Macrophage depletion by liposome-encapsulated clodronate suppresses seizures but not hippocampal damage after acute viral encephalitis

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Abbreviations: ANOVA, analysis of variance; B6, C57BL/6; DA, Daniel; DAPI, 4',6-diamidino-2-phenylindole; FJC, fluoro-Jade C; Gr-1, anti-granulocyte receptor-1; Iba1; ionized calcium-binding adaptor molecule 1; IHC, immunohistochemistry; IL-6, interleukin-6; PBS, phosphate-buffered saline; TMEV, Theiler's murine encephalomyelitis virus; TLE, temporal lobe epilepsy

Abstract

Viral encephalitis is a major risk factor for the development of seizures and epilepsy, but the underlying mechanisms are only poorly understood. Mouse models such as viral encephalitis induced by intracerebral infection with Theiler's virus in C57BL/6 (B6) mice allow advancing our understanding of the immunological and virological aspects of infection-induced seizures and their treatment. Previous studies using the Theiler's virus model in B6 mice have indicated that brain-infiltrating inflammatory macrophages and the cytokines released by these cells are key to the development of acute seizures and hippocampal damage in this model. However, approaches used to prevent or reduce macrophage infiltration were not specific, so contribution of other mechanisms could not be excluded. In the present study, we used a more selective and widely used approach for macrophage depletion, i.e., systemic administration of clodronate liposomes, to study the contribution of macrophage infiltration to development of seizures and hippocampal damage. By this approach, almost complete depletion of monocytic cells was achieved in spleen and blood of Theiler's virus infected B6 mice, which was associated with a 70% decrease in the number of brain infiltrating macrophages as assessed by flow cytometry. Significantly less clodronate liposome-treated mice exhibited seizures than liposome controls (P<0.01), but the development of hippocampal damage was not prevented or reduced. Clodronate liposome treatment did not reduce the increased Iba1 and Mac3 labeling in the hippocampus of infected mice, indicating that activated microglia may contribute to hippocampal damage. The unexpected mismatch between occurrence of seizures and hippocampal damage is thought-provoking and suggests that the mechanisms involved in degeneration of specific populations of hippocampal neurons in encephalitis-induced epilepsy are more complex than previously thought.

Introduction

Infections of the central nervous system (CNS) are among the most common risk factor for seizures and acquired epilepsy (Vezzani et al., 2016). Over 100 different neurotropic viruses can cause encephalitis in humans, which is often associated with early (insult-associated) and late (spontaneous) seizures (Getts et al., 2008; Misra et al., 2008; Singhi, 2011). Mesial temporal lobe epilepsy (TLE) is the most common type of viral encephalitis-induced epilepsy in adult patients and is typically associated with hippocampal sclerosis (Vezzani et al., 2016). As yet there is no prophylactic treatment to prevent epilepsy after infection, which presents a major unmet clinical need (Löscher et al., 2013). Animal models of viral encephalitis-induced seizures are useful to understand the processes leading from encephalitis to early and late seizures (Vezzani et al., 2016; DePaula-Silva et al., 2017).

One emerging animal model of viral encephalitis-induced seizures is based on infection with Theiler's virus (also termed Theiler's murine encephalomyelitis virus [TMEV]), a non-enveloped, positive-sense, single-stranded RNA virus of the *Picornaviridae* family and *Cardiovirus* genus that is a naturally occurring enteric pathogen of the mouse (Libbey and Fujinami, 2011; DePaula-Silva et al., 2017). In C57BL/6 (B6) mice, intracerebral inoculation of this virus induces encephalitis, particularly in the hippocampus, leading to early and late seizures and hippocampal damage resembling mesial TLE in humans (Libbey and Fujinami, 2011; Vezzani et al., 2016; DePaula-Silva et al., 2017). Two groups independently reported that brain-infiltrating inflammatory monocytes/macrophages damage the hippocampus (Howe et al., 2012a) and are key to the development of acute seizures (Cusick et al., 2013) following infection with Theiler's virus in B6 mice. Howe et al. (2012a) used treatment with an anti-granulocyte receptor-1 (Gr-1) antibody, which depletes inflammatory monocytes and neutrophil granulocytes, while Cusick et al. (2013) used treatment with minocycline or wogonin to reduce infiltration of macrophages into the brain. However,

minocycline and wogonin exert a variety of other effects that could suppress encephalitis-associated acute seizures, including anti-inflammatory, anti-oxidant, neuroprotective and anti-seizure effects as well as modulatory effects on various cell signaling pathways, including the PI3K/AKT/NF-κB pathway (Park et al., 2007; Leite et al., 2011; Chen et al., 2012; Bialer et al., 2015; Safdari et al., 2015). Therefore, it is difficult to explain effects of these treatments on acute seizures in Theiler's virus model solely by a reduction in the extent of macrophage infiltration. Furthermore, Cusick et al. (2013) did not report the effect of drug treatment, if any, on hippocampal damage, while Howe et al. (2012a) did not report whether treatment with the Gr-1 antibody affected the development of seizures, although there is some evidence that hippocampal damage and occurrence of acute seizures might be correlated in the Theiler's virus model (Libbey et al., 2008; Bröer et al., 2016).

In the present study, we used a more selective approach to decrease macrophage brain infiltration after infection with Theiler's virus and studied consequences for both occurrence of early seizures and hippocampal damage. This approach is the so-called liposome-mediated macrophage 'suicide' approach, which is frequently applied in studies aimed at unravelling macrophage function (van Rooijen and Sander, 1994; van Rooijen and van Kesteren-Hendrikx, 2002; van Rooijen and Hendrikx, 2010; Frediani and Bertoldi, 2015). In this approach, upon systemic administration of liposome-encapsulated clodronate (dichloromethylene diphosphonate) the liposomes are phagocytosed and digested by peripheral monocytes/macrophages, followed by intracellular release and accumulation of clodronate, causing >90% depletion of these cells by apoptosis within 24–36 h (van Rooijen et al., 1994). Administration of clodronate liposomes does not affect neutrophils or lymphocytes (van Rooijen et al., 1989; van Rooijen and Sanders, 1994; Alves-Rosa et al., 2000). Unexpectedly, when using this more selective approach for reducing brain infiltration of macrophages, we found no reduction in hippocampal damage, whereas the occurrence of early seizures was significantly reduced.

Materials and methods

Animals

Six-week-old female C57BL/6J (B6) mice (body weight 18-20 g) were purchased from Charles River (Sulzfeld, Germany) and kept in groups of five to eight animals under controlled environmental conditions (22-24°C, 50-60% humidity, 12/12 light/dark cycle) and ad libitum access to standard rodent diet (Altromin 1324 standard diet; Altromin, Lage, Germany) and tap water. Environmental enrichment was ensured by group houses and nesting material. Female mice were used to allow group housing (to enable normal social behavior and interactions between the animals) over several months without the problems owing to the naturally aggressive behavior of male mice. Libbey et al. (2008) have shown previously that the consequences of infection with Theiler's virus do not differ between male and female B6 mice. All animal experiments were conducted in accordance with the German Animal Welfare Law and were authorized by the local government (LAVES, Oldenburg, Germany, permission number 33.12-42502-04-15/1892).

Infection

At the age of 7 weeks, mice were either intracerebrally infected with 20 µl of TMEV (Daniel's strain [DA], 2.44 x 10^7 PFU [plaque forming units] diluted in cell culture medium [Dulbecco's Modified Eagle's Medium; PAA Laboratories, Cölbe, Germany] with 2 % fetal calf serum and 50 µg/kg gentamicin) or mock-infected with 20 µl of virus-free medium. The dose of TMEV was based on previous experiments of our group in B6 mice, to ensure a high incidence of early seizures in this mouse strain (Bröer et al., 2016). For infection, mice were deeply anesthetized with isoflurane inhalation and slowly injected into the left parietal cortex using a free-hand method of injection according to Libbey et al. (2008), using a 26G cannula und 50 µl syringe (Hamilton, Bonaduz, Switzerland). Depth of entry was limited to 2 mm by

using a stop collar. Mice were allowed to recover from anesthesia, before being returned to their home cages.

Drug treatment

In preliminary experiments, different dosages and routes of administration of clodronate liposomes were evaluated, based on previously described protocols (cf., van Rooijen et al., 1996; Seiler et al., 1997; Zattoni et al., 2011; Ma et al., 2016). One group of animals received clodronate liposomes intravenously (i.v.) in two different dosages (either 100 µl per injection per mouse, or 200 µl per injection per mouse, corresponding to ~54-63 and ~108-125 µl per 10 g body weight, respectively), and the other group received the substance intraperitoneally (i.p.), in the same dosages. Each animal received three doses of clodronate liposomes at intervals of 3 days. For comparison, other mice received the same injection volume of liposomes containing phosphate buffered saline (PBS) either i.p. or i.v.. Liposomes containing PBS or clodronate were purchased from Liposoma B.V. (Amsterdam, The Netherlands). The concentration of clodronate was ~5mg clodronate per ml. Following treatment, mice were closely observed for adverse effects. For this purpose, the Irwin screen was performed 1 h after each treatment as described in detail recently (Klee et al., 2015). Based on the results of these experiments (see Results), we decided to use a dosing protocol with an initial i.v. injection of 200 µl per mouse. To avoid toxicity, the dose for the second and third injection was lowered to 100 µl per 10 g bodyweight. The injections were repeated every three days, resulting in three injections per mouse in total. The substances were administered i.v. via the tail vein and, in some single cases of venous occlusion, i.p.

Three experiments were performed with this dosing protocol in the Theiler model: (1) The first, preliminary experiment was done to assess the tolerability of the dosing protocol in infected mice and determine its efficacy to deplete macrophages in spleen and blood. (2) The second experiment was the pivotal experiment for the present study and evaluated the effects

6

of clodronate on acute seizures as well as hippocampal pathology. (3) The third experiment was performed to determine whether the major effect of the treatment with clodronate liposomes, suppression of seizures, is reproducible.

One day before infection with TMEV, animals were randomly divided into control and treatment groups. Mice in the treatment group received clodronate liposomes whereas mice in the control groups received either PBS liposomes (same injection volume as the mice with clodronate liposomes) or no treatment at all. The latter group served to exclude that liposomes exerted any effect of their own, independent of clodronate. As shown in Fig. 1, TMEV was injected one day after the first clodronate liposome administration. Administration of clodronate liposomes was repeated twice at an interval of 3 days. This protocol was based on a study of Zattoni et al. (2011), in which administration of clodronate liposomes was started 1 day before intrahippocampal injection of kainate and repeated twice at 3–4 d intervals. Similar protocols with starting clodronate liposome administration one day before experimental brain injury were also used in models of neuroinflammation (Zito et al., 2001), stroke (Ma et al., 2016), and Parkinson's disease (Coté et al., 2015), based on the observation that injection of clodronate liposomes ensures the depletion of macrophages within 24 to 36 h after systemic administration (Seiler et al., 1997).

Surveillance

In order to assess the development of acute seizures, mice were monitored twice daily for one hour each (randomly between 7 a.m and 6 p.m) by experienced researchers over one week following the infection. Acute seizures were graded by the Racine seizure scale (1972): 1, mouth and facial movements; 2, head nodding; 3, forelimb clonus; 4, rearing; 5, rearing and falling. During monitoring, mice were weighed and clinically scored for general appearance, activity level and gait as described earlier (Bröer et al. 2016). During this procedure mice were handled with increased intensity, including mild cage shaking, exposure to loud noises

and repeated tail-lifting to trigger seizures in accordance with our earlier studies in this model (Bröer et al., 2016).

Perfusion

Seven days after infection animals were deeply anesthetized with chloralhydrate i.p. and transcardially perfused with PBS followed by 4% paraformaldehyde; animals that were used for flow cytometry were perfused with 4°C PBS only. Brains and spleens were removed and left in 10% formalin overnight before being embedded in paraffin and cut into 2 μm slice sections. Furthermore, blood was sampled for determination of monocytes. While brains were harvested and stored in 4°C PBS until being further processed for flow cytometry, spleens were left in paraformaldehyde overnight for histological processing.

Histology and immunohistochemistry

In accordance with our earlier studies (Bröer et al., 2016; Bröer et al., 2017) we compared inflammation and neurodegeneration in mice after mock infection and after TMEV infection. We also analyzed seizing and non-seizing mice separately. As the viral-induced encephalitis in this model is associated mainly with pathological changes within the hippocampal formation (Libbey et al., 2008), we immunostained sections of the dorsal hippocampus (at 2.08 ± 0.38 mm from bregma) by NeuN (for details see Polascheck et al., 2010). Damage in the hemisphere ipsilateral to the virus infection was scored as described recently (Bröer et al. 2016): 0 = no obvious damage; 1 = slightly abnormal appearance of the structure without clear evidence of visible neuron loss; 2 = lesions involving <20 % of neurons of the whole structure; 3 = lesions involving 20-50 % of neurons; 4 = lesions involving > 50 % of neurons. Intermediate scores (e.g., 0.5) were obtained if one person scored a finding as 1 and the other scientist as 0, resulting in an average score of 0.5. Based on scoring hippocampal damage in individual mice, the average and maximum damage in each animal were estimated. Maximum

damage was the maximum loss of neurons observed within a damaged area of the hippocampus, whereas average damage depicts the average degree of degeneration in the complete hippocampal subregion. In addition to scoring neurodegeneration, neurons were counted in the dentate hilus of NeuN-stained sections as previously described (Polascheck et al., 2010). Data from both hemispheres were analyzed separately. Furthermore, the size of lesion in CA1 and CA2 was morphometrically assessed in the NeuN-stained sections with Axiovision SE64 Rel. 4.9 (Carl Zeiss AG) using the microscope Leica DM LB (Leica Microsystems).

Additional sections (at 2.08 ± 0.38 mm from bregma) were stained by Fluoro-Jade C (FJC), a sensitive and specific fluorescent marker of neuronal degeneration (Schmued et al., 2005), as described in detail previously (Gröticke et al., 2007). FJC-positive neurons were counted in 8 square fields of a defined size (90,890 μm²) that were posed subsequently in the respective region of each section at each section level to cover a large part of the region (resulting in different numbers of square fields per region): CA1, four fields; CA3a, two fields; CA3c, one field; dentate hilus, one field (for details see Polascheck et al., 2010).

For labelling activated microglia and macrophages, a monoclonal rat anti-Mac3 (1:200, AbD Serotec, Oxford, UK) was used. Mac3 (also known as CD107b and LAMP-2) is a glycoprotein that is expressed at the plasma membrane of both activated microglia and infiltrated macrophages (Ho and Springer, 1983) and does not allow a differentiation of the two cell populations. Mac3 immunohistochemistry (IHC) was analysed in different hippocampal regions (CA1, CA2, CA3, dentate gyrus, hilus) and also in the cortex and thalamic/hypothalamic region, which exhibited particularly high signal. The number of Mac3-positive cells in different areas of the hippocampus as well as some other brain regions was assessed semi-quantitatively with the following score: 0, absent (no positive, activated cells within the region); 1, mild (single positive cells found in the region); 2, moderate (up to 30 % of region populated with positive cells); 3, severe (more than 30 % of region populated with

positive cells). Data from both hemispheres were analyzed separately except for thalamus/hypothalamus and cerebral cortex, where Mac3-positive cells were often in medial portions, so that these portions in both hemispheres were analyzed.

To further assess the activation status of myeloid cells in the brain, paraffin embedded sections (at 2.08 ± 0.38 mm from bregma) were stained with Iba1 (ionized calcium-binding adaptor molecule 1; Wako, Neuss, Germany) and Mac3 and analysed by fluorescence microscopy using the microscope Leica DM LB (Leica Microsystems) and Axiovision SE64 Rel. 4.9 (Carl Zeiss AG). DAPI (4',6-diamidino-2-phenylindole) was used for staining of cell nuclei. Pictures were taken and cells were counted using ImageJ software. Cell counting was performed based on the previously performed FJC analysis (Polascheck et al., 2010). Square fields of 250 μm x 250 μm were used and distributed in the hippocampal area as followed: CA1/2 - four square fields, CA3a - two square fields, CA3c - one square field, hilus - one square field and the vessel layer in between the pyramidal cell layer - three square fields. Initially Iba1 positive cells were counted within the square fields and thereafter cells were double stained for Iba1 and Mac3. Statistics were calculated for the number of Iba1 positive cells, the number of Iba1/Mac3 positive cells and for percentage of Mac3 positive cells within the Iba1 positive cells.

Spleen sections were stained with Iba1 for validation of depletion efficiency (see Peng et al., 2016 for details) and analysed with ImageJ (Schneider et al., 2012), where the positive pixel were counted.

Viral antigen labelling for Theiler's virus in brain sections was performed via IHC as well (see Kummerfeld et al., 2012 for details), using a polyclonal rabbit anti-TMEV capsid protein VP1-specific antibody (Kummerfeld et al., 2009), and the positive areas and the distribution of these within the brain were evaluated. Positive labelling in the hippocampus was scored as follows: 0, no stained cells; 1, few positively stained cells (<10%); 2, 10-25 % of cells stained; 3, >25-45 % of cells stained; 4, >45 % of cells stained.

All brain histological analyses were performed in a blinded fashion by two scientists. In case that the two persons obtained slightly different scores, the average score was used for final analysis of data.

Isolation of CNS immune cells and flow cytometry

CNS immune cells were isolated using neural tissue dissociation kit (P) as specified by the manufacturer (Miltenyi Biotec, Bergisch Gladbach, Germany). After preparing homogenous brain suspensions, cells were separated using a 30%-37%-70% percoll gradient and centrifugation for 30 min at 500 G without brakes. Percoll solutions of different densities were created mixing Percoll (Sigma-Aldrich Chemie Gmbh, Munich, Germany) and PBS (Thermo Fisher Scientific, Schwerte, Germany). Myelin and debris were discarded and the layer containing immune cells was collected, washed using MACS buffer (Miltenyi Biotec). Samples were stained for 30 minutes at 4°C using the following antibodies: CD45.2 Pacific blue (Biolegend, San Diego, CA, USA), CD11b APC-Cy7 (BD Biosciences; Heidelberg, Germany), CD86 PE-Cy5 (Biolegend), CD206 PE-Cy7 (Biolegend), and Ly6C Alexaflour700 (Biolegend). Unstained samples or samples incubated with respective isotype control antibodies were used to set gates.

For flow cytometry of blood cells, 25 μL whole EDTA blood was stained with different antibodies for 15 min at 4°C using following antibodies: B220 Pacific Blue, CD3 AF700, CSF-1R BV605 from Biolegend and CD11b APC-Cy7. Thereafter, erythrocytes were lysed and cells fixed by incubating blood samples with flow cytometry lysing solution (BD Biosciences) for 30 min at RT. The reaction was stopped by adding 1 ml of flow cytometry buffer. After centrifugation for 6 min at 300g supernatants were discarded.

Data were acquired on an LSRII flow cytometer (BD Biosciences) and analyzed with FlowJo software (Tree Star; Ashland, OR, USA). All flow cytometric analyses were performed in a blinded fashion.

Statistics

In all experiments, mice were randomly assigned to the drug and vehicle groups. Depending on whether data were normally distributed or not, either parametric or nonparametric tests were used for statistical evaluation. For comparison of two groups, either Student's t-test or the Mann-Whitney U-test was used. In case of more than two groups we used analysis of variance (ANOVA) with post hoc testing and correction for multiple comparisons. Depending on data distribution, either the ANOVA F-test, followed posthoc by Dunnett's multiple comparisons test, or the Kruskal-Wallis test followed posthoc by Dunn's multiple comparisons test were used. For comparison of frequencies in a 2 x 2 table, Barnard's unconditional test (Barnard, 1947) was used, because this test preserves the significance level and generally is more powerful than Fisher's exact test for moderate to small samples (Lydersen et al., 2009). Except Barnard's unconditional test (for which the online calculator SciStatCalc version 1.5 was used), all statistical analyses were performed with the Prism 6 software from GraphPad (La Jolla, CA, USA). All tests were used two-sided; a $P \leq 0.05$ was considered significant.

Results

Preliminary experiments with clodronate liposomes

Following repeated i.p. administration of clodronate liposomes (either 3x100 or 3x200 µl per mouse), weight loss and infrequently impaired general appearance (bend backwards position, sometimes even globe-shaped posture, reduced explorative behavior and reduced activity in general, piloerection) were observed, most likely due to irritation of the peritoneum after i.p. injection, especially after the higher dosage (200 µl) of clodronate liposomes. Post mortem examination showed that, particularly after i.p. administration of 200 µl, large amounts of the milky liposome preparation were found inside the abdominal cavity, indicating poor

absorption. IHC analysis of mouse spleens showed that a dose of 100 μl per injection does not lead to a sufficient depletion of macrophages (data not shown), independent of administration route (i.p. or i.v.), whereas a much more marked depletion was obtained with 200 μl (corresponding to ~1 mg liposome-encapsulated clodronate per mouse). The best result in depletion efficiency and tolerability was achieved by repeated i.v. administration of 200 μl. Thus, based on these results, we decided to use the following dosing protocol for treatment of infected mice: an i.v. bolus dose of 200 μl clodronate liposomes per mouse on day 1 (~ 108-125 μl/10 g bodyweight) , followed by two subsequent doses of 100 μl/10 g body weight at intervals of 3 days (Fig. 1). This dosing protocol was evaluated in a preliminary experiment in mice infected with Theiler's virus, showing that the animals tolerated the clodronate liposome treatment without any observable adverse effect or body weight loss other than the moderate (~7-10%) loss observed shortly after virus infection independent of liposome treatment. Furthermore, the treatment had almost completely depleted macrophages in spleen and blood when animals were killed 7 days after infection. Therefore, this dosing protocol was used for the experiments with clodronate liposomes described in the following (and illustrated in Fig. 1). Controls either received PBS liposomes or no treatment (to control for any effects of the liposomes alone). Because the two control groups did not differ significantly, they were combined for most data, with the exception of results on seizure occurrence (see below).

In the absence of clodronate liposome treatment, peripheral macrophages infiltrate the brain of virus-infected mice but not mock-infected mice

As described previously (Howe et al., 2012a; Cusick et al., 2013), flow cytometry was used to differentiate $CD45^{hi}$ $CD11b^+$ cells, which are considered to be mainly brain-infiltrating macrophages, from $CD45^{low}$ $CD11b^+$ cells, which are considered to be mainly resident microglia, in the brain (Fig. 2). Therefore, we will name these two cell populations infiltrating monocytes (macrophages) and microglia in the following. Seven days after infection, we

observed a marked (2100%) increase in the number of brain-infiltrating macrophages in Theiler virus-infected mice vs. mock-infected controls (Fig. 3A). In contrast, microglia was only increased by 60%, which did not reach statistical significance ($P = 0.0557$; Fig. 3B).

Treatment with clodronate liposomes depletes peripheral monocytes and macrophages and lowers brain infiltration of macrophages

As shown in Fig. 3C, treatment with clodronate liposomes induced an almost complete (-92%) depletion of Iba1-labelled macrophages in the spleen. Furthermore, CSF-1R positive monocytes in the blood were decreased by 97% (Fig. 3D). As reported previously (van Rooijen et al., 1989; van Rooijen and Sanders, 1994; Alves-Rosa et al., 2000), administration of clodronate liposomes did not affect neutrophils or lymphocytes as indicated by investigating the flow cytometry data of the blood using the side- and forward scatter (SSC-A, FSC-A)(not illustrated).

The depletion of peripheral monocytes and macrophages was associated with a significant (-72%) decrease in the number of brain-infiltrating macrophages ($P<0.01$; Fig. 3A). Furthermore, the number of resident microglia was significantly lower (-29%; $P<0.05$) compared to infected controls (Fig. 3B). Further evaluation of the flow cytometry data focused on the expression of Ly6C, CD86 and CD206 to determine the activation status of the myeloid cells. Data indicated activation of microglia as well as infiltrating monocytes by changes in morphology and by expression of activation marker such as CD86 and CD206 and presence of Ly6C on infiltrating monocytes in infected mice (not illustrated). There was no difference in the expression of any of these markers between control animals and animals that were given clodronate liposomes.

Treatment with clodronate liposomes decreases the occurrence of acute seizures

As reported previously (Libbey et al., 2008; Stewart et al., 2010; Bröer et al., 2016), the

encephalitis induced by intracerebral infection with Theiler's virus is associated with the occurrence of acute (early) seizures in the first week after infection. In the present experiments, 84% of untreated control mice exhibited such seizures. As shown in Fig. 4A, the occurrence of seizures was not affected by treatment with PBS liposomes when mice were compared with non-treated but infected controls. Treatment with clodronate liposomes significantly reduced seizure incidence by 40-44% when compared to treatment with PBS liposomes ($P<0.01$) or untreated controls ($P<0.05$). However, 50% of the clodronate-treated mice still exhibited acute seizures, which was significantly different from mock-infected controls ($P<0.05$; Fig. 4A). The severity of seizures was not different among groups (Fig. 4B). When seizure burden was calculated as the number of seizures per mouse observed over 7 days after infection, clodronate-treated mice exhibited a significantly lower seizure burden compared to either treatment with PBS liposomes ($P<0.05$) or untreated controls ($P<0.001$).

In addition to reducing the incidence of seizures, treatment with clodronate liposomes also affected the temporal occurrence of seizures in infected mice (Fig. 5). In untreated and PBS liposome-treated mice, most seizures occurred between 1-3 days after infection with a peak at 2 days, whereas peak of seizure incidence shifted to 3 days in mice treated with clodronate liposomes.

To evaluate whether the effect of clodronate liposomes on seizure occurrence is reproducible, the experiment was performed twice. In both experiments, treatment with clodronate liposomes decreased seizure occurrence by the same extent, so that the data were combined in Figs. 4 and 5.

Treatment with clodronate liposomes does not prevent hippocampal damage

Hippocampal pyramidal neurons, particularly in the CA1 and CA2 sectors, are rapidly injured in B6 mice after intracerebral infection with the DA strain of Theiler's virus, and it has been suggested that infiltration of inflammatory monocytes is critically involved in this damage

(Howe et al., 2012a). In the present experiments, we observed a large variation in the extent of hippocampal damage, when pyramidal neurons in CA1/CA2 were immunolabelled with NeuN (Fig. 6A-C). It has been previously reported that mice with acute seizures have more severe hippocampal damage than mice without such seizures (Libbey et al., 2008; Bröer et al., 2017), so we illustrate mice with and without seizures in Fig. 6. Indeed, a maximum damage score of 4 was observed in 5/13 mice with early seizures compared to 0/4 without early seizures, which, however, was not statistically significant because of the small group size of the animals without acute seizures (P = 0.1879). In contrast to the marked neurodegeneration in the CA1/CA2 layers of the hippocampus, only few mice exhibited damage in the CA3a and CA3c layers, and none of the mice showed damage in the dentate gyrus (Figs. 6 and 7). Also, the number of hilar neurons was not reduced by infection (Fig. 6D).

As shown in Fig. 6A-C, treatment with clodronate liposomes did not reduce the hippocampal damage in infected mice. Typical examples of severe hippocampal damage in mice with vs. without clodronate treatment are shown in Fig. 7B,C. For further evaluating potential effects of treatment, degenerating neurons were labelled with FJC. As shown in Fig. 7D,E and Fig. 8A, most FJC labelled neurons were observed in the CA1 and CA2 regions of the hippocampus, but some FJC-positive neurons were also seen in the CA3 and dentate hilus (Fig. 8B-D). Furthermore, some FJC positive neurons were irregularly observed in cerebral cortex and thalamus (data not shown). Treatment with clodronate liposomes did not significantly alter the number of FJC positive neurons in any region (Fig. 7D,E; Fig. 8).

Treatment with clodronate liposomes does not prevent the infection-induced increase in Mac3-staining in the hippocampus

We have previously reported that infection of B6 mice with Theiler's virus significantly increases Mac3-positive cells in the hippocampus (Bröer et al., 2016; Bröer et al., 2017). Mac3 labels both activated microglia and blood-borne macrophages that infiltrated into the

brain (Ho and Springer, 1983). Infected mice with acute seizures had significantly higher Mac3 labelling than mice without such seizures (Bröer et al., 2017). In the present study, a large variation in Mac3 labelling was observed in the hippocampal CA1 and CA2 layers of infected mice (Fig. 9A,B). Examples are shown in Fig. 7G,H. Treatment with clodronate liposomes did not reduce Mac3 labelling in the hippocampus (Fig. 7G,H; Fig. 9A,B). Increased Mac3 labelling was also observed in other brain regions, including the thalamus and hypothalamus and the cerebral cortex adjacent to the dorsal hippocampus (Fig. 9C,D). Again, no effect of clodronate liposomes was observed.

Clodronate liposome-treated mice exhibit increases in Iba1/Mac3-double labeled cells in the hippocampus

For further evaluating the activation status of myeloid cells in the brain, the amount of Iba1- and Mac3-immunolabelled cells was compared in the hippocampus. While Ib1 labels both resting and activated microglia and infiltrating macrophages, Mac3 labels only activated microglia and macrophages (Jeong et al., 2013; Goldmann et al., 2015; Skripuletz et al., 2015). Regarding Iba1 labeling, almost no such labeling was observed in mock-infected controls (Fig. 10A,D-F), while the number of Iba1-positive cells significantly increased in both infected controls and infected clodronate-treated mice in all hippocampal subfields examined (($P<0.05$ to <0.01, depending on region; Fig. 10B-F; CA1/CA2 and hilus shown as examples in Fig. 10D,E). There was no significant difference in the number of Iba1-positive cells per mm² between infected controls and clodronate-treated mice in any of the hippocampal regions. Depending on the hippocampal subregion and treatment, about 5-30% of the Iba1-labeled cells were also positive for Mac3 (Fig. 10B,C,F). By comparing the number of Iba1 and Mac3 double labelled cells between infected controls and clodronate-treated mice, a significantly ($P = 0.05$) higher number of double stained cells per mm² was determined in the dentate hilus of clodronate-treated animals (Fig. 10F), indicating more

activated myeloid cells (most likely microglia) within the hilus. A trend for more activated myeloid cells in clodronate-treated mice was also observed in all other hippocampal regions examined, but this did not become statistically significant when each region was separately examined (not shown). However, when we combined the data from all examined hippocampal subfields (CA1/CA2, CA3a, CA3c, hilus, vessel layer), a highly significant difference in activated (Iba1/Mac3-double labelled) myeloid cells was obtained for infected controls and infected mice treated with clodronate lioposomes. In infected controls, on average $9.2 \pm 2.4\%$ of the Iba1-positive cells were also positive for Mac3, compared to $19.4 \pm 2.9\%$ of the clodronate-treated infected mice ($P = 0.0026$).

During visual inspection of Iba1-labelled sections of infected mice, we observed Iba1-labeled myeloid cells not only in the hippocampus but also observed accumulation of such cells within the cortex, corpus callosum and thalamus/hypothalamus (not illustrated), indicating that these regions contributed to the inflammatory process. No difference was observed between infected controls and clodronate-treated mice.

Treatment with clodronate liposomes does not alter viral antigen presence in the hippocampus

Seven days after infection, most cells positive for virus antigen were seen in the hippocampus; only few positive cells were also seen in the cerebral cortex, thalamus and hypothalamus. Within the hippocampus, positive cells were always located in the CA1 sector (Fig. 11A,B) and, less frequently, in CA2 and CA3a. Based on morphology, viral antigen was exclusively located in pyramidal cells. No positive cells were observed in CA3c, dentate gyrus and dentate hilus. Labelled cells were observed more frequently in the ipsilateral than contralateral hippocampus (Fig. 11C,D). In several mice, no viral antigen labeling was seen in any region, indicating that the virus had already been eliminated. Treatment with clodronate liposomes did not alter viral antigen presence in the hippocampus (Fig. 11).

Discussion

Administration of liposome-encapsulated clodronate is one of the most selective procedures to deplete systemic macrophages (van Rooijen et al., 1994; van Rooijen et al., 2002; van Rooijen and Hendrikx, 2010). Free clodronate, which is not a toxic drug in itself, does not easily pass cell membranes, but clodronate liposomes are recognized as foreign particles and rapidly phagocytosed by macrophages (van Rooijen and Hendrikx, 2010). Clodronate, once delivered into phagocytic cells using liposomes as a Trojan horse strategy, will not escape from the cell but, after disruption of the phospholipid bilayers of the liposomes by lysosomal phospholipases in the macrophage, the drug accumulates intracellularly leading to cell death by apoptosis (van Rooijen and Hendrikx, 2010). Free clodronate, e.g. released from dead macrophages, has an extremely short half-life in the circulation, explaining the lack of any pronounced nonspecific effects of this drug following administration of the relatively low doses used for the liposome preparation (van Rooijen and Hendrikx, 2010). Clodronate liposomes do not pass the blood-brain barrier, so that macrophage depletion is restricted to the periphery (Huitinga et al., 1990). One limitation with clodronate liposomes, however, was the toxicity that we experienced in B6 mice. Indeed, the needed dose of clodronate to completely deplete macrophages was too toxic for B6 mice, so we could not completely prevent macrophage infiltration into the brain by the clodronate liposome dosing protocol used in the present study.

In the present study, systemic administration of tolerable doses of liposome-encapsulated clodronate led to an almost complete depletion of monocytic cells in spleen and blood of Theiler's virus infected mice, while treatment with PBS liposomes had no effect. Brain analysis of infiltrated macrophages and activated microglia by flow cytometry in Theiler's virus infected B6 mice confirmed previous reports (Howe et al., 2012a; Cusick et al., 2013), stating that the viral encephalitis is associated with a marked infiltration of

peripheral monocytes/macrophages into the brain. Howe et al. (2012a) showed that inflammatory monocytes are found in the brain of infected mice within 12 h after infection with the DA strain. Our own experiments on the time course of macrophage infiltration indicated significant infiltration at both 2 and 7 days after infection (unpublished). As shown here, at 7 days after infection, infected mice treated with clodronate liposomes had an about 70% decrease in the number of brain-infiltrated macrophages. In addition, a small but statistically significant decrease in microglia was observed when analyzing the whole brain. The incidence of acute seizures was significantly reduced by 40-44% in mice treated with clodronate liposomes. Interestingly, this effect is very similar to the 44% decrease in seizures reported for wogonin treatment by Cusick et al. (2013) in Theiler's virus infected B6 mice. However, in contrast to our expectation, hippocampal damage was not reduced by treatment with clodronate liposomes in the present study.

Intracerebral infection of B6 mice with TMEV leads to a circumscribed damage in the hippocampal formation that is characterized by a profound loss of CA1 and CA2 pyramidal neurons in the hippocampus, while other hippocampal regions, e.g., the CA3 and the dentate gyrus are spared (DePaula-Silva et al., 2017). This is a striking difference to other rodent models of TLE, including the intrahippocampal kainate model in mice, and patients with TLE, which typically exhibit hippocampal sclerosis with neuronal damage in CA1, CA3c and dentate hilus, while the CA2 sector is spared (Jefferys et al., 2016; Levesque et al., 2016). As in TLE, neurodegeneration in B6 mice with TMEV infection is not restricted to the hippocampus, but also occurs in the periventricular thalamic nuclei, the lateral entorhinal cortex, and the parietal cortex of infected mice (Stewart et al., 2010a,b). The present data are consistent with these findings.

In the study of Howe et al. (2012a), depletion of inflammatory monocytes and neutrophils with a Gr-1 (RB6-8C5) antibody resulted in hippocampal neuroprotection, while specific depletion of neutrophils with a 1A8 antibody (mouse anti-Ly6G) failed to preserve

neurons, suggesting that inflammatory monocytes are required for the loss of CA1/CA2 pyramidal neurons that occurs in the first few days of infection with Theiler's virus in B6 mice. In addition, immunodepletion of inflammatory monocytes and neutrophil granulocytes preserved cognitive function in the Morris water maze, which is otherwise impaired in this model (Howe et al., 2012a). It is important to note that the treatment with Gr-1 reduced inflammatory monocytes (counted by flow cytometry) in the brain by 98% (Howe et al., 2012a) which is higher than the 70 % reduction obtained with clodronate liposomes in the present study or the 49-58 % reduction reported by Cusick et al. (2013) for treatment with wogonin or minocycline.

Additional studies of Howe et al. (2012b) substantiated that neuronal loss during viral encephalitis is not primarily due to direct virus-mediated injury but much of the damage is associated with immune-mediated bystander pathology in response to infiltration of inflammatory monocytes/macrophages. Furthermore, the latter group showed that hippocampal neuron death in these animals is associated with calpain activation (Buenz et al., 2009), leading to the working model that infiltrating inflammatory cells release cytokines and other effector molecules that disrupt hippocampal circuitry, triggering seizures and inducing further disruption of the hippocampal network (Howe et al., 2016). Indeed, treatment with the calpain inhibitor ritonavir significantly reduced calpain activity in the hippocampus, protected hippocampal neurons from death, preserved cognitive performance, and suppressed seizure escalation in the Theiler' virus model (Howe et al., 2016). Among the cytokines released by infiltrating inflammatory macrophages, interleukin-6 (IL-6) has been proposed to be critically involved in viral encephalitis-induced seizures in the Theiler's virus model (Libbey et al., 2011a,b; Cusick et al., 2013; DePaula-Silva et al., 2017). Indeed, IL-6 can lead to increased exposure to, and extended availability of, the excitatory neurotransmitter glutamate in the synapse, which in turn can result in increased excitation and excitotoxicity (DePaula-Silva et al., 2017). In the present study it was not possible to measure IL-6 by a cytokine array due to

the lack of frozen brain tissue samples. Attempts to measure IL-6 by IHC failed because two different polyclonal anti-mouse IL-6 antibodies turned out to be nonselective in IHC (unpublished data).

An open question is why the present study did not indicate any neuroprotective effect of treatment with clodronate liposomes. One explanation is that the reduction of brain macrophage infiltration was less marked than obtained with the immunodepletion approach performed by Howe et al. (2012a). However, a similar reduction of macrophage brain infiltration by clodronate liposomes as observed in the present study has been reported to exert neuroprotective activity in a stroke model (Ma et al., 2016), while it did not protect the basal ganglia in a mouse model of Parkinson's disease (Coté et al., 2015). In the intrahippocampal kainate mouse model of TLE, systemic clodronate liposome administration did not reduce the kainate-induced hippocampal lesion pattern in CA1 and CA3, but reduced granule cell dispersion (Zattoni et al., 2011). Thus, the effects of treatment with clodronate liposomes on neurodegeneration critically depend on the type of neurodegeneration examined. This is also illustrated by experiments with Theiler's virus in T cell defective SJL mice, in which cerebral administration of the virus results in persistent infection that lasts throughout the lifespan of the mouse, resulting in slowly progressing inflammatory encephalomyelitis in the spinal cord, which is widely used as a model of multiple sclerosis (DePaula-Silva et al., 2017). Treatment of SJL mice with mannosylated liposomes containing clodronate prevented infiltration of blood-born macrophages and demyelination in the spinal cord of infected SJL mice (Rossi et al., 1997), consistent with the observation that in infected SJL controls most of the viral burden during persistent infection is in spinal cord macrophages, which replicate the virus and are thus responsible for its persistence in this mouse strain (Lipton et al., 2005). In contrast to SJL mice, Theiler's virus does not persist in B6 mice, but is cleared during the first 2-4 weeks following infection, probably through activation of antiviral CD8^{+} T cells and antibodies, explaining that B6 mice do not develop an

inflammatory demyelinating disease, but only encephalitis during the acute stage of the infection (DePaula-Silva et al., 2017). Interestingly, during acute encephalitis, macrophage invasion into the brain is markedly higher in B6 than SJL mice (Howe et al., 2012a), which may explain why acute seizures are only observed in B6 mice (DePaula-Silva et al., 2017).

Remarkably, while flow cytometry in the present experiments in B6 mice indicated a 70% decrease in brain infiltration of macrophages by clodronate liposomes and a small (30%) decrease in microglia, Mac3 labeling of activated microglia and infiltrated macrophages was not different between infected controls and mice with clodronate liposome treatment. First, brightfield Mac3 IHC is not as sensitive as flow cytometry. Furthermore, because the whole brain was used for flow cytometric analysis, this may indicate that although clodronate liposome treatment reduced the number of infiltrated macrophages in the brain, this effect occurred predominantly in brain regions other than the hippocampus, which, however, is not substantiated by the present data. By using FJB labelling of dying neurons, Stewart et al. (2010a,b) have reported that neurodegeneration after infection with Theiler's virus is not restricted to the hippocampus, but also occurs in the periventricular thalamic nuclei, the lateral entorhinal cortex, and the parietal cortex of infected mice. We looked for Mac3 labeling in these regions, but did not observe any effect of clodronate liposome treatment on the intensity of the signal. Another possible explanation for the apparent mismatch between the flow cytometry data and the results of Mac3 staining would be a compensatory increase in the number of activated microglia in specific brain regions such as the hippocampus in response to inhibition of macrophage infiltration by clodronate liposome treatment. For further evaluation of this possibility, we combined Iba1 and Mac3 labeling of myeloid cells and found a significantly increased percentage of Iba1/Mac3-positive cells in the hippocampus of clodronate-treated mice, most likely reflecting a compensatory increase in the number of activated microglia.

The significant increase of Iba1/Mac3 double stained cells in the dentate hilus of

clodronate-treated mice is interesting, because the dentate gyrus is thought to protect

hippocampal circuits from overexcitation and thus play a role as a gate in seizure propagation

(Krook-Magnuson et al., 2015). Thus, presuming that the Iba1/Mac3-positive cells in the hilus

represent microglia, a protective effect of microglia within the hilus, which is not damaged in

the TMEV model, may play a role in seizure inhibition as observed in our experiments.

Indeed, depending on the pathological condition and cellular environment, microglia can

release anti-inflammatory cytokines and neurotrophic factors that can provide tissue repair

and, due to direct cell-to-cell interactions, modulate neuronal functions and synaptic plasticity

(Gomes-Leal, 2012; Ferreira and Bernardino, 2015). This interesting possibility obviously

deserves further studies.

Another explanation for the lack of a neuroprotective effect of clodronate treatment

could be that such effect is only transient. However, preliminary data from mice that were

killed 2 days after intracerebral infection with Theiler's virus did not indicate that treatment

with clodronate liposomes retarded the increase in Mac3 positivity or the degeneration of

hippocampal neurons.

The present finding that partial depletion of macrophages in the brain protects part of

the infected mice against seizures but does not reduce hippocampal damage argues against a

direct correlation between hippocampal damage and occurrence of acute seizures as suggested

previously (Libbey et al., 2008; Bröer et al., 2016; Bröer et al., 2017). Instead, the fact that

mice with acute seizures often exhibit more severe hippocampal neurodegeneration than mice

without such seizures may simply relate to individual differences in the severity of the

infection. Indeed, acute (early) insult-associated seizures, which occur less than one week

after injury, are known to reflect particularly severe brain insults, which, in turn, are likely to

be associated with more severe hippocampal damage in models of acquired epilepsy (Löscher

et al., 2015). Thus, although hippocampal damage starts within one day after infection with

Theiler's virus (Buenz et al., 2009), the present data may indicate that this damage is not a

prerequisite or consequence of early seizures, because severe damage was also observed in some mice without early seizures, substantiating previous findings of our group in this model with other substrains of TMEV (Bröer et al., 2017). Using different strains of Theiler's virus for intracerebral infection of B6 mice, Libbey et al. (2011c) came to a similar conclusion that elevated levels of cytokines, rather than neuronal cell death, seemed to play the dominant role in seizure induction. Furthermore, in our previous studies with different strains of Theiler's virus and different B6 substrains, we also found a mismatch between induction of seizures and hippocampal damage (Bröer et al., 2016 and 2017).

In conclusion, similar to a previous study with liposome-entrapped clodronate in the kainate model of TLE (Zattoni et al., 2011), this approach of macrophage depletion did not prevent hippocampal damage in a viral encephalitis model of TLE. Instead, macrophage depletion by clodronate liposomes suppressed the occurrence of acute seizures in the encephalitis model, substantiating previous experiments of Cusick et al (2013) with less selective treatments, which indicated that infiltrating macrophages are critically involved in the development of seizures following virus infection. In a next step we plan to study whether treatment with clodronate liposomes also affects the development of chronic spontaneous seizures in Theiler's virus model in B6 mice. The mismatch between hippocampal damage and occurrence of acute seizures found in the present study with clodronate liposomes was unexpected, and it will be important to examine whether this mismatch also holds true for development of epilepsy with spontaneous recurrent seizures.

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Legends to the figures

Fig. 1

Experimental protocol of the experiments. For details see the Methods section. In short, groups of mice were either treated with PBS or clodronate liposomes, starting one day before intracerebral infection with TMEV (Da strain). Treatment was repeated twice at intervals of 3 days. Mock-infected mice were used as additional controls. Encephalitis-associated early (acute) seizures occurring in the first week after infection were monitored daily. Seven days after infection, mice were perfused for blood, spleen and brain analyses.

Fig. 2

Differentiation between microglia and macrophages in the brains of Theiler's virus-infected mice. The figures present representative flow cytometry plots of (A) a mock-infected control mouse, (B) a Theiler's virus (TMEV) infected mouse without any treatment, (C) a Theiler's virus infected mouse treated with PBS liposomes, and (D) a Theiler's virus infected mouse treated with clodronate liposomes. Animals were killed one week after infection as shown in Fig. 1. Microglia are indicated by $CD45^{low}$ $CD11b^+$ positive cells (black circle in each dot plot), while brain-infiltrating bone marrow-derived macrophages are indicated by $CD45^{high}$ $CD11b^+$ positive cells (red circle in each dot plot). Note the scarcity of infiltrating macrophages in mock control (A) and the marked infiltration of such macrophages in the brain following virus infection (B). Also moderate microglia activation is seen in B, C and D. Treatment with clodronate liposomes clearly suppresses macrophage infiltration, while microglia activation is hardly affected in the mouse shown in D.

Fig. 3

Quantification of flow cytometry data. Data are shown as boxplots with whiskers from

31

minimum to maximal values; the horizontal line in the boxes represents the median value. In addition, individual data are shown. Significant differences to mock-infected mice are indicated by asterisks (**P<0.01) while significant differences between infected mice treated with clodronate liposomes and infected controls are indicated by circles (°P<0.05; °°P<0.01). (A) Brain-infiltration of inflammatory monocytes (CD45^{hi} CD11b$^+$) as analyzed by fluorescence-activated cell sorting. Virus infection increased macrophage infiltration >20fold vs. mock, while treatment with clodronate liposomes markedly (>70%) reduced macrophage infiltration. Sample size: 4 mock-infected controls; 12 infected controls; 6 infected mice with clodronate. (B) Microglia (CD45^{low} CD11b$^+$) as analyzed by fluorescence-activated cell sorting. A small non-significant (P = 0.0557) increase in microglia occurred in infected mice, while clodronate liposome treated mice did not show such increase. Sample size: 4 mock-infected controls; 12 infected controls; 6 infected mice with clodronate. (C) In the spleen, monocytes/macrophages were immunostained by Iba1, demonstrating almost complete (-92%) macrophage depletion by treatment with clodronate liposomes. Sample size: 4 mock-infected controls; 6 infected controls; 6 infected mice with clodronate. (D) Flow cytometric analysis of blood macrophages positive for colony stimulating factor 1 receptor (CSF-1R$^+$) substantiated the marked macrophage-depleting effect of clodronate liposome treatment. Sample size: 3 mock-infected controls; 8 infected controls; 6 infected mice with clodronate.

Fig. 4

Occurrence of early (acute) seizures in mice during the first week following intracerebral infection with Theiler's DA virus. The experiment was performed twice; because both experiments resulted in a similar effect of treatment with clodronate liposomes, data were combined. (A) illustrates the number of mice exhibiting acute seizures (indicated by the striped part of the columns; exact percentages are indicated at the bottom of the columns). Data are from 5 (mock), 19 (infected/no treatment, and infected/PBS liposomes), and 24

(infected/clodronate liposomes) mice, respectively. Significant differences to mock are indicated by asterisk (*P<0.05; ***P<0.001), while significant differences between infected mice treated with clodronate liposomes and infected mice treated either with PBS liposomes or untreated are indicated by circles ($^{\circ}$P<0.05; $^{\circ\circ}$P<0.01). (B) Average seizure score of the acute seizures observed in infected mice (see above for sample size). The average seizure score was calculated over the seven day's monitoring period including every seizure each mouse had. Some mice seized multiple times during one monitoring period, so every seizure was taken into account. Data are shown as boxplots with whiskers from minimum to maximal values; the horizontal line in the boxes represents the median value. In addition, individual data (average seizure score determined in each mouse) are shown. Mice treated with clodronate liposomes did not significantly differ from the two control groups. (C) Seizure burden calculated in infected mice (see above for sample size). For calculating seizure burden, the total number of acute seizures observed in each infected mice in the week after infection was used. Data are shown as boxplots with whiskers from minimum to maximal values; the horizontal line in the boxes represents the median value. In addition, individual data are shown. Significant differences between infected mice treated with clodronate liposomes and infected mice treated either with PBS liposomes or untreated are indicated by circles ($^{\circ}$P<0.05; $^{\circ\circ}$P<0.01).

Fig. 5

Occurrence of acute (early) seizures in the 7 days after infection. Data are shown as percent of mice with seizures per mice infected with Theiler's DA virus. Group size was 19 (infected/no treatment), 19 (infected/PBS liposomes), and 24 (infected/clodronate liposomes) mice, respectively. Significant differences between infected mice treated with clodronate liposomes and infected but untreated mice are indicated by asterisks (*P<0.05; ***P<0.001), while significant differences between infected mice treated with clodronate liposomes and infected

mice treated with PBS liposomes are indicated by circles ($^{oo}P<0.01$; $^{ooo}P<0.001$).

Fig. 6

Neuronal damage in the hippocampus determined one week after infection with Theiler's DA virus. All data are from the ipsilateral hippocampus; data from the contralateral hemisphere indicated less severe damage (not illustrated). Data are shown as boxplots with whiskers from minimum to maximal values; the horizontal line in the boxes represents the median value. In addition, individual data are shown. Open circles indicate mice without acute seizures, while closed circles indicate mice with such seizures; mice with vs. without seizures did not differ significantly for any parameter shown in A-D. Significant group differences to mock-infected mice are indicated by asterisks (*P<0.05; **P<0.01); infected mice treated with clodronate liposomes did not differ significantly from infected controls. (A) Size of lesion in the CA1/CA2 sectors. Sample size: 5 mock-infected controls; 16 infected controls; 6 infected mice with clodronate. (B) Average damage in CA1/CA2. Sample size: 5 mock-infected controls; 16 infected controls; 6 infected mice with clodronate. (C) Maximum damage in CA1/CA2. Sample size: 5 mock-infected controls; 17 infected controls; 6 infected mice with clodronate. (D) Density of neurons in the dentate hilus. Sample size: 4 mock-infected controls; 22 infected controls; 6 infected mice with clodronate. For none of the data shown in A-C, significant differences were observed between mice with seizures vs. mice without seizures. See Fig. 7A-C for representative photomicrographs of NeuN-labeled neurons.

Fig. 7

Representative photomicrographs illustrating neurodegeneration and neuroinflammation in the ipsilateral hippocampus following intracerebral infection of C57BL/6J (B6) mice with the DA strain of Theiler's virus. (A) NeuN-labeled hippocampal neurons of a mock-infected control mouse. (B) NeuN-labeled hippocampal neurons of an infected control mouse. (C)

NeuN-labeled hippocampal neurons of an infected clodronate-treated mouse. (D) Fluoro-Jade C (FJC)-positive neurons in the hippocampus of an infected control mouse; no FJC labeling was seen in mock-infected mice (not illustrated). (E) FJC-positive neurons in the hippocampus of an infected clodronate-treated mouse. (F) Absence of Mac3-positive microglia/macrophages in the ipsilateral hippocampus of a mock-infected control mouse. (G) Mac3-positive microglia/macrophages in the ipsilateral hippocampus of an infected control mouse; (H) Mac3-positive microglia/macrophages in the ipsilateral hippocampus of an infected clodronate-treated mouse. Scale bars in C, E and H = 200 μm.

Fig. 8

Number of Fluoro-Jade C (FJC) positive neurons in the ipsilateral hippocampus at one week after infection. Data are shown as boxplots with whiskers from minimum to maximal values; the horizontal line in the boxes represents the median value. In addition, individual data are shown. Sample size: 8 infected controls; 6 infected mice with clodronate. No FJC-positive neurons were observed in the hippocampus of mock-infected mice (not illustrated). The highest number of FJC-positive neurons was observed in the CA1/CA2 regions (A), while fewer neurons were labeled in CA3a (B), CA3C (C) and hilus (D). Similar results were obtained in the contralateral hippocampus (not illustrated). Furthermore, FJC-stained neurons were also observed in other brain regions in both hemispheres (see text). There was no significant difference between infected mice treated with clodronate liposomes and infected controls. See Fig. 7D,E for representative photomicrographs of FJC-labeled neurons.

Fig. 9

Analysis of Mac3-positive cells at one week following infection. All data are from the ipsilateral hippocampus; data from the contralateral hemisphere indicated less severe Mac3-labeling (not illustrated). Data are shown as boxplots with whiskers from minimum to

maximal values; the horizontal line in the boxes represents the median value. In addition, individual data are shown. Sample size: 5 mock-infected controls; 17-18 infected controls; 6 infected mice with clodronate. Open circles indicate mice without acute seizures, while closed circles indicate mice with such seizures. Significant differences to mock-infected mice are indicated by asterisks (*P<0.05; **P<0.01; ***P<0.001); infected mice treated with clodronate liposomes did not differ significantly from infected controls. Data for CA1 (A) and CA2 (B) are from the ipsilateral hippocampus, while data for thalamus/hypothalamus (C) and cerebral cortex (D) are from the medial portions of these regions in both hemispheres. See Fig. 7G,H for representative photomicrographs showing Mac3-labeled activated microglia and infiltrated macrophages.

Fig. 10

Analysis of Iba1/Mac3-positive cells at one week following infection. All data are from the ipsilateral hippocampus. A (mock infected control), B (infected, control) and C (infected, clodronate) show representative illustrations of the Iba1/Mac3-stained hippocampal sections; scale bar in C 200 μm. Blue indicates nuclear staining by DAPI, green Iba1-labeled myeloid cells, red Mac3-labeled activated myeloid cells and yellow/orange Iba1/Mac3-double labeled myeloid cells. D-F show data from semi-quantitative analyses of immunohistochemical labeling. Data are shown as boxplots with whiskers from minimum to maximal values; the horizontal line in the boxes represents the median value. In addition, individual data are shown. Sample size: 5 mock-infected controls; 6 infected controls; 6 infected mice with clodronate. Open circles indicate mice without acute seizures, while closed circles indicate mice with such seizures. Significant differences to mock are indicated by asterisk (*P<0.05; **P<0.01), while significant differences between infected mice treated with clodronate liposomes and infected control mice are indicated by circles (°P<0.05). In all examined hippocampal regions of infected mice, a significant increase in Iba1-labeled myeloid cells was

observed without significant difference between infected controls and mice treated with clodronate; D and E show the CA1/CA2 and dentate hilus regions as examples. In the hilus, the percentage of Mac3-positive cells within the Iba1-positive cell population was significantly higher in clodronate-treated mice (F) which was not observed in other hippocampal regions. See text for further analyses.

Fig. 11

Theiler's virus antigen in the hippocampus (HC) of acutely infected B6 mice indicated by immunohistochemistry of horizontal sections taken from infected mice 7 days post infection. (A) Virus antigen in an infected control mouse. Note the intense labeling in the CA1 region of the ipsilateral hippocampus. (B) Virus antigen in an infected clodronate liposome-treated mouse. Scale bar in B = 200 µm. C and D show data from semi-quantitative analyses of immunohistochemical virus antigen labeling. Data are shown as boxplots with whiskers from minimum to maximum values; the horizontal line in the boxes represents the median value. In addition, individual data are shown. Sample size is 8 infected controls and 6 infected mice with clodronate. Open circles indicate mice without acute seizures, while closed circles indicate mice with such seizures. No significant differences between controls and clodronate liposome-treated mice were observed.