

Etanercept Reduces Neuroinflammation and Lethality in Mouse Model of Japanese Encephalitis

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Background. Japanese encephalitis virus (JEV) is a neurotropic flavivirus that causes Japanese encephalitis (JE), which leads to high fatality rates in human. Tumor necrosis factor alpha (TNF- α) is a key factor that mediates immunopathology in the central nervous system (CNS) during JE. Etanercept is a safe anti-TNF- α drug that has been commonly used in the treatment of various human autoimmune diseases.

Methods. The effect of etanercept on JE was investigated with a JEV-infected mouse model. Four groups of mice were assigned to receive injections of phosphate-buffered saline, etanercept, JEV, or JEV plus etanercept. Inflammatory responses in mouse brains and mortality of mice were evaluated within 23 days post infection.

Results. The in vitro assay with mouse neuron/glia cultures showed that etanercept treatment reduced the inflammatory response induced by JEV infection. In vivo experiments further demonstrated that administration of etanercept protected mice from JEV-induced lethality. Neuronal damage, glial activation, and secretion of proinflammatory cytokines were found to be markedly decreased in JEV-infected mice that received etanercept treatment. Additionally, etanercept treatment restored the integrity of the blood–brain barrier and reduced viral load in mouse brains.

Conclusions. Etanercept effectively reduces the inflammation and provides protection against acute encephalitis in a JEV-infected mouse model.

Keywords. etanercept; Japanese encephalitis virus; viral encephalitis; TNF- α ; inflammation.

Viral encephalitis is a devastating illness that claims several thousands human lives every year, often leaving survivors to suffer from permanent neurological deficit [1]. Japanese encephalitis virus (JEV), which belongs to the genus *Flavivirus* in the family *Flaviviridae*, is the most prevalent type of viral encephalitis, with 30 000–50 000 cases (mostly children) and a high fatality rate of 30% being reported annually. Clinical symptoms related to Japanese encephalitis (JE) include headache, fever, vomiting, diarrhea, reduced levels of consciousness,

and signs of meningeal irritation [2, 3]. Fortunately, both inactivated and live attenuated JEV vaccines have been developed and used in Asia. However, few therapies beyond intensive supportive care and no antiviral agent are available to treat patients with JE.

JE is characterized by profound neuronal damage along with intense microgliosis and astrogliosis. During infection, neurons can directly undergo apoptosis due to lytic replication or through a bystander mechanism where overactivation of astrocytes and glial cells leads to emancipation of numerous proinflammatory cytokines, including tumor necrosis factor alpha (TNF- α), interleukin-1 β (IL-1 β), and IL-6 [4–6]; TNF- α is regarded as a key factor that mediates immunopathology in the central nervous system (CNS). It has been reported that TNF- α is the main culprit in the neurotoxic cascade of JEV [4], and the increased levels of TNF- α in cerebrospinal fluid and serum have been correlated with cases of severe disease during JEV infection [7]. TNF- α directly mediates neuronal apoptosis by the

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engagement of TNF receptor (TNFR) and the TNFR-associated death domain (TRADD). Neuronal death contributes to glial activation and subsequent neuroinflammation. TNF- α is also a known trigger of intercellular adhesion molecule 1 and vascular cell adhesion molecule 1 expression on neurovascular endothelial cells, leading to leukocyte extravasation in the CNS [8, 9]. Although the mechanism by which neurotropic viruses cross the blood–brain barrier (BBB) is largely unknown, TNF- α -mediated changes in the BBB are considered to be a mediator of viral entry into the CNS [10–12]. TNF- α -mediated regulation of the major histocompatibility complex II molecules is also believed to regulate the persistency of West Nile virus (WNV) infection in brain [13, 14]. Whereas in cases of Borna disease virus infection, TNF- α is responsible for triggering epileptic seizures [15]. The multiple downstream effects of TNF- α along with other chemokine-mediated destruction of neurons and demyelination are well established in cases of JEV and human immunodeficiency virus-induced CNS pathology [16, 17]. In addition, TNF- α plays an essential role in the initiation and regulation of different cytokine cascades. Due to the augmented significance of TNF- α in viral neuropathogenesis, anti-TNF- α treatment can serve as a potential therapeutic strategy in cases of viral encephalitis.

Etanercept (Enbrel; Pfizer, NY) is a soluble TNF- α -binding protein with a long half-life. It directly binds to TNF- α , reducing its biological effectiveness [18, 19]. Etanercept is frequently used to treat autoimmune diseases, such as rheumatoid arthritis [20], ankylosing spondylitis [21], psoriasis, and psoriatic arthritis [22], by acting as a TNF- α inhibitor. It has also been used safely in patients who have both psoriasis and hepatitis C virus infection [23]. The long-term safety of etanercept in children is well established [24].

In the present study, we investigated the effect of etanercept as an anti-TNF- α therapy using a JEV-infected mouse model and demonstrated the significant neuroprotection offered by etanercept through reduction of inflammation.

METHODS

Preparation of Primary Mouse Neuron/Glia Cultures and Virus

Neuron/glia cultures were prepared from the cerebral cortices of 1-day-old Balb/c mice and plated on polylysine-coated (20 mg/mL) dishes at a density of 10^5 cells per well in Dulbecco's modified Eagle medium supplemented with 5% fetal bovine serum. After 8 hours for seeding, the culture medium was replaced with neurobasal medium supplemented with 2% B-27, 0.5% streptomycin and penicillin, and 0.5 mM L-Glu. The cells were used for subsequent experiments after incubation for 7 days. The neuron/glia cultures were mock infected or infected with JEV at a multiplicity of infection of 0.1. Etanercept (10 ng/mL, 100 ng/mL, and 1000 ng/mL) was added at 6 hours and phosphate-buffered saline (PBS) was added at 12 hours post infection (hpi).

The JEV wild-type strain P3 used in this study was propagated in suckling mouse brain. The titer of virus was determined by plaque assay on Baby Hamster Syrian Kindey (BHK-21) cells as described previously [25].

Etanercept Administration to JEV-Infected Mice

Adult Balb/c mice (10 weeks old) were purchased from the Hubei Provincial Center for Disease Control and Prevention (Wuhan, China). Mice were randomly divided into 4 groups: control group (PBS; $n = 35$); only etanercept-treated group (Etan; $n = 35$); JEV-infected group (Jev; $n = 35$); and JEV- and etanercept-treated group (Jev + Etan; $n = 35$). Mice belonging to the Jev and Jev + Etan groups were intraperitoneally injected with 10^6 pfu of JEV P3 strain in 200 μ L PBS. Etanercept (100 μ g in 100 μ L physiological saline per mouse) [26] was intravenously administered to mice belonging to the Jev + Etan group on day 3 and day 5 post infection [27]. Mice in the PBS group received PBS and those in the Etan group received Etanercept.

Twenty mice of each group were monitored daily to assess behavior and mortality. Behavioral scoring was performed in a masked manner to avoid bias toward any one group of animals. All neurological parameters were recorded visually, and the total score was calculated on the basis of the appearance of symptom [28, 29]. The remaining mice were sacrificed on day 6 and day 23 post infection, and brain samples were collected for additional experiments. All experiments were performed following the protocols recommended by the Research Ethics Committee, College of Veterinary Medicine, Huazhong Agricultural University, Hubei, China.

Quantification of Cytokine Production by Enzyme-Linked Immunosorbent Assay

Enzyme-linked immunosorbent assay (ELISA) kits (Ebioscience, USA) were used to determine the secretion of TNF- α , IL-1 β , IL-6, and chemokine (C-C motif) ligand 2 (CCL-2) in cell cultures or mouse brain tissue lysates according to the manufacturer's instructions.

Hematoxylin–Eosin and Immunohistochemistry Staining

The standard hematoxylin–eosin staining protocol was followed for tissue staining. For immunohistochemical staining, sections were incubated overnight at 4°C with primary antibodies against ionized calcium binding adapter molecule 1 (IBA-1) (Wako, Japan), glial fibrillary acidic protein (GFAP) (Dako, Denmark), and neuronal nuclei (NeuN; Chemicon, USA). After washing, slides were incubated with appropriate secondary antibodies, washed, and cover protected. The number of positive cells for each antibody was analyzed for the integrated optical density index in 3 fields at $\times 200$ magnification using ImagePro Plus software. Then, the ratio of positive cells for each antibody was calculated.

Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling (TUNEL) Assay

To detect the extent of cell death, a TUNEL assay was performed using an in situ cell death detection kit (Roche, Mannheim, Germany) according to the manufacturer's instructions. For each experiment, TUNEL-positive cells and 4,6 diamidino-2-phenylindole (DAPI)-stained cells were counted in 5 fields/well/slide with 3 wells/slide/sample, and the percentage of TUNEL-positive cells was calculated.

Immunoblot Analysis

A Western blot analysis was performed with protein isolated from brain tissues from all groups of animals. The nuclear proteins were extracted using an NE-PER(R) nuclear and

cytoplasmic extraction kit (Thermo, USA). Each sample was electrophoresed and transferred onto a nitrocellulose membrane. Membranes were then blocked and probed with primary antibodies, including anti-caspase 3 (Abclonal Technology, China), nuclear factor-KappaB (NF- κ B; Cell Signaling Technology, USA), activation protein-1 (AP-1; Cell Signaling Technology), as well as β -tublin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and lamin A (Abclonal Technology, China) antibodies. After washing, membranes were incubated with the appropriate peroxidase-conjugated secondary antibodies (Boster, China). The blots were processed for development using SuperSignal West Femto (Thermo). The protein levels were quantified by immunoblot scanning and normalized with respect to the amount of β -tublin, GAPDH, or lamin A.

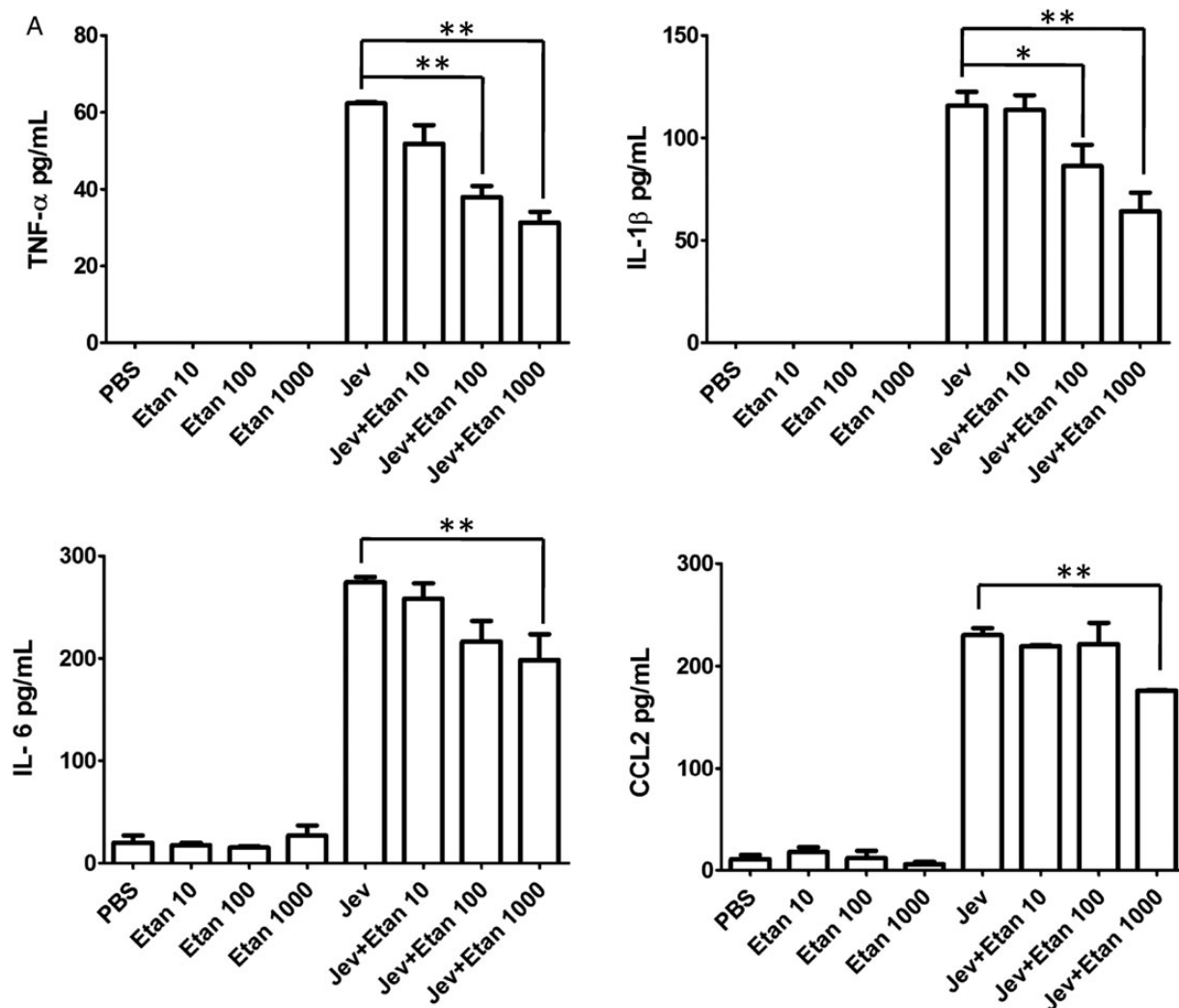


Figure 1. Etanercept attenuates Japanese encephalitis virus (JEV)-induced inflammation in neuron/glia cultures. Neuron/glia cultures were prepared from the cerebral cortexes of 1-day-old mice. Cells were plated in a 24-well plate at a density of 1×10^5 cells/well and mock infected or infected with JEV (0.01 multiplicity of infection). Cells were treated with either etanercept (10 ng/mL, 100 ng/mL, 1000 ng/mL) or phosphate-buffered saline (PBS) at 6 hours and 12 hours post infection. A, At 48 hours post infection (hpi), the supernatant of mixed cells was harvested and the concentrations of tumor necrosis factor-alpha, interleukin-1 β , IL-6, and CCL2 were determined by enzyme-linked immunosorbent assay.

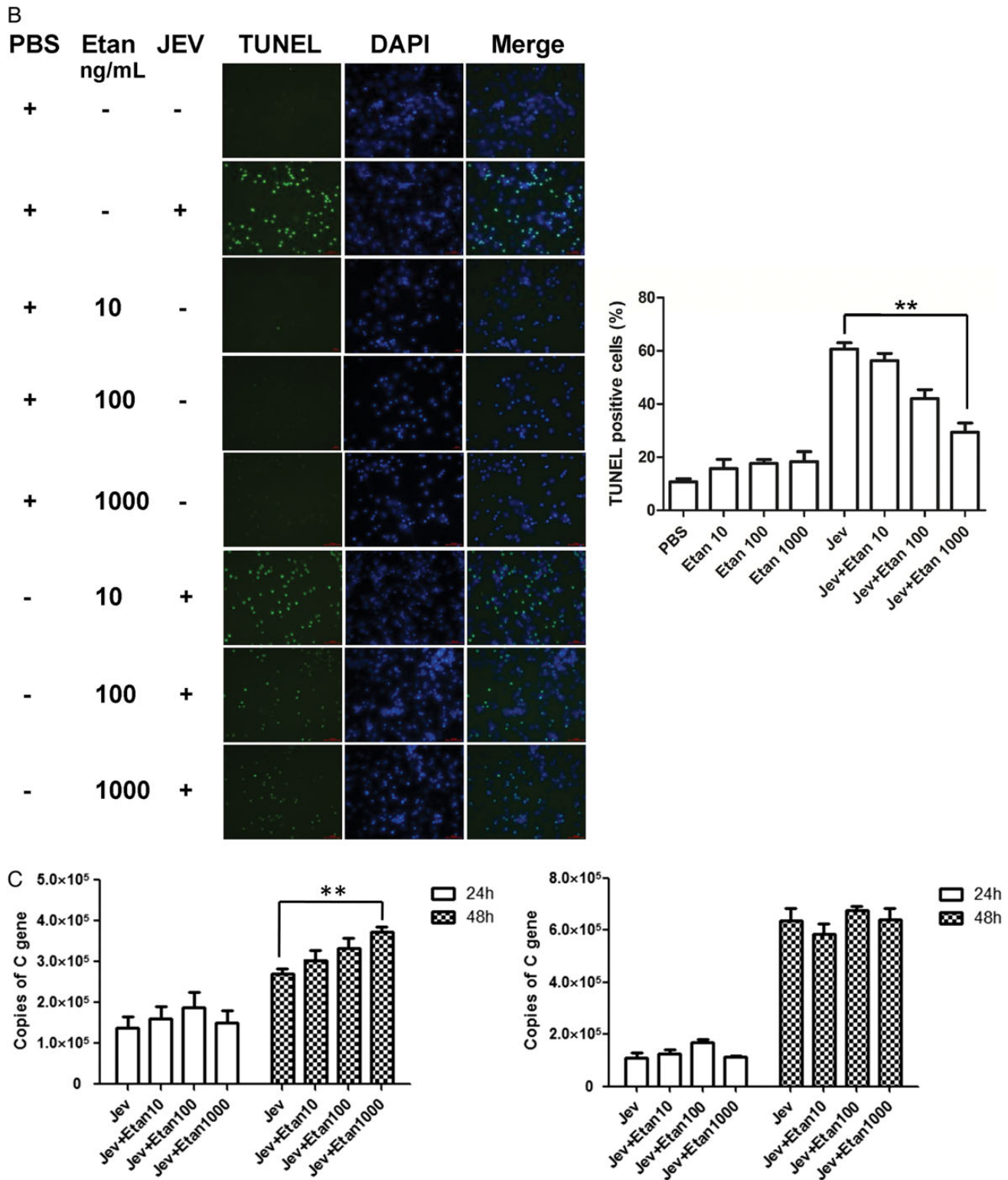


Figure 1 continued. *B*, Cell death was measured by TUNEL assay. TUNEL-stained cells and total cells (4,6-diamidino-2-phenylindole) were randomly photographed. Scale bar represents 100 μ m. The numbers of TUNEL-positive cells compared with total cells were counted (right graph). *C*, Neuron/glia cultures were either infected with JEV followed by treatment with etanercept or PBS as described above (left panel) or infected with JEV that had been preincubated with etanercept or PBS for 1 hour (right panel). Cells were harvested at 24 and 48 hpi and the *C* gene copies were determined by quantitative reverse-transcription polymerase chain reaction. Data represents mean + standard error of the mean for 3 independent experiments (* P < .05, ** P < .01).

BBB Permeability Assay

BBB permeability was assessed by sodium fluorescein (NaF) uptake assay. To this end, mice were injected intraperitoneally with NaF (2% in 200 μ L PBS) and allowed to circulate for 30 minutes. The brain tissues containing hippocampus, frontal cortex, and putamen regions were harvested and immediately immersed in liquid nitrogen. The tissues were homogenized in PBS followed by protein measurement. The samples were then precipitated in 10% trichloroacetic acid. The pH was adjusted by adding 8.33 μ L 5 M sodium hydroxide to 100 μ L supernatant aliquots, and fluorescence was detected using a fluorescence plate reader with excitation at 485 nm and emission at 530 nm. BBB permeability was expressed as pg NaF/ μ g protein.

Detection of JEV mRNA Level by Quantitative Reverse-Transcription Polymerase Chain Reaction

Total cellular RNA was isolated and reversely transcribed using the ReverTra Ace- α kit (TOYOBO, Japan) according to the manufacturer's instructions. Quantitative reverse-transcription polymerase chain reaction (qRT-PCR) experiments were carried out using SYBR Green Real-Time PCR Master Mix (TaKaRa, Japan) according to the manufacturer's instructions. Plasmid pcDNA-HA-C was used to construct the standard curve for quantitation of viral load in 10-fold dilution with the initial concentration of 4×10^{14} copies/mL. Specific forward and reverse primers targeting the JEV C gene were as follow: 5'-GGCTCTTATCACGTTCTTCAAGTTT-3'; 5'-TGCTTTCATCGGCCTAAAA-3'.

Statistical Analyses

Statistical analyses were performed using GraphPad Prism 5 software. Statistical differences between the experimental groups were determined using the Student *t* test. *P* values < .05 were considered significant. Data represent mean \pm standard error of the mean.

RESULTS

Etanercept Reduces Secretion of Proinflammatory Cytokines and Viral-Mediated Neuronal Death In Vitro

In order to assess the role of etanercept in attenuating the massive inflammatory response induced by JEV, a series of in vitro experiments were performed with mouse neuron/glia cultures. JEV- or mock-infected cells were treated with etanercept or PBS at 6 hours and 12 hours post infection, and levels of proinflammatory cytokines (TNF- α , IL-1 β , IL-6, and CCL-2) in the culture media were measured. As expected, viral infection triggered the release of a large amount of proinflammatory cytokines, whereas etanercept treatment significantly decreased the cytokine production of neuron/glia cultures (Figure 1A). To further evaluate the significance of etanercept in preventing neuronal death during infection, a TUNEL assay was performed. As anticipated, etanercept prevented cell death in neuron/glia cultures in a dose-dependent manner (Figure 1B). Next, the effect of

etanercept on viral replication was validated by detecting the viral genome copies. Results showed that treatment with etanercept after viral infection slightly increased the viral replication at 48 hpi (Figure 1C). However, incubation of JEV with etanercept

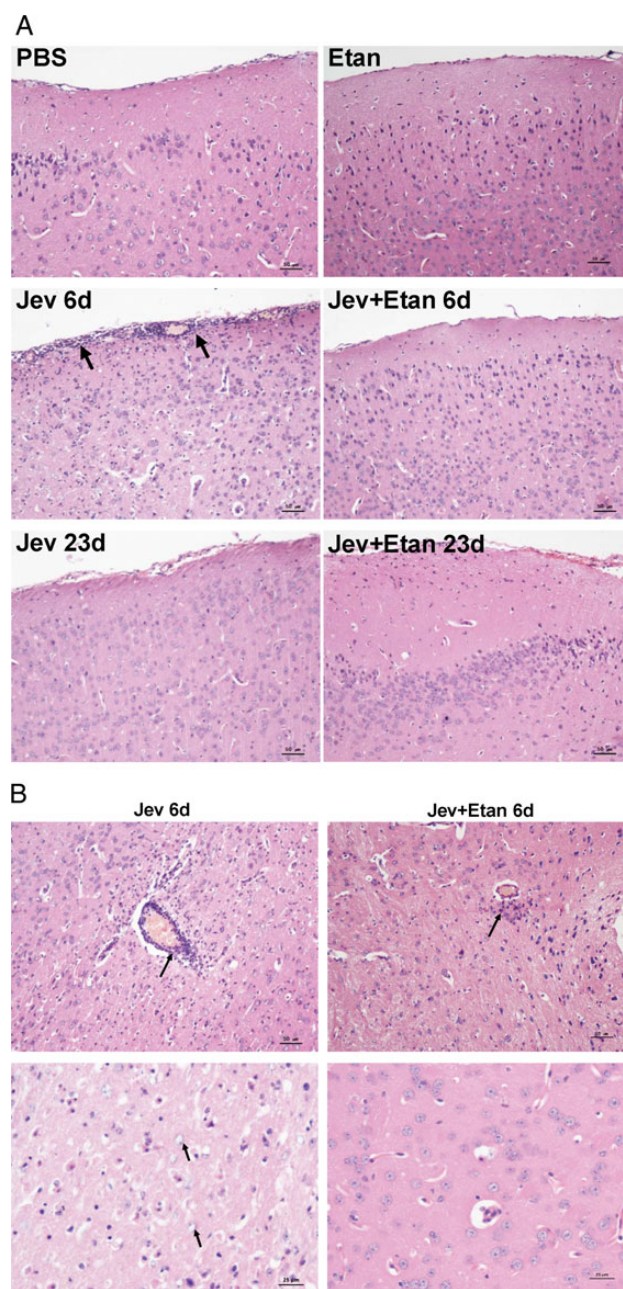


Figure 2. Etanercept treatment contributes to the improved histopathological changes of mice caused by Japanese encephalitis virus (JEV) infection. Mice in each group were sacrificed on day 6 and day 23 post infection, and brain samples were collected. Hematoxylin–eosin staining of brain sections was performed to observe the pathological changes. *A*, Meningitis appeared in the JEV-infected group (indicated by arrows), while no apparent inflammatory cell accumulation was seen on day 6. *B*, Vacuolar degeneration of neuron also appeared in JEV-infected mice, while normal neuronal morphology was observed following etanercept treatment (indicated by arrows) (400 \times).

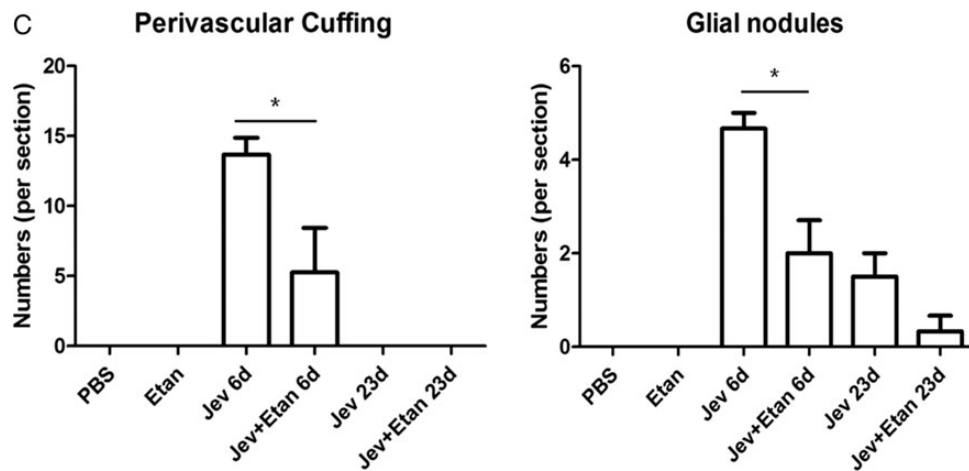


Figure 2 continued. C, Numbers of glial nodules and perivascular cuffing on brain sections were counted and calculated. Data represent mean \pm standard error of the mean of 3 sections from 3 mice from each group; 5 fields for each section were tested ($*P < .05$).

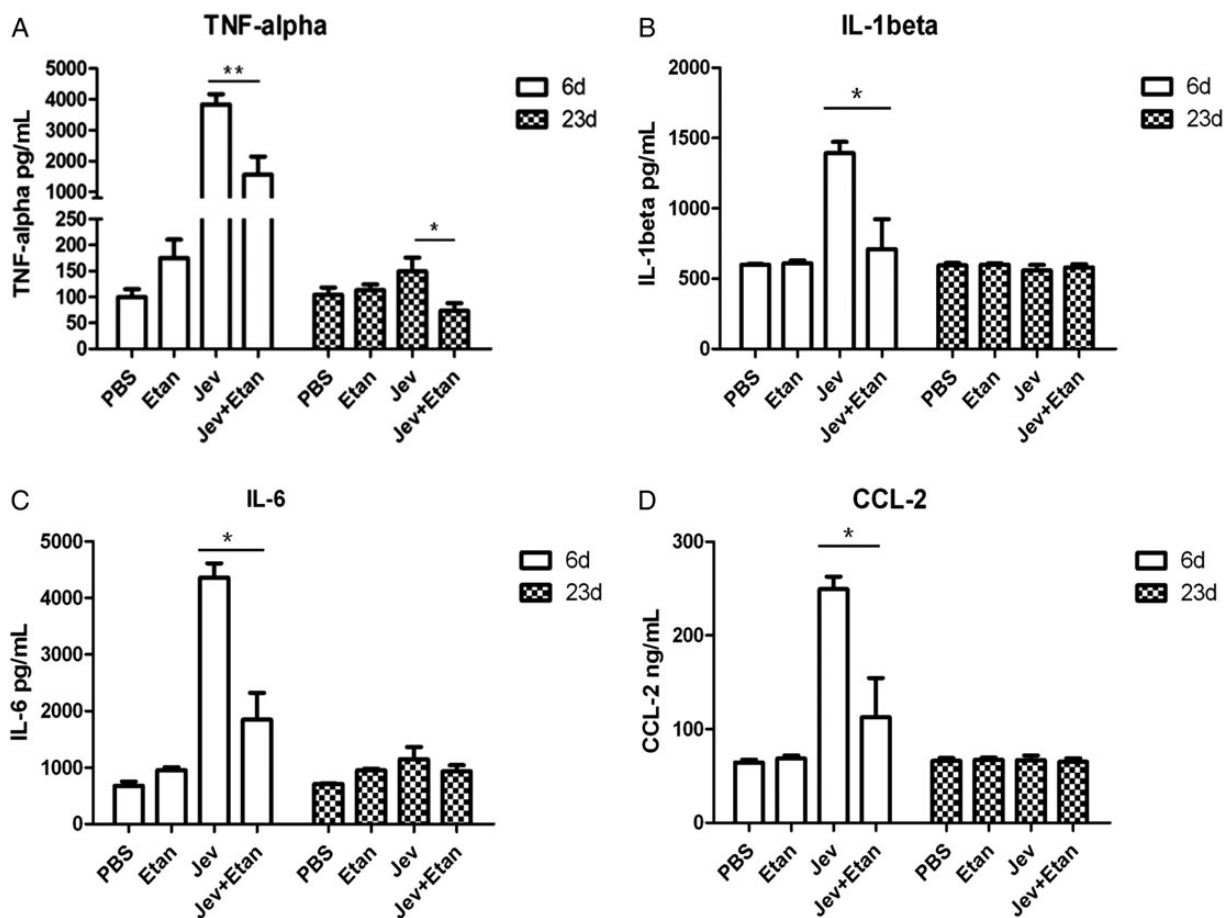


Figure 3. The effect of etanercept on the expression of tumor necrosis factor- α (TNF- α), interleukin-1 β (IL-1 β), IL-6, and CCL-2 following Japanese encephalitis virus infection of mice was determined by enzyme-linked immunosorbent assay (ELISA). Mice in each group were sacrificed on day 6 and day 23 post infection, and brain samples were collected. The production of TNF- α (A), IL-1 β (B), IL-6 (C), and CCL-2 (D) in brain lysates was determined by ELISA. Significant reductions of TNF- α , IL-1 β , IL-6, and CCL-2 were observed in the etanercept-treated group on day 6 and day 23 post infection ($**P < .01$, $*P < .05$). No apparent difference was found in phosphate-buffered saline and Etanercept control groups from all the cytokines/chemokines. Data represent mean \pm standard error of the mean of 5 experiments with 5 mice from each group.

prior to infection did not affect viral replication. It suggests no direct antiviral activity of etanercept.

Etanercept Treatment Attenuates Inflammatory Response in Mouse Brain

To ensure the effectiveness of etanercept in JEV-induced encephalitis, *in vivo* experiments were performed with a JE mouse model. Brain tissues were collected at day 6 and day 23 post infection since mice started to show signs of infection on day 5 and most of the living mice were recovered at day 23. Histological brain alterations revealed severe meningitis in JEV-infected mice on day 6 post infection, while etanercept treatment significantly alleviated this phenomenon (Figure 2A). Both groups showed no evidence of meningitis on day 23 post infection. Vacuolar degeneration and liquefactive necrosis were also observed in neurons of the Jev group but not the Jev + Etan group (Figure 2B). For glial nodules, an apparent decline appeared in JEV-infected mice with etanercept treatment on day 6 post infection, while no significant difference between the 2 groups was seen on day 23 post infection (Figure 2C). In addition, a significant reduction of perivascular cuffings was also found in etanercept-treated mice on day 6 post infection (Figure 2C). These results indicate the curative effects of etanercept on attenuating JEV-mediated inflammation.

To quantify the levels of proinflammatory cytokines and chemokines, ELISA with brain homogenates was performed. As expected, a significant reduction in the level of TNF- α in the Jev + Etan group was found compared with that of the Jev group on day 6 post infection (Figure 3A). On day 23 post infection, the level of TNF- α in the Jev group was similar to that in the control but significantly higher than that in the Jev + Etan group. A remarkably decreased expression of IL-1 β , IL-6, and CCL-2 was also shown in the Jev + Etan group compared with that in the Jev group on day 6 post infection (Figure 3B–D). These results clearly indicate the etanercept-mediated reduction in the release of proinflammatory cytokines.

Etanercept Treatment Abrogates Microglia/Astrocyte Activation and Neuronal Death in Mouse Brain

To assess the role of etanercept in JEV-mediated glial activation, brain sections were immunohistochemically stained. Plentiful star-shaped, activated microglia were observed in the Jev group on day 6 post infection, while etanercept treatment downregulated microglial proliferation substantially (Figure 4A and 4D). Similarly, fewer activated astrocytes were seen in the Jev + Etan group (Figure 4B and 4D). In addition, etanercept treatment inhibited the degeneration of neurite processes caused by JEV infection and also increased the number of visible neurons on day 6

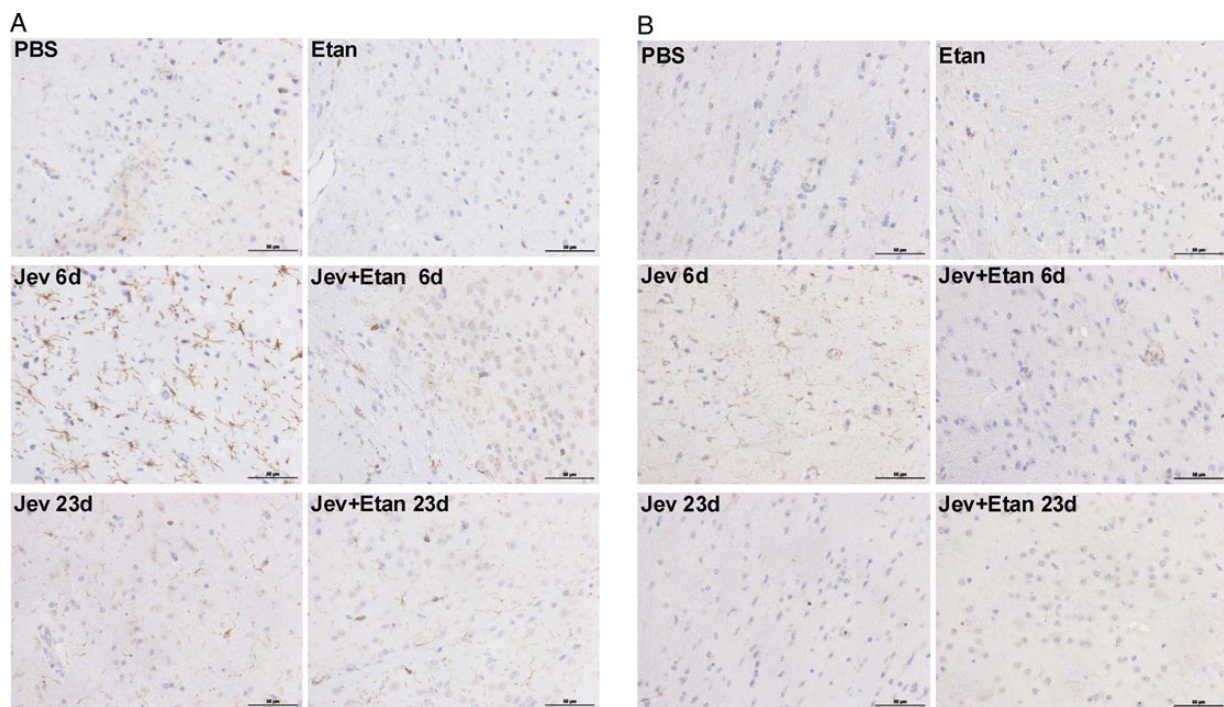


Figure 4. Etanercept treatment reduces glial activation and neuronal death in Japanese encephalitis virus–infected mice. Mice in each group were sacrificed on day 6 and day 23 post infection, and brain samples were collected. Sections of brain tissue were analyzed by immunohistochemical staining. *A*, Activation of microglia was detected by anti-IBA antibody. Etanercept treatment resulted in reduction of activated microglia by a decrease in IBA-1 expression following day 6 post infection. *B*, Activation of astrocytes was analyzed by staining with anti–glial fibrillary acidic protein (GFAP) antibody. Activated astrocyte was also found to be reduced following treatment, as evidenced by a change in GFAP expression following day 6 post infection.

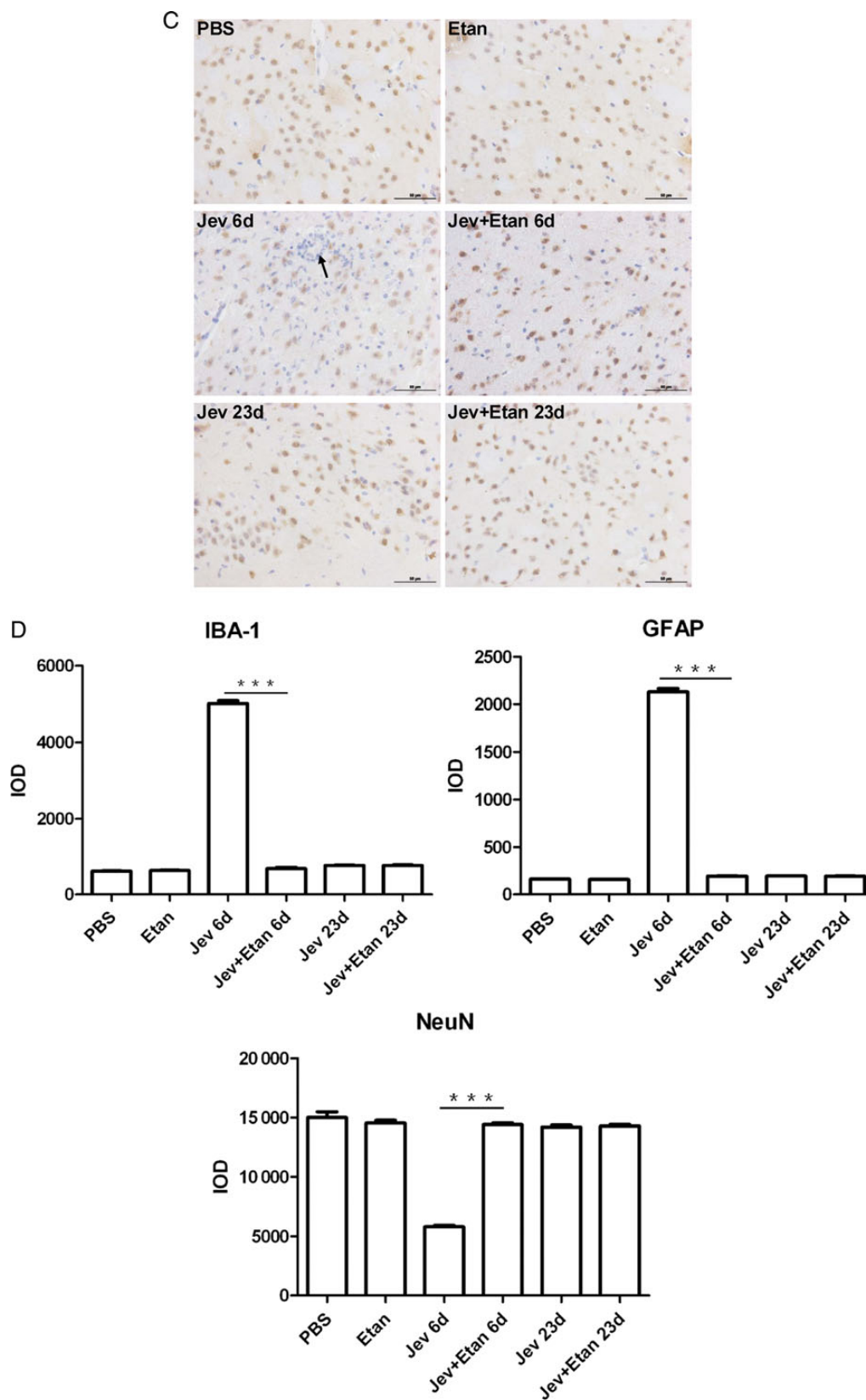


Figure 4 continued. *C*, Following immunohistochemical staining of neuronal nuclei (NeuN) representing the amount of survived neurons, treatment with etanercept was also found to improve neuron survival. *D*, Integrated optical density analysis was performed to quantify the results of immunohistochemical staining. Data represent mean \pm standard error of the mean (SEM) of 3 sections from 3 mice from each group; 5 fields for each section were tested (***) $P < .001$.

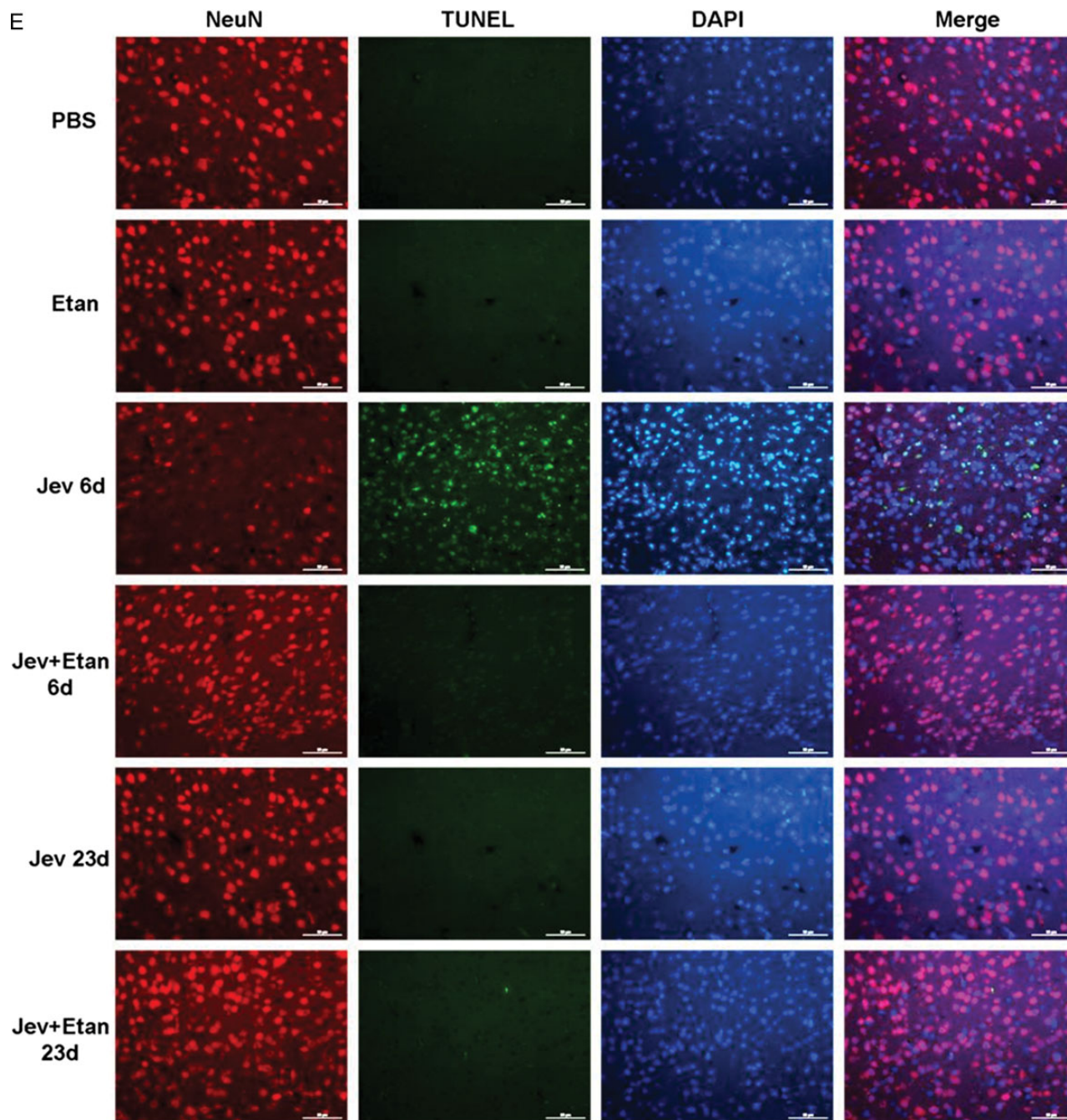


Figure 4 continued. E, TUNEL assay was performed to detect neuronal death. Neurons were stained with anti-NeuN antibody and apoptotic cells were stained using the TUNEL assay kit.

post infection (Figure 4C and 4D). These results indicate that reduction in inflammatory cytokines was indeed accompanied by reduced activation of microglia and astrocytes.

To determine whether etanercept could reduce neuronal damage, brain tissue sections were processed for TUNEL assay. The number of NeuN-positive cells was significantly increased with etanercept treatment in JEV-infected mice on day 6 post infection, which is consistent with the result of

immunohistochemical staining (Figure 4E and 4F). It suggests that etanercept could prevent JEV-caused neuronal death, which is the hallmark of pathogenesis.

Etanercept Blocks the Activation of Signaling Cascades Related to Inflammation and Apoptosis in Mouse Brain

Binding of TNF- α to TNFR induces intracellular signaling cascades that can lead to the activation of caspases and the

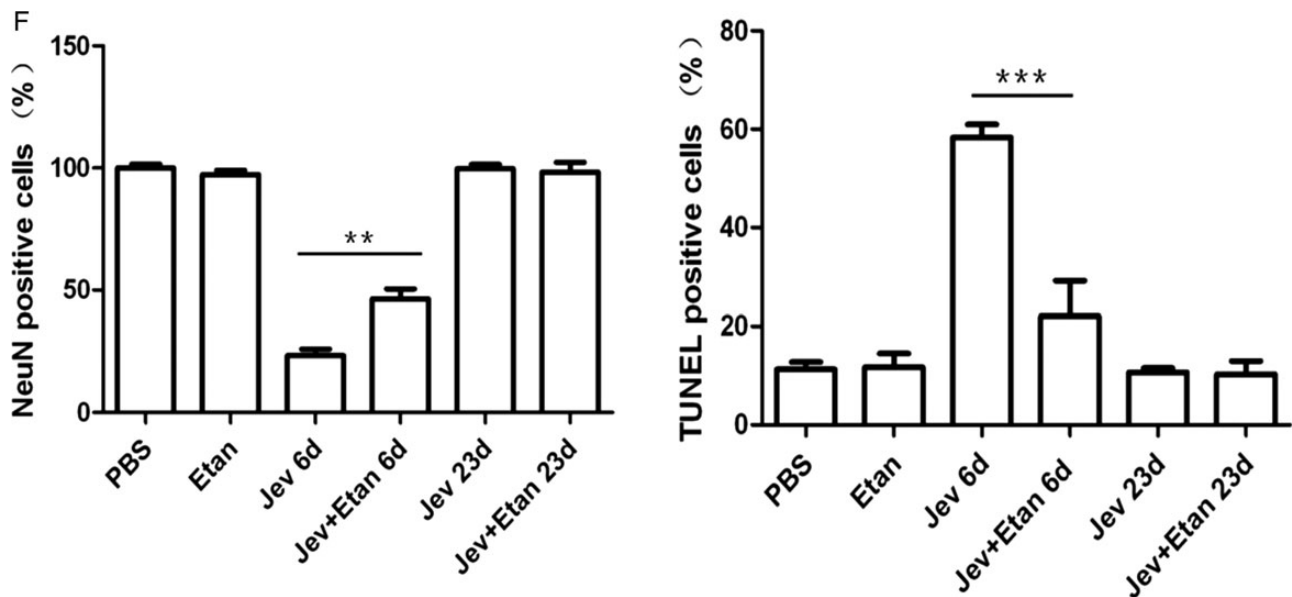


Figure 4 continued. F. The numbers of NeuN-positive cells and TUNEL-positive cells were counted and represented as mean + SEM of 3 sections from 3 mice from each group; ad 5 fields for each section were tested (** $P < .01$, *** $P < .001$).

2 transcription factors NF- κ B, and AP-1, which induce apoptosis and inflammation, respectively. To evaluate the regulatory effect of etanercept on the downstream signaling of TNF- α , the activation of caspase 3 and the nuclear translocation of NF- κ B and AP-1 in mouse brain were examined. A significant increase in active caspase 3 was observed in the JEV-infected mice. However, etanercept treatment effectively reduced the level of active caspase 3 (Figure 5A). Similarly, the nuclear translocation of NF- κ B and AP-1 was significantly upregulated in JEV-infected mice, whereas this effect was inhibited in response to etanercept treatment (Figure 5B and 5C). These results indicate that etanercept suppresses the apoptosis and inflammatory signaling induced by JEV infection.

Etanercept Treatment Regulates Viral-Induced BBB Disintegration and Reduces Viral Load in Mouse Brain

A key factor in the regulation of viral pathogenesis in brain infection is damage to the BBB caused by neuroinflammation. Therefore, an NaF uptake assay was performed to further determine whether etanercept plays a role in the maintenance of BBB integrity. Our results revealed that JEV infection markedly increased BBB permeability at day 6 post infection, whereas etanercept treatment restored BBB integrity (Figure 6A).

To further relate these curative effects with viral load in a mouse brain, mouse brain samples were subjected to qRT-PCR and plaque assays. It is important to note that etanercept treatment significantly reduced the viral titers and mRNA transcripts in JEV-infected mice (Figure 6B). These results suggest an etanercept-related decrease in the viral load in mouse brain.

Etanercept Confers Protection Against JEV Infection-Induced Lethality

To further validate the role of etanercept in viral encephalitis, we examined etanercept's ability to protect mice from JEV-induced lethality. High mortality was observed in mice that succumbed to JEV infection within 5–7 days post infection (Figure 7A). All mice in the Etan and PBS groups survived during observation. As anticipated, most mice in the Jev group died within 14 days post infection (30% survival). In contrast, mortality was decreased by 50% in the Jev + Etan group (80% survival), suggesting etanercept provided effective protection against JEV-induced mortality.

To verify the effect of etanercept on neurological sequelae and brain function, behavioral scoring was performed during the course of observation [28, 29]. An improved behavior was observed in the Jev + Etan group compared with that in the Jev group (Figure 7B), suggesting that etanercept treatment alleviated suffering in animals with JE. Similar to the mortality pattern, high scores concentrated within 5–7 days post infection. The scores of living mice reflected a gradual decrease in the progression of disease, and mice in the Etan and PBS groups did not show any alterations in behavior.

DISCUSSION

JE is characterized by profound neuronal damage along with intense microgliosis and astrogliosis. During the course of the disease, TNF- α is believed to play a significant role in the development of neuropathology by mediating neuronal apoptosis with the engagement of TNFR and TRADD. The increasing

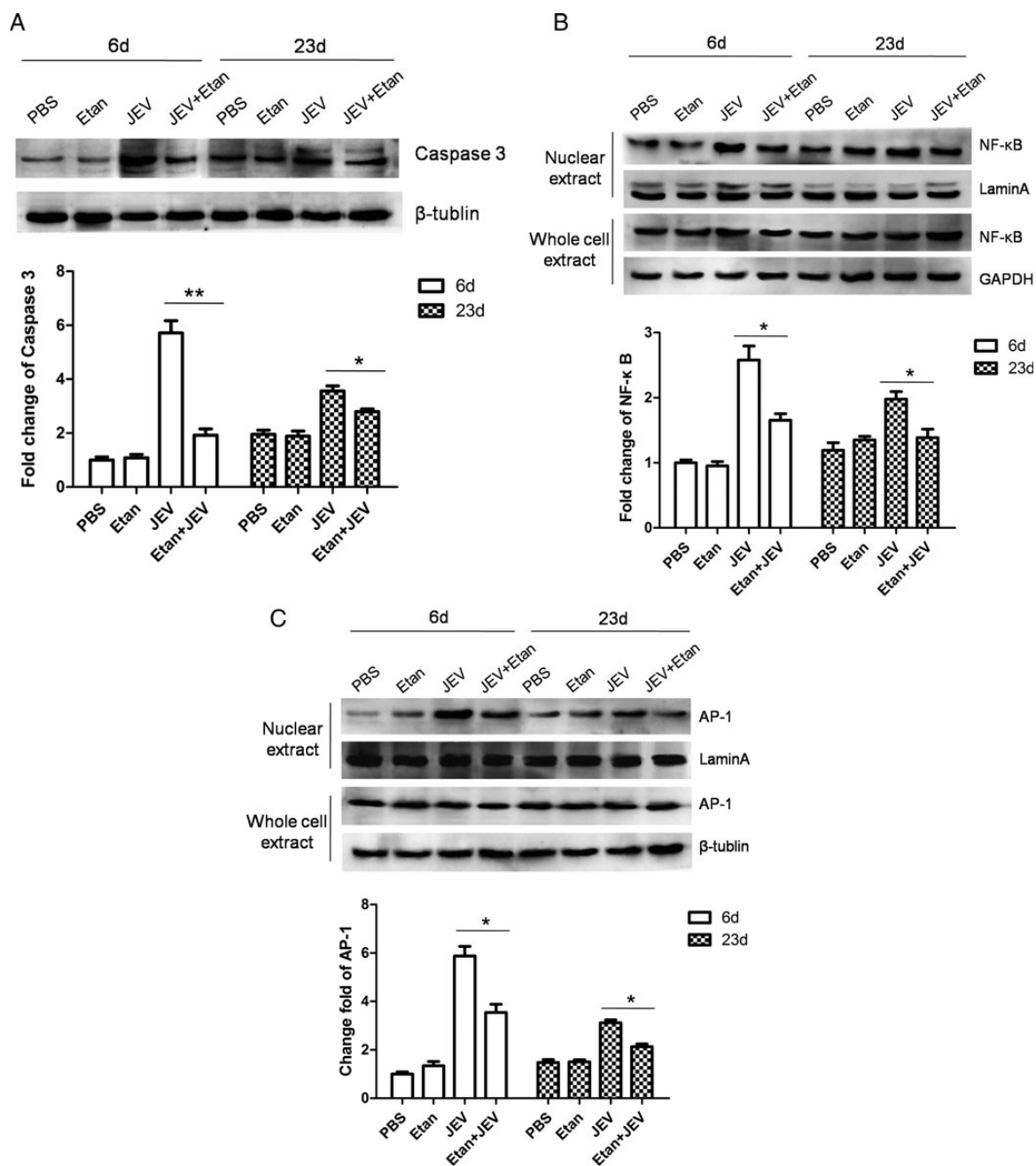


Figure 5. Etanercept treatment inhibits caspase 3 activation and nuclear localization of nuclear factor-kappaB (NF- κ B) and activation protein-1 (AP-1). Whole cell protein and nuclear protein were isolated from mouse brain tissue at 6 and 23 dpi. The expressions of active caspase 3 (A), NF- κ B (B), and AP-1 (C) in nucleus and whole cell extract were detected by Western blot. The protein levels were quantified by immunoblot scanning and normalized with respect to the amount of β -tubulin, glyceraldehyde-3-phosphate dehydrogenase, and lamin A. The ratio of NF- κ B and AP-1 in the nucleus to that in whole cell extract was calculated. Error bars represent the standard deviations of results from 3 independent assays with 3 mice from each group (* $P < .05$, ** $P < .01$, *** $P < .001$).

mortality rate with increasing concentrations of TNF- α in serum and cerebrospinal fluid was shown in JE patients [7]. Additionally, it has been reported that proinflammatory mediators released by activated microglia induce neuronal death during JE. Also, release of glutamate by JEV-infected microglia involves TNF- α signaling, which contributes to neuronal death [30, 31].

On the other hand, TNF- α has a protective role against encephalitic virus infection. For example, TNF- α was shown to provide protection against WNV infections and restrict WNV pathogenesis by promoting the trafficking of mononuclear leukocytes into the CNS [32, 33]; a study on herpes simplex virus (HSV) also demonstrated a protective role of TNF- α against HSV-induced

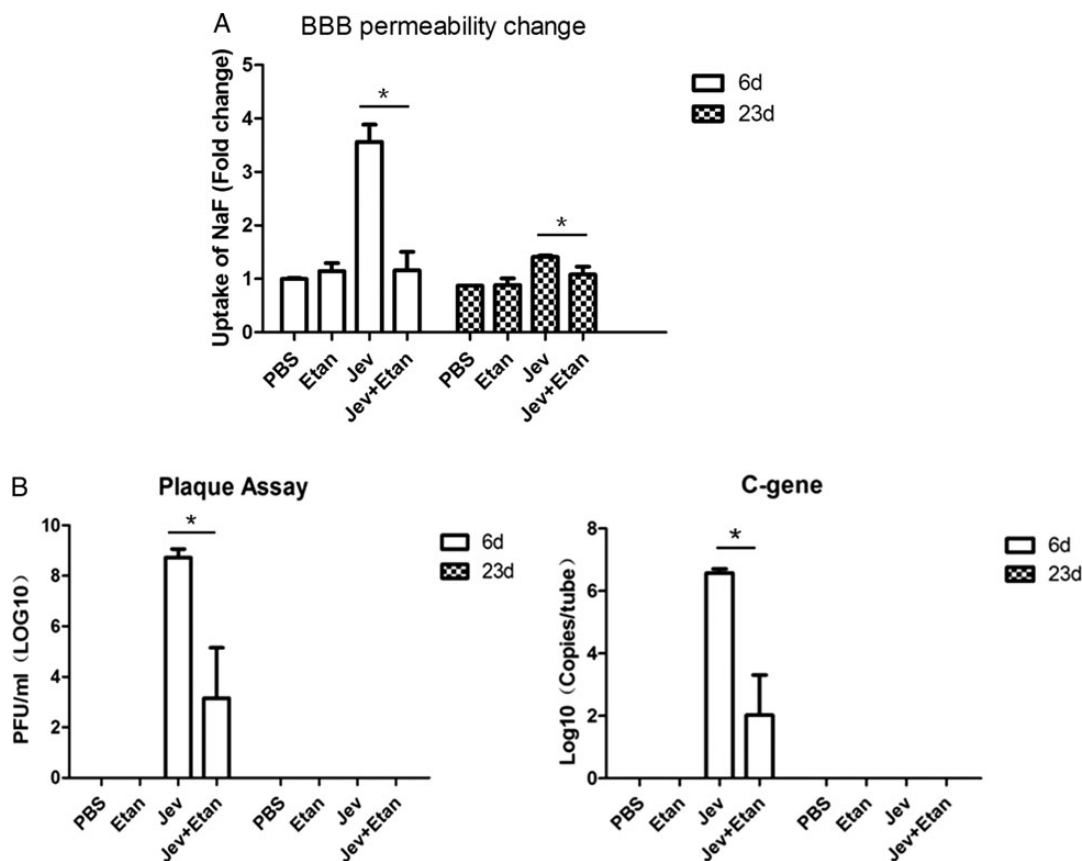


Figure 6. Etanercept treatment regulates viral induced blood–brain barrier (BBB) disintegration and reduces viral load in mouse brain. *A*, BBB permeability was assessed by sodium fluorescein (NaF) uptake assay. Mice were injected intraperitoneally with NaF on day 6 and day 23 post infection. The brain tissues containing hippocampus, frontal cortex, and putamen regions were collected. The fluorescence was detected using a fluorescence plate reader. BBB permeability was expressed as pg sodium fluorescein/ μ g protein. Data represent mean \pm standard error of the mean (SEM) of 3 independent experiments ($*P < .05$). *B*, Plaque-forming unit (left panel) and *C* gene copies (right panel) of virus in mouse brain were measured by plaque assay and quantitative reverse-transcription polymerase chain reaction. Data represent mean \pm SEM of 5 independent experiments with 5 mice from each group ($*P < .05$).

encephalitis [34]. This suggests that TNF- α acts as both neurodegenerator and neuroprotector during viral encephalitis. Therefore, the regulation between neurodegeneration and neuroprotection functions of TNF- α may be important for the pathogenesis and clinical outcome of viral encephalitis. Initiation of immunoregulation by TNF- α is an important protective mechanism in the CNS, whereas unrestrained TNF- α production may result in irreparable brain damage. In this study, we focused on the effect of the anti-TNF- α agent on a mouse model with well-established JEV infection and activated TNF- α signaling, which is quite different from the studies on WNV and HSV that used TNFR-deficient or TNF- α -depleted mice for viral challenge.

A number of anti-TNF- α agents, including infliximab, adalimumab, and etanercept, have been licensed for a diverse set of human inflammatory disorders such as ankylosing spondylitis [20, 21], rheumatoid arthritis [20], chronic asthma [35], cholestasis [36], and Crohn's disease [37]. Etanercept is a competitive TNF- α inhibitor that has been shown to be effective in acute CNS injury [38]. It has been reported that anti-TNF- α

treatment is effective in preventing initiation of pathology along with amelioration of disease progression in cases of autoimmune encephalomyelitis [39]. Another recent study demonstrated that even the peripheral administration of etanercept is effective in reducing neutrophil recruitment to IL-1 β - or LPS-induced brain lesions with an intact BBB [40]. However, this drug has never been tested as a therapeutic measure against any neurotropic virus. To the best of our knowledge, this is the first report to demonstrate the curative effects of etanercept on viral encephalitis.

It has been reported that the outcome of JEV pathogenesis is significantly influenced by microglial activation, which triggers bystander damage by release of inflammatory mediators [16]. Therefore, inhibition of neuroinflammatory factors seems to be a practical and curative measure against JEV infection. In this study, a decrease in microglial activation along with a reduction in proinflammatory cytokines occurred with etanercept treatment following JEV infection. This may be an important factor that contributes to the reduced neuronal death observed

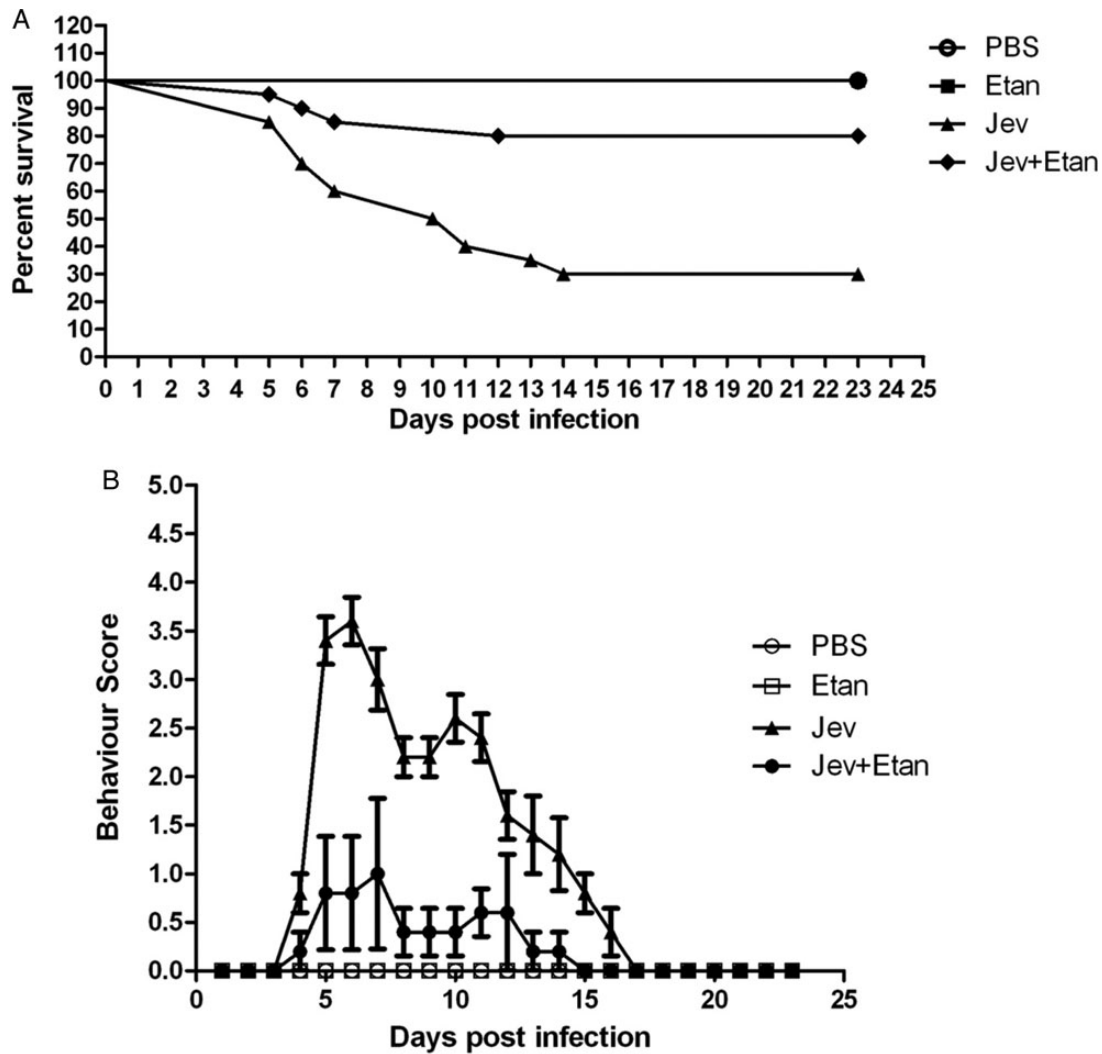


Figure 7. Etanercept treatment protects mice from Japanese encephalitis virus (JEV) infection. *A*, Survival of mice in each group was monitored for 23 days after intraperitoneal inoculation of JEV. Data were collected and shown as Kaplan–Meier survival curves ($n = 20$ for each group). *B*, Behavior score chart showing the gradual alleviation of sufferings following JEV infection. Note: Behavior score. 0 = no restriction of movement; no blink frequently; no body stiffening; no hind limb paralysis. 1 = no restriction of movement; blink frequently; no body stiffening; no hind limb paralysis. 2 = restriction of movement; blink frequently; no body stiffening; no hind limb paralysis. 3 = restriction of movement; body stiffening; no hind limb paralysis. 4 = restriction of movement; eyes closed; body stiffening; hind limb paralysis, sometimes tremor.

in the Jev + Etan group. Inhibition of the activation of the signaling cascades caspase 3, NF- κ B and AP-1 by etanercept further adds to the evidence that etanercept could attenuate JEV-induced apoptosis and inflammation by blocking the downstream signaling pathways of TNF- α .

JEV is characterized by disruption of the BBB and enhanced infiltration of immune cells into the CNS. Crossing the BBB is an important factor in the increased pathogenesis and clinical outcome of JEV infection. Our results showed that etanercept can inhibit the permeability change of the BBB induced by JEV infection. This may be influenced by the effect of etanercept on the inhibition of the release of inflammatory mediators, which are critical to the regulation of BBB permeability.

Compared with its antiinflammatory property, the antiviral effect of etanercept in mouse brain is a new phenomenon. Treatment with etanercept after JEV infection slightly increased the viral replication in neuron/glia cultures, which may be caused by reduced inflammation and neuronal death. However, no obvious change in viral replication was observed under the condition of JEV incubation with etanercept prior to infection. These results suggest that etanercept has no direct antiviral activity against JEV. Therefore, the etanercept-related reduction of viral load in mouse brain may be influenced by the effect of etanercept on restoring BBB integrity, which blocks JEV entry to the CNS. Consequently, etanercept may alleviate the sufferings caused by JE not only due to the inhibited

inflammatory response but also due to the reduced viral load in mouse brain.

In the present study, we demonstrate that etanercept administration rescued 50% of mice with well-established JEV infection. Significant reduction of symptoms by etanercept treatment was also shown by the preservation of brain tissue and improved behaviors. As observed, high scores concentrated within 5–7 days post infection, which is the acute period of infection, suggesting the neurological dysfunction of JEV-infected mice. The mice in the Etan and PBS groups did not show any alteration in behaviors, indicating no specific toxicity of drug.

In conclusion, the major finding in this study is that etanercept treatment provides effective protection against acute, established viral encephalitis in a JEV-infected mouse model. This drug-related neuroprotective effect is found to be associated with marked decrease in the level of proinflammatory cytokines, neuronal apoptosis, microgliosis, BBB permeability, viral titer, and mortality of mice. Since etanercept is a safe and readily available drug that is commonly used for the treatment of various human inflammatory diseases, it may be considered as an attractive candidate for human trial against JE. Moreover, this study may also provide insight into the use of etanercept against other viral encephalitis.

Notes

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Potential conflicts of interest. All authors: No reported conflicts.

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