EFFECTS OF TREM-1 EXPRESSION ON ACUTE PHASE SEIZURES IN A MOUSE MODEL OF VIRAL-INDUCED EPILEPSY

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ABSTRACT

Around 70 million people worldwide suffer from epilepsy, a neurological disorder that causes unprovoked and persistent seizures. A dominant cause of acquired epilepsies are from injuries to the central nervous system (CNS), such as viral infection of the CNS that results in brain inflammation (encephalitis). To study how viral encephalitis results in seizures, we use a mouse model of viral-induced epilepsy. In this model, C57BL/6J mice are intracerebrally injected with Theiler's murine encephalomyelitis virus (TMEV). Disease progression is demonstrated by an acute seizure phase followed by a latent phase, where no seizures are observed, followed by spontaneous recurrent seizures (epilepsy). It has been established that the innate immune system is implicated in the CNS inflammation that induces seizures. Specifically, activation of microglia and infiltration of peripheral macrophages into the brain together with the secretion of pro-inflammatory cytokines are associated with seizure development. The Triggering Receptor Expressed on Myeloid cells-1 (TREM-1) was found to be exclusively and highly expressed by peripherally infiltrating macrophages during acute phase seizures. TREM-1 activation has been related to pro-inflammatory cytokine secretion. In this project, we used the mouse model of viral-induced epilepsy to study whether TREM-1 infiltrating macrophages play a role in seizure development by utilizing genetically modified mice lacking TREM-1. We evaluated both

spontaneous and handling behavioral seizures. While there was no significant difference in spontaneous seizures, we found decreased behavioral seizure incidence and seizure severity in mice lacking TREM-1 during the acute phase of the infection. This suggests that TREM-1 plays a role in the development of acute phase seizures following viral encephalitis. Moreover, we found that cellular infiltration and macrophage activation in the CNS did not differ between wild type mice and mice lacking TREM-1. These studies allow us to learn more about how the inflammatory immune response to viral encephalitis relates to epileptogenesis and may reveal a novel therapeutic target to treat seizures and epilepsy in high-risk groups.

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INTRODUCTION

Viral Encephalitis and Epilepsy

Epilepsy, a neurological disorder that causes unprovoked recurrent seizures, affects 70 million people worldwide (Ngugi et al., 2010). Although treatments are available, one-third of patients are resistant to anti-seizure drugs (Laxer et al., 2014). Thus, developing new disease-modifying therapies is critical to treat and prevent seizures in high-risk groups. There are many different types of epilepsy, however, temporal lobe epilepsy (TLE) is the most common form of epilepsy in adult patients and is the most difficult to pharmacologically treat (Laxer et al., 2014).

Many factors are associated with the development of TLE, and brain inflammation (encephalitis) that occurs as a result of infection of the central nervous system (CNS) is an important cause of TLE. Over 100 viruses have been implicated in viral encephalitis, and many of these are implicated in the development of seizures and epilepsy, including human herpes virus type 6 and influenza virus (Millichap & Millichap, 2006), herpes simplex virus (Misra et al., 2008), cytomegalovirus (Myrzaliyeva, 2008), Japanese encephalitis virus (Sehgal et al., 2012), West Nile virus (Getts et al., 2007), and enteroviruses (de Crom et al., 2016). There are many ways viruses can enter the CNS: endothelial cells lining the blood may become infected during viremia, through axonal transport along peripheral nerves, or by infecting mononuclear cells that enter the CNS (Libbey et al., 2008; Vezzani et al., 2016).

Disease onset begins within one to two weeks of the initial infection, when the patient may present with acute seizures. After months to years of no seizure activity, spontaneous and recurrent seizures, or epilepsy, may develop (DePaula-Silva et al., 2021). The presence of acute seizures does not guarantee late-onset epilepsy, but their presence indicates up to 22 times more risk for developing epilepsy (Annegers et al., 1988). Further, there is a 16 times increased risk of

developing epilepsy for those that survive viral encephalitis (Misra et al., 2008). A predictor for refractory epilepsy is the occurrence of frequent seizures in the beginnings of disease onset (Laxer et al., 2014). Therefore, preventing or treating seizures during the acute phase of the infection may inhibit the development of epilepsy and improve treatment outcomes. However, the precise mechanisms of how inflammation induced by viral infection leads to epilepsy are unclear. To study the role of inflammation in the development of acute seizures, we used the Theiler's murine encephalomyelitis virus (TMEV) model of viral-induced epilepsy, a model for TLE.

The TMEV Model

While there are multiple animal models of viral encephalitis, a significant limitation of these models is their low survival rate during the acute phase of the infection (Getts et al., 2007; Sehgal et al., 2012; Wang et al., 2012; Wu et al., 2003). This does not allow for the assessment of long-term effects of viral encephalitis, including the potential onset of epilepsy. Further, most models make use of electrical or toxin stimulations to induce status epilepticus, as to which the host immune system plays no role in the development of seizures (Libbey et al., 2008). The TMEV model, however, allows for evaluation of seizures at the acute, latent, and post-infection epileptic phase due to the survivability of animals, and allows for experiments evaluating the contribution of the immune system on seizure development. The strain of mouse and virus used in this model of viral-induced epilepsy allows for effective viral clearance, mirroring that of infection acquired TLE.

TMEV is a single stranded RNA virus from the picornavirus family that naturally occurs as an enteric pathogen of the mouse (reviewed in DePaula-Silva et al., 2017). The virus was originally found to cause rare, spontaneous, and flaccid hind-limb paralysis in lab-bred mice

(Theiler, 1937). Subsequent intracerebral (i.c.) injections of brain and spinal cord suspensions from infected mice into healthy mice resulted in similar presentations of encephalomyelitis. Overtime, different strains of TMEV have been isolated. Two subgroups have emerged, with one being more neurovirulent (GDVII and FA strains) than the other (DA, WW, TO4, BeAn 8386, and Yale strains) (Lorch et al., 1981). It was found that larger plaques and more apoptotic neurons were present in the more virulent strains, which explains their increased severity of disease presentation (Lipton, 1980; Tsunoda et al., 1997). Daniel's strain of TMEV, or DA, was isolated from mice with chronic spinal cord demyelination in addition to paralysis (Daniels et al., 1952). After Swiss mice were i.c. injected with this strain, they acutely presented with flaccid paralysis. Inflammatory lesions in the leptomeninges and white matter in the spinal cord followed, which presented before patchy demyelination of the spinal cord (Lipton, 1975). However, i.c. injection of the DA virus into different mouse strains leads to varying disease presentations and severity. Specifically, the SJL/J strain was found to have less mortality, more pronounced inflammation, and earlier demyelination than in Swiss mice (Lipton & Dal Canto, 1976). This mouse strain, along with the DA strain of TMEV, creates an animal model that mimics histopathological features of multiple sclerosis (MS) that is now widely used (reviewed in DePaula-Silva et al., 2017). A month after infection, the mice develop demyelinating disease and paralysis without recovery, matching that of progressive MS in human patients. Importantly, the "susceptible" SJL/J mice are unable to clear the viral infection (chronically infected) and the DA virus and viral antigen will persist in the spinal cord (DePaula-Silva et al., 2017; Lipton & Dal Canto, 1979).

Conversely, the use of a different mouse strain with the DA strain of virus allows for the mouse model of viral-induced epilepsy (Fig. 1). Here, C57BL/6J mice are i.c. inoculated with
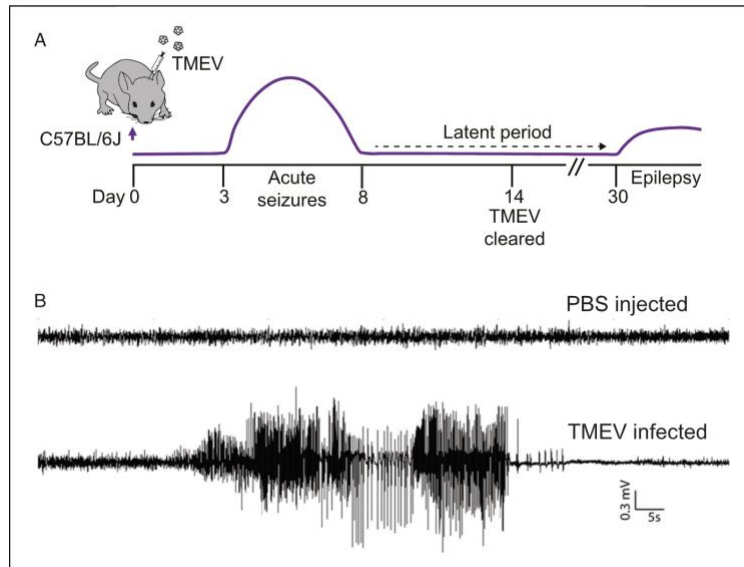

Figure 1 (DePaula-Silva et al., 2021). Mouse model of viral-induced epilepsy. (A) C57BL/6J mice are i.c. injected with Theiler's murine encephalomyelitis virus, a neurotropicvirus member of the Picornaviridae family that can cause encephalitis in infected mice. Between 3 and 8 days post-infection (d.p.i.), mice develop acute seizures, followed by a latent period in which seizures are no longer observed. Between 30 and 100 d.p.i., some mice that experienced acute seizures develop spontaneous recurrent seizures (epilepsy). (B) Representative electroencephalogram from a phosphate-buffered saline-injected mouse, showing normal electroencephalogram recording, and Theiler's murine encephalomyelitis virus-injected mouse, 5 d.p.i., showing generalized convulsive seizures.

4×10^5 PFU of the DA strain, where the virus will be cleared by the mouse's immune system and disease progression mirrors that of viral encephalitis and late-stage epilepsy (Libbey et al., 2008). After 3 days post infection (d.p.i.), about 50-60% of infected mice will develop acute behavioral seizures accompanied by weight loss. Seizure frequency and severity will peak at 6 d.p.i, and will cease by 8 d.p.i. However, when animals are monitored via continuous video electroencephalogram (vEEG),

75% of the mice show electrographic activity, therefore demonstrating that seizure frequency is probably higher than seen in handling-induced behavioral seizures (Stewart et al., 2010b). After cessation of seizures, virus is cleared from the CNS and mice will continue to not seize during a latent period. At least a month post-infection, roughly 50% of mice who experienced a seizure in the acute phase will develop persistent and spontaneous seizures, mirroring that of TLE (DePaula-Silva et al., 2017; Libbey et al., 2008). Moreover, the physical damage done to the neurons, as well as glial reactivity, resembled that in people with TLE (Libbey et al., 2008).

This is the model utilized in the current study to evaluate the effects of the immune system response to viral encephalitis. Seizures begin at 3 d.p.i., which is before the adaptive immune system can initiate its response. Therefore, it is reasoned that the innate immune system is implicated in seizure development (DePaula-Silva et al., 2021; Misra et al., 2008; Vezzani et al., 2016).

Response of the Innate Immune System

Within the CNS, contributions from both the innate and adaptive immune systems are heavily controlled (Choi & Koh, 2008; Ransohoff & Brown, 2012). Under normal conditions, the CNS is "protected" from the periphery and to the rest of the circulatory system by the blood-brain barrier (BBB). Resident glial cells, such as astrocytes, NG-2 cells, and microglia, primarily upkeep the CNS under homeostatic conditions. It is the inflammatory response of the resident cells and breakdown of the BBB during brain injury, such as in viral encephalitis, that contributes to seizure development (Choi & Koh, 2008).

When resident microglia and astrocytes are activated and/or peripheral macrophages infiltrate the CNS, they release pro-inflammatory cytokines such as interferon (IFN)-γ, interleukin (IL)-1, IL-2, IL-6, and tumor necrosis factor-alpha (TNF-α). In turn, these cytokines can modify the glutamate processing system on astrocytes. Glutamate is an excitatory neurotransmitter that must be efficiently removed from the synaptic cleft after neuron firing to cease excitation. Thus, failure to remove glutamate can lead to excessive extracellular glutamate and hyperexcitable conditions (Choi & Koh, 2008). When neurons undergo a state of imbalanced excitability, they begin to degenerate. Moreover, viruses implicated in viral encephalitis seem to have an affinity for limbic structures, such as the hippocampus (Getts et al., 2008). There is significant neuronal death

in the CA1 pyramidal layer in both animal models and postmortem human tissue, which is thought to contribute to seizure development (Getts et al., 2008), and hippocampal sclerosis is a hallmark of TLE (Vezzani et al., 2016; Zhang et al., 2021). Regarding the TMEV model, there is severe hippocampal sclerosis, degradation of the CA1 and CA2 layers, and astrogliosis in TMEV-infected mice with seizures (Gerhauser et al., 2019; Stewart et al., 2010a).

Of the cytokines that are found to be highly expressed in the CNS during viral encephalitis, IL-6 and TNF-α seem to play a large role in the development of seizures. In fact, increased levels of plasma and brain IL-6 is found in patients with TLE (DePaula-Silva et al., 2021; Liimatainen et al., 2013). Additionally, IL-6 can play a role in glutamate clearance and would thus contribute to neuronal hyperexcitability (reviewed in DePaula-Silva et al., 2017). It was found that TMEV infected mice lacking TNF receptor or IL-6 led to significant seizure reduction (Cusick et al., 2013; Kirkman et al., 2010). It was also found that elevated levels of peripheral IL-6 may still be enough to initiate seizures even in the absence of viral replication in the CNS, suggesting an important link between neuronal excitation and inflammatory cytokines (Cusick et al., 2017).

Importantly, while microglia and macrophages have been implicated in seizure development, other immune cells such as neutrophils and natural killer (NK) cells are not associated with the development of seizures. Mice infected with TMEV were given specific antibodies to either deplete neutrophils cells or NK cells, which led to no significant difference in seizure incidence (Libbey et al., 2011). Further, mice deficient in complement protein C3 did experience seizures, but were delayed by a few days. C3 has been demonstrated to instigate release of TNF-α and IL-6. These mice were also unable to clear the virus from the CNS. Therefore, complement may help exacerbate the pro-inflammatory response and play a role in viral clearance but seem to not be directly correlated with seizures (Libbey & Fujinami, 2011). Regarding the

adaptive immune response, the use of the TMEV model demonstrated that mice with inactivated CD8+ T cells did not affect seizure incidence, just viral clearance from the CNS (Kirkman et al., 2010). Similarly, RAG$^{-/-}$ mice, which lack B and T cells, showed no difference in seizure incidence compared to wild type mice (DePaula-Silva et al., 2018).

Macrophages and TREM-1

As discussed above, the innate immune system is implicated in the development of viral encephalitis and acute phase seizures following TMEV infection. The response to viral infection includes the release of chemokines and an increase in BBB permeability, leading to the entry of peripheral immune cells into the CNS and engaging in the injury response (Bosco et al., 2020; Turner et al., 2014; Vezzani et al., 2013). A high number of peripheral macrophages, derived from blood monocytes, infiltrates the CNS after TMEV infection (Cusick et al., 2013). The level of infiltration correlates with the peak of seizures in the acute phase (DePaula-Silva et al., 2018). Moreover, seizure incidence was decreased in wogonin and minocycline-treated mice as well as in clodronate liposome-treated mice. While wogonin and minocycline have anti-inflammatory properties and decreased immune cell infiltration into the CNS (Cusick et al., 2013), clodronate liposome treatment depletes macrophages (Waltl et al., 2017; DePaula-Silva et al., 2018). Since seizures were drastically reduced in the absence of macrophages, this finding suggests a key role for macrophages in seizure generation (Cusick et al., 2013; Waltl et al., 2017). Inflammatory macrophages were found to enter the brain within 12 hours after inoculation, and their depletion by antibody showed preservation of CA1 neurons, therefore implicating their role in hippocampal damage (Howe et al., 2012). Taken together, infiltrating macrophages play a dominant role in acute phase seizures in viral encephalitis.

Macrophages can be found in varying forms of activation states, depending on the environmental stimuli. Once thought to be discrete states of being, we now know these states of activation can reside on a spectrum, and additionally, one cell may be able to express more than one state at a time (Boche et al., 2013; Li & Barres, 2018; Yin et al., 2017). The M1 state is defined by the production of pro-inflammatory cytokines, such as TNF-α and IL-6 (Yin et al., 2017), while the M2 state is defined by the increased production of anti-inflammatory cytokines. Flow cytometry analysis of TMEV infected mice show infiltrating macrophages are activated towards the M1 phenotype at 7 d.p.i. (DePaula-Silva et al., 2019).

To better understand the biology of infiltrating macrophages, both microglia and macrophages were isolated from the brains of PBS injected and TMEV-infected mice during the peak of seizure activity and RNA sequencing was performed (DePaula-Silva et al., 2019). The Triggering receptor expressed on myeloid cells-1 (TREM-1) was found to be highly expressed by infiltrating macrophages. Flow cytometry confirmed that TREM-1 was exclusively expressed on infiltrating macrophages (Fig. 2). Activation of the TREM-1 receptor amplifies the inflammatory response and has been shown to be exclusively expressed on blood neutrophils and monocytes and is up-regulated by foreign stimuli such as bacteria and fungi. Its activation releases pro-inflammatory cytokines, including Il-6, and will increase surface expression of cell activation markers (Bouchon et al., 2000; Weber et al., 2014). Macrophages expressing high levels of TREM-1 are found in inflamed tissues, such as those in the intestine in inflammatory bowel disease (Schenk et al., 2007).

Additionally, higher levels of TREM-1 expressing macrophages were found at 3 d.p.i. versus 7 d.p.i, showing there is a coincidence with high levels of TREM-1 expression and the beginning of the acute seizure phase (DePaula-Silva et al., 2019). TREM-1 is also associated with the M1 phenotype of infiltrating macrophages (Lo et al., 2014), potentially implicating the inflammatory conditions seen in the acute phase with M1 macrophages and TREM-1.
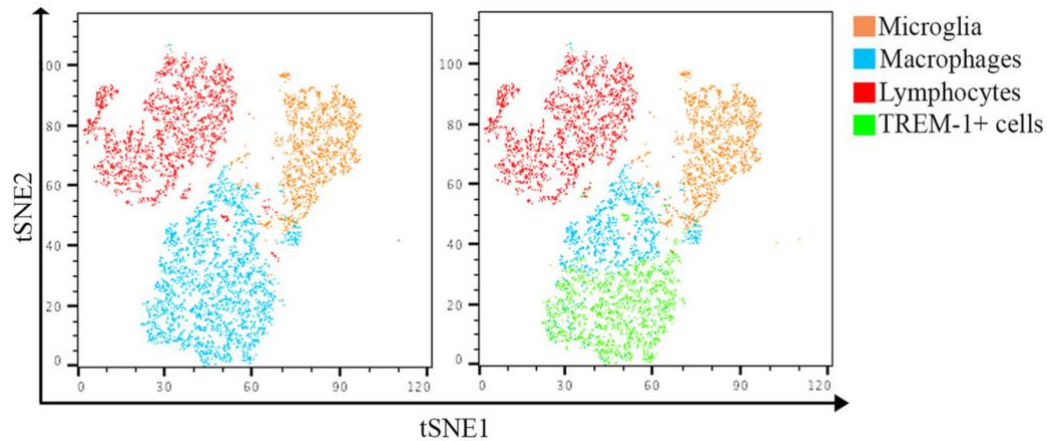

Figure 2 (DePaula-Silva et al., 2019). TREM-1 is expressed by infiltrating macrophages. C57BL/6J mice were intracerebrally infected with TMEV or PBS-injected and mice were euthanized at 3 d.p.i. Overlay of manually gated microglia, macrophages, lymphocytes, and TREM-1 expressing cells, demonstrating TREM-1 is exclusively expressed in infiltrating macrophages.

High levels of expression of TREM-1 on infiltrating macrophages suggests that these cells contribute to the profound cytokine storm observed in the brain following CNS infection. Therefore, the present work was designed to test the hypothesis that acute seizure incidence in TMEV infection would decrease in the absence of TREM-1. To test the effects of TREM-1 on acute seizure development, we used C57BL/6J mice genetically modified without TREM-1, referred here as TREM-1 knockout (KO) mice (Weber et al., 2014). Our results show that TREM-1 KO mice have decreased seizure incidence and severity in handling behavioral seizures compared to the wild type (WT) mice. There was no significant difference of spontaneous seizure incidence between TREM-1 KO and WT mice. We also began to investigate how the immune response in TREM-1 KO mice differs than in WT. While cellular infiltration and macrophage

activation, measured here only by MHC-II expression, are similar between TREM-1 KO and WT after TMEV infection, it remains unclear whether TREM-1 KO is affecting cytokine secretion. Further studies will be required to elucidate the exact mechanism TREM-1 plays in acute seizure development following viral encephalitis.

METHODS

Animals and Infection

C57BL/6J TREM-1 KO mice were obtained from Dr. Christoph Müller at the University of Bern. All animal experiments were reviewed and approved by the University of Utah Institutional Animal Care and Use Committee (IACUC protocol number 20-05007) and conducted in accordance with the guidelines prepared by the Committee on Care and Use of Laboratory Animals Resources, National Research Council. All efforts were made to minimize suffering. C57BL/6J wild type (WT) and TREM-1 KO mice were anesthetized with isoflurane by inhalation and i.c. infected with either 4×10^5 plaque forming units (20 µL) of the DA strain of TMEV or PBS (control).

Video Electroencephalograph (vEEG)

Mice underwent surgery to implant EEG recording electrodes into their brains. A drill was used to create two holes in the skull. The depth electrode was placed in the CA1 region through a hole over the right hemisphere dorsal hippocampus. The reference electrode was placed through a hole over the cerebellum. After surgery, mice were individually housed. Two weeks later, mice were connected to continuous vEEG monitoring. After recording baseline responses, mice were i.c. infected with 20 µL TMEV and monitored 24 hours per day for 7 days. Electrographic activity

was then analyzed and matched with the video recording demonstrating a Racine behavioral seizure as previously described (Patel et al., 2017): 1- facial movements, 2- head nodding, 3- forelimb clonus, 4- forelimb clonus and rearing, 5- rearing and falling, and 6- running, jumping, and repeated falling. The seizures captured in these experiments are termed "spontaneous seizures." Mice were sacrificed at 7 d.p.i.

Behavioral Seizures

C57BL/6J TREM-1 KO and WT mice were i.c. infected with TMEV and handling-induced behavioral seizures were determined from 3-7 d.p.i. Twice a day, each cage was exposed to noise and mice were handled to invoke seizures. Seizures were also scored based on the modified Racine scale. Mice were also weighed once a day, as an extra assessment of animal health. Mice were sacrificed at 7 d.p.i.

Flow Cytometry

At 7 d.p.i. mice were euthanized through an overdose of isoflurane and perfused with PBS. Brains were removed and placed in tubes with PBS and kept on ice. Brains were finely chopped using razor blades and pieces were digested in 2 ml of collagenase I (1 mg/ml) and DNAseI solutions (0.1mg/ml) for 20 minutes at 37°C. After digestion, tubes were placed on ice to inactivate collagenase. Then, the solution was homogenized using a 10 ml pipet and washed with 8 ml of DMEM containing 10% of Cosmic calf serum (CCS) (Hyclone, Logan, UT). Suspensions were centrifuged for 7 minutes at 500 g at 4°C. The pellet was resuspended in 10 ml of 37% Percoll prepared in 1X PBS and centrifuged at 700 g for 30 minutes at 15°C. The top layer containing myelin was removed, and the pellet was washed and resuspended in 1 ml of 1x PBS + 3% CCS.

Cells were counted and the determined number of cells were washed with 1X PBS, 0.5% bovine serum albumin (BSA), and 2 mM EDTA and treated with Fc blocking reagent (Miltenyi Biotec, Auburn, CA). Lastly, cells were immunostained with the following anti-mouse antibodies: anti-mouse CD45 V500, APC anti-mouse CD11b, Pe anti-mouse TREM-1, and Brilliant Violet 412 anti-mouse MHC-II. The stained cells were analyzed by flow cytometry using a BD LSRFortessa X-20 Cell Analyzer (BD Bioscience, San Jose, CA). Gating was determined from fluorescence-minus-one (FMO), by creating different controls including each staining antibody except one. Flow cytometry data analysis was performed using FlowJo software (Tree Star, Inc., Ashland, OR).

Statistical Analysis

Statistical analysis was performed using the program Prism 5 (GraphPad Software, La Jolla, CA). Fisher's exact test was used for seizure frequency comparison. The two-way ANOVA and Sidak's multiple comparison tests were used to compare cumulative seizure burden. The Mann-Whitney test was used for seizure severity comparison. Differences were considered to be statistically significant at $p < 0.05$.
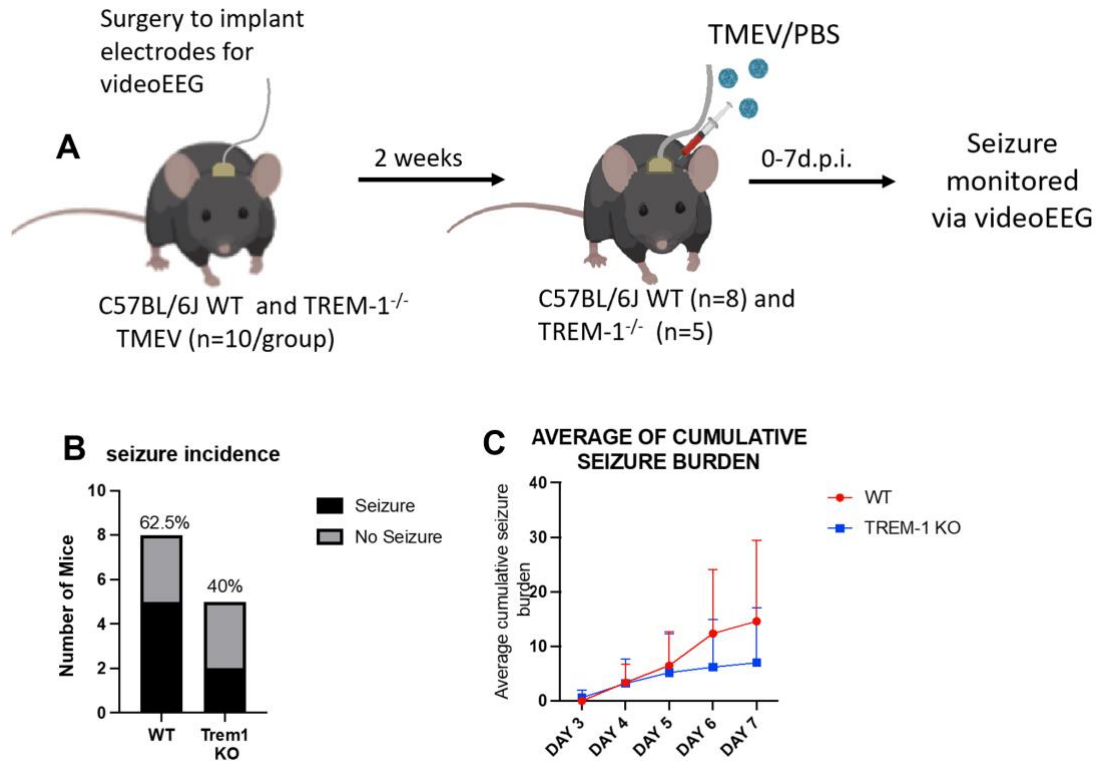
RESULTS

Spontaneous Seizure Incidence and Burden After TMEV Infection in TREM-1 KO Mice

Figure 3. (A) Experiment schematic representation (B-C) TREM-1 KO and WT C57BL/6J were i.c infected with TMEV and spontaneous seizures were measured continuously by vEEG for 7 days (B) Number of mice having seizures in WT and TREM-1 KO mice (C) Cumulative seizure burden calculated by adding Racine score to previous scores.

In the first set of experiments, C57BL/6 TREM-1 KO (n = 5) and WT (n = 8) mice were i.c. inoculated with the DA strain of TMEV and brain electrographic activity was continuously monitored by vEEG (Fig. 3A). EEG activity was matched with video of the seizure if seizures were observed, and they were scored from 1 to 6 based on the modified Racine scale (Patel et al., 2017). While 2 out of the 5 (40%) TREM-1 KO mice seized, 5 out of the 8 (62.5%) WT mice seized (Fig. 3B). The cumulative seizure burden is calculated by adding all previous Racine scores up to that d.p.i. (Fig. 3C). While the data trends towards a decreased seizure incidence in TREM-1 mice, no significant difference was found, most likely due to the very small sample size present.

Performing additional experiments with more mice will allow us to determine if deleting the TREM-1 gene results in changes to spontaneous seizure incidence.

TREM-1 KO Mice Show Decreased Behavioral Seizure Incidence and Burden after TMEV
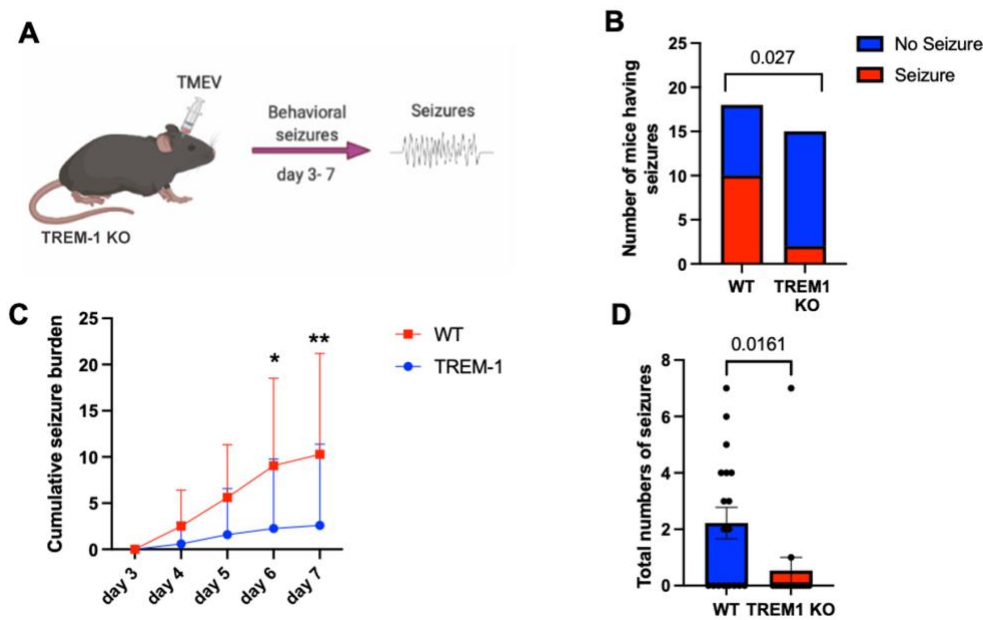
Infection

Figure 4. (A) Experiment schematic representation (B-D) TREM-1 KO (n = 15) and WT (n = 18) C57BL/6J were i.c infected with TMEV. Handling-induced seizures were determined twice a day for 3-7 d.p.i. (B) Number of mice having seizures was significantly reduced in TREM-1 KO mice compared to WT control mice (p<0.05, Fisher's exact test). (C) Cumulative seizure burden was also significantly reduced in the TREM-1 KO mice (p<0.05, two-way ANOVA and Sidak's multiple comparison test). (D) The total number of seizures observed per mice was significantly reduced in TREM-1 KO mice (p<0.05, Mann-Whitney test).

In the next set of experiments, we followed the same procedure of TMEV inoculation of C57BL/6J TREM-1 KO and WT mice, as described earlier, but handling-induced behavioral seizures were evaluated and vEEG was not recorded. Twice a day, the cage of animals would be gently shaken and introduced to noise and handling to invoke behavioral seizures from 3-7 d.p.i. (Fig. 4A). Seizures were scored using the modified Racine scale. In TREM-1 KO mice, only 2 of 15 (13.3%) mice demonstrated seizures. On the other hand, 10 of 18 (55.5%) WT mice demonstrated seizures. Therefore, seizure incidence was significantly decreased in TREM-1 KO

mice (p < 0.05, Fischer's exact test) (Fig. 4B). By day 6 and through day 7, there was a significant

difference in cumulative seizure burden between the two groups (p < 0.05, two-way ANOVA)

(Fig. 4C). We also determined that the number of times each mouse seized is significantly

decreased in the TREM-1 KO group compared to WT (p < 0.05, Mann-Whitney test) (Fig. 4D).

This experiment was conducted two independent times and similar results were obtained.

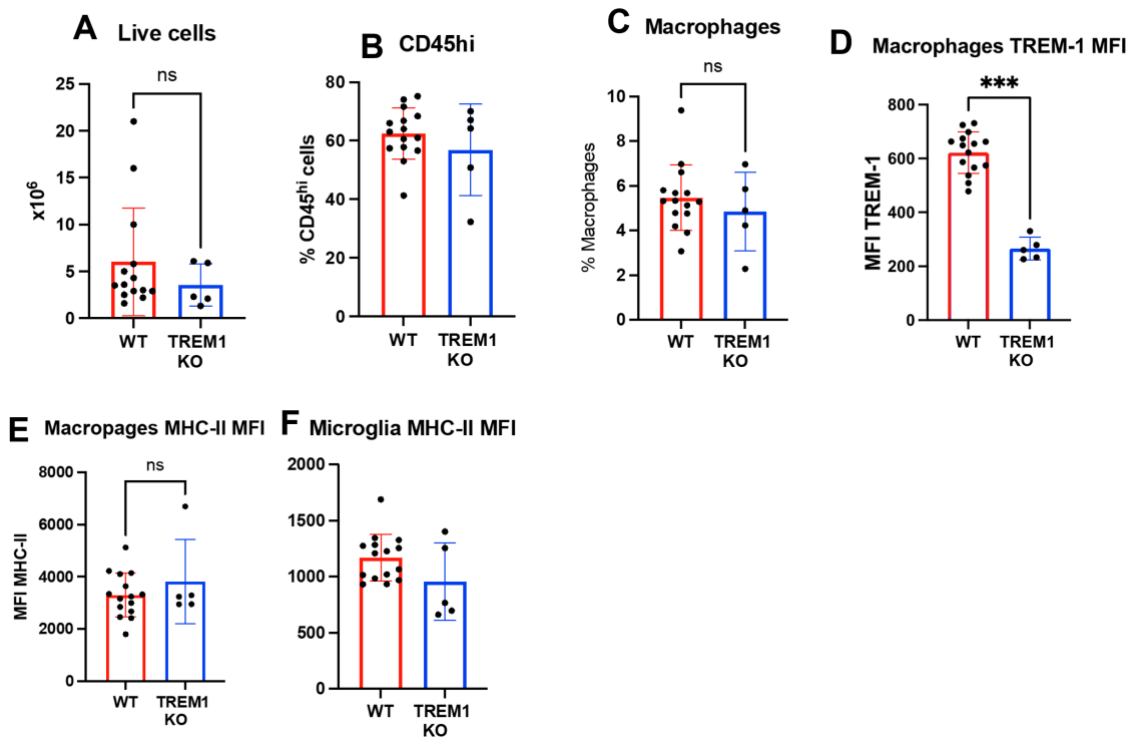*Infected TREM-1 Mice Have Similar CNS Infiltration of Macrophages Compared to WT*

Figure 5. Cellular infiltration and macrophage activation in TREM-1 KO mice is similar to that seen in WT after viral-encephalitis. PBS-perfused brains were removed from TREM-1 KO and WT mice infected with TMEV, at 7d.p.i. Cells were immunostained and analyzed by flow cytometry. A) Total live cells; B) Percentage of CD45hi cells; C) Percentage of macrophages; D) Mean fluorescent intensity (MFI) of TREM-1 expression in macrophages; E) MFI of MHC-II expression in macrophages, and F) MFI of MHC-II expression in microglia. Only significant difference seen between the two mouse groups is in macrophage TREM-1 MFI, as expected.

Lastly, we performed flow cytometry on seized mice in both the TREM-1 KO and WT

mouse groups to evaluate if macrophage infiltration in viral encephalitis varied between the

groups. Our results show that there was no difference in cellular infiltration, as demonstrated by

CD45high (Fig. 5B). There was no significant difference in macrophage infiltration and activation, as demonstrated by CD11b+CD45high and MHC-II (Fig. C,E). We also did not find a difference in microglia activation (CD11b+CD45low and MHC-II) (Fig. 5F). TREM-1 KO mice showed no expression of the TREM-1 protein, as expected (Fig. 5D). Therefore, deletion of TREM-1 does not seem to affect the number of macrophages that infiltrate the CNS.

DISCUSSION

Viral infection of the CNS resulting in encephalitis is a cause for acquired TLE. Disease presentation is usually marked by an acute phase of seizures, a latent stage with no seizures, and potentially followed by spontaneous and recurrent seizures, or, epilepsy. While the presence of acute seizures does not guarantee late-onset epilepsy, their presence does indicate up to a 22 times increased risk for developing late-onset epilepsy (Annegers et al., 1988). Up to one-third of epileptic patients are resistant to anti-seizure drugs, and TLE is the most difficult type of epilepsy to pharmacologically treat (Laxer et al., 2014). Therefore, models of viral-induced epilepsy are needed to discover new therapeutic targets to improve patient outcomes.

While these animal models exist, most are marked by a high level of mortality and cannot be evaluated past the acute stage. The TMEV model, however, not only allows for evaluation of late-onset epilepsy, but the histopathological effects seen resemble that in human patients (DePaula-Silva et al., 2017; Libbey et al., 2008). Using this model, it has been hypothesized that the innate immune system in response to viral infection is implicated in the development of seizures during the acute phase (Kirkman et al., 2010). Specifically, infiltrating macrophages and their subsequent release of IL-6 may lead to imbalanced neuronal conditions resulting in hyperexcitability (Cusick et al., 2013; Waltl et al., 2017). TREM-1 is a receptor found on neutrophils and blood macrophages

involved in the release of pro-inflammatory cytokines, including IL-6 (Bouchon et al., 2000). RNA-sequencing of cells extracted from the brains of mice infected with TMEV showed a subpopulation of infiltrating macrophages that highly express TREM-1 during acute seizures (DePaula-Silva et al., 2019). Since TREM-1 is associated with amplification of the inflammatory response, and since inflammation is an important contributor of seizure development, we hypothesized that seizure incidence and severity will be diminished in mice lacking the TREM-1 receptor (Weber et al., 2014).

We evaluated two instances of seizures: spontaneous and behavioral. Spontaneous seizures are monitored by continuous vEEG and can detect seizures at every level on the modified Racine scale. While our data trends towards a lower seizure incidence in TREM-1 KO mice compared to WT mice after TMEV infection, we found no significant difference between the groups. This is most likely due to a low number of mice used in our experiment. This procedure should be repeated in the future to obtain a higher sample volume and so differences can be better evaluated.

We also evaluated handling-induced behavioral seizures between TREM-1 KO and WT C57BL/6J mice. We found that TREM-1 KO mice demonstrated decreased seizure incidence and cumulative seizure burden than WT in this set of experiments. Further, 6 and 7 d.p.i was found to demonstrate a significantly higher cumulative seizure burden for TREM-1 KO mice compared to WT. These results indicate that TREM-1 expressed on infiltrating macrophages may play a role in acute phase seizure development in viral encephalitis.

To begin determining how TREM-1 affects immune cellular response in TREM-1 KO mice, we performed flow cytometry on the brains of seized mice in both groups. Interestingly, we found no significant difference in cellular infiltration into the CNS between TREM-1 KO and WT groups and no difference in microglia or macrophage activation, as measured by MHC-II expression. So,

TREM-1 deficient mice respond to viral encephalitis with the same level of macrophage infiltration into the CNS as seen in WT mice. Instead, TREM-1 may contribute to an increased state of activation of the inflammatory macrophage itself, which is not measured by MHC-II. To elucidate the difference in immune response to encephalitis, cytokine profiles and immunohistochemistry on brains will be performed on seized TREM-1 KO mice.

These studies will help us to further explore the relationship of the inflammatory innate immune system response and the incidence of seizures after viral encephalitis. Here, we have demonstrated that TREM-1 expressed on a population of infiltrating macrophages may play a role in seizure development. Macrophages and TREM-1 may be a possible therapeutic target to prevent seizures and late-onset epilepsy, which would benefit the one-third of epileptic patients that are resistant to pharmacological intervention.

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