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Etanercept reduces neuroinflammation and lethality in mouse model of Japanese encephalitis

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Conclusions. Etanercept effectively reduces the inflammation and provides protection against acute encephalitis in JEV infected mouse model.
Dear Editor,

We would like to thank JID for giving us the opportunity to revise our manuscript. We thank the reviewers for their careful read and thoughtful comments on previous draft. We have carefully taken all of the comments into consideration for revising our manuscript. A point by point response to the reviewers’ comments was listed in the attached file “Response to Editor/Reviewer Comments”.

This manuscript has been read and approved by all authors and all persons listed as authors have contributed to preparing the manuscript. The contents of this manuscript are our original work and have not been published, in whole or in part, prior to or simultaneous with our submission of the manuscript to JID.

We appreciate if you can reconsider it for publication.

Best regards,

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Etanercept reduces neuroinflammation and lethality in mouse model of Japanese encephalitis

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Abstract

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

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

**Results.** *In vitro* assay with mouse neuron/glia cultures showed that etanercept treatment reduced the inflammatory response induced by JEV infection. *In vivo* experiments further demonstrate 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 with etanercept treatment. Additionally, etanercept treatment restored the integrity of blood brain barrier and reduced viral load in mouse brains.

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

**Keywords:** Etanercept; Japanese encephalitis virus; viral encephalitis; TNF-α; inflammation
Viral encephalitis is a devastating human illness claiming several thousands of human lives every year and 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 contributor of viral encephalitis with 30,000-50,000 cases (mostly children) and a high fatality rate of 30% being reported annually. Clinical symptoms related with 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 Japanese encephalitis.

Japanese encephalitis 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 bystander mechanism where over activation of astrocytes and glial cells lead to emancipation of numerous proinflammatory cytokines including tumor necrosis factor alpha (TNF-α), interleukin-1β (IL-1β) and interleukin-6 (IL-6) [4-6], among which, TNF-α is regarded as one of the key factors that mediate immunopathology in the CNS. It has been reported that TNF-α is the main culprit in neurotoxic cascade of JEV [4], and the increased levels of TNF-α in cerebrospinal fluid (CSF) and serum has been correlated with cases of severe disease during JEV infection [7]. TNF-α directly mediates neuronal apoptosis by the engagement of TNFR and the TNFR-associated death domain (TRADD) and neuronal death contributes to glial activation and subsequent neuroinflammation. TNF-α is also a known trigger of ICAM-1 and VCAM-1 expression on neurovascular endothelial cells leading to leukocyte extravasation in CNS [8, 9]. Although the mechanism by which neurotropic viruses cross the blood brain barrier
(BBB) is largely unknown, TNF-α mediated changes in BBB are considered as mediator of viral entry in CNS [10-12]. TNF-α mediated regulation of the MHC II molecules is also supposed to regulate the persistency of WNV infection in brain [13, 14]. Whereas in case of BDV infection, TNF-α is blamed to trigger epileptic seizures [15]. The multiple downstream effects of TNF-α along with other chemokines mediated destruction of neurons and demyelation is well established in case of JEV and HIV induced CNS pathology [16, 17]. In addition, TNF-α plays an essential role in initiating and regulating different cytokines cascades. Due to the augmented significance of TNF-α in viral neuropathogenesis, anti-TNF-α treatment can serve as a potential therapeutic strategy in case 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 the biological effectiveness of TNF-α [18, 19]. Etanercept is frequently used to treat autoimmune disease like rheumatoid arthritis [20], ankylosing spondylitis [21], psoriasis and psoriatic arthritis [22] by acting as a TNF-α inhibitor. It has also been used as a safe drug in patients having psoriasis along with HCV infection [23]. The long-term safety of etanercept in children is well established [24]. In present study, we investigated the effect of etanercept as an anti-TNF-α therapy on JEV infected mouse model and demonstrate the significant neuroprotection offered by etanercept through reduction of inflammation.

METHODOLOGY

Preparation of primary mouse neuron/glia cultures and Virus

Neuron/glia cultures were prepared from cerebral cortices of 1-day-old Balb/c mice and plated on poly-lysine coated (20mg/ml) dishes at a density of $10^5$ cells per well in DMEM
supplemented with 5% FBS. After 8 hours for seeding, the culture medium was replaced with neurobasal medium supplemented with 2% B-27, 0.5% streptomycin and penicillin and 0.5mM 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 (MOI) of 0.1. Etanercept (10ng/ml, 100ng/ml and 1000ng/ml) or PBS was added at 6h and 12h post infection (hpi), respectively.

JEV wild type strain P3 used in this study was propagated in suckling mouse brain. Titer of virus was determined by plaque assay on BHK-21 cells as described previously [25].

**Etanercept administration to JEV-infected mice**

Adult Balb/c mice (10 weeks old) were purchased from Hubei Provincial Center for Disease Control and Prevention (Wuhan, China). Mice were randomly divided into four groups: Control group (PBS) (n= 35); only etanercept treated group (Etan) (n= 35); JEV infected group (Jev) (n= 35); JEV and etanercept treated group (Jev+Etan) (n= 35). Mice belonging to Jev and Jev+Etan groups were intraperitoneally injected with $10^6$ pfu of JEV P3 strain in 200μl phosphate buffered saline (PBS). Etanercept (100μg in 100μl physiological saline per mouse) [26] was intravenously administered to mice belonging to Jev+Etan group on day 3 and 5 post-infection [27]. Mice in PBS and Etan groups respectively received PBS and 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 the neurological parameters were recorded visually and the total score was calculated on the basis of the appearance of the symptoms [28, 29]. The rest of mice were sacrificed on day 6 and
23 post-infection and brain samples were collected for additional experiments. All experiments were performed following the protocols recommended by Research Ethics Committee of College of Veterinary Medicine, Huazhong Agricultural University, Hubei, China.

Quantification of cytokine production by ELISA

ELISA kits (Ebioscience, USA) were used to determine the secretion of TNF-α, IL-1β, IL-6 and CCL-2 in cells cultures or mouse brain tissue lysates according to the manufacturer’s instructions.

H&E and immunohistochemistry staining

Standard H&E staining protocol was followed for tissue staining. For immunohistochemical staining, sections were incubated overnight at 4°C with primary antibodies against IBA-1 (Wako, Japan), Glial Fibrillary Acidic Protein (GFAP) (Dako, Denmark) and neuronal nuclei (NeuN) (Chemicon, USA), respectively. After washing, slides were incubated with appropriate secondary antibodies, washed and cover protected. The numbers of positive cells for each antibody were analyzed for IOD (Integrated Option Density) index in 3 fields at ×200 magnification by software ImagePro Plus. At the end, ratio of positive cells for each antibody was calculated.

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 DAPI stained cells were counted in 5 fields per well/slide with 3 wells/slides per sample, and the percentage of TUNEL positive cells was calculated.

**Immunoblot analysis**

A Western-blot analysis was performed with protein isolated from brain tissues of all groups of animals. The nuclear proteins were extracted by 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), NF-κB (Cell signaling technology, USA), AP-1(Cell signaling technology, USA), β-tublin, GAPDH and Lamin A (Abclonal technology, China) antibodies. After washing, membranes were incubated with appropriate peroxidase-conjugated secondary antibodies (Boster, China). The blots were processed for development using SuperSignal West Femto (Thermo, USA). The protein levels were quantified by immunoblot scanning and normalized with respect to the amount of β-tublin, GAPDH or Lamin A.

**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 μl PBS) and allowed to circulate for 30 min. 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 8.33μl 5M NaOH to 100μl 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 sodium fluorescein/μg protein.

Detection of JEV mRNA level by qRT-PCR

Total cellular RNA was isolated and reversely transcribed by using ReverTra Ace-α-kit (TOYOBO, Japan) according to the manufacturer’s instructions. qRT-PCR experiments were carried out by SYBR Green Real time PCR Master Mix (TaKaRa, Japan) according to the manufacturer’s instructions. Moreover, 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 to JEV C gene are as follow: 5’-GGCTCTTATCAGTTCTTCTTCAAGTTT-3; 5’-TGCTTTCATCGGCCTAAAA-3’.

Statistical Analysis
Statistical analyses were performed using GraphPad Prism 5 Software. Statistical differences between the experimental groups were determined using student’s t-test. P values less than 0.05 were considered significant. Data represents mean±SEM.

RESULTS

Etanercept reduces secretion of proinflammatory cytokines and viral mediated neuronal death in vitro

In order to assess the role of etanercept in attenuating 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 6h and 12h post infection and levels of proinflammatory cytokines (TNF-α, IL-1β, IL-6 and CCL-2) in the culture media were measured. As expected, viral infection triggered the release of plentiful amount of proinflammatory cytokines, whereas etanercept treatment significantly decreased the cytokine production of neuron/glia cultures (Fig. 1A). To further evaluate the significance of etanercept in preventing neuronal death during infection, TUNEL assay was performed. As anticipated, etanercept rescued cell death in neuron/glia cultures in a dose dependent manner (Fig. 1B). Then the effect of etanercept on viral replication was validated by detecting the viral genome copies. Results showed that treatment of etanercept after viral infection slightly increased the viral replication at 48 hpi (Fig. 1C). However, incubation of JEV with etanercept prior to infection did not affect the viral replication. It suggests no direct anti-viral 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 JE mouse model. Brain tissues were collected at day 6 and 23 post infection since mice started to show the signs of infection from day 5 and most of living mice were recovered at day 23. Histological alterations of brain revealed severe meningitis in JEV infected mice on day 6 post infection, while etanercept treatment significantly alleviated the phenomenon (Fig. 2A). Both groups showed no evidence of meningitis on day 23 post infection. Vacuolar degeneration and liquifactive necrosis was also observed in neurons of Jev group but not in Jev+Etan group (Fig. 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 two groups on day 23 post infection (Fig. 2C). In addition, a significant reduction of perivascular cuffings was also found in etanercept-treated mice on day 6 post infection (Fig. 2C). These results indicate curative effects of etanercept on attenuating JEV mediated inflammation.

To quantify the levels of proinflammatory cyto/chemokines, ELISA with brain homogenates was performed. As expected, a significant reduction in the level of TNF-α in Jev+Etan group was found as compared to Jev group on day 6 post infection (Fig. 3A). On day 23 post infection, level of TNF-α in Jev group was similar to control but significantly higher than Jev+Etan group. A remarkably decreased expression of IL-1β, IL-6, CCL-2 also was also shown in Jev+Etan group compared with that in Jev group on day 6 post infection (Fig. 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, immunohistochemical staining of brain sections was performed. Plentiful star shaped, activated microglia were observed in Jev group on day 6 post-infection, while etanercept treatment downregulated microglial proliferation substantially (Fig. 4A and D). Similarly, less activated astrocyte was also shown in Jev+Etan group (Fig. 4B and D). In addition, etanercept treatment inhibited the degeneration of neurite processes caused by JEV infection along with increased number of visible neurons on day 6 post infection (Fig. 4C and D). These results indicate that reduction in inflammatory cytokines was indeed accompanied with the reduced activation of microglia and astrocytes.

To further evaluate whether etanercept could reduce neuronal damage, brain tissue sections were processed for TUNEL assay. Number of NeuN-positive cells was significantly increased upon etanercept treatment in JEV-infected mice on day 6 post infection, consistent with the result of immunohistochemical staining (Fig. 4E and F). It suggests that etanercept could rescue 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 two transcription factors, Nuclear Factor-KappaB (NF-κB) and Activation Protein-1 (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 nuclear translocation of NF-κB and AP-1 in mouse brain was examined. A significant increase of active Caspase 3 was observed in the JEV-infected mice. However, etanercept treatment effectively reduced the level of active Caspase 3 (Fig. 5A). Similarly, the nuclear translocation of NF-κB and AP-1 was significantly upregulated in JEV-infected mice, whereas this effect was inhibited in response to etanercept treatment (Fig. 5B and C). 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**

One of the key factors that regulate viral pathogenesis in brain infection is the damage of BBB caused by neuro-inflammation. Therefore, NaF uptake assay was performed to further evaluate whether etanercept can play a role in maintaining BBB integrity. Our results revealed that JEV infection markedly increased BBB permeability at day 6 post infection, whereas etanercept treatment restored BBB integrity (Fig. 6A).

To further relate these curative effects with viral load in 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 (Fig. 6 B). These results suggest an etanercept related decrease of viral load in mouse brain.
Etanercept confers protection against JEV infection induced lethality

To further validate the role of etanercept on viral encephalitis, we examined its 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 (Fig. 7A). All the mice in Etan and PBS groups survived during observation. As anticipated, most of mice in Jev group died within 14 days post infection (30% survival). In contrast, mortality was decreased by 50% in Jev+Etan group (80% survival), suggesting etanercept provided effective protection against JEV-induced mortality.

To verify the effect of etanercept on the neurological sequelae and brain function, behavioral scoring was performed during the course of observation [28, 29]. An improved behavior was observed in Jev+Etan group compared with that in Jev group (Fig. 7B), suggesting that etanercept treatment alleviated animal’s suffering from JE. Similar as 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 the mice in Etan and PBS groups did not show any alteration in behaviors.

DISCUSSION

Japanese encephalitis is characterized by profound neuronal damage along with intense microgliosis and astrogliosis. During 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 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 induces neuronal death during
JE and glutamate released by JEV-infected microglia involves TNF-α signaling contributing to neuronal death [30, 31]. On the other hand, TNF-α also has a protective role against encephalitic virus infection. For example, TNF-α was shown to protect WNV infections and restrict WNV pathogenesis by promoting trafficking of mononuclear leukocytes into the CNS [38,39]; a study on herpes simplex virus (HSV) also demonstrated a protective role of TNF-α against HSV-induced encephalitis [32]. It 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 immuno-regulation 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 focus on the effect of anti-TNF-α agent on mouse model with well-established JEV infection and activated TNF-α signaling, which is quite different from the studies on WNV and HSV using 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 [33], cholestasis [34] and Crohn’s disease [35]. Etanercept is a competitive TNF-α inhibitor that has been shown to be effective in acute CNS injury [36]. It has been previously reported that anti-TNF-α treatment is effective in preventing initiation of pathology along with amelioration of disease progression in case of autoimmune encephalomyelitis [37]. Another recent study demonstrated that even the peripheral administration of etanercept is effective in reducing neutrophils recruitment to an IL-1β or LPS induced brain lesions with an intact BBB [38]. However, this drug has never been tested as a
therapeutic measure against any neurotropic virus. As far as could be ascertained, this is the first report to demonstrate the curative effects of etanercept on viral encephalitis.

It has already 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 neuro-inflammatory factors seems to be a practical and curative measure against JEV infection. In this study, decrease in microglial activation along with reduction of proinflammatory cytokines has been shown with etanercept treatment following JEV infection. This may be an important factor that contributes to the reduced neuronal death observed in Jev+Etan group. Inhibition of the activation of signaling cascades, Caspase 3, NF-κB and AP-1, by etanercept further strengthens the evidence that etanercept could attenuate JEV-induced apoptosis and inflammation by blocking the downstream signaling pathways of TNF-α.

JEV-associated encephalitis 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 the JEV infection. Our results showed that entanercept can inhibit the permeability change of BBB induced by JEV infection. This may be contributed by the effect of entanercept on inhibiting the release of inflammatory mediators which are critical to regulate BBB permeability.

Compared with the anti-inflammatory property, antiviral effect of etanercept in mouse brain is a new phenomenon. The treatment of etanercept after JEV infection slightly increased the viral replication in neuron/glia cultures, which may be caused by the reduced inflammation and neuronal death. However, no obvious change on viral replication was observed under the
condition of incubation of JEV 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 contributed by the effect of etanercept on restoring
the BBB integrity which blocked CNS entry of JEV. So etanercept may alleviate individual’s
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 infection of JEV. Significant recovery of symptoms by etanercept treatment was
also shown by the preservation of brain tissue and the 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 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 JEV-infected mouse model. This drug
related neuroprotective effect is found to be associated with marked decrease in (i) the level of
proinflammatory cytokines, (ii) neuronal apoptosis, (iii) microgliosis, (iv) BBB permeability, (v)
viral titer and (vi) mortality of mice. Since etanercept is a safe and easily available drug
commonly used for the treatment of various human inflammatory diseases, it may be considered
as an attractive candidate for human trial against Japanese encephalitis. Moreover, this study
may also provide insight into the use of etanercept for the therapy against other viral
encephalitis.
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Conflict of Interests
All authors report no potential conflict of interests.

References


Figure legends

Figure 1. Etanercept attenuates 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
24-well plate at a density of $1 \times 10^5$ cells/well and mock-infected or infected with JEV (0.01 MOI). Cells were treated with either Etan (10ng/ml, 100ng/ml, 1000ng/ml) or PBS at 6h and 12h post infection. A. At 48hpi, the supernatant of mixed cells were harvested and the concentrations of TNF-α, IL-1β, IL-6 and CCL2 were determined by ELISA. B. Cell death was measured by TUNEL assay. TUNEL-stained cells and total cells (DAPI) were randomly photographed. Scale bar represents 100 um. The numbers of TUNEL-positive cells compared with total cells were counted (left graph). C. Neuron/glia cultures were either infected with JEV followed by treatment of etanercept or PBS as described above (left panel) or infected with JEV that had been pre-incubated with etanercept or PBS for 1h (right panel). Cells were harvested at 24 and 48 hpi and the C gene copies were determined by qRT-PCR. Data represents mean + SEM for three independent experiments (*P < 0.05, **P < 0.01).

Figure 2. Etanercept treatment contributes to the improved histopathological changes of mice caused by JEV infection. Mice in each groups were sacrificed on day 6 and 23 post-infection and brain samples were collected. H&E staining of brain sections was performed to observe the pathological changes. A. Meningitis appeared in JEV infected group (indicated by arrows) while no apparent inflammatory cell accumulation was seen on day 6. B. Vacuolar degeneration of neuron was also appeared in JEV infected mice, while normal neuronal morphology was observed following Etanercept treatment (indicated by arrows) (400×). C. Numbers of glial nodules and perivascular cuffing on brain sections were counted and calculated. Data represents mean ± SEM of 3 sections from 3 mice of each group and 5 different fields for each section were performed (*P < 0.05).
Figure 3. The effect of Etanercept on the expression of TNF-α, IL-1b, IL-6 and CCL-2 following JEV infection of mice was determined by ELISA. Mice in each group were sacrificed on day 6 and 23 post-infection and brain samples were collected. The productions of TNF-α (A), IL-1β (B), IL-6 (C) and CCL-2 (D) in brain lysates were determined by ELISA. Significant reductions of TNF-α, IL-1b, IL-6 and CCL-2 were observed in Etanercept treated group on day 6 and 23 post infection (**P < 0.01, *P < 0.05). No apparent difference was found in PBS and Etanercept control groups from all the cyto/chemokines. Data represents mean ± SEM of five independent experiments with 5 mice from each group.

Figure 4. Etanercept treatment reduces glial activation and neuronal death in JEV-infected mice. Mice in each group were sacrificed on day 6 and 23 post-infection and brain samples were collected. Sections of brain tissues 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-GFAP antibody. Activated astrocyte was also found to be reduced following treatment as evidence by change in GFAP expression following day 6 post infection. C. Following immunohistochemical staining of NeuN representing the amount of survived neurons, treatment of etanercept was also found to improve neuron survival. D. IOD analysis was performed to quantify the results of immunohistochemical staining. Data represent mean ± SEM of 3 sections from 3 mice of each group and 5 different fields for each section were performed (***P < 0.001). E. TUNEL assay
was performed to detect the neuronal death. Neurons were stained with anti-NeuN antibody and apoptotic cells were stained by using TUNEL assay kit. F. The numbers of NeuN-positive cells and TUNEL-positive cells were counted and represented as mean + SEM of 3 sections from 3 mice of each group and 5 different fields for each section were performed ( **P < 0.01, ***P < 0.001).

**Figure 5.** Etanercept treatment inhibits Caspase 3 activation and nuclear localization of NF-κB and AP-1. Whole cell protein and nuclear protein was isolated from mouse brain tissue at 6 and 23 dpi. The expressions of active Caspase 3 (A), NF-κB (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-tublin, GAPDH and Lamin A. The ratio of NF-κB and AP-1 in 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 < 0.05, **P < 0.01, ***P < 0.001).

**Figure 6.** Etanercept treatment regulates viral induced BBB disintegration and reduces viral load in mouse brain. A. BBB permeability was assessed by sodium fluorescein uptake assay. Mice were injected i.p. with sodium fluorescein on day 6 and 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/μg protein. Data represent mean ± SEM of 3 independent experiments (*P<0.05). 

Plaque-forming unit (left panel) and C gene copies (right panel) of virus in mouse brain were measured by plaque assay and qRT-PCR. Data represents mean ± SEM of 5 independent experiments with 5 mice from each group (*P<0.05).

Figure 7. Etanercept treatment protects mice from 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
Etanercept reduces neuroinflammation and lethality in mouse model of Japanese encephalitis

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Abstract

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

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

**Results.** *In vitro* assay with mouse neuron/glia cultures showed that etanercept treatment reduced the inflammatory response induced by JEV infection. *In vivo* experiments further demonstrate 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 with etanercept treatment. Additionally, etanercept treatment restored the integrity of blood brain barrier and reduced viral load in mouse brains.

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

**Keywords:** Etanercept; Japanese encephalitis virus; viral encephalitis; TNF-α; inflammation
Viral encephalitis is a devastating human illness claiming several thousands of human lives every year and 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 contributor of viral encephalitis with 30,000-50,000 cases (mostly children) and a high fatality rate of 30% being reported annually. Clinical symptoms related with 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 Japanese encephalitis.

Japanese encephalitis 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 bystander mechanism where over activation of astrocytes and glial cells lead to emancipation of numerous proinflammatory cytokines including tumor necrosis factor alpha (TNF-α), interleukin-1β (IL-1β) and interleukin-6 (IL-6) [4-6], among which, TNF-α is regarded as one of the key factors that mediate immunopathology in the CNS. It has been reported that TNF-α is the main culprit in neurotoxic cascade of JEV [4], and the increased levels of TNF-α in cerebrospinal fluid (CSF) and serum has been correlated with cases of severe disease during JEV infection [7]. TNF-α directly mediates neuronal apoptosis by the engagement of TNFR and the TNFR-associated death domain (TRADD) and neuronal death contributes to glial activation and subsequent neuroinflammation. TNF-α is also a known trigger of ICAM-1 and VCAM-1 expression on neurovascular endothelial cells leading to leukocyte extravasation in CNS [8, 9]. Although the mechanism by which neurotropic viruses cross the blood brain barrier
(BBB) is largely unknown, TNF-α mediated changes in BBB are considered as mediator of viral entry in CNS [10-12]. TNF-α mediated regulation of the MHC II molecules is also supposed to regulate the persistency of WNV infection in brain [13, 14]. Whereas in case of BDV infection, TNF-α is blamed to trigger epileptic seizures [15]. The multiple downstream effects of TNF-α along with other chemokines mediated destruction of neurons and demyelation is well established in case of JEV and HIV induced CNS pathology [16, 17]. In addition, TNF-α plays an essential role in initiating and regulating different cytokines cascades. Due to the augmented significance of TNF-α in viral neuropathogenesis, anti-TNF-α treatment can serve as a potential therapeutic strategy in case 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 the biological effectiveness of TNF-α [18, 19]. Etanercept is frequently used to treat autoimmune disease like rheumatoid arthritis [20], ankylosing spondylitis [21], psoriasis and psoriatic arthritis [22] by acting as a TNF-α inhibitor. It has also been used as a safe drug in patients having psoriasis along with HCV infection [23]. The long-term safety of etanercept in children is well established [24]. In present study, we investigated the effect of etanercept as an anti-TNF-α therapy on JEV infected mouse model and demonstrate 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 cerebral cortices of 1-day-old Balb/c mice and plated on poly-lysine coated (20mg/ml) dishes at a density of $10^5$ cells per well in DMEM
supplemented with 5% FBS. After 8 hours for seeding, the culture medium was replaced with neurobasal medium supplemented with 2% B-27, 0.5% streptomycin and penicillin and 0.5mM 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 (MOI) of 0.1. Etanercept (10ng/ml, 100ng/ml and 1000ng/ml) or PBS was added at 6h and 12h post infection (hpi), respectively.

JEV wild type strain P3 used in this study was propagated in suckling mouse brain. Titer of virus was determined by plaque assay on BHK-21 cells as described previously [25].

**Etanercept administration to JEV-infected mice**

Adult Balb/c mice (10 weeks old) were purchased from Hubei Provincial Center for Disease Control and Prevention (Wuhan, China). Mice were randomly divided into four groups: Control group (PBS) (n= 35); only etanercept treated group (Etan) (n= 35); JEV infected group (Jev) (n= 35); JEV and etanercept treated group (Jev+Etan) (n= 35). Mice belonging to Jev and Jev+Etan groups were intraperitoneally injected with $10^6$ pfu of JEV P3 strain in 200μl phosphate buffered saline (PBS). Etanercept (100μg in 100μl physiological saline per mouse) [26] was intravenously administered to mice belonging to Jev+Etan group on day 3 and 5 post-infection [27]. Mice in PBS and Etan groups respectively received PBS and 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 the neurological parameters were recorded visually and the total score was calculated on the basis of the appearance of the symptoms [28, 29]. The rest of mice were sacrificed on day 6 and
23 post-infection and brain samples were collected for additional experiments. All experiments were performed following the protocols recommended by Research Ethics Committee of College of Veterinary Medicine, Huazhong Agricultural University, Hubei, China.

Quantification of cytokine production by ELISA

ELISA kits (Ebioscience, USA) were used to determine the secretion of TNF-α, IL-1β, IL-6 and CCL-2 in cells cultures or mouse brain tissue lysates according to the manufacturer’s instructions.

H&E and immunohistochemistry staining

Standard H&E staining protocol was followed for tissue staining. For immunohistochemical staining, sections were incubated overnight at 4°C with primary antibodies against IBA-1 (Wako, Japan), Glial Fibrillary Acidic Protein (GFAP) (Dako, Denmark) and neuronal nuclei (NeuN) (Chemicon, USA), respectively. After washing, slides were incubated with appropriate secondary antibodies, washed and cover protected. The numbers of positive cells for each antibody were analyzed for IOD (Integrated Option Density) index in 3 fields at ×200 magnification by software ImagePro Plus. At the end, ratio of positive cells for each antibody was calculated.

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 DAPI stained cells were counted in 5 fields per well/slide with 3 wells.slides per sample, and the percentage of TUNEL positive cells was calculated.

**Immuno blot analysis**

A Western-blot analysis was performed with protein isolated from brain tissues of all groups of animals. The nuclear proteins were extracted by 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), NF-κB (Cell signaling technology, USA), AP-1(Cell signaling technology, USA), β-tublin, GAPDH and Lamin A (Abclonal technology, China) antibodies. After washing, membranes were incubated with appropriate peroxidase-conjugated secondary antibodies (Boster, China). The blots were processed for development using SuperSignal West Femto (Thermo, USA). The protein levels were quantified by immunoblot scanning and normalized with respect to the amount of β-tublin, GAPDH or Lamin A.

**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 μl PBS) and allowed to circulate for 30 min. 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 8.33μl 5M NaOH to 100μl 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 sodium fluorescein/μg protein.

Detection of JEV mRNA level by qRT-PCR

Total cellular RNA was isolated and reversely transcribed by using ReverTra Ace-α-kit (TOYOBO, Japan) according to the manufacturer’s instructions. qRT-PCR experiments were carried out by SYBR Green Real time PCR Master Mix (TaKaRa, Japan) according to the manufacturer’s instructions. Moreover, 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 to JEV C gene are as follow: 5’-GGCTCTTATCACGTTCTTCAAGTTT-3’; 5’-TGCTTTCCATCGGCCTAAAA-3’.

Statistical Analysis
Statistical analyses were performed using GraphPad Prism 5 Software. Statistical differences between the experimental groups were determined using student’s t-test. P values less than 0.05 were considered significant. Data represents mean±SEM.

RESULTS

Etanercept reduces secretion of proinflammatory cytokines and viral mediated neuronal death in vitro

In order to assess the role of etanercept in attenuating 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 6h and 12h post infection and levels of proinflammatory cytokines (TNF-α, IL-1β, IL-6 and CCL-2) in the culture media were measured. As expected, viral infection triggered the release of plentiful amount of proinflammatory cytokines, whereas etanercept treatment significantly decreased the cytokine production of neuron/glia cultures (Fig. 1A). To further evaluate the significance of etanercept in preventing neuronal death during infection, TUNEL assay was performed. As anticipated, etanercept rescued cell death in neuron/glia cultures in a dose dependent manner (Fig. 1B). Then the effect of etanercept on viral replication was validated by detecting the viral genome copies. Results showed that treatment of etanercept after viral infection slightly increased the viral replication at 48 hpi (Fig. 1C). However, incubation of JEV with etanercept prior to infection did not affect the viral replication. It suggests no direct anti-viral activity of etanercept.
To ensure the effectiveness of etanercept in JEV induced encephalitis, in vivo experiments were performed with JE mouse model. Brain tissues were collected at day 6 and 23 post infection since mice started to show the signs of infection from day 5 and most of living mice were recovered at day 23. Histological alterations of brain revealed sever meningitis in JEV infected mice on day 6 post infection, while etanercept treatment significantly alleviated the phenomenon (Fig. 2A). Both groups showed no evidence of meningitis on day 23 post infection. Vacuolar degeneration and liquifective necrosis was also observed in neurons of Jev group but not in Jev+Etan group (Fig. 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 two groups on day 23 post infection (Fig. 2C). In addition, a significant reduction of perivascular cuffings was also found in etanercept-treated mice on day 6 post infection (Fig. 2C). These results indicate curative effects of etanercept on attenuating JEV mediated inflammation.

To quantify the levels of proinflammatory cyto/chemokines, ELISA with brain homogenates was performed. As expected, a significant reduction in the level of TNF-α in Jev+Etan group was found as compared to Jev group on day 6 post infection (Fig. 3A). On day 23 post infection, level of TNF-α in Jev group was similar to control but significantly higher than Jev+Etan group. A remarkably decreased expression of IL-1β, IL-6, CCL-2 also was also shown in Jev+Etan group compared with that in Jev group on day 6 post infection (Fig. 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, immunohistochemical staining of brain sections was performed. Plentiful star shaped, activated microglia were observed in Jev group on day 6 post-infection, while etanercept treatment downregulated microglial proliferation substantially (Fig. 4A and D). Similarly, less activated astrocyte was also shown in Jev+Etan group (Fig. 4B and D). In addition, etanercept treatment inhibited the degeneration of neurite processes caused by JEV infection along with increased number of visible neurons on day 6 post infection (Fig. 4C and D). These results indicate that reduction in inflammatory cytokines was indeed accompanied with the reduced activation of microglia and astrocytes.

To further evaluate whether etanercept could reduce neuronal damage, brain tissue sections were processed for TUNEL assay. Number of NeuN-positive cells was significantly increased upon etanercept treatment in JEV-infected mice on day 6 post infection, consistent with the result of immunohistochemical staining (Fig. 4E and F). It suggests that etanercept could rescue 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 two transcription factors, Nuclear Factor-KappaB (NF-κB) and Activation Protein-1 (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 nuclear translocation of NF-κB and AP-1 in mouse brain was examined. A
significant increase of active Caspase 3 was observed in the JEV-infected mice. However,
etanercept treatment effectively reduced the level of active Caspase 3 (Fig. 5A). Similarly, the
nuclear translocation of NF-κB and AP-1 was significantly upregulated in JEV-infected mice,
whereas this effect was inhibited in response to etanercept treatment (Fig. 5B and C). These
results indicate that etanercept suppresses the apoptosis and inflammatory signaling induced by
JEV infection.

10 Etanercept treatment regulates viral induced BBB disintegration and reduces viral load in
11 mouse brain

One of the key factors that regulate viral pathogenesis in brain infection is the damage of BBB
caused by neuro-inflammation. Therefore, NaF uptake assay was performed to further evaluate
whether etanercept can play a role in maintaining BBB integrity. Our results revealed that JEV
infection markedly increased BBB permeability at day 6 post infection, whereas etanercept
treatment restored BBB integrity (Fig. 6A).

To further relate these curative effects with viral load in 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 (Fig. 6 B).
These results suggest an etanercept related decrease of viral load in mouse brain.
Etanercept confers protection against JEV infection induced lethality

To further validate the role of etanercept on viral encephalitis, we examined its 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 (Fig. 7A). All the mice in Etan and PBS groups survived during observation. As anticipated, most of mice in Jev group died within 14 days post infection (30% survival). In contrast, mortality was decreased by 50% in Jev+Etan group (80% survival), suggesting etanercept provided effective protection against JEV-induced mortality.

To verify the effect of etanercept on the neurological sequelae and brain function, behavioral scoring was performed during the course of observation [28, 29]. An improved behavior was observed in Jev+Etan group compared with that in Jev group (Fig. 7B), suggesting that etanercept treatment alleviated animal’s suffering from JE. Similar as 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 the mice in Etan and PBS groups did not show any alteration in behaviors.

DISCUSSION

Japanese encephalitis is characterized by profound neuronal damage along with intense microgliosis and astrogliosis. During 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 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 induces neuronal death during
JE and glutamate released by JEV-infected microglia involves TNF-α signaling contributing to neuronal death [30, 31]. On the other hand, TNF-α also has a protective role against encephalitic virus infection. For example, TNF-α was shown to protect WNV infections and restrict WNV pathogenesis by promoting trafficking of mononuclear leukocytes into the CNS [38,39]; a study on herpes simplex virus (HSV) also demonstrated a protective role of TNF-α against HSV-induced encephalitis [32]. It 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 immuno-regulation 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 focus on the effect of anti-TNF-α agent on mouse model with well-established JEV infection and activated TNF-α signaling, which is quite different from the studies on WNV and HSV using 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 [33], cholestasis [34] and Crohn’s disease [35]. Etanercept is a competitive TNF-α inhibitor that has been shown to be effective in acute CNS injury [36]. It has been previously reported that anti-TNF-α treatment is effective in preventing initiation of pathology along with amelioration of disease progression in case of autoimmune encephalomyelitis [37]. Another recent study demonstrated that even the peripheral administration of etanercept is effective in reducing neutrophils recruitment to an IL-1β or LPS induced brain lesions with an intact BBB [38]. However, this drug has never been tested as a
therapeutic measure against any neurotropic virus. As far as could be ascertained, this is the first report to demonstrate the curative effects of etanercept on viral encephalitis.

It has already 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 neuro-inflammatory factors seems to be a practical and curative measure against JEV infection. In this study, decrease in microglial activation along with reduction of proinflammatory cytokines has been shown with etanercept treatment following JEV infection. This may be an important factor that contributes to the reduced neuronal death observed in Jev+Etan group. Inhibition of the activation of signaling cascades, Caspase 3, NF-κB and AP-1, by etanercept further strengthens the evidence that etanercept could attenuate JEV-induced apoptosis and inflammation by blocking the downstream signaling pathways of TNF-α.

JEV-associated encephalitis 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 the JEV infection. Our results showed that entanercept can inhibit the permeability change of BBB induced by JEV infection. This may be contributed by the effect of entanercept on inhibiting the release of inflammatory mediators which are critical to regulate BBB permeability.

Compared with the anti-inflammatory property, antiviral effect of etanercept in mouse brain is a new phenomenon. The treatment of etanercept after JEV infection slightly increased the viral replication in neuron/glia cultures, which may be caused by the reduced inflammation and neuronal death. However, no obvious change on viral replication was observed under the
condition of incubation of JEV 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 contributed by the effect of etanercept on restoring the BBB integrity which blocked CNS entry of JEV. So etanercept may alleviate individual’s 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 infection of JEV. Significant recovery of symptoms by etanercept treatment was also shown by the preservation of brain tissue and the 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 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 JEV-infected mouse model. This drug related neuroprotective effect is found to be associated with marked decrease in (i) the level of proinflammatory cytokines, (ii) neuronal apoptosis, (iii) microgliosis, (iv) BBB permeability, (v) viral titer and (vi) mortality of mice. Since etanercept is a safe and easily available drug commonly used for the treatment of various human inflammatory diseases, it may be considered as an attractive candidate for human trial against Japanese encephalitis. Moreover, this study may also provide insight into the use of etanercept for the therapy against other viral encephalitis.
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Conflict of Interests

All authors report no potential conflict of interests.

References


Figure legends

Figure 1. Etanercept attenuates 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
24-well plate at a density of $1 \times 10^5$ cells/well and mock-infected or infected with JEV (0.01 MOI). Cells were treated with either Etan (10ng/ml, 100ng/ml, 1000ng/ml) or PBS at 6h and 12h post infection. A. At 48hpi, the supernatant of mixed cells were harvested and the concentrations of TNF-α, IL-1β, IL-6 and CCL2 were determined by ELISA. B. Cell death was measured by TUNEL assay. TUNEL-stained cells and total cells (DAPI) were randomly photographed. Scale bar represents 100 um. The numbers of TUNEL-positive cells compared with total cells were counted (left graph). C. Neuron/glia cultures were either infected with JEV followed by treatment of etanercept or PBS as described above (left panel) or infected with JEV that had been pre-incubated with etanercept or PBS for 1h (right panel). Cells were harvested at 24 and 48 hpi and the C gene copies were determined by qRT-PCR. Data represents mean ± SEM for three independent experiments (*P < 0.05, **P < 0.01).

**Figure 2.** Etanercept treatment contributes to the improved histopathological changes of mice caused by JEV infection. Mice in each groups were sacrificed on day 6 and 23 post-infection and brain samples were collected. H&E staining of brain sections was performed to observe the pathological changes. A. Meningitis appeared in JEV infected group (indicated by arrows) while no apparent inflammatory cell accumulation was seen on day 6. B. Vacuolar degeneration of neuron was also appeared in JEV infected mice, while normal neuronal morphology was observed following Etanercept treatment (indicated by arrows) (400×). C. Numbers of glial nodules and perivascular cuffing on brain sections were counted and calculated. Data represents mean ± SEM of 3 sections from 3 mice of each group and 5 different fields for each section were performed (*P < 0.05).
**Figure 3.** The effect of Etanercept on the expression of TNF-α, IL-1β, IL-6 and CCL-2 following JEV infection of mice was determined by ELISA. Mice in each group were sacrificed on day 6 and 23 post-infection and brain samples were collected. The productions of TNF-α (A), IL-1β (B), IL-6 (C) and CCL-2 (D) in brain lysates were determined by ELISA. Significant reductions of TNF-α, IL-1β, IL-6 and CCL-2 were observed in Etanercept treated group on day 6 and 23 post infection (**P < 0.01, *P < 0.05). No apparent difference was found in PBS and Etanercept control groups from all the cytokines. Data represents mean ± SEM of five independent experiments with 5 mice from each group.

**Figure 4.** Etanercept treatment reduces glial activation and neuronal death in JEV-infected mice. Mice in each group were sacrificed on day 6 and 23 post-infection and brain samples were collected. Sections of brain tissues 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-GFAP antibody. Activated astrocyte was also found to be reduced following treatment as evidence by change in GFAP expression following day 6 post infection. C. Following immunohistochemical staining of NeuN representing the amount of survived neurons, treatment of etanercept was also found to improve neuron survival. D. IOD analysis was performed to quantify the results of immunohistochemical staining. Data represent mean ± SEM of 3 sections from 3 mice of each group and 5 different fields for each section were performed (***P < 0.001). E. TUNEL assay
was performed to detect the neuronal death. Neurons were stained with anti-NeuN antibody and apoptotic cells were stained by using TUNEL assay kit. F. The numbers of NeuN-positive cells and TUNEL-positive cells were counted and represented as mean + SEM of 3 sections from 3 mice of each group and 5 different fields for each section were performed (**P<0.01, ***P<0.001).

**Figure 5.** Etanercept treatment inhibits Caspase 3 activation and nuclear localization of NF-κB and AP-1. Whole cell protein and nuclear protein was isolated from mouse brain tissue at 6 and 23 dpi. The expressions of active Caspase 3 (A), NF-κB (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-tublin, GAPDH and Lamin A. The ratio of NF-κB and AP-1 in 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<0.05, **P<0.01, ***P<0.001).

**Figure 6.** Etanercept treatment regulates viral induced BBB disintegration and reduces viral load in mouse brain. A. BBB permeability was assessed by sodium fluorescein uptake assay. Mice were injected i.p. with sodium fluorescein on day 6 and 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/μg protein. Data represent mean ± SEM of 3 independent experiments (*P<0.05). B. Plaque-forming unit (left panel) and C gene copies (right panel) of virus in mouse brain were measured by plaque assay and qRT-PCR. Data represents mean ± SEM of 5 independent experiments with 5 mice from each group (*P<0.05).

Figure 7. Etanercept treatment protects mice from 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
Dear Editor,

We would like to thank JID for giving us the opportunity to revise our manuscript. We thank the reviewers for their careful read and thoughtful comments on previous draft. We have carefully taken all of the comments into consideration for preparing our revision, and a point by point response to the comments was listed in this letter.

We appreciate if you can reconsider it for publication.

Best regards,

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Response to the reviewer comments

Reviewer #1

Major concern:
The experiments conducted in this manuscript are straight-forward and the data consistently show a protective role of blocking TNF-alpha (by etanercept, a fusion protein of TNF receptor 2 and the Fc of IgG1) in JEV-induced encephalitis. However, TNF is a multifunctional cytokine that has a role in induction and regulation of host innate and adaptive immune response; previous studies on other models of virus-induced encephalitis have shown a different role of TNF-alpha in viral encephalitis than reported here. For example, in a study of West Nile virus (WNV), a flavivirus related to JEV, TNF-alpha plays a protective role in controlling CNS infection of WNV (Journal of Virology 82: 8956-8964, 2008). A fatal case of WNV with meningoencephalitis and acute flaccid paralysis after anti-TNF-alpha antibody (infliximab) therapy has been reported (J Rheumatol 33: 191-192, 2006). A study on herpes simplex virus (HSV) also showed a protective role of TNF against HSV-induced encephalitis (Journal of Virology 81: 1454-1460, 2007) and 3 cases of HSV-encephalitis in patients receiving TNF-alpha inhibitors for rheumatologic disorders have been reported (Clin Infect Dis 49: 924-927, 2009). Thus, the authors should only focus on JEV and should not extend their conclusion to viral encephalitis in general (as shown in the title). The previous studies showing contradictory results should be mentioned and discussed. Furthermore, to strengthen their results on JEV, they should provide other lines of evidences supporting a pathological role of TNF-alpha in JEV-induced encephalitis; for example, TNF receptor-deficient mice and TNF-alpha-depleted mice have been used to test the biological significance of TNF-alpha in WNV infection. Other TNF-alpha inhibitors should also be tested to verify whether it is an "etanercept"-specific or "anti-TNF"-mediated therapeutic event.

Reply:
Thanks for mentioning the critical issues. Many studies have reported that TNF-α acts both as degenerator and protector of neurons. On one hand, TNF-α directly mediates neuronal apoptosis by the engagement of TNF receptor 1 (TNFR1), the TNFR-associated death domain (TRADD) and neuronal death contributes to glial activation and subsequent neuroinflammation; on the other hand, TNF-α was shown to protect against the infections of many encephalitic virus and restricts pathogenesis. In our perspective, the duel roles of TNF-α may coexist in the host. Although initiation of immuno-regulation by TNF-α is an important protective mechanism in the CNS, unrestrained TNF-α production may result in irreparable brain damage. In case of JEV infection, the over-activation of glial cells along with the high production of pro-inflammatory cyto/chemokines has been demonstrated to contribute significantly to the fatal outcome, and it was reported that the mortality rate increases with increasing concentrations of TNF-α in serum and cerebrospinal fluid in JE
patients [1], suggesting the high level of TNF-α is one of the key factors that mediate the immunopathogenesis of JEV infection. In both studies on WNV and HSV, TNFR deficient or TNF-α depleted mice were used for evaluating the roles of TNF-α on viral infection, which means that the TNF-α signaling was eliminated before viral challenge. However, in our study, we focused on the effect of anti-TNF-α agent on mouse model with well-established JEV infection, suggesting that the anti- TNF-α treatment was performed after the TNF-α signaling had been activated by viral infection. These may explain the contradictory results between previous researches on WNV and HSV and our research on JEV. Considering the different results in previous studies with other models of virus-induced encephalitis, we agree that it is better to focus our study only on JEV but not extend the conclusion to viral encephalitis in general. We have revised the manuscript according to reviewer’s suggestion on this point. And the previous studies showing contradictory results has been mentioned and discussed in the discussion section of the revised manuscript.

Recently, increasing evidences have been reported to support the role of TNF-α in JEV pathogenesis. As mentioned in the manuscript, proinflammatory mediators released by activated microglia induces neuronal death in Japanese Encephalitis [2]; TNF-α released by microglia in JEV infection stimulates microglial glutamate release which contributes to neuronal death [3]. Moreover, it has been reported that increased levels of TNF-α in cerebrospinal fluid (CSF) and serum correlate with cases of severe disease in JE patients [1]. All these evidences are mentioned in the revised manuscript. In addition, our study which demonstrates the anti-TNF-α agent can provide protection against acute encephalitis in JEV infected mice would also be regarded as strong evidence supporting a pathological role of TNF-α in JEV-induced encephalitis. However, at this stage of our knowledge, the precise role of the TNF-α response in encephalitic flaviviral pathogenesis remains to be clarified.

Etanercept is a recombinant protein which fuses the TNF receptor to the constant end of the IgG1 antibody. It is a commercial biopharmaceutical that treats autoimmune diseases by acting as a TNF inhibitor. The only mechanism of action is functioning as a decoy receptor that binds to TNF and reducing the effect of naturally present TNF. Till now, no other role of etanercept except TNF-α inhibitor was reported. Therefore, the therapeutic effect of etanercept in this study is believed to be an "anti-TNF"-mediated therapeutic event. To further confirm this, another TNF-α inhibitor, WP9QY, was employed to test its effect on JE according to reviewer’s suggestion. WP9QY is a cyclic peptide which mimics the critical recognition loop on TNF-R1, hence possesses similar function as etanercept. The in vitro experiments demonstrated that treatment of neuron/glia culture with WP9QY also reduced the release of proinflammtory cytokines and neuronal death induced by JEV infection. It suggests that the therapeutic effect on JE is not “etanercept specific”. This data was shown as follow but not shown in the manuscript, since this study mainly focuses on etanercept which has been widely used as an anti-TNF agent with well established safety in human use, and the key point may be confused by adding the experiments
with another TNF-α antagonist.

Figure 1. WP9QY attenuates JEV-induced inflammation in neuron/glia cultures.
Neuron/glia cultures were prepared from the cerebral cortices of 1-day-old mice. Cells were plated in 24-well plate at a density of $1\times10^5$ cells/well and mock-infected or infected with JEV (0.01 MOI). Cells were treated with either WP9QY (10ng/ml, 100ng/ml, 1000ng/ml) or PBS at 6h and 12h post infection. A. At 48hpi, the supernatant of mixed cells were harvested and the concentrations of TNF-α, IL-1β, IL-6 and CCL2 were determined by ELISA. B. Cell death was measured by TUNEL assay. TUNEL-stained cells and total cells (DAPI) were randomly photographed. Scale bar represents 100 um. The numbers of TUNEL-positive cells compared with total cells were counted and represented as mean + SEM (left graph) for three independent experiments (*P < 0.05, **P < 0.01, ***P < 0.001).

Other points:

1. Figure 1 shows that etanercept attenuates JEV-induced inflammation in neuron/glia culture, but no data are presented regarding its effect on viral replication. The authors claim that their study clearly indicates an obvious antiviral role of etanercept against JEV (page 15 lines 19-20). However, they only show a reduction of viral load in mouse brain after etanercept treatment (Figure 6), which can be a result of either an antiviral effect of etanercept or from an anti-inflammatory effect blocking CNS entry of JEV. To clarify these possibilities, they should study the effect of etanercept on JEV replication in their in vitro culture system.

Reply:

Our results show an obvious reduction of viral load in mouse brain upon etanercept treatment, so we claim that etanercept has an antiviral role against JEV. But as we discussed, the antiviral role of etanercept may be indirect and contributed by its effect on restoring the BBB permeability which blocks the viral entry into CNS. To further clarify this possibility, we performed the qRT-RCR to exam the viral replication in neuron/glia cultures under different conditions. Our results show that viral replication was increased slightly at 48 hpi in condition of etanercept treatment following JEV infection (may be caused by the reduced antiviral response and neuronal death); no significant changes on viral replication when virus was incubated with etanercept prior to infection. It indicates that etanercept has no direct antiviral effect on JEV infection. Thus it is most likely that the antiviral role of etanercept is contributed by the restored BBB permeability. This data was shown in figure 1C in the revised manuscript.

2. Figure 3D shows the data for CCL-2; however, in the legend of Figure 3, both RANTES (also called CCL5) and CCL-2 (also called MCP-1) are mentioned. Which one is correct?

Reply:

We are sorry for this mistake. We just checked the expression of CCL-2 (MCP-1) but
not CCL-5 (RANTES). We have revised this point in the METHOD and figure legend.

3. What is "NaF" mentioned in page 12 (line 19) and in Figure 6A, sodium fluoride? Furthermore, it is mentioned in the legend of Figure 6A that different brain regions (hippocampus, frontal cortex, and putamen) were isolated, but only one data point is shown in the figure. Which part of the brain does this data point come from?

Reply:
We are sorry for the confusion. NaF mentioned in page 12 (line 19) and Figure 6A means sodium fluorescein (also called fluorescein sodium) which is widely used as a fluorescent tracer for many applications. To check the BBB permeability of mice, mouse brain tissues containing different regions were harvested and subjected to NaF uptake assay. It means that the regions mentioned above were collected together and the data shown in figure 6A indicates the total NaF uptake level of all these regions of mouse brain. We have modified this point in the METHODS and FIGURE LEGEND sections of revised manuscript.

Reviewer #2

1. Linguistic and spelling errors are frequent and annoying. The paper needs proofreading and editing.

Reply:
We have modified the language errors in the revised manuscript.

2. Five mice were from the initial group of 25 per condition in the mortality/behavior studies. How were these mice chosen? Was this random or could it have affected scoring/mortality (e.g., if sicker or less sick mice were taken)?

Reply:
As mentioned in the METHODS section, 20 mice from each group of 35 mice were monitored for mortality and behavior studies. All the mice were purchased from Hubei Provincial Center for Disease Control and Prevention with the guarantee of health, and the selection in each step was random, so it would not affect the scoring of behavior and mortality.

3. The primary neuron/glial culture data add little, as the bulk of the studies use an in vivo mouse model; please eliminate these.

Reply:
Thanks for the suggestion. However, in our opinion, the in vitro data is still necessary for this study. The in vivo environment is complex. The primary neuron/glial culture
is a proper material to investigate the anti-inflammation effect of etanercept on CNS directly. The in vitro data can serve as a strong evidence for this study and also a basis for the following in vivo experiments.

4. Please specify in methods or figure legends the exact number of mice used for different experiments and if the same mice were used to generate several figures, please make this clear if the figures have different numbers (it is fine if same mice were, for example, used in different panels of the same figure with the number specified in the legend...).

Reply:
As described in METHODS, 20 mice of each group (35 mice) were monitored daily to access behavior and mortality. The rest of 15 mice were sacrificed at day 6 and 23 post-infection and brain samples were collected for additional experiments. Among these, 8 mice were sacrificed at day 6, 3 of which were used for histopathological (figure 2) and immunohistochemical analysis (figure 4), and 5 of which were used for cyto/chemokine production assay (figure 3), signal molecule analysis (figure 5), BBB permeability assay and viral load determination (figure 6). At day 23 post-infection, most of mice in Jev group and some mice in Jev+Etan group were dead, so all the survival mice for behavior and mortality analysis were also sacrificed to supplement other experiments. As same as day 6, 8 mouse brains in each group were collected at day 23 for the following experiments. 3 of them were used for histopathological (Figure 2) and immunohistochemical analysis (figure 4), and the other 5 were used for cyto/chemokine production assay (figure 3), signal molecule analysis (figure 5), BBB permeability assay and viral load quantification (figure 6). In the revised manuscript, the exact number of mice used for different experiments were specified in the figure legends.

5. The behavioral score seems arbitrary. Has this been validated? How were the individual steps selected? Were the animals evaluated by reviewers blinded to their treatment group?

Reply:
The behavioral scoring system applied in this study is a well-established method which has been wildly used in many studies for analyzing the symptom progression of JEV infection [4,5]. The group of mice for observation was selected in a masked manner to avoid bias toward any treatment condition. All the neurological parameters were recorded visually and the total score was calculated on the basis of the appearance of the symptoms. The scores shown in figure 7 represent the average scores of the mice in each group. This point has been explained in the METHODS section of the revised manuscript.

References:


Figure 2B

Click here to download high resolution image
Figure 2C
Figure 4A
Figure 4D

Click here to download high resolution image
Figure 4F
Figure 5B

Click here to download high resolution image
**Figure 5C**

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