance and distended clubfoot-like endings or a stellate morphology with larger cell bodies and coarse processes.

3.6.3 | Early chronic phase

Many clusters of reactive microglia were observed in the dentate gyrus (Figure 2F), CA3, and CA1, and CD11b/c immunoreactivity was higher compared to controls (Table S4 and Figure S7F).

3.6.4 | Late chronic phase

In chronic epileptic rats that had a nonprogressive form of epilepsy, a few reactive microglia cells with strong immunoreactivity were present. Similarly, in rats that had a progressive form of epilepsy, a few CD11b/c-IR cells were

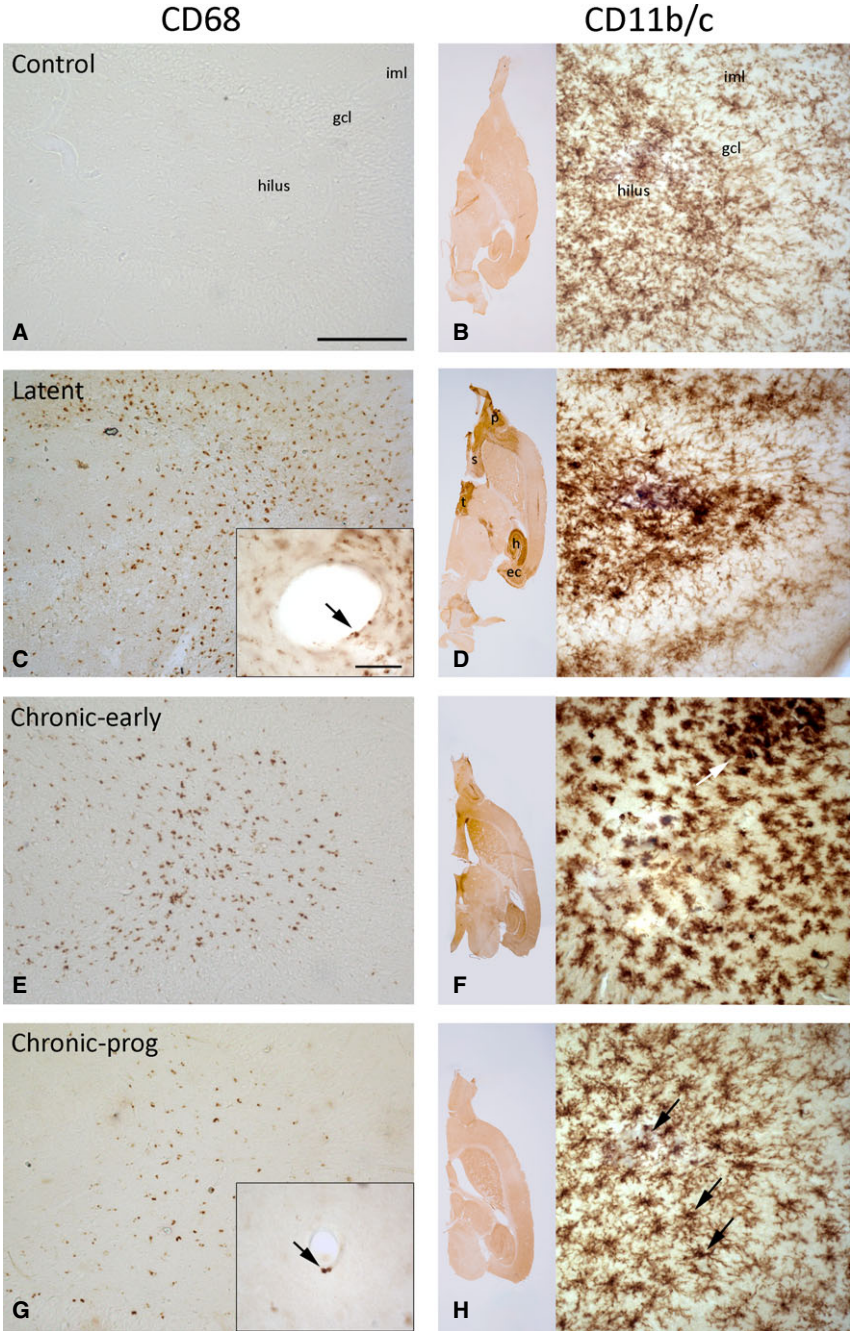

FIGURE 2 CD68 and CD11b/c immunoreactivity in rat brain. CD68 staining in the dentate gyrus of a control rat (A), and in rats sacrificed during the latent period (C; 1 week after status epilepticus [SE]), early during the chronic period (E; 6 weeks after SE), and late during the chronic period (G; 7 months after SE). The latter rat had a progressive form of epilepsy. CD68 was not observed in controls. Higher expression was observed in the latent period (C) and throughout epileptogenesis, compared to controls. CD68-immunoreactive (IR) cells were present throughout the parenchyma, and some were in close proximity to blood vessels (arrows in the insets of C and G). Quantification of the number of CD68 cells (Table S4) showed that they were more abundant compared to controls at all time points throughout epileptogenesis. In addition, more CD68-IR cells were observed in rats with a progressive form of epilepsy (Chronic-prog) compared to rats that did not have a progressive form of epilepsy. CD11b/c staining in a control rat (B), and in rats sacrificed during the latent period (D; 1 week after SE), early during the chronic period (F; 6 weeks after SE), and late during the chronic period (H; 7 months after SE). The latter rat had a progressive form of epilepsy. In controls, CD11b/c-IR cells had the morphology of resting microglia cells. In contrast, CD11b/c-IR cells had the morphology of activated microglia cells during the latent period (D) and throughout epileptogenesis (F, H). Quantification of the number of CD11b/c cells (Table S4) in the hippocampus revealed that they were more abundant compared to controls at all time points throughout epileptogenesis. ec, entorhinal cortex; gcl, granule cell layer; h, hippocampus; iml, inner molecular layer; p, piriform cortex. Scale bar: A-H, 250 μm; insets, 20 μm

found with activated morphology in all hippocampal layers (Figure 2H). Both chronic nonprogressive and progressive rats had higher CD11b/c immunoreactivity compared to controls. No differences were found between nonprogressive and progressive rats (Table S4 and Figure S7F).

3.7 | CCL2 and osteopontin immunoreactivity in human and rat brain

Higher expression of CCL2 and osteopontin (OPN) was observed in epileptogenic human and rat hippocampus compared to controls. For details, see Appendix S1 (Materials and Methods).

3.8 | CD163 immunoreactivity in human brain

3.8.1 | Control

CD163 specifically labels mature macrophages and monocytes. In controls, a low number of CD163-positive cells was observed in the hippocampus (50 ± 10 cells, mean ± SEM). CD163-IR cells had an inactive morphology and were located in perivascular regions and around the meninges (Figure 1B).

3.8.2 | Status epilepticus

The number of CD163-IR cells was higher in the hippocampus of people who died after SE (4119 ± 2401). Perivascular CD163-IR cells had an activated morphology, with large cell bodies and coarse processes. Additionally, CD163 immunoreactivity was also observed in the parenchyma (Figure 1D).

3.8.3 | Temporal lobe epilepsy

A higher number of CD163-IR cells were observed in patients with TLE (no-HS, 122 ± 20; HS, 299 ± 90) as compared to controls. In patients with HS, CD163-IR cells were activated and immunoreactivity was observed around blood vessels as well as in the parenchyma (Figure 1F, H).

3.9 | CD163 immunoreactivity in rat brain

3.9.1 | Control

A low number of CD163-IR cells were observed in the hippocampus (31 ± 7 cells, mean ± SEM). These mature tissue macrophages were exclusively present around blood vessels and the meninges (Figure 3A) and not in the parenchyma.

3.9.2 | Acute and latent phase

The number of CD163-IR cells in the acute phase (77 ± 7) and latent phase (98 ± 5) was higher compared to controls, and these cells had the morphology of activated macrophages (Figure 3B and C). CD163-IR cells were only present around blood vessels and the meninges and not in the parenchyma.

3.9.3 | Early chronic phase

Less perivascular and meningeal CD163-IR cells were observed (81 ± 4) compared to the latent phase; however, more cells were present compared to controls and these cells had the morphology of activated macrophages (Figure 3D).

3.9.4 | Late chronic phase

The number of CD163-IR cells in the hippocampus of rats with a progressive form of epilepsy (105 ± 5) was higher compared to rats with a nonprogressive form of epilepsy (61 ± 3), and CD163-IR cells seem to favor the vascular wall (inset in Figure 3E) and the pia or vascular interface (lower left corner of Figure 3E). Both groups had more CD163-positive cells compared to controls.

3.10 | BBB leakage in relation to CD163 expression in rat brain

BBB permeability was assessed in the hippocampus by FSC entry and albumin immunohistochemistry. FSC and albumin were not detected in the hippocampus of control rats (Figure 4A, D), indicating an intact BBB, and a few CD163 cells were observed around blood vessels (red cells in Figure 4A, green cells in Figure 4D). Extravasation of FSC (green parenchymal staining in Figure 4B) and albumin (red parenchymal staining in Figure 4E) was evident during the acute phase, which coincided with an increase in the number of CD163 cells that had the morphology of activated macrophages, with large cell bodies and coarse processes (red cells in Figure 4B, green cells in Figure 4E). During the chronic epileptic phase, BBB leakage was still evident, although to a much smaller extent than in the acute phase. FSC was found as particles with high fluorescence intensity close to cell nuclei (Figure 4C). These particles were most evident in rats with a high seizure frequency. Immunostainings with anti-CD163 showed that FSC colocalized (yellow staining in Figure 4C) with CD163 (red cells in Figure 4C) in most cases. Albumin extravasation was more abundant compared to FSC (red staining in Figure 4F) and albumin also colocalized with CD163 (yellow staining in Figure 4F); however, it was also detected outside CD163-IR cells (green staining in Figure 4F). A significant positive correlation between the FSC

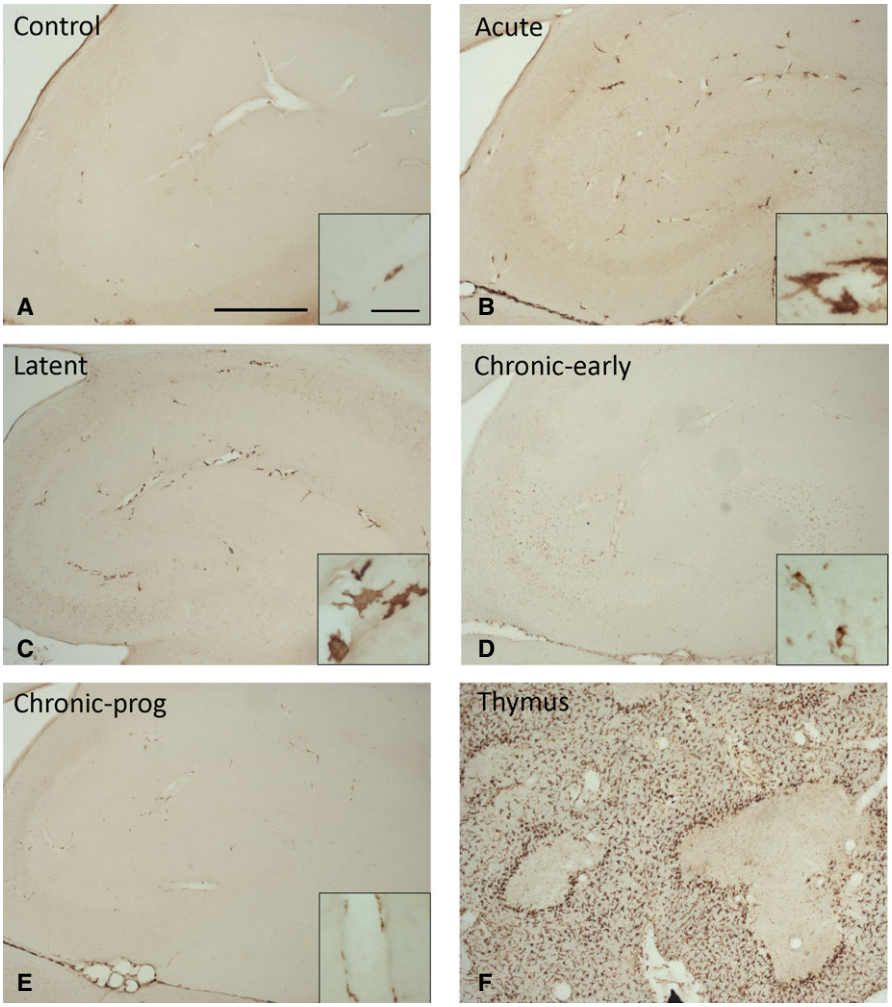

FIGURE 3 CD163 immunoreactivity in rat brain. CD163 staining in the hippocampus of a control rat (A), in rats sacrificed during the acute period (B; 1 day after status epilepticus [SE]), latent period (C; 1 week after SE), early during the chronic period (D; 6 week after SE), and late during the chronic period (7 months after SE), and in a rat that developed a progressive form of epilepsy (E; Chronic-prog). F, The thymus served as positive control. A low number of CD163-immunoreactive (IR) cells was observed in the hippocampus of controls (A). These mature tissue macrophages were exclusively present around blood vessels and the meninges and not in the parenchyma. During the acute phase, the number of CD163-IR cells was higher compared to controls and had the morphology of activated macrophages (B, C). CD163-IR cells were only present around blood vessels and the meninges and not in the parenchyma. During the early chronic phase, fewer perivascular and meningeal CD163-IR cells were observed compared to the acute and latent phase; however, they were more numerous compared to controls and had the morphology of activated macrophages (D). During the chronic phase, the number of IR cells in rats with a progressive form of epilepsy was higher compared to controls, but the morphology of CD163-IR macrophages was comparable to controls (E). Scale bar: A-F, 500 μm; insets, 20 μm

index (BBB permeability) and the number of hippocampal CD163-positive cells was observed (Figure 4G); Spearman rank, $\rho = 0.95$, $P < 0.05$.

3.11 | Brain inflammation and seizure activity

3.11.1 | Humans

To correlate protein expression of CD68 and CCL2 with epilepsy, the number of seizures per month, the duration of epilepsy, and the age of onset were related to protein expression for all patients who underwent surgery (both HS and no-HS cases). A positive correlation between duration of epilepsy and protein expression was found for CD68 (Figure 5A), CCL2 in neurons (Figure 5B), and CCL2 in glia (Figure 5C); Spearman rank order correlation, respectively: $\rho = 0.74$, 0.33, 0.60; $P < 0.05$. The seizure frequency and the age of onset were not correlated to CD68 or CCL2 expression.

3.11.2 | Rats

To correlate protein expression of CD68, CCL2, and CD163 with the induced seizure activity (the initial insult),

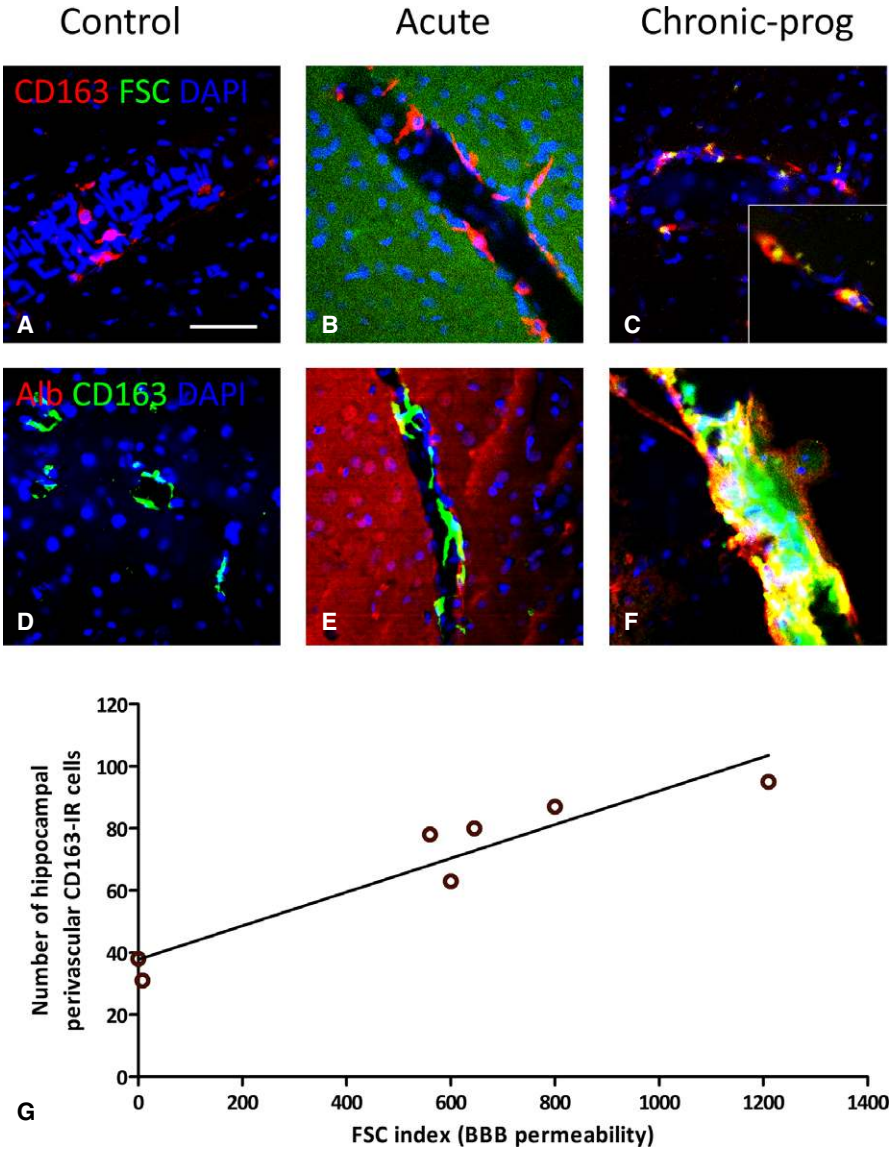

FIGURE 4 Blood-brain barrier (BBB) permeability and CD163 expression. BBB permeability was assessed in the hippocampus by fluorescein (FSC) entry and albumin immunohistochemistry. Sections were counterstained with 4,6-diamidino-2-phenylindole (DAPI; blue) to visualize nuclei. FSC was not detected in the hippocampus of control rats (A), indicating an intact BBB, and a few CD163-immunoreactive (IR) cells were observed around blood vessels (red cells in A, green cells in D). Extravasation of FSC (green parenchymal staining in B) and albumin (red parenchymal staining in E) was evident during the acute phase, which coincided with an increase in the number of CD163 cells that had the morphology of activated macrophages, with large cell bodies and coarse processes (red cells in B, green cells in E). During the chronic epileptic phase, BBB leakage was still evident, although to a much smaller extent than in the acute phase. FSC was found as particles with high fluorescence intensity close to cell nuclei (C). These particles were most evident in rats with a high seizure frequency. Immunostainings with anti-CD163 showed that FSC colocalized (yellow staining in C) with CD163 (red cells in C) in most cases. Albumin extravasation was more abundant compared to FSC (red staining in F), and albumin also colocalized with CD163 (yellow staining in F), although it was also detected outside CD163-IR cells (green staining in F). G, A significant positive correlation between the FSC index (BBB permeability) and the number of hippocampal CD163-positive cells was observed; Spearman rank order correlation, $\rho = 0.95$, $P < 0.05$. Scale bar: A-F, 20 μm

the duration of SE was related to protein expression for all rats that were sacrificed during the acute (1 day after SE) and latent (1 week after SE) phase. A positive correlation between SE duration and protein expression was found for CD68 (Figure 6A) and CCL2 in glia (Figure 6E) and CD163 (Figure 6G); Spearman rank order correlation, respectively: $\rho = 0.91$, 0.89, 0.87; $P < 0.05$. A negative correlation between SE duration and protein expression was found for CCL2 in neurons (Figure 6C); Spearman rank order correlation, $\rho = 0.89$; $P < 0.05$.

To correlate protein expression of CD68, CCL2, and CD163 with spontaneous seizures, the average number of

seizures per day during the week before the rats were sacrificed was related to protein expression for chronic epileptic rats (7-9 months after SE). A positive correlation between the seizure frequency and protein expression was found for CD68 (Figure 6B), CCL2 in neurons (Figure 6D), CCL2 in glia (Figure 6F), and CD163 (Figure 6H); Spearman rank order correlation, respectively: $\rho = 0.66, 0.51, 0.80, 0.78$; $P < 0.05$.

4 | DISCUSSION

The main finding of this study is that monocytes or macrophages and other cells of the innate immune response are persistently activated in the epileptogenic human and rat hippocampus, which is positively correlated with BBB dysfunction and seizure activity, whereas T cells and dendritic cells, which are closely associated with the adaptive immune response, were sparsely detected in the hippocampus during epileptogenesis. Furthermore, higher expression of CCL2 and OPN was observed. We will discuss this in more detail in the following paragraphs.

4.1 | Adaptive and innate immune response

Monocyte infiltration, microglia, and perivascular macrophage activation were persistently increased in both epileptogenic human and rat hippocampus, which coincided with increased protein expression of CCL2 and OPN, whereas T lymphocytes and dendritic cells were not or were scarcely detected. In TLE patients, it has been reported that peripheral T cells that are associated with the adaptive immune system can be detected around blood vessels, but they are scarce.[4,15,20] Furthermore, penetration of green fluorescent protein–labeled peripheral white blood cells into brain parenchyma was limited after pilocarpine-induced SE.[20] The scarce presence of these cells suggests that they play a less prominent role than the dramatically increased activated cells of the innate immune response (eg, monocytes, microglia, and astrocytes).[4,7] However, in patients with focal cortical dysplasia or Rasmussen encephalitis, activation of both the adaptive and innate immunity was shown,[6,21] and a prominent role for lymphocytes in epilepsy was suggested in mice and rats in which epilepsy was induced by the chemoconvulsant pilocarpine.[8,15] In contrast to these findings, an antiepileptogenic and neuroprotective role for both innate and adaptive immune cells has been suggested after kainic acid–induced SE in mice.[22] Taken together, the innate immune response is mainly activated in the epileptogenic brain, suggesting a prominent role during epileptogenesis, whereas activation of the adaptive immune response may depend on the type of epilepsy, animal model, species, or neuropathology.

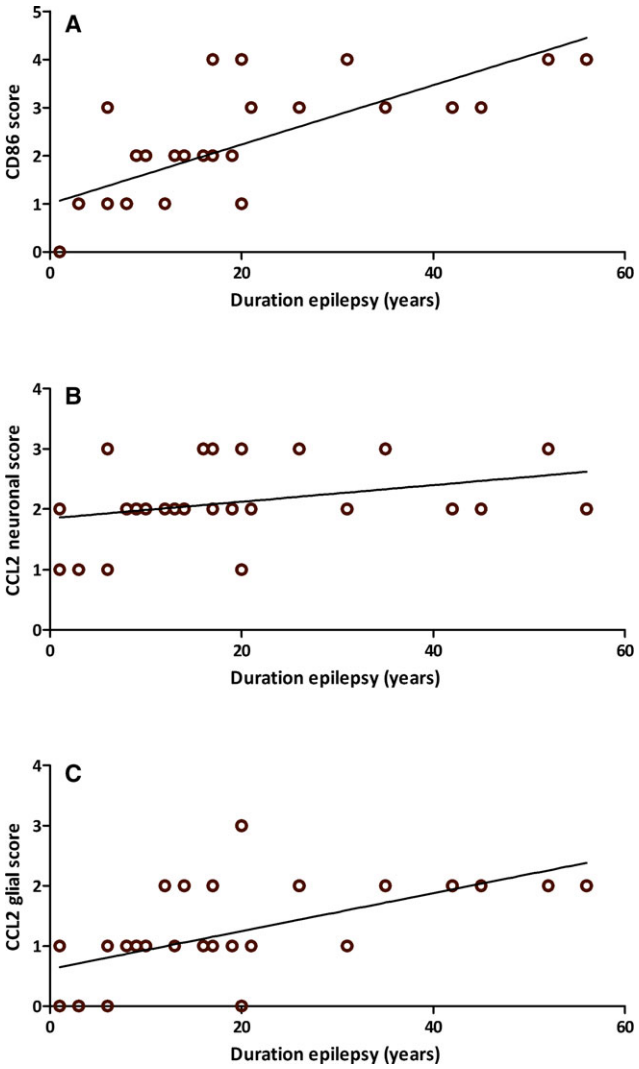

FIGURE 5 CD68 and CCL2 expression in the human brain in relation to seizure activity. To correlate protein expression of CD68 and CCL2 with epilepsy, the number of seizures per month, the duration of epilepsy, and the age of onset were related to protein expression for all patients who underwent surgery (both hippocampal sclerosis [HS] and no-HS cases). A positive correlation between duration of epilepsy and protein expression was found for CD68 (A), CCL2 in neurons (B), and CCL2 in glia (C); Spearman rank order correlation, $P < 0.05$, ρ values, respectively: 0.74, 0.33, and 0.60

4.2 | Brain inflammation in relation to seizure activity

In rats, the expression of CD68, CCL2, and the perivascular macrophage marker CD163 was related to the duration of SE, indicating that higher expression of these proteins is triggered by the initial insult. Furthermore, higher expression was also evident during the latent phase, when seizures are absent, and during the chronic epileptic phase, when recurrent seizures are detected. The expression of CD68, CCL2, and CD163 was related to the number spontaneous seizures during the chronic phase in the rat,

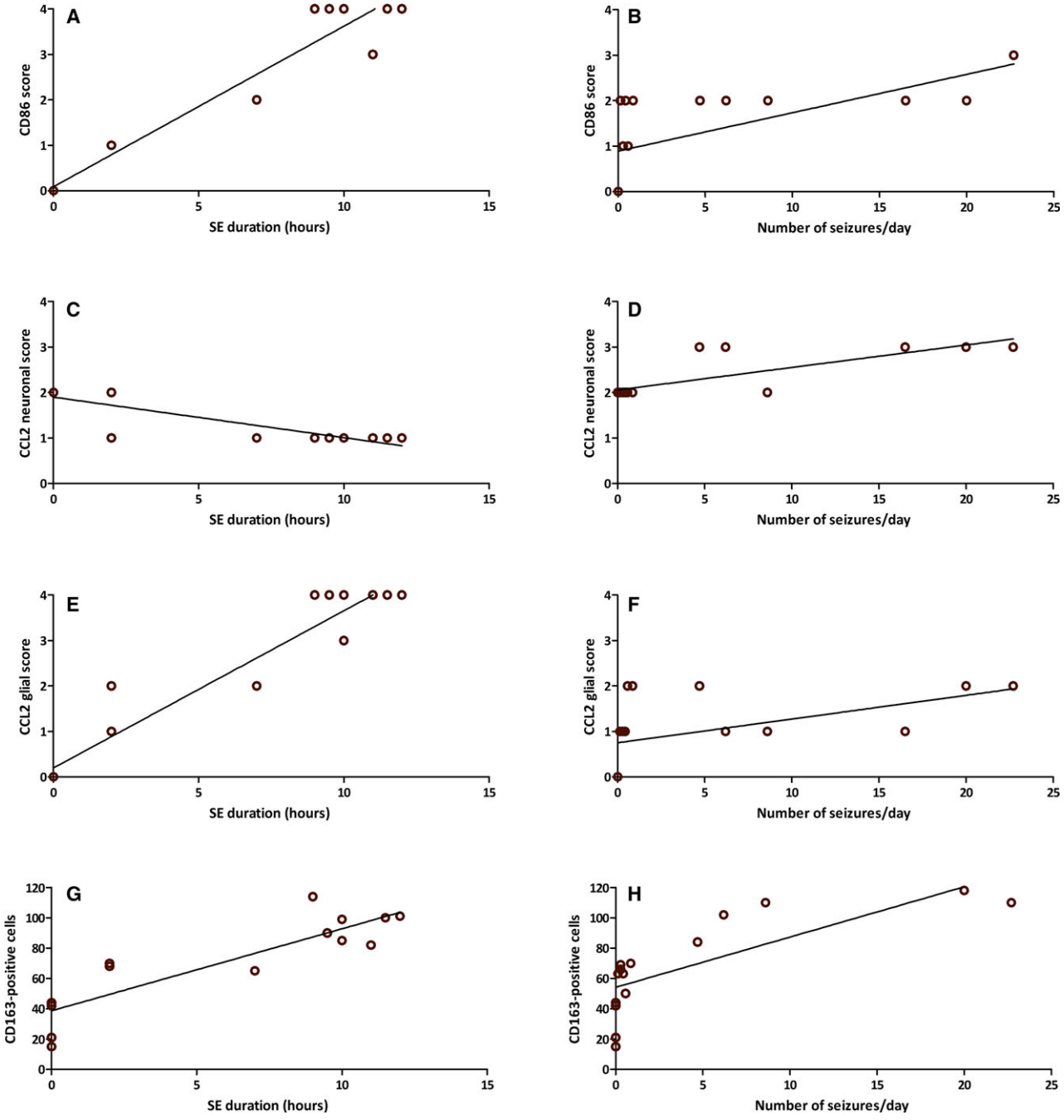

FIGURE 6 CD68, CCL2, and CD163 expression in the rat brain in relation to seizure activity. To correlate protein expression of CD68, CCL2, and CD163 with the induced seizure activity (the initial insult), the duration of status epilepticus (SE) was related to protein expression for all rats that were sacrificed during the acute (1 day after SE) and latent (1 week after SE) phase. A positive correlation between SE duration and protein expression was found for CD68 (A) and CCL2 in glia (E) and CD163 (G); Spearman rank order correlation, $P < 0.05$, ρ values, respectively: 0.91, 0.89, and 0.87. C, A negative correlation between SE duration and protein expression was found for CCL2 in neurons; Spearman rank order correlation, $P < 0.05$, $\rho = 0.89$. To correlate protein expression of CD68, CCL2, and CD163 with spontaneous seizures, the average number of seizures per day during the week before the rats were sacrificed was related to protein expression for chronic epileptic rats (7-9 months after SE). A positive correlation between the seizure frequency and protein expression was found for CD68 (B), CCL2 in neurons (D), CCL2 in glia (F), and CD163 (H); Spearman rank order correlation, $P < 0.05$, ρ values, respectively: 0.66, 0.51, 0.80, and 0.78

suggesting that these proteins can contribute to epileptogenesis and progression of epilepsy. In humans, the expression of CD68 and CCL2 was related to the duration of epilepsy and the type of pathology. Although the expression of CD68 and CCL2 was not related to seizure frequency in humans (which can be due to patients taking antiepileptic

drugs), a strong positive correlation ($\rho = 0.74$) was found between CD68 expression and the duration of epilepsy and a weak to moderate correlation between CCL2 in respective neurons ($\rho = 0.33$) and glia ($\rho = 0.60$) and the duration of epilepsy. In addition, the activation of the inflammatory response was related to the neuropathology, as inflammation was more pronounced in patients with hippocampal sclerosis compared to patients without hippocampal sclerosis. Likewise, more extensive inflammation was found in rats that had progressive development of epilepsy. Clinical observations and data from animal studies propose that brain inflammation is a common factor contributing to, or predisposing one to, the occurrence of seizures in various forms of epilepsy, especially TLE.[6,23] Because brain inflammation was already present during the acute and latent period in our study, which was also reported by others[4] and preceded the onset of epilepsy, our data support a causal role of inflammation in the generation of spontaneous seizures. Furthermore, our data suggest that macrophages or monocytes, as detected by the specific markers CD68 and CD163,[24] can play a prominent role and contribute to epileptogenesis.

4.3 | OPN and CCL2 expression

OPN, a glycoprotein that promotes macrophage migration,[25] was persistently increased in astrocytes and microglia during epileptogenesis in humans and rats, which confirmed previous findings.[13,26] Interestingly, it has been reported that upregulation of OPN was markedly suppressed in the absence of the cytokine CCL2 (also known as monocyte chemoattractant protein-1),[27] indicating that CCL2 can induce OPN expression. We previously showed that CCL2 was the most activated gene, which peaked early during epileptogenesis in rats.[13] In agreement with this, we detected a persistent increased expression of CCL2 in astrocytes of the epileptogenic human and rat brain. CCL2 is a potent chemoattractant for cells of the monocyte lineage (including macrophages, monocytes, and microglia),[28-31] which may explain the increase of macrophages or monocytes that we observed in the epileptogenic human and rat brain. Increased expression of CCL2 has also been observed in mice and rats 1-3 days after intrahippocampal–kainic acid injection,[32,33] in mice in which pilocarpine was used to induce SE,[34] and in the human epileptogenic brain.[31] CCL2 can induce the release of proinflammatory cytokines (eg, interleukin-1β) and lead to neurodegeneration and BBB disruption.[30,32,35-37] Because these changes can contribute to the occurrence of seizures, this suggests an important role for CCL2 during epileptogenesis.

4.4 | BBB dysfunction

Increased secretion of CCL2 by astrocytes and monocytes or macrophages in the epileptogenic brain can lead to opening of the BBB.[37] Furthermore, we showed that the number of CD163-positive perivascular macrophages was positively correlated to BBB permeability in chronic epileptic rats, suggesting an important role for these cells in BBB dysfunction. This is in line with a previous study in which resected brain tissue from patients with TLE was used, showing a strong presence of inflammatory mediators and BBB leakage in cases with epilepsy.[38] Because disruption of the BBB can affect epileptogenesis and may lead to progression of epilepsy,[5,9,11,20] inhibition of brain inflammation may decrease CCL2 secretion from astrocytes, monocytes, and macrophages and can be a potential therapy to reduce seizures.

Interestingly, dexamethasone reduced the number of seizures after pilocarpine-induced SE in rats and improved BBB integrity. In addition, rats that received CD163-targeted liposomes were partially protected against 6-hydroxydopamine–induced dopaminergic neurodegeneration.[39] Furthermore, glucocorticosteroids reduced the occurrence of seizures in pediatric drug-resistant epilepsy patients in the same study.[10] Limiting the entry of blood monocytes via blockade of CCL2 synthesis or its receptor CCR2 reduced lipopolysaccharide-induced seizures,[31] and attenuated BBB disruption and neuronal damage after pilocarpine-induced SE.[40] Therefore, it is tempting to speculate that blood monocytes that enter the brain during epileptogenesis are proepileptogenic. Future studies should reveal whether more specific manipulation (eg, aimed at CCL2) can modify or prevent epileptogenesis in TLE. Drugs blocking CCL2/CCR2 signaling are currently under development for several inflammatory brain disorders[31] and could also be tested in drug-resistant epilepsy.

In conclusion, these data show that the innate immune response is persistently activated in human TLE and in the electrical post-SE rat model. This suggests a proepileptogenic role of the innate immune response, particularly for monocytes and (perivascular) macrophages possibly via increased leakage of the BBB, whereas the adaptive immune system does not seem to play a major role. Future studies should reveal whether specific manipulation of the innate immune response can modify or prevent epileptogenesis in TLE.

ACKNOWLEDGMENTS

The research leading to these results has received funding from the European Union's Seventh Framework Program (FP7/2007-2013) under grant agreement 602102 (EPITARGET; E.A.v.V., J.A.G., E.A.) and the Dutch Epilepsy Foundation, project 16-05 (D.W.M.B., E.A.v.V.).

DISCLOSURE

The authors have no conflicts of interest to report. We confirm that we have read the Journal's position on issues

involved in ethical publication and affirm that this report is consistent with those guidelines.

REFERENCES

1. Vezzani A, French J, Bartfai T, et al. The role of inflammation in epilepsy. Nat Rev Neurol. 2011;7:31–40.
2. van Vliet EA, Aronica E, Vezzani A, et al. Review: neuroinflammatory pathways as treatment targets and biomarker candidates in epilepsy: emerging evidence from preclinical and clinical studies. Neuropathol Appl Neurobiol. 2018;44:91–111.
3. Holtman L, van Vliet EA, van Schaik R, et al. Effects of SC58236, a selective COX-2 inhibitor, on epileptogenesis and spontaneous seizures in a rat model for temporal lobe epilepsy. Epilepsy Res. 2009;84:56–66.
4. Ravizza T, Gagliardi B, Noe F, et al. Innate and adaptive immunity during epileptogenesis and spontaneous seizures: evidence from experimental models and human temporal lobe epilepsy. Neurobiol Dis. 2008;29:142–60.
5. van Vliet EA, da Costa Araújo S, Redeker S, et al. Blood-brain barrier leakage may lead to progression of temporal lobe epilepsy. Brain. 2007;130:521–34.
6. Vezzani A, Granata T. Brain inflammation in epilepsy: experimental and clinical evidence. Epilepsia. 2005;46:1724–43.
7. Crespel A, Coubes P, Rousset MC, et al. Inflammatory reactions in human medial temporal lobe epilepsy with hippocampal sclerosis. Brain Res. 2002;952:159–69.
8. Marchi N, Johnson AJ, Puvenna V, et al. Modulation of peripheral cytotoxic cells and ictogenesis in a model of seizures. Epilepsia. 2011;52:1627–34.
9. Marchi N, Angelov L, Masaryk T, et al. Seizure-promoting effect of blood-brain barrier disruption. Epilepsia. 2007;48:732–42.
10. Marchi N, Granata T, Freri E, et al. Efficacy of anti-inflammatory therapy in a model of acute seizures and in a population of pediatric drug resistant epileptics. PLoS One. 2011;6:e18200.
11. Seiffert E, Dreier JP, Ivens S, et al. Lasting blood-brain barrier disruption induces epileptic focus in the rat somatosensory cortex. J Neurosci. 2004;24:7829–36.
12. van Gassen KL, de Wit M, Koerkamp MJ, et al. Possible role of the innate immunity in temporal lobe epilepsy. Epilepsia. 2008;49:1055–65.
13. Gorter JA, Van Vliet EA, Aronica E, et al. Potential new antiepileptogenic targets indicated by microarray analysis in a rat model for temporal lobe epilepsy. J Neurosci. 2006;26:11083–110.
14. Uva L, Librizzi L, Marchi N, et al. Acute induction of epileptiform discharges by pilocarpine in the in vitro isolated guinea-pig brain requires enhancement of blood-brain barrier permeability. Neuroscience. 2008;151:303–12.
15. Fabene PF, Navarro Mora G, Martinello M, et al. A role for leukocyte-endothelial adhesion mechanisms in epilepsy. Nat Med. 2008;14:1377–83.
16. Balosso S, Maroso M, Sanchez-Alavez M, et al. A novel non-transcriptional pathway mediates the proconvulsive effects of interleukin-1beta. Brain. 2008;131:3256–65.
17. Vezzani A, Conti M, De Luigi A, et al. Interleukin-1beta immunoreactivity and microglia are enhanced in the rat hippocampus by focal kainate application: functional evidence for enhancement of electrographic seizures. J Neurosci. 1999;19:5054–65.
18. Vezzani A, Friedman A. Brain inflammation as a biomarker in epilepsy. Biomark Med. 2011;5:607–14.
19. Cerri C, Caleo M, Bozzi Y. Chemokines as new inflammatory players in the pathogenesis of epilepsy. Epilepsy Res. 2017;136:77–83.
20. Marchi N, Teng Q, Ghosh C, et al. Blood-brain barrier damage, but not parenchymal white blood cells, is a hallmark of seizure activity. Brain Res. 2010;1353:176–86.
21. Iyer A, Zurolo E, Spliet WG, et al. Evaluation of the innate and adaptive immunity in type I and type II focal cortical dysplasias. Epilepsia. 2010;51:1763–73.
22. Zattoni M, Mura ML, Deprez F, et al. Brain infiltration of leukocytes contributes to the pathophysiology of temporal lobe epilepsy. J Neurosci. 2011;31:4037–50.
23. Yang T, Zhou D, Stefan H. Why mesial temporal lobe epilepsy with hippocampal sclerosis is progressive: uncontrolled inflammation drives disease progression? J Neurol Sci. 2010;296:1–6.
24. Dijkstra CD, Dopp EA, Joling P, et al. The heterogeneity of mononuclear phagocytes in lymphoid organs: distinct macrophage subpopulations in the rat recognized by monoclonal antibodies ED1, ED2 and ED3. Immunology. 1985;54:589–99.
25. Scatena M, Liaw L, Giachelli CM. Osteopontin: a multifunctional molecule regulating chronic inflammation and vascular disease. Arterioscler Thromb Vasc Biol. 2007;27:2302–9.
26. Kim SY, Choi YS, Choi JS, et al. Osteopontin in kainic acid-induced microglial reactions in the rat brain. Mol Cells. 2002;13:429–35.
27. Dewald O, Zymek P, Winkelmann K, et al. CCL2/monocyte chemoattractant protein-1 regulates inflammatory responses critical to healing myocardial infarcts. Circ Res. 2005;96:881–9.
28. Izikson L, Klein RS, Luster AD, et al. Targeting monocyte recruitment in CNS autoimmune disease. Clin Immunol. 2002;103:125–31.
29. Mahad D, Callahan MK, Williams KA, et al. Modulating CCR2 and CCL2 at the blood-brain barrier: relevance for multiple sclerosis pathogenesis. Brain. 2006;129:212–23.
30. Deng YY, Lu J, Ling EA, et al. Monocyte chemoattractant protein-1 (MCP-1) produced via NF-kappaB signaling pathway mediates migration of amoeboid microglia in the periventricular white matter in hypoxic neonatal rats. Glia. 2009;57:604–21.
31. Bozzi Y, Caleo M. Epilepsy, seizures, and inflammation: role of the C-C motif ligand 2 chemokine. DNA Cell Biol. 2016;35:257–60.
32. Sheehan JJ, Zhou C, Gravanis I, et al. Proteolytic activation of monocyte chemoattractant protein-1 by plasmin underlies excitotoxic neurodegeneration in mice. J Neurosci. 2007;27:1738–45.
33. Manley NC, Bertrand AA, Kinney KS, et al. Characterization of monocyte chemoattractant protein-1 expression following a kainate model of status epilepticus. Brain Res. 2007;1182:138–43.
34. Turrin NP, Rivest S. Innate immune reaction in response to seizures: implications for the neuropathology associated with epilepsy. Neurobiol Dis. 2004;16:321–34.
35. Dinkel K, MacPherson A, Sapolsky RM. Novel glucocorticoid effects on acute inflammation in the CNS. J Neurochem. 2003;84:705–16.

36. Jiang Y, Beller DI, Frendl G, et al. Monocyte chemoattractant protein-1 regulates adhesion molecule expression and cytokine production in human monocytes. J Immunol. 1992;148:2423–8.
37. Yadav A, Saini V, Arora S. MCP-1: chemoattractant with a role beyond immunity: a review. Clin Chim Acta. 2010;411:1570–9.
38. Liu JY, Thom M, Catarino CB, et al. Neuropathology of the blood-brain barrier and pharmaco-resistance in human epilepsy. Brain. 2012;135:3115–33.
39. Tentillier N, Etzerodt A, Olesen MN, et al. Anti-inflammatory modulation of microglia via CD163-targeted glucocorticoids protects dopaminergic neurons in the 6-OHDA Parkinson's disease model. J Neurosci. 2016;36:9375–90.
40. Varvel NH, Neher JJ, Bosch A, et al. Infiltrating monocytes promote brain inflammation and exacerbate neuronal damage after status epilepticus. Proc Natl Acad Sci U S A. 2016;113:E5665–74.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

How to cite this article: Broekaart DWM, Anink JJ, Baayen JC, et al. Activation of the innate immune system is evident throughout epileptogenesis and is associated with blood-brain barrier dysfunction and seizure progression. *Epilepsia*. 2018;59:1931–1944. https://doi.org/10.1111/epi.14550